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FOREWORD OF THE ASSISTANT DEAN (EXTERNAL & GLOBAL ENGAGEMENT) OF FACULTY OF SCIENCE

Bismillahirrahmanirrahim,

Assalamualaikum warahmatullahi wabarakatuh,

I would like to thank all academic and non-academic staffs who have been involved in the Biosciences Symposium of the Final Year Undergraduate Project 2018 which was held at the Department of Biosciences (T02), Faculty of Science (formerly known as Department of Biosciences (T02), Faculty of Biosciences & Medical Engineering). Congratulations to all students for successfully presenting their respective projects.

The published proceedings and abstracts from this symposium are the results of students' research assisted by the academic staff as supervisors and co-supervisors. This activity will encourage the undergraduate students to continue working in the field of research. In addition, students are not only able to carry out projects in the laboratory but they also have the ability to deliver their projects in the form of scientific writing and oral presentation.

Finally, I hope that this symposium will be continued in the future to enhance the faculty's research activities through scientific writing and also to elevate the UTM's image nationally and globally.

PROF. DR. FAHRUL ZAMAN HUYOP
Assistant Dean (External & Global Engagement)

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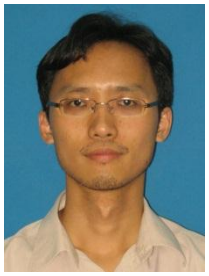

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SYMPOSIUM SCHEDULE

DAY 1

AL-HAZEN: Bacteria-Fungus-Yeast/Phage/Bacteriology/Bacteria Isolation				14th MAY 2018 (MONDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Eka Zanariah binti Dzulkarnain	Adibah binti Yahya		Optimization Of Protease Production By Bacteria Co-Culture Utilizing Chicken Viscera As Substrate
2.20 PM	Mariatul Hajar binti Salim	Adibah binti Yahya		Isolation And Screening Of Lignocellulolytic Bacteria And Fungi From Partialy Matured Compost (EFB)
2.40PM	Nur Hidayah binti Membron	Adibah binti Yahya		Isolation And Characterization Of Bacteria And Fungi From Palm Oil Mill Effluent (POME)
3.00 PM	Nur Syamimi Nadiah binti Baharudin	Nor Azimah binti Mohd Zain		Isolation And Characterization Of Antibiotic Resistant Bacteria From Rivers In Melaka
3.20 PM	Syahidatul Fazlina binti Samsul Rizal	Nor Azimah binti Mohd Zain		Isolation And Characterization Of Antibiotic Resistant Bacteria From Rivers In Negeri Sembilan
3.40 PM	Nur Roslina bt Peer Muhammad Hussain	Nor Azimah binti Mohd Zain		Study On The Emergence Of Antibiotic Resistance Bacteria From Rivers In Kuala Lumpur
4.00 PM	Fatin Shahira binti Othman	Nor Azimah binti Mohd Zain		Study On Emergence Of Antibiotic Resistance Bacteria From Rivers In Selangor

AL-BIRUNI: Bacteria-Fungus-Yeast/Phage/Bacteriology/Bacteria Isolation				14th MAY 2018 (MONDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Kevin bin Jaffery	Madihah binti Md Salleh		Isolation And Screening Of Marine Yeast For Bioethanol Production In Batch Culture
2.20 PM	Cheah Wai Jun	Chong Chun Shiong		Charaterization *Nesterenkonia* sp. CL21 And Its Protease Activity
2.40PM	Ching Xin Huey	Chong Chun Shiong		Characterization Of Xylanase-Producing *Nesterenkonia* Species CL21
3.20 PM	Mohamad Hamizan bin Abd Karim	Chong Chun Shiong		Isolation, Identification And Characterisation Of Lignocellulolytic Bacteria From Saline Environment
3.40 PM	Mohammad Azrul Izham bin Awang	Fahrul Zaman bin Huyop		Screening Of Physical Characteristics, Antioxidant And Cytotoxicity Activities Of Black Root And Suri Extracts
4.00 PM	Wan Nur Afdhilla binti Wan Mazelan	Nurliyana binti Ahmad Zawawi	Zarita binti Zakaria	The Effect Of Silver Nanoparticles-Multiwalled Carbon Nanotubes (AgNP-MWCNT) On Bioaerosol Bacteria Contamination Of Air-Conditioner Filter

AL-KINDI: Plant-Related Sciences				**14th MAY 2018 (MONDAY)**
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Fatin Naziha Binti Rosli	Alina binti Wagiran		Callus Induction And Morphology Of Wetland Rice Cultivar Mr297 Via Scanning Electron Microscopy
2.20 PM	Nur Laila Natasya binti Hamzah	Alina binti Wagiran		Morphological Characteristic Of MR297 Malaysian Wetland Rice Callus Using Amino Acids And Maltose
2.40PM	Nur Aida Syasya binti Azlan	Alina binti Wagiran		Suspension Cell Culture Development Of Malaysian Mr297 Rice Cultivar
3.00 PM	Dona binti Abd Karim	Fazilah binti Abd Manan		Morphology And Photosynthetic Properties Of Different *Elaeis guineensis* Planting Materials Treated With NK Fertilizer
3.20 PM	Dharshyini A/P V.Sivananda	Fazilah binti Abd Manan		Antioxidant Properties In Six *Elaeis guineensis* Planting Materials Treated With Different Levels Of Nitrogen-Potassium Fertilizer
3.40 PM	Intanku Noorfatin bt Rozman	Fazilah binti Abd Manan		Vegetative Growth And Biochemical Response Of *Elaeis guineensis* Treated With Different Amount Of Fertilizer
4.00 PM	Amelia Nabila binti Affendi	Zaidah binti Rahmat		Antioxidant Activity And Trace Elements From *Orthosiphon stamineus* White And Purple Varieties
4.20 PM	Muhamad Azhanizam bin Mohd Salleh	Zaidah binti Rahmat		Antioxidant Activity And Trace Elements From *Orthosiphon stamineus* Tea Product

PBL 1: Environmental Sciences				**14th MAY 2018 (MONDAY)**
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Muhd Hafiz bin Jusoh	Nik Ahmad Nizam bin Nik Malek		Removal Of Herbicide Paraquat By Cetyltrimethyl Ammonium Bromide Modified Pineapple Leaves
2.20 PM	Punitawathy A/P Palanisamy	Zaidah binti Rahmat		Antioxidant Activity And Trace Elements From Fresh And Commercialized *Moringa oleifera* Seeds
2.40PM	Nur Nadia Fazira binti Makimin	Zaharah bt Ibrahim		Development Of Lightweight Macrocomposite For Treatment Of Polluted River Water
3.00 PM	Norazian binti Abu Bakar	Zaharah bt Ibrahim		Isolation And Characterization Of Bacteria Found In Mix-Culture Of Microalgae Bioreactor
3.20 PM	Siti Nadhrah binti Shaharudin	Zaharah bt Ibrahim		Isolation And Characterization Of Halotolerant Lignocellulolytic Bacteria From Marine Environment
3.40 PM	Normaliana binti Rosli	Norahim bin Ibrahim		Isolation And Identification Of Rhizobacteria From *Coriandrum sativum* By Verticulture System
4.00 PM	Nadhirah binti Omar	Norahim bin Ibrahim		Bioelectricity Generation From PMFC By *Mentha spicata* Using Nutri-Pot Technique With Different Growth Media
4.20 PM	Nuranis Shabirin binti Ismail	Norahim bin Ibrahim		Electricity Generation Using *Oenanthe javanica* In Plant Microbial Fuel Cell With Fertigation Method

PBL 2: Eukaryotes/Animal Cell/Cancer/Virus				**14th MAY 2018 (MONDAY)**
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Oi Jing Yi	Khairunadwa binti Jemon		Antibacterial And *In Vitro* Wound Healing Activities Of *Helichrysum italicum* And *Leptospermum scoparium* Essential Oils
2.20 PM	Liew Zi Ming	Khairunadwa binti Jemon		Antibacterial And In Vitro Wound Healing Activity Of Essential Oils
2.40PM	Nik Muhammad Aizat bin Nik Ali	Mohd Helmi bin Sani		Screening Of Cellonsphere Microcarrier In A Microwell Attachment Plates For Mammalian Cell
3.00 PM	Nurul Hidayah binti Khairuddin	Mohd Helmi bin Sani		Screening Of Cytodex 3® Microcarrier In A Microwell Attachment Plates For Mammalian Cell
3.20 PM	Nurul'ashikin binti Haamdan	Mohd Helmi bin Sani		Screening Of Cellonsphere™3 Microcarrier In Microwell Attachment Plates For Mammalian Cell
3.40 PM	Yeo Zhin Leng	Praseetha A/P Prabhakaran	Faezah binti Mohd Salleh	Effect Of Combined Cisplatin And *Clinacanthus nutans* On Gene Expression Of MDA-MB-231 Breast Cancer Cells
4.00 PM	Siti Nur Diyana binti Azmi	Praseetha A/P Prabhakaran		Effects Of Combined Retinoic Acid-*Clinacanthus nutans* Treatment In Hela Cancer Cells
4.20 PM	Nazera Hana Masrin	Praseetha A/P Prabhakaran		Effects Of Combined Retinoic Acid - Curcumin Treatment In Hela Cancer Cells

PBL 3: Multidisipline – Molecular Biology/Genetic Eng/Metagenomics/Eukaryotes/Animal Cell/Cancer/Virus				**14th MAY 2018 (MONDAY)**
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Thong Hui Yee	Faezah binti Mohd Salleh		DNA Barcoding Using Chloroplastic And Nuclear Marker In *Ficus deltoidea*
2.20 PM	Nur Umairah binti Abdul Razak	Faezah binti Mohd Salleh		Development Of Reference DNA Barcodes For *Labisia pumila* Using Chloroplastic And Nuclear Marker
2.40PM	Fatin Liyana binti Yusli	Faezah binti Mohd Salleh		DNA Barcoding Using *ITS2* For Identification of *Labisia pumila* In Selected Malaysia Herbal Medicinal Product
3.00 PM	Nur Shahamimi bt. Harisfadzilah	Nur Izzati binti Mohd Nor		cDNA Synthesis Of Flavanones Biosynthetic Genes Of *Citrus* Plant Of Local Variety
3.20 PM	Siti Nabilah binti Nor Basri	Razauden bin Mohd Zulkifli		Development Of Loop-Mediated Isothermal Amplification (LAMP) Technique For Detection Of Porcine DNA For Food Quality
3.40 PM	Noor Amylina binti Nawawe	Razauden bin Mohd Zulkifli		Development Of Loop-Mediated Isothermal Amplification (LAMP) Technique For Food Safety: Detection Of Porcine Gene
4.00 PM	Alnaseri, Zubaida Jalal M.	Haryati binti Jamaluddin		Sub-Cloning Of *Cyclin-Dependent Kinase 3* (*CDK3*) Gene Into A Mammalian Expression Vector4
4.20 PM	Koh Ting Ting	Haryati binti Jamaluddin		Sub-Cloning Of Brain Expressed X-linked 3 Into Mammalian Expression Vector

DAY 2

AL-HAZEN (LEVEL 2): Plant Related & Environmental Sciences				15th MAY 2018 (TUESDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Farah Shahirah binti Suhaimi	Salehhuddin bin Hamdan		Antibacterial Activity Of *Citrus microcarpa* (Limau Kasturi) Leaves Extract
2.20 PM	Nur Fateha binti Muhamad Asfar	Salehhuddin bin Hamdan		Antibacterial Activity Of *Murraya koenigii* Leaves Extract
2.40PM	Nur Shazwani binti Shaary	Salehhuddin bin Hamdan		Antibacterial Activity Of *Citrus hystrix* Leaves Extract
3.00 PM	Nur Hasnanie binti Jaaman	Azman bin Abd Samad		Effect Of Plant Growth Regulator On *In Vitro* Regeneration Of Banana cv. Berangan
3.20 PM	Nur Syafiqah binti Hafiz	Azman bin Abd Samad		Effect Of Growth Regulators On Adventitious Root Induction Of *Justicia gendarussa*
3.40 PM	Nadia bt Jamaludeen	Norahim bin Ibrahim		Degradation Of Palm Oil Mill Effluent (POME) At Different ph Using Single Strain *Bacillus* sp. And Pome Treatment Via Microbial Fuel Cell (MFC)
4.00 PM	Nur Kamilah binti Mohd. Nordin	Madihah binti Md Salleh		Isolation And Partial Characterization Of Amylolytic Bacteria During Food Waste Fermentation Using Solid State Fermentation (SSF)

AL-BIRUNI (LEVEL 3): Bacteria-Fungus-Yeast/Phage/Bacteriology/Bacteria Isolation & Fermentation Technology				15th MAY 2018 (TUESDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Nur Azreen binti Saidon	Saleha binti Shahar		Isolation Of Bacteriophage Infecting Enterobacteriaceae From Wastewater
2.20 PM	Mega Rajiman	Saleha binti Shahar		Identification Of Sewage Bacteriophage Using Sequencing Method
2.40PM	'Aina Nabilah Faizah bt Ahmad Bustamam	Saleha binti Shahar		Isolation Of Bacteriophages Infecting Enterobacteriaceae From Sheep Stool
3.00 PM	Norahimah binti Ismail	Shafinaz binti Shahir		Screening And Identification Of Bacteria From Copper Ores Effluent
3.20 PM	Nur Nadhirah binti Khalid	Shafinaz binti Shahir		Screening and Identification of Copper-Tolerant Bacteria from Copper Concentrate
3.40 PM	Siti Zulaikha binti Abdullah	Shafinaz binti Shahir		Screening And Identification Of Copper Tolerant Bacteria
4.00 PM	Siti Baziah binti Baharin	Wan Rosmiza Zana bt Wan Dagang		Marine Bacterial Biofilm Of *Salinimonas lutimaris* sp. On Paint Surfaces Under Hydrodynamic Conditions
4.20 PM	Norfaziera bt Mohammad Khai	Wan Rosmiza Zana bt Wan Dagang		Effect Of Surface Roughness Of Stainless Steel On The Formation Of *Salinimonas lutimaris* Biofilm

AL-KINDI (LEVEL 4):Bacteria-Fungus-Yeast/Phage/Bacteriology/Bacteria Isolation & Fermentation Tehnology/Environmental Sciences				15th MAY 2018 (TUESDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Nurazlin binti Ahamad	Nur Izzati binti Mohd Noh		Isolation And Screening Of Thermophilic Cyanobacteria From Ulu Slim Hot Spring
2.20 PM	Norhafizah binti Hatta	Nur Izzati binti Mohd Noh		Comparative Study Of Fungal Growth Inhibition By Encapsulated Fungicide In MWCNTs And Activated Carbon
2.40PM	Wan Sujaihah binti Meor Hissan	Zarita binti Zakaria		Isolation And Identification Of Thermophilic Protease-Producing Bacteria From Kuala Woh Hot Springs, Perak
3.00 PM	Nurul Husna binti Md Rozaman	Wan Rosmiza Zana bt Wan Dagang		Formation Of *Salinimonas lutimaris* Biofilm On Wood With Epoxy Coating Surfaces Under Hydrodynamic Condition
3.20 PM	Nurliyana Adlina bt Abdullah	Zarita binti Zakaria		Isolation And Identification Of Thermophilic Protease-Producing Bacteria From Ulu Slim Hot Spring, Perak
3.40 PM	Nur Amira Syahirah binti Mazlan	Zarita binti Zakaria		Optimisation Of Thermophilic *Bacillus licheniformis* Strain MEA-01's Proteolytic Activity Towards Casein Based On Temperature And Incubation Period
4.00 PM	Evernessah Eva Dominus	Huszalina binti Hussin	Madihah Md Salleh	Biosugar And Biovanillin Productions From Pineapple Crown In Solid State Fermentation By *Phanerochaete chrysosporium*
4.20 PM	Norfazilah binti Md Zaki	Huszalina binti Hussin	Madihah Md Salleh	Optimization Of Ferulic Acid Recovery From Lemongrass Leaves For Biovanillin Production By *Phanerochaete chrysosporium*

PBL 1 (LEVEL 3): Biochemistry/Chemistry/Food/Analytical Chem-Biochemisty & Plant Related Sciences				15th MAY 2018 (TUESDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Lim Chai Hui	Nik Ahmad Nizam bin Nik Malek		Preparation, Characterization And Antibacterial Activity Of Gentamicin Loaded Surfactant-Kaolinite
2.20 PM	Wan Nur Aryantie binti Wan Othman	Nik Ahmad Nizam bin Nik Malek		Adsorption Of Streptomycin On Organo-Kaolinite And Its Antibacterial Activity
2.40PM	Nurul Hidayu binti Abdul Karim	Nurriza binti Ab. Latif	Khairunadwa binti Jemon	Antibiofilm And Antiadherence Activities Of *Amaranthus* spp. Leaf Extracts Against *Streptococcus mutans*
3.00 PM	Nurul Husna binti Ab Rahman	Nurriza binti Ab. Latif	Khairunadwa binti Jemon	Antimicrobial Activity Of *Pithecellobium jiringa* Against Oral Bacteria
3.20 PM	Jobeca Jone	Nurriza binti Ab. Latif		Antibiofilm And Antiadhesion Activities Of *Clinacanthus nutans* (Burm. F.) Lindau Leaves Extract Against Oral
3.40 PM	Nur'ain binti Mohammad Tahir	Mohd Firdaus bin Abdul Wahab		Optimization Of Cassava Wastewater For Efficient Starch Degradation By *Bacillus* sp. Strain LFSF-20

PBL 2 (LEVEL 3): Biochemistry/Chemistry/Food/Analytical Chem-Biochemisty & Plant Related Sciences				15th MAY 2018 (TUESDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Nurulain Syamimi binti Mohamad	Syazwani Itri binti Amran		Screening Of *Nigella sativa*-Based Herbal Medical Products Using Analytical Methods
2.20 PM	Nurul Ain Syukriyah binti Ahmad Muhamud	Syazwani Itri binti Amran		Screening Of Epigallocatechin-3-gallate (EGCG) Content In Commercialized *Camellia sinensis* Products
2.40PM	Nur Azian binti Zubir	Syazwani Itri binti Amran		Antibacterial Activity Of Thymoquinone (TQ) And Epigallocatechin-3-gallate (EGCG) On *Staphylococcus aureus*
3.00 PM	Siti Sapinah binti Bani Yamin	Azman bin Abd Samad	Wan Rosmiza Zana bt Wan Dagang	Propagation Of Local Pineapple (*Ananas Comosus L.*) In Temporary Immersion System
3.20 PM	Nur Atikah bt Mustfa Kamal	Muhammad Arshad Javed	Fazilah binti Abd Manan	Response Of Rice Varieties To Salinity Stress At Seedling Stage
3.40 PM	Neshalini A/P P.Ragavan	Muhammad Arshad Javed	Fazilah binti Abd Manan	Combined Effects Of Salinity And Alkalinity On Rice Seedlings

PBL 3 (LEVEL 4): Multidiscipline – Proteins/Enzymology/Proteomics/Bioinformatics & Protein Structural Analyses				15th MAY 2018 (TUESDAY)
TIME	**STUDENT**	**SUPERVISOR**	**CO-SUPERVISOR**	**TITLE**
2.00 PM	Nur Azimah Hafsyah binti Ariffin	Mohd Shahir Shamsir bin Omar		Molecular Modelling And Bioinformatics Analysis Of Twin Arginine Translocation (TatD) Deoxyribonuclease
2.20 PM	Nor Amira bt Mohd Lazim	Mohd Shahir Shamsir bin Omar		Homology Modeling And Adaptation Study Of N-acyl-homoserine Lactonase From Extremophiles
2.40PM	Nazatul Aida bt Md Saad	Mohd Shahir Shamsir bin Omar	Khairunadwa binti Jemon	Modelling And Adaptation Study Of Alkaline Phosphatase
3.00 PM	Hamizah binti Abdul Kadir	Haryati binti Jamaluddin		Expression Of Recombinant Serine Protease From *Acinetobacter baumannii* In *Escherichia coli*
3.20 PM	Chai Pei Wen	Goh Kian Mau		Purification And Characterisation Of An *Anoxybacillus* β-Glucosidase With Lactase Activity
3.40 PM	Tan Sing Ngoh	Mohd Firdaus bin Abdul Wahab		Expression And Solubility Of H368A Human HSP47 Mutant
4.00 PM	Yeoh Sin Nie	Goh Kian Mau		Purification And Characterization Of Endoglucanase From *Dictyoglomus* sp.

ABSTRACTS

Optimization of Protease Production by Bacteria Co-Culture Utilizing Chicken Viscera as Substrate

Eka Zanariah Dzulkarnain [1] and Adibah Yahya [2,*]

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Abstract: Proteolytic enzymes are one of the most significant group of commercially manufactured enzyme. Protease have been chosen as the enzyme of interest in this study as they are remarkable for its ability in various industries sector such as leather industry, pharmaceutical industry, detergent industry, and food industry. Protease producing co-culture bacteria were isolated from chicken viscera (chicken waste). This study aims to investigate the relationship between bacterial growth and protease enzyme production as well as to optimize physical parameters requires for maximum protease production. Sample was cultures for 16 hours and harvested periodically, every half an hour for the first three hours, one hour for the following four hours and two hours for the remaining nine hours. Temperature, pH, and inoculation size were the parameters selected for the optimization study. Bacteria co-culture were grown in different temperature (27 °C, 37 °C, 46 °C, 56 °C and 60 °C), pH (4, 5, 6, 7 and 8), and inoculation size (5% (v/v), 10% (v/v), 15% (v/v), 20% (v/v), and 25% (v/v)). This study demonstrated that maximum production occurred during the exponential phase of bacterial growth. In addition, the optimum culture condition for protease production were at 37 °C, pH 6, and inoculation size of 10% (v/v), incubated with shaking at 180 rpm with value of protease activity is 0.480 U/mL. In conclusion, this study has successfully revealed that chicken viscera could be used as a potential substrate to synthesis protease enzyme and maximum production could be obtained with incubation under optimized conditions indicated above. Indeed, protease unique properties which makes it attractive and useful to be utilized in diverse biotechnological applications.

Keywords: Protease enzyme; Chicken viscera; Bacteria co-culture; Parameter

Isolation and Screening of Lignocellulolytic Bacteria and Fungi from Partially Matured Compost (EFB)

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Abstract: Oil Palm Empty Fruit Bunch (EFB) is the suitable feedstock for composting. The microbiological components of EFB compost consist of bacteria and fungi. The addition of thickened Palm Oil Mill Effluent (POME) sludge throughout the composting process could simultaneously provide nutrients and source of microorganisms for biodegradation of the lignocellulosic EFB. This study aimed to investigate the characteristic of isolated microbes from EFB compost hence to determine the lignocellulolytic activity of the isolated microbes. Four strains of B12, B14, FG and FB were successfully isolated. Both strains of bacteria are Gram-positive species. This qualitative screening showed that all isolated strain was positive in producing xylanase and ligninase. The assays for enzymatic activity of CMCase, Fpase, ß-glucosidase, xylanase and lignin peroxidase (LiP) were determined based on the value obtained from the calculation of enzyme production. Among those five mechanisms of enzyme assay, Lignin peroxidase (LiP) shows the higher production value by B12, B14, FG and FB which are 0.928 U/mL, 0.945 U/mL, 0.389 U/mL and 0.331 U/mL respectively. However, all the isolates have a potential as a cellulase, hemicellulase and ligninase producers with in different intensity.

Keywords: EFB; Compost; Bacteria; Fungi; Lignocellulolytic

Isolation and Characterization of Bacteria and Fungi from Palm Oil Mill Effluent (POME)

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Abstract: Palm oil mill effluent (POME) is the liquid form of the oil palm residue and was recognized as the most harmful waste. POME contain lignocellulosic biomass which the most abundant renewable biomass, consist of cellulose, hemicellulose and lignin. A variety of microorganism such as bacteria and fungi have been evolved the ability in producing lignocellulolytic enzyme of biodegrading lignocellulosic in oily wastewater. The aim of this study is to isolate and characterization of microorganism such as bacteria and fungi in their ability to produce the higher amount of lignocellulolytic enzyme from POME. Bacterial and fungal strains were cultivated on selective agar medium supplemented with carboxymethylcellulose (CMC), xylan and alkaline lignin as carbon source. The lignocellulolytic enzyme activity was screening qualitatively by monitored the formation of clear zones on selective agar plates. The largest diameter of clearing zones was showed by bacterial BX4, BX5 and FX1 from xylan-agar that indicates the ability to produce xylanase activity. Further screening on quantitative assay were analysed in the enzyme assay, which were CMCase, FPase, ß-glucosidase, xylanase and lignin peroxidase (LiP). Among the four enzymes, lignin peroxidase (LiP) for detect ligninolytic enzymes shows the highest reading at 7 hour for bacterial BL5 strain with 0.8224 U/mL and at day 4 for fungi FL1 strain with 0.6305 U/mL. From the qualitative screening results, there was no obvious correlation with the value of quantitative screening.

Keywords: Palm oil mill effluent (POME); Lignocellulolytic enzyme; Qualitative screening; Enzyme assay; Lignin peroxidase (LiP)

Isolation and Characterization of Antibiotic Resistant Bacteria from Rivers in Melaka

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Abstract: Contamination of antibiotics in water environment becomes are great worldwide concern. The widespread uses of antibiotics in many fields are contributing to the emergence of pathogenic antibiotics resistance bacteria. The emergence of these antibiotic resistance bacteria become one of the most serious challenges in diseases treatment. The aim of this study is to determine the presence of antibiotic resistance bacteria from rivers in Melaka. A total of 7 bacterial strains were successfully isolated from Melaka River and 8 bacterial strains were successfully isolated from Tuang River, Melaka respectively. The isolated bacteria were tested for resistance against six different types of antibiotics including ampicillin, gentamicin, ciprofoxalin, chloramphenicol, rifamicin and tetracycline using antibiotic susceptibility test by disc diffusion. Most of the isolated bacteria show highest resistance effect toward chloramphenicol and tetracycline, less in rifamicin, gentamicin and ampicilin. None of the isolated bacteria showed resistance to ciprofoxalin. The MAR index was calculated and 6 isolated bacteria have the MAR index more than 50% were selected to be further analyzed by using 16S rRNA sequencing in order to determine their species. The characterization of bacteria has been conducted by Gram staining and biochemical tests. All the bacterial strains from Tuang River were identified as *Escherichia vulneris* sp., *Escherichia fergusonii* sp., *Citrobacter freundii* sp. and *Bacillus thuringiensis* sp.. While, antibiotic resistance bacteria identify from Melaka River were *Burkholderia multivorans* sp. and *Escherichia vulneris* sp..

Keywords: Antibiotic resistance bacteria; River; Melaka

Isolation and Characterization of Antibiotic Resistant Bacteria from Rivers in Negeri Sembilan

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Abstract: The antibiotic utilization in the environment may have escalated the occurrence of antibiotic resistant bacteria in the aquatic environment. The emergence of antibiotic resistant bacteria in the aquatic environment especially rivers increases the health risks associated with the waterborne bacteria in animals and humans that have becoming a worldwide concern nowadays. The mostly considered hot spot for becoming the widespread of the antibiotic resistance is the aquatic environment due to pollution with emerging antibiotics as the contaminants that were derived from the human activities. In this study, a total of 35 bacterial isolates were isolated from the water samples collected from point sources (near to residential areas) and from the non-point sources (soil leaching waters from agricultural and poultry farms) from Linggi and Jempol River, Negeri Sembilan. The bacterial isolates were tested for their susceptibility towards 6 antibiotics; gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin respectively by using disk diffusion method. From the test, 45.7% of the bacterial isolates were antibiotic resistant (AR) that were resistant to at least one antibiotic and another 54.3% were multiple antibiotic resistant (MAR) that were resistant to at least two antibiotics. Hence, a multiple antibiotic resistant (MAR) index was calculated for each water samples and 5 bacterial isolates that have the MAR index for more than 50% were selected for further identification by 16S rRNA sequencing. The bacteria identified were *Ralstonia pickettii*, *Ochrobactrum ciceri* and *Staphylococcus kloosii*. Based on this study, the existence of antibiotic resistant bacteria in the rivers revealed that the antibiotic contamination was indeed occurred in the Linggi River and Jempol River, Negeri Sembilan and their dispersion in the nature may elevate serious threats toward both public and environmental health.

Keywords: Antibiotic resistance bacteria; Multiple antibiotics resistant; River waters; Disk diffusion method

Study on the Emergence of Antibiotic Resistance Bacteria from Rivers in Kuala Lumpur

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Abstract: Currently dispersion of antibiotic resistance bacteria has elevated in the environment which set-up a serious worldwide impact in both environment and public health. The purpose of this study was to determine the presence of antibiotic resistance bacteria from rivers in Kuala Lumpur, Malaysia which is Batu River and Gombak River. Water samples were collected from two different points of both rivers respectively. Isolated bacteria were tested for resistance against six different antibiotics (ampicillin, chloramphenicol, ciproflaxin, gentamicin, tetracycline and rifampicin) using antibiotic susceptible testing by disc diffusion method. From both of the rivers, the greatest frequency of resistances in isolates was against chloramphenicol and ampicillin but less often in rifampicin, gentamicin and tetracycline. However, there is zero resistance of the bacteria isolates against ciproflaxin. Hence, most of the bacteria show Multiple Antibiotic Resistance (MAR) index value more than 20% which indicates the sampling areas are in high risk with antibiotic resistant bacteria contamination. Seven over forty-one isolates that shows MAR index value more than 50% were further identified by performing 16S rRNA polymerase chain reaction, PCR. Those isolates that showed resistance to most antibiotics were identified as *Pseudomonas* sp., *Acinetobacter baumannii* sp., *Pseudomonas putida* sp. and *Uncultured Ralstonia* sp.. From this study, it reveals the existence of antibiotic resistance bacteria which also proves the antibiotic contamination in the rivers (Batu River and Gombak River), Kuala Lumpur and dispersion of these kinds of bacteria in environment could lead to serious threat to agriculture, human and animal population.

Keywords: Antibiotic resistance bacteria; River; Kuala Lumpur

Study on Emergence of Antibiotic Resistance Bacteria from Rivers in Selangor

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Abstract: The emergence of antibiotic resistant is primarily due to excessive and often unnecessary use of antibiotic in animals and humans especially from clinical and agriculture. The widespread uses of antibiotic will contribute the development of antibiotic resistant bacteria (ARB). This study was conducted to determine the presence of ARB strains present in the Chemubong River and Langat River in Selangor. It was performed by collecting the water samples from both rivers. The bacteria were isolated and their morphological and biochemical analysis was conducted. Morphological structure and Gram staining study were conducted to differentiate between Gram-negative bacteria and Gram-positive bacteria. Antibiotic susceptibility test was also performed to indicate their resistances by using disk diffusion method. Identification of the bacterial isolates were determined from their 16S rRNA gene sequencing. A total of 38 bacteria were isolated and tested against six different antibiotic; chloramphenicol, ciprofloxacin, rifampicin, gentamycin, tetracycline and ampicillin. In this study, nine ARB strains were successfully isolated based on their susceptibility and MAR Index for more than 50% toward different antibiotics. It was identified that Chemubong River and Langat River waters are contaminated with *Burkholderia* sp., *Pseudomonas* sp., *Bacillus* sp., *Vogesella* sp. and *Staphylococcus hominis*, which are human opportunistic pathogens and may affect human health. Thus, this research can be useful to bring awareness to the Malaysian Government and public about the safety precaution and measures to be taken into contamination of ARB prevention in water sources in Selangor.

Keywords: Antibiotic resistance bacteria; Antibiotic; River; Selangor

Isolation and Screening of Marine Yeast for Bioethanol Production in Batch Culture

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Abstract: Fossil fuels such as oil, coal and natural gases have become the prime sources of energy in the current era. Rising of fossil fuel consumption due to the rapid growth of industries and the concern effect of the these fossil fuel combustion and also the uses of limited freshwater as the medium of fermentation is widely upsetting as this freshwater is highly valuable in certain country. Hence, an alternative have been seek out which using the seawater to replace the freshwater as the fermentation medium. The analysis of sea water component shows sea water consisted of macro and micro elements which are required for the growth and bioethanol production by halotolerent yeast. Three different types of mangrove soils and sea water were used as a source for isolation of potential halophilic yeast for bioethanol production. The yeast strain was isolated on Glucose yeast peptone (GYP) agar with additional of 0.1% (w/v) chloramphenicol using technique streak plate technique. Out of 11 total isolates, only 7 strains shows budding and positive in sugar fermentative during durham tube test. Screening of 7 potential halotolerant yeast for bioethanol production in glucose yeast peptone medium shows strain A3P1 shows the highest ethanol production 3.4 g/L ethanol after 24 hour incubation at 37 °C. Optimization of bioethanol production by strain A3PI was studied based on the effect of seawater concentrations, initial culture pH and glucose concentrations. The optimum condition for the highest bioethanol production was obtained at initial pH 4 with additional of 40 g/L of glucose and 50% (v/v) sea water. Approximately, 16.6 g/L of bioethanol with maximum productivity 0.24 g/L/h and overall productivity 0.298 g/L/h. This result showed the yields of bioethanol (Yp/s) and cell (Yx/s) are 0.415 g/g and 0.00019 g/g respectively. The yield efficiency of bioethanol production per gram of cell (Yp/x) is 2.2 g/g. The potential application of sea water as a medium for bioethanol production can be applied for replacement of fresh water during biorefinery processing.

Keywords: Bioethanol; Seawater; Halophilic

Charaterization *Nesterenkonia* sp. CL21 and Its Protease Activity

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Abstract: *Nesterenkonia* sp. CL21 is a bacterium isolated by previous researcher. Bacteria under genus *Nesterenkonia* are relatively less studied. Thus, this study focused on the phenotypic and enzymatic characterization of *Nesterenkonia* sp. CL21. Biochemical tests were performed to investigate the physiological characteristics of the bacterium. Antibiotic resistance test was carried out and discovered *Nesterenkonia* sp. CL21 was resistant to ampicillin, tetracycline, penicillin G and novomycin. Proteases are the enzyme which is ubiquitous in nature and used to catalyse protein. Crude protease was extracted from *Nesterenkonia* sp. CL21 and characterized by azocasein assay in this study. Protease showed the optimal activity at 60 °C, pH 10 in 2% (w/v) salt concentration and stable in the presence of metal ions (Ca^{2+}, Cu^{+}, Fe^{2+} and Fe^{3+}), organic solvent (ethanol), detergent (Tween 20, Tween 40, Tween 60, Tween 80, H_2O_2, Triton X-100, sodium deoxycholate, sodium carbonate) and commercial detergent (Axion, Brozip, G-10, Breeze, Top, Dynamo). The protease from *Nesterenkonia* sp. CL21 can be considered as potential enzyme for industrial application.

Keywords: Protease; *Nesterenkonia* sp. CL21; Phenotypic and enzymatic characterization

Characterization of Xylanase-Producing *Nesterenkonia* Species CL21

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Abstract: Xylan is a source of carbohydrate (hemicellulose) found in most of the lignocellulosic materials that can be obtained from various types of biomass wastes. Xylose produced from xylanase-degradation of lignocellulosic materials served as a potential renewable feedstock in production of biofuel; while xylanase is also widely used in industrial application such as facilitating process of lignin removal. *Nesterenkonia* sp. strain CL21 was identified as a halotolerant bacterium that grows optimally at 50 °C and pH 9, in the presence of 2% salt concentration. Strain CL21's xylanase exhibited stability at a wide range of salt concentration (0 – 14% w/v) and temperatures (30 – 60 °C), meanwhile display a limited stability at pH (10 – 11). The maximal xylanase activity was obtained at 10% NaCl (w/v), 35 °C and pH 10. The xylanase activity was found to be stable in a range of organic solvents (25%, v/v) like acetone, chloroform, DMSO, ethanol, isopropanol and methanol (107 – 161% relative activity). Series of metal ions (Al^{3+}, Cd^{2+}, Ca^{2+}, Co^{2+}, Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, K^{+}, Zn^{2+}) showed enhancement in xylanase activity, while Mg^{2+} and Ni^{2+} reduced the xylanase activity. High stability (>70%) of xylanase was shown in the presence of various types of detergents with highest stability exhibited in the addition of SDS (414.9% relative activity). However, presence of hydrogen peroxide inhibited xylanase activity. The xylanase produced from strain CL21 was found to be compatible with commercial detergents. The findings suggested that the xylanase from strain CL21 has the potential to be applied in industries.

Keywords: Xylanase; *Nesterenkonia* sp. strain CL21; Biomass wastes; Biofuel

Isolation, Identification and Characterisation of Lignocellulolytic Bacteria from Saline Environment

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Abstract: In Malaysia, approximately 168 million tons of lignocellulose biomass was produced annually including biomass from oil palm waste, sugarcane waste, rice husks and timber. Besides, there is extremely overflowing research in bioconversion of lignocellulosic biomass through microbial enzymes to produce fermentable sugars and others high value products. Therefore, with the need to reduce lignocellulosic biomass by converting them into various high added value products, efforts in searching new lignocellulose degrading enzymes is one of the key focuses. In this study, two bacterial strains have been isolated from plant material and sea water samples collected from Tioman Island at Mersing, Johor. Strain CM1 and CM2 showed positive degradation on lignin and xylan were selected for further characterisation and subsequently identified as *Shewanella* sp. and *Exiguobacterium* sp. based on 16S rRNA gene sequence analysis. Strain CM1 is Gram-negative, non-endospore forming and grew at 37 °C and pH 7.0 with 2% sodium chloride (NaCl). Strain CM1 positive for catalase, oxidase, urease, ligninase, casease, while utilising sucrose, lactose and glucose as a source carbon. As for strain CM2, it is Gram-positive, non-endospore forming and grew at 37 °C and pH 7.0. Strain CM2 was positive for catalase, oxidase, gelatinase, xylanase, amylase and casease. Antibiotic resistance was exhibited by *Shewanella* sp. CM1 and *Exiguobacterium* sp. CM2 and these included nobobiocin, kanamycin, ampicillin, erythromycin, chloramphenicol, bacitracin, tetracycline, streptomycin, lincomycin, oleandomycin, gentamicin, polymyxin, neomycin, and penicillin. Both strain CM1 and CM2 positive for lignocellulotic activity suggesting that both strains have potential application value in industries.

Keywords: Lignocellulose biomass; Bioconversion; Microbial enzymes

Screening of Physical Characteristics, Antioxidant and Cytotoxicity Activities of Black root and Suri Extracts

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Abstract: Overproduction of reactive oxygen species (ROS) such as superoxide anion, hydroxyl and hydrogen peroxide can increase the chronic diseases such as diabetes, cardiovascular diseases and cancer. Natural antioxidant that can be found in plant shown inhibitory effect on these free radicals. Black root and Suri are traditional medicine that contains combination of several herbs as its ingredient. Black root ingredient contains mixture of temulawak (*Curcuma zanthorrhiza*), temu hitam (*Curcuma aeruginosa rosb*), akar lalang *(Imperata cylindrica*), halia (*Zingiber officinale roscoe*), biji kembang semangkuk (*Sterculia lynchnophora* seed), kulit bawang merah (*Allium sepa* skin) and kulit bawang putih (*Allium sativum* skin). For Suri, it is a mixture of kayu manis (*Cinnamomum verum*), temu hitam (*Curcuma aeruginosa rosb*), kunyit putih *(Curcuma zedoaria*) and cucur atap (*Beackea frutescens*). In this study, functional group, active compound, antioxidant activity and cytotoxicity effect on cancer cell lines (MCF7) was examined. Results show that both extract with water have same functional group compared to raw sample but for ethanol extract, it contains difference functional group. Suri ethanol extract showed the highest antioxidant activity which is EC_{50} value 0.2 mg/mL compared to other extracts. For cytotoxicity activity, Suri ethanol extract with 26 µg/mL value of IC_{50} and Black root ethanol extract with 15.4 µg/mL extract have moderate cytotoxicity activity. Volatile compounds are detected in both extracts of Black root and Suri. These compounds have shown involved in several biological activities. Black root and Suri extracts can be compared in term of antioxidant and cytotoxicity activity to determine which one is better to use as the herbal medicinal product.

Keywords: Black root extract; Suri extract; Physical characteristics; Antioxidant activity; Cytotoxicity activity

The Effect of Silver Nanoparticles-Multiwalled Carbon Nanotubes (AgNP-MWCNT) on Bioaerosol Bacteria Contamination of Air-conditioner Filter

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Abstract: Hybrid nanostructure has been widely studied due to its high potential in biotechnology. This study reports the development of nanohybrid composed of silver nanoparticles (AgNP) and oxidized multi-walled carbon nanotubes (ox-MWCNT) for its antibacterial effect on bioaerosol bacteria from air-conditioner filter. It was first prepared by treating pristine multi-walled carbon nanotubes (p-MWCNT) with concentrated sulphuric and nitric acid mixture (3:1) to generate ox-MWCNT. Afterwards, the AgNP, produced from silver ion reduction method was attached on ox-MWCNT by sonication in ethanol. The successful attachment was verified using Fourier transform infrared spectroscopy (FTIR), energy dispersive X-ray spectroscopy (EDX), transmission electron microscopy (TEM), zeta potential and dispersion test. The antibacterial effect of AgNP-MWCNT was confirmed by measuring the inhibition zone against isolated bacteria culture, and was compared to ox-MWCNT. From the results, AgNP showed successful attachment to ox-MWCNT, confirmed by the changes of C=O intensity peak at 1720 cm^{-1} in AgNP-MWCNT, as compared to ox-MWCNT. AgNP-MWCNT also showed noticable peaks at 1401, 1065 and 6707 cm^{-1} that indicates the interaction of silver with functional group. Silver element in AgNP-MWCNT was at high percentage (69.5%) in EDX results, proved by TEM analysis that showed AgNP embedded on surface of ox-MWCNT. AgNP-MWCNT also showed good stability in water as ox-MWCNT, confirmed by zeta potential results with value of -31.23 mV and -29.99 mV, respectively. In the antibacterial test, 100 µg/mL AgNP-MWCNT yielded the most efficient inhibitory zone against the Gram-positive Bacillus bacteria. The results are promising to show synergistic activity of AgNP-MWCNT compared to ox-MWCNT alone. This would be beneficial for researchers who interested to study requirement of AgNP-MWCNT needed to hinder growth of bacteria. Further studies are required to determine bacterial strain inhibited by 100 µg/mL AgNP-MWCNT as lower concentration is not effective for antibacterial treatment modality.

Keywords: Antibacterial; MWCNT; Silver nanoparticles; Hybrid nanostructure; Bioaerosol

Callus Induction and Morphology of Wetland Rice Cultivar MR297 via Scanning Electron Microscopy

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Abstract: The purposes of this research are to determine the effect of different 2,4-D concentrations (1 mg/L – 4 mg/L) and media type (N_6 and MS) on percentages of callus induction and induction of embryogenic callus for wetland rice, MR297 cultivar. Dehusked seed were cultured on MS medium supplemented with 1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L of 2,4-D separately were performed. Then, the seeds were cultured on N_6 media with optimum concentration of 2,4-D (3 mg/L). The morphology of embryogenic callus from these treatments then was determined using scanning electron microscopy (SEM). Results showed that percentage of callus induction varied depending on the different concentrations of 2,4-D applied on the MS media. After 4 weeks, the study revealed that treatment of 2,4-D added onto MS media showed positive effect on percentage of callus induced. It was observed that the optimum concentration of 2,4-D was 3 mg/L with highest percentage of 86.67% for callus induction. Furthermore, treatment with optimum 2,4-D concentration (3 mg/L) on N_6 media showed lower percentage of callus induction as compared to MS media (78.57%). The callus obtained were cream to pale yellow in colour with dry, compact and organized structure showing embryogenic potential. The morphology characteristic was confirmed with SEM analysis. The SEM results showed that embryogenic callus shape was compact cell mass with dome-like structure and globular. The study demonstrated that MR297 cultivar gave highest response to 3 mg/L 2,4-D and usage of MS medium for callusing showed better results on the percentage of callus induction for MR297 while morphology of embryogenic callus of MR297 were confirmed with SEM.

Keywords: MR297; Wetland rice cultivar; MS media; N_6 media; 2,4-D plant growth regulator; Percentage of callus induction; Scanning electron microscopy; Embryogenic callus; Nonembryogenic callus

Morphological Characteristic of MR297 Malaysian Wetland Rice Callus Using Amino Acids and Maltose

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Abstract: *Oryza sativa* L. (paddy) is the most important crop in the world and Asia. The aims of this study are to evaluate the effect of different amino acids (proline and casein hydrolysate) and different maltose concentrations on the percentage and morphological characteristic of callus induced from potential of Malaysian wetland rice, MR297 cultivar. Dehusked seed was treated on MS medium supplemented with constant 3 mg/L of 2,4-D but with three different concentrations of proline or casein hydrolysate separately. Then the optimal concentrations of amino acids were used in the maltose experiment. The embryogenic callus was then immersed in 1% of Evans blue to validate the viability of cells. The research revealed that callus induction were varied depend on amino acid supplied. The treatment with casein hydrolysate showed positive effect on percentage of callus induction after 4 weeks of culture. It was observed that the optimum concentration of 1.5 g/L casein hydrolysate show the highest callusing percentage (70%) compare to the control treatment (56.7%). However treatment with proline does not showed positive effect as the percentage of callus induction was lower than without proline treatment. The use of different maltose concentrations did not show promotive effect on callus induction. Morphology of proliferated embryogenic callus was whitish and yellowish in colour and has nodular appearance showing embryogenic potential. This study concludes that only casein hydrolysate did have promotive effect on callusing but not for proline and maltose.

Keywords: Wetland rice; Callus; Amino acid; Maltose; Evans blue

Suspension Cell Culture Development of Malaysian MR297 Rice Cultivar

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Abstract: The aim of the study is to evaluate the effect of different concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction of Malaysia rice MR297 cultivars. Then the calli induced were used for developing growth curve of suspension cell culture. Mature seeds were dehusked and placed on MS medium supplemented with four different concentrations of 2,4-D (1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L) separately. The results showed that after four weeks in culture, percentage of callus induction was optimal on MS medium supplemented with 2,4-D at 3 mg/L (73.33%). The callus morphology was yellowish in colour with compact and nodular appearance showing embryogenic potential. The four weeks old calli was selected to determine the growth of suspension cell culture. Therefore, two media type was studied such as N_6 media and MS media with constant supplementation of 3 mg/L 2,4-D and 1 mg/L kinetin for two weeks with regular subculture. To minimize aggregation between cells, the effect of pectinase was also determine. Finally, the growth curve was determined by measuring the fresh weight. To differentiate viable cell with nonviable cell, Evans blue staining was used Based on growth curve, the fresh weight of suspension cell culture in N_6 medium was higher compared to MS media. The present study showed addition of 0.005% pectinase resulted in higher fresh weight (2.18 ± 0.005 g) of suspension cells and fine cell compared to without pectinase (2.14 ± 0.01 g) on day 10 and decreased after day 11. Using 1% Evans blue the suspension cell viability has been shown with colourless while non embryogenic suspension cells were dark blue. This study concludes that MR297 prefer 3 mg/L of 2,4-D on callusing. The use of N_6 media and pectinase proven to produce higher suspension cell culture. The growth curve has been established for MR297 varieties and can be used for future as reference protocol.

Keywords: Suspension cell culture; *O. sativa* L.; *Indica* rice; Evans blue

Morphology and Photosynthetic Properties of Different *Elaeis guineensis* Planting Materials Treated with NK Fertilizer

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Abstract: Oil palm *Elaeis guineensis* yields is influenced by the agronomic practices and the quality of planting materials. *Deli x Avros* planting materials are widely used for seed production as it has unique and good fruit qualities particularly in Indonesia and Malaysia. Nutrients such as nitrogen (N) and potassium (K) is very crucial as it will contribute to the yields production of oil palm. Moreover, it also could influence the morphology and the biochemical activities in the plants. This study aimed to investigate the effects of different levels of NK fertilizer on the morphology and photosynthetic properties in the leaves of *Elaeis guineensis*. Total soluble sugar content and starch content were determined using Anthrone assays while the total chlorophyll content was analyzed using acetone assay. Five planting materials known as A, B, C, D and E that were treated with the lowest amount of NK fertilizer resulted in total soluble sugar content which were 78.91 mg/g, 69.91 mg/g, 81.71 mg/g, 227.82 mg/g and 132.89 mg/g respectively. Meanwhile, for planting material F that was treated with the highest amount of NK fertilizer recorded 113.9 mg/g amount of soluble sugar content. Planting materials A, B, C and D also had the highest total chlorophyll content with the lowest treatment of NK fertilizer which were 48.99 µg/mL, 47.68 µg/mL, 40.67 µg/mL and 35.04 µg/mL respectively. As the NK fertilizer treatment increased the production of total soluble sugar and total chlorophyll decreased. This finding is crucial as it provides good opportunity to increase the effectiveness of fertilizer application on the planting materials (*Elaeis guineensis*) in order to obtain high yields in the future and ensure that excessive fertilizer applications are avoided.

Keywords: NK fertilizer; Total soluble sugar; Starch; Chlorophyll; Morphology

Antioxidant Properties in Six *Elaeis guineensis* Planting Materials Treated with Different Levels of Nitrogen-Potassium Fertilizer

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Abstract: There are many types of fertilizers that have been used as source of nutrients for oil palm. In this study, the effects of Nitrogen-Potassium (NK) fertilization on the morphology of different planting materials and the antioxidant properties in the leaves of *Elaeis guineensis* were studied. NK fertilizer used in this research comprises muriate of potash (MOP), bayovar rock phosphate (BRP), Foliar Boron (F'Bor), Kieserite (KS), and ammonium sulphate (AS). Fertilizer treatment were indicated T1 which is control, T2 and T3. Six planting materials tested were A, B, C, D, E and F. Oil palm morphology was recorded *in situ* and samples were collected for antioxidant analysis. Morphology observed were the heights of the oil palm tree, the stem girth, and length of fronds in the tree. Antioxidant activity was determined by the accumulation of total phenolic content (TPC) and total flavonoid content (TFC). For TPC, planting material A, T2 showed the highest value which is 59.04 µg/g. Planting material B, T1 showed the highest TPC (46.10 µg/g). Planting material C, T2 showed highest TPC (77.87 µg/g). Planting material D, T2 showed highest TPC (35.59 µg/g). Planting material E, T3 showed highest TPC (43.23 µg/g). Planting material F, T2 showed highest TPC (36.65 µg/g). For TFC, leaves showed similar variations according to treatments. TPC and TFC will be varied for each planting material according to the amount of fertilizer the leaves received.

Keywords: *Elaeis guineensis*; Antioxidant properties; NK fertilizer; Planting materials

Vegetative Growth and Biochemical Response of *Elaeis guineensis* Treated with Different Amount of Fertilizer

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Abstract: Oil palm (*Elaeis guineensis*) is one of the largest economic crop cultivated for its nutritional oil in several tropical countries. Since it produce one of world highest nutritional content oils, oil palm become the most profitable commercial cultivation mainly in humid tropics. However, the industry encounters difficulties to determine the suitable planting materials, types and ratio of fertilizers to produce high quality of palm oil based on morphology basis. So, the objectives of this study are to measure the palm vegetative growth from different planting materials at the oil palm plantation and to determine amount of total soluble protein and proline content in oil palm leaves, based on various ratio of NK fertilizers treatment using colorimetric assay. Based on the result, the best NK fertilizer ratio for all planting materials can be predicted. After the vegetative measurement of oil palm plants, treatment 2 for planting material B showed the best result with the means and standard error for; number of green fronds (47.27 ± 1.17), number of pinnae (118.33 ± 3.99) and frond length (371.41 ± 7.60) cm. For the widest size of trunk (274 ± 14.53) cm recorded in planting material D, treatment 2. Planting material F with fertilizer treatment 2, dominated as the tallest trunk (613.33 ± 6.69) cm. For biochemical analysis, the highest total soluble protein was found in planting material F treated with treatment 3 (140.74 ± 2.02) µg while planting material A with fertilizer treatment 2 recorded the most abundant proline content (4.75 ± 1.17) ng. In conclusion, fertilizer treatment 2 is the best ratio for vegetative growth. The biochemical test showed planting material F treated with treatment 3 has the highest total soluble protein content while planting material A with fertilizer treatment 2 resulted in the most abundant proline content in the oil palm leaf tissues.

Keywords: *Elaeis guineensis*; NK fertilizers; Total soluble protein; Proline

Antioxidant Activity and Trace Elements from *Orthosiphon stamineus* White and Purple Varieties

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Abstract: *Orthosiphon stamineus* is one of the most well-known Malaysian local herbs and considered as an important traditional folk medicine. There is substantial interest in *O. stamineus* because it is believed that *O. stamineus* leaves have special chemical and pharmacological properties such as antioxidant activity, anti-inflammatory activity and antihypertensive activity. Although there are great interests in *O. stamineus*, however researches focus more on white variety with scarce research in purple variety. It would be beneficial for people to know the correlation of antioxidant activity in *O. stamineus* varieties to their tea product. Furthermore, there are also certain trace elements that can act directly or as cofactor in antioxidant processes. Therefore, this study focused on determination of antioxidant activity and trace elements from *O. stamineus* white and purple varieties. Antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity & Ferric Reducing Antioxidant Power (FRAP) assays while trace elements were detected by using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES) analysis. Purple variety of *O. stamineus* has higher antioxidant activity value for both radical scavenging activity, 74.02 ± 0.58% and ferric reducing activity, 11541 ± 35.91 Fe^{2+}/g compared to white variety which were 56.81 ± 4.45% and 8531 ± 463.2 Fe^{2+}/g respectively. Potassium (K), calcium (Ca), Magnesium (Mg) and Sodium (Na) were detected in both varieties while Selenium (Se) was undetectable. Both varieties was observed to contain the trace elements in the order of K > Ca > Mg > Na > Se.

Keywords: *Orthosiphon stamineus*; Antioxidant activity; Trace elements

Antioxidant Activity and Trace Elements from *Orthosiphon stamineus* Tea Product

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Abstract: *Orthosiphon stamineus* or known as Cat's Whisker is a famous herbal plant that contains antihypertensive, anti-allergic, anti-inflammatory and antioxidant. This herbal plant has been commercialized widely as tea product. *O. stamineus* tea product sold in the market was found to contain both leaf and stem. Previously, the leaves of *O. stamineus* have been proven to contain the highest antioxidant activity among other parts of the plant. In this study, leaves were separated from sachet of *O. stamineus* tea product and compared with mixture of leaves and stems. Analysis of the leaves and mixture extract have been conducted to determine the trace elements potassium (K), magnesium (Mg), calcium (Ca), sodium (Na) and selenium (Se) by Inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis and the antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Besides, antioxidant analysis *O. stamineus* tea drink at three different water temperatures which were boiled (100 °C), cold (4 °C) and room temperature (28 °C). For trace elements determination, K, Mg, Na, and Ca showed higher concentration in leaves compared to mixture of tea product while Se was undetectable for both samples. In both sample, the order of elements concentration were as follows: K > Ca > Mg > Na > Se in descending order. For antioxidant activity, leaves extract of *O. stamineus* tea product contained higher antioxidant activity for booth DPPH and FRAP assays. Lastly, boiled tea drink contained the highest antioxidant activity followed by cold and room temperature for both DPPH and FRAP assays. This research showed that the antioxidant activity is higher in leaves and under boiled water temperature. This work can serve as the reference for *O. stamineus* tea drink preparation for consumers.

Keywords: *Orthosiphon stamineus*; Trace elements; Antioxidant activity

Removal of Herbicide Paraquat by Cetyltrimethyl Ammonium Bromide Modified Pineapple Leaves

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Abstract: Paraquat pesticide is categorized as contaminants of emerging concern (CEC) that can cause serious environmental problem and toxic effects toward human and animals. The decomposing by burning pineapple leaves could create environmental problems such as air pollution. Therefore, in the present study, the pineapple leaves powder was utilized as a low-cost adsorbent to remove paraquat from aqueous solution. The adsorption of paraquat from aqueous solution by pineapple leaf powder (PLP) and surfactant modified pineapple leaf powder (SMPLP) was examined. SMPLP was prepared by reacting PLP with different concentrations of cationic surfactant, cetyltrimethyl ammonium bromide (CTAB) (0.5, 1.0, 2.5 and 4.0 mM). The PLP and SMPLP were characterized using Fourier transform infrared (FTIR) spectroscopy after the modification process with CTAB and after adsorption process with paraquat. The result shows that there are no significant changes in the chemical structure of pineapple leaves after modification. The SMPLP exhibited higher adsorption affinity toward cationic herbicide. The adsorption experiments of paraquat were carried out in a batch mode at room temperature. The effect of paraquat concentration (2 – 20 mg/mL) on the adsorption capacity of PLP and SMPLP were investigated. The suitability of adsorbent was tested by fitting the adsorption data into Langmuir and Freundlich isotherm equilibrium models. The experimental adsorption data fitted well with Freundlich isotherm with multilayer adsorption capacity of 13.0 mg/g. The highest removal of paraquat was obtained by SMPLP treated with CTAB 2.5 mM while the lowest removal was found for PLP. As a conclusion, the utilization surfactant modified pineapple leaves powder can become an alternative adsorbent for the removal of herbicide compound in aqueous solution.

Keywords: Paraquat; Adsorption; Pineapple leaves; Surfactant

Antioxidant Activity and Trace Elements from Fresh and Commercialized *Moringa oleifera* seeds

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Abstract: *Moringa oleifera* is a humble tree that contributes to high amount of antioxidant activity and mineral compositions from all parts especially leaves and seeds. People consumed *M. oleifera* fresh seeds through variety of cooking and also as commercialized form of supplement to obtain antioxidants and minerals including trace elements. Antioxidant is a substance capable to inhibit oxidation in order to protect human against infection, degenerative diseases and oxidative damage. Previous studies from other countries shown antioxidant properties and mineral compositions of *M. oleifera* that contribute to pharmaceutical and therapeutic applications. None of the study was from Malaysia. Since geographical location plays a role in nutritional value and antioxidant capabilities, this study was carried out to determine and compare the trace elements and antioxidant activity from fresh and commercialized *M. oleifera* seeds from Malaysia. Both types of seeds were extracted by acid digestion method and analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis to determine the mineral composition of Calcium (Ca), Magnesium (Mg), Potassium (K), Sodium (Na) and Selenium (Se). With the exception of Se, the trace elements were detected at varied level from both sources. The trace elements detected in overall seed samples are in the order of K > Ca > Mg > Na > Se. To determine antioxidant activity from both fresh and commercialized *M. oleifera* seeds methanol extraction was done followed by Ferric Reducing Antioxidant Power (FRAP) assay and 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) assay for determining the antioxidant activity. The result of this study indicates that fresh *M. oleifera* seeds contain high amount of antioxidant activity through FRAP (190.63 ± 86.82 Fe^{2+}/g) and DPPH assays (1.64 ± 0.08%) compared to commercialized *M. oleifera* seeds that contain lower amount of antioxidant activity through FRAP (36.85 ± 5.26 Fe^{2+}/g) and DPPH (1.87 ± 0.39%) assays.

Keywords: Fresh and commercialized *M. oleifera* seeds; Trace elements; Antioxidant activity

Development of Lightweight Macrocomposite for Treatment of Polluted River Water

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Abstract: River water is an important source of water for the household, agricultural and industries. The increase in human population, rapid urbanization and industrialization has resulted in river water being heavily polluted. It has become a big challenge to Malaysia to have access for clean and safe drinking water. In this study, a newly developed lightweight macrocomposite which mainly made up from pumice rock, zeolite, activated carbon and cement was used in the treatment of polluted river water in Johor. Pumice rock originates from volcanic eruptions; it is lightweight and porous compared to aggregates and is anticipated to provide high surface for the formation of biofilm. To evaluate the performance of the lightweight macrocomposite, two samples from water treatment plant in Skudai and Batu Pahat were used and the parameters such as COD, pH, colour and NH_3-N were analysed on a period of 10 days. Results showed that the reduction of COD, colour and NH_3-N for water treatment plant in Skudai were 97%, 97%, and 99% (initial COD = 704 mg/L; colour = 190 ADMI; NH_3-N = 1.05 mg/L), respectively. While for the water treatment plant in Batu Pahat were 81%, 99%, and 96% (initial COD = 700 mg/L; colour = 497 ADMI; NH_3-N = 1.19 mg/L) respectively. It can be concluded that the lightweight macrocomposite showed good potential application for treatment of polluted river water

Keywords: Polluted river water; Lightweight macrocomposite; Water treatment plant

Isolation and Characterization of Bacteria Found in Mix-culture of Microalgae Bioreactor

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Abstract: Coal-fired power plants make use of the combustion of coal to generate electricity and are also reported to emit greenhouse gases such as carbon dioxide (CO_2) and methane (CH_4), that contribute to climate change. At present, microalgae are mainly used for the treatment of greenhouse gases. Since the microalgae bioreactor allows the inflow of seawater into the bioreactor, it will be of great interest to study other types of microbes that can survive in this adverse environment such as those found in the bioreactor. Hence this study was undertaken to investigate the presence of other microbes found in the mix-culture of microalgae bioreactor. In this study, marine bacteria were isolated from mix-culture of microalgae bioreactor using two techniques, enrichment culture technique and direct plating technique, in the presence of light at 30 °C. Amongst the twelve bacteria isolated, five were pigmented bacteria, rod-shaped and showed positive results in catalase, oxidase, nitrate reduction, starch hydrolysis and able to survive at a high salt concentration 7.5% (w/v). The specific growth rate and doubling time for ADW, BAY, BDMW, ABW and BAC were 0.242 h^{-1}, 0.257 h^{-1}, 0.297 h^{-1}, 0.331 h^{-1} and 0.357 h^{-1}, followed by doubling time of 2.868 h, 2.7 h, 2.337 h, 2.094 h and 1.943 h respectively. Five pigmented bacteria were characterized using UV-Vis spectrophotometer for photosynthetic pigments. All the characterized bacteria contained carotenoid pigment absorbed at peaks within range 400 to 500 nm, with no peak at 600 to 800 nm for bacteriochlorophyll pigments. Based on 16S rRNA identification, it revealed that bacteria ABW, ADW, BAC, BAY and BDMW could be grouped as *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Bacillaceae bacterium*, *Bacillus cereus sp.* and *Bacillus flexus* respectively.

Keywords: Microalgae; Photosynthetic pigments; Marine bacteria; Greenhouse gases

Isolation and Characterization of Halotolerant Lignocellulolytic Bacteria from Marine Environment

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Abstract: Lignocellulolytic bacteria are capable of degrading lignocellulosic material such as agricultural waste and plant materials. The marine environment with high salt concentration have been found to be the largest source of microorganism producing enzyme that is essential for industries. Enzymes from this type of environment could give more advantages compared to the traditional enzymes because of their ability to survive in an extreme environmental conditions. The aim of this project was to isolate halotolerant bacteria from marine environment at Tanjung Piai, Pontian, Johor. Among the twelve bacteria isolated, only three bacteria that were HB 3, HB 5 and HB 7 showed positive lignocellulolytic degradation of carboxymethylcellulose (CMC), xylan and lignin. All these bacteria were Gram-negative and rod-shaped. Total of selected biochemical was eight and carried out for all three strains. Out of the eight selected biochemical test, bacteria HB 3 showed a positive result towards catalase and MacConkey test. Meanwhile for bacteria HB 7 was positive in catalase, oxidase, simmon citrate, MacConkey and oxidative fermentative test and for bacteria HB 5 it is positive on starch hydrolysis and mannitol salt agar test (MSA). All the selected strains were identified using 16S rRNA gene sequence analysis and bacterial strains HB 3 and HB 5 isolated and characterized in this study was deduced as *Shewanella* sp, while for HB 7 as *Pseudomonas* sp..

Keywords: Lignocellulolytic; Halotolerant bacteria; Marine environment; Biochemical test; 16S rRNA analysis

Isolation and Identification of Rhizobacteria from *Coriandrum sativum* by Verticulture System

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Abstract: The lack of land area for growing crops for agricultural purposes has been a universal issue especially in urban areas in Malaysia. As such, many new techniques have been introduced to enable edible plant farming indoors including verticulture which is an alternative method in growing short-term plants. In this study, *Coriandrum sativum* a globally known culinary herb has been chosen to be grown using two vertical plant towers with 1.2 meter in length each, made out of two polyvinyl chloride (PVC) pipes connected to an automated water supplying system. In order to probe the effect of growth media efficiency on the plant growth, two different types of growth media were used; tower A contains 100% coco peat while tower B was filled up with 50% coco peat and 50% organic compost. Based on the parameters such as number of leaves, length of main stem, diameter of stem, the number of big and the number of small branches proved to have yielded better results. The presence of plant growth promoting rhizobacteria from the addition of organic compost in tower B was observed to have positively affected the growing pattern of *C. sativum*. In order to clarify the effect of these bacteria, growth media samples from both towers and root of *C. sativum* were taken for isolation. Out of seven isolates rhizobacteria, three isolates were chosen for identification as two were revealed to fix the nitrogen on selected medium and one isolate mostly positive for all biochemical tests. Based on BLAST analysis, 16S rRNA sequence of C3 isolate demonstrated 99% similarity to *Pantoea dispersa*, C1B isolate showed 97% similar to *Serratia liquefaction* and R2 isolate 99% to *Klebsiella* species. Results of the biochemical tests for those three strains were compatible to the outcomes of genotypic identification. These bacteria inoculants proved to promote the growth of *C. sativum* plant by fixing the atmospheric nitrogen and alter into more bioavailable nutrient forms.

Keywords: Rhizobacteria; Verticulture; *Coriandrum sativum*; Nitrogen

Bioelectricity Generation from PMFC by *Mentha spicata* Using Nutri-Pot Technique with Different Growth Media

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Abstract: Plant Microbial Fuel Cell (PMFC) is one of the alternative methods to generate electricity. The process involves the utilization of electrochemically active bacteria as biocatalyst. In this study, *Mentha spicata* or usually known as mint plants was used as source of electrons for the electricity generation through PMFC. Three different types of growth media was used to examine the growth of mint plants and the performance of PMFC; i) 100% coco peat, ii) 50% coco peat:50% compost and iii) 70% coco peat:30% compost. The coco peat has good water and air retention capacity thus is a good substitute to soil as growth media. The combination of 50% coco peat and 50% compost gave the fastest growth rate for mint plants among these three types of combination. The PMFC performance was determined through all three sets of growth media using graphite electrode. The maximum Open Circuit Voltage (OCV) produced was 159.6 mV from 50% coco peat:50% compost. When the external loads were applied, the maximum Closed Circuit Voltage (CCV) produced was 64.27 mV at 510 kΩ resistance from 100% coco peat growth medium. The current and power density generated was 0.1331 μA/cm^2, and, 8.5571 μW/cm^2, respectively. This study revealed that *Mentha spicata* has a potential to produce electricity through PMFC system however further study need to be done to improve the generation of current density and power density.

Keywords: Plant Microbial Fuel Cell; *Mentha spicata*; Coco peat; Electrochemically active bacteria

Electricity Generation Using *Oenanthe javanica* in Plant Microbial Fuel Cell with Fertigation Method

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Abstract: Plant Microbial Fuel Cell (PMFC) is a recent approach to generate electricity from the roots of living plants without affecting their growth. This study investigates the influence of the growth of plant species which is *Oenanthe javanica* by using fertigation system on bioelectricity generation in PMFC. Electrons generated from PMFC will then be converted into electricity. The PMFC performance was optimized by elucidating the effects of uncoated electrode and pyrochlore coating material electrode, different external resistance, and electrode size. Electricity generation measurement by this specific species was conducted for 19 days. Based on the Open Circuit Voltage (OCV) measurement conducted, the uncoated electrode gave the highest voltage production which was 122.87 mV. When external loads were applied, the highest voltage reading for uncoated electrode produced was 48.067 mV using 510 000 Ω resistance for Closed Circuit Voltage (CCV), respectively. When pyrochlore was coated on the anode surface, the maximum voltage produced showed 53.83 mV for Open Circuit Voltage (OCV) and 41.6 mV for Closed Circuit Voltage (CCV) using resistance 510 000 Ω. The maximum current density measured was 100 μA and the maximum power density was 550 μW at day 17. For 16S rRNA gene sequence and phylogenetic tree analysis, strain PA of isolated bacteria from pyrochlore coated electrode is much related to *Bacillus megaterium* with 93% identity. Strain GA of isolated bacteria from uncoated electrode is closely related to *Stenotrophomonas maltophilia* with 97% identity. Finally, strain GB of isolated bacteria from uncoated electrode has a close affiliation with *Achromobacter piechaudii* showing 98% identity.

Keywords: Plant Microbial Fuel Cell; *Oenanthe javanica*; Electricity generation; Uncoated electrode; Pyrochlore; Scanning electron microscopy

Antibacterial and *In Vitro* Wound Healing Activities of *Helichrysum italicum* and *Leptospermum scoparium* Essential Oils

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Abstract: The excessive use of ionic silver and silver nanoparticles in current wound care products has created some concern regards to the cytotoxic effects on various mammalian cells. This circumstance has stimulated modern medicine to concentrate on natural products with antibacterial and wound healing properties. Plant essential oils have long been used for various medicinal purposes. *Helichrysum italicum* and *Leptospermum scoparium* essential oils (EOs) contain phytochemical compounds which have been reported to inhibit bacterial growth and promote wound healing. The present study was designed to evaluate the antibacterial and wound healing activities of *Helichrysum italicum* and *Leptospermum scoparium* EOs *in vitro*. The antibacterial activity of *Helichrysum* and *Leptospermum scoparium* EOs was evaluated by disc diffusion assay against selected Gram-positive and Gram-negative bacteria. The *in vitro* wound healing activity of *Helichrysum italicum* and *Leptospermum scoparium* EOs was investigated by cytotoxicity and scratch wound assays on human skin fibroblast (HSF 1184) cells. Cells were treated with different concentrations of EO. The gap closure were monitored at different time intervals and images were then analysed using ImageJ™ software. It was observed that both *Helichrysum italicum* and *Leptospermum scoparium* EOs inhibited the growth of all Gram-positive bacteria but not Gram-negative bacteria, thus reflected the selective antibacterial potencies of these EOs. Results from cytotoxicity assay revealed that both EOs were proved to be non-toxic to the fibroblast cells. In addition, both EOs enhanced the wound closure progression as compared to untreated cells in scratch wound assay when treated with concentrations of EO ranging from 0.125 µl/mL to 0.25 µl/mL. Based on the results, it was suggested that *Helichrysum italicum* and *Leptospermum scoparium* EOs possess antibacterial and wound healing capacities which could be useful for the advancement of antibacterial and wound healing treatment in the future.

Keywords: Essential oils; Wound healing activity; Antibacterial activity

Antibacterial and *In Vitro* Wound Healing Activity of Essential Oils

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Abstract: Essential oils extracted from traditional medicinal plants have various biological activities, including antibacterial activity, fighting infections as well as for wound healing. Despite this, there are limited data available on the cytoxicity of the essential oils to human cells as well as their antibacterial activities. This project was carried out to investigate the antibacterial activity of essential oils as well as their cell viability effects and wound healing activity *in vitro*. Cinnamon oil, basil oil, *Melaleuca* oil and their combinations were tested against four types of common pathogenic wound bacteria; *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* through disc diffusion test. It was observed that most of the essential oils showed potent inhibitory activity against all tested bacteria, except for *P. aeruginosa* that showed resistance towards basil oil. In cell viability assay, all essential oils did not induce any cytotoxicity effects towards fibroblasts, suggesting the tested concentration is safe to be used. For *in vitro* wound scratch assay, basil and *Melaleuca* oils promoted wound healing activity. Mixed essential oils did not demonstrated improved effects in wound healing activity *in vitro*. Results from this study indicate that there were different antibacterial activities and wound healing capacity exhibited in each tested essential oil. This provided promising data for future references especially in formulation of topical products designed for chronic wound.

Keywords: Essential oils; Antibacterial activity; MTT Cytotoxicity Assay; Wound Scratch Assay; Human dermal fibroblasts

Screening of Cellonsphere Microcarrier in a Microwell Attachment Plates for Mammalian Cell

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Abstract: Chinese Hamster Ovary (CHO) cells culture system has been used generally for production process of therapeutic protein in industrial scale. However, the downside of monolayer cell culture has been proven to use a lot of raw materials, expose to high risk of contamination and particularly time consuming. Hence, this study was carried out to determine the effectiveness of microwell based systems and the use of Cellonsphere microcarrier to increase the number of CHO cells culture. To understand the effect of microcarrier on CHO cell, the experiment was performed by comparing two different batch of CHO cell with microcarrier and without microcarrier. All the variables during experiment were controlled with temperature at 37 °C, 5% of carbon dioxide and 2 g/L glucose content in the RPMI medium. The data of chemical analysis and morphology was collected for seven consecutive day and the results from the studies between the CHO cell culture without microcarrier and CHO cell culture with microcarrier shows a comparable of maximum viable cell concentration of 2.91 x 10^6 cell/mL and 7.8 x 10^6 cell/mL respectively. Whereas, the doubling time between normal CHO cell culture and CHO cell culture with microcarrier are T_d = 1.6321d and T_d = 1.273d respectively. On the other hand, the glucose consumption for CHO cell culture with microcarrier are slightly higher compared to normal CHO cell culture when the glucose concentration detected at day 7 by 0.097 mg/mL and 0.193 mg/mL respectively. Overall, the results obtained from this project demonstrated the impact of microcarrier on CHO cells viability as the surface area of microcarrier for cell growth increase along with metabolism rate. As a conclusion, the use of Cellonsphere microcarrier has the potential for the production of CHO cell and can be useful for large production of therapeutic protein.

Keywords: Microcarrier; Cellonsphere; CHO cell; Microwell

Screening of Cytodex 3® Microcarrier in a Microwell Attachment Plates for Mammalian Cell

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Abstract: Microcarrier technology provides advantage to the culture of anchorage-dependent cells especially in the biopharmaceutical industry. Microcarrier technology not only provides a larger surface area than monolayer cell culture, but also it has the potential ease of scalability in large scale processes. However, the initial screening of a suitable microcarrier in small scale process is quite laborious with the use of flasks and bioreactors. In contrast, static cell culture experiments in microwell plates offer a great feasibility for screening of the suitable microcarrier. Hence, this study focused on the investigation of cell attachment and cell growth of Chinese Hamster Ovary (CHO) cells cultivated with Cytodex 3 microcarriers in 24-well plates. Experiments were performed in static condition for seven days for both monolayer and microcarrier cell culture. The viable cell number for Cytodex 3 culture is significantly increased than the monolayer culture. The maximum viable cell number was 6 x 10^6 and 2 x 10^6 for Cytodex 3 microcarrier and monolayer culture, respectively. In the Cytodex 3 microcarriers culture, the CHO cells thoroughly proliferate at the surface of Cytodex 3 microcarriers and exhibiting a spherical morphology until day 6; further growth was limited by the depleted glucose concentration. The glucose consumption rate by cells slowed down when glucose was limiting. The specific growth rate, μ was relatively higher in Cytodex 3 microcarriers culture which is 0.675 d^{-1}, than the monolayer cell culture, 0.380 d^{-1}, indicating that the cells survived and attached to a greater extend in the microcarrier culture. Thus, the results provide a valuable data as an initial screening of Cytodex 3 microcarriers that can be used further to develop and scale up the production of anchorage-dependent cells.

Keywords: Microcarrier; Chinese Hamster Ovary (CHO); Cytodex; Microwell plate

Screening of Cellonsphere™3 Microcarrier in Microwell Attachment Plates for Mammalian Cell

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Abstract: Mammalian cell culture especially Chinese Hamster Ovary (CHO) has been used widely for production of therapeutic protein. However, the conventional technique is time consuming, labour intensive and not cost effective. Therefore, microcarrier culture technique has been applied to improve the attachment and growth culture for an anchorage dependent cell. This study was carried out to determine the ability of the microcarrier for the attachment of adherent CHO cell. Cellonspehere which used as the microcarrier was treated with Ultraviolet/ozone coated with gelatin to enhance the attachment of the cell onto it. The Cellonsphere microcarrier was compared with the 2D monolayer cell culture. Cell concentration of 1×10^5 was used for both monolayer and Cellonsphere culture. The cells were cultured for 7 consecutive days in microwell plate and growth rate of cells were monitored. There was significant difference on the specific growth rate between monolayer and Cellonsphere which are 0.3884/day and 0.3593/day respectively. The doubling time was inversely proportional to the specific growth rate of the cell, hence the doubling time are 1.7846 day for monolayer, 1.9292 day for Cellonsphere. The number of viable cell count was higher in the Cellonsphere because of its 3D dimension that gives more surface area for the cell to attach and survive. Furthermore, the cells can live up to seven days on the surface of Cellonsphere, while for monolayer culture, the cells started to die from day five. As a conclusion, Cellonsphere microcarrier could be used as preliminary studies for cell attachment and have potential for higher number of cell viability and productivity.

Keywords: Microcarrier; Microwell plate; Cellonsphere; Chinese Hamster Ovary (CHO)

Effect of Combined Cisplatin and *Clinacanthus nutans* on Gene Expression of MDA-MB-231 Breast Cancer Cells

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Abstract: Triple Negative Breast Cancer (TNBC) is the most invasive breast cancer enriched with cancer stem cells (CSCs). Absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) make TNBCs difficult to be targeted by existing chemotherapy treatment. This study was aimed at identifying the effects of the combined therapy on the gene expression of Triple-Negative Claudin-low MBA-MD-231 breast cancer cell line. Both cisplatin and *C. nutans* especially as combined treatment exhibited potent anticancer effects on MDA-MB-231 breast cancer cells. In this research, cisplatin concentration at (0.76, 1.52, 3.05, 4.57, 6.10, and 15.23 µg/mL), *C. nutans* concentration at (2.5, 5, 10, 20, 30, and 50 µg/mL) and combination of cisplatin (3.05 µg/mL) with *C. nutans* concentration at (0, 2.5, 5, 10, 20, 30, and 50 µg/mL) were tested on MDA-MB-231 cell viability. From the findings, both cisplatin and *C. nutans* reduced cell viability in a dose-dependent manner by 7.05 – 57.47% (cisplatin), 7.02 – 50.29% (*C. nutans*) and 8.36 – 75.79% (combined cisplatin-*C. nutans*). The study also revealed that mono cisplatin and *C. nutans* induced differentiation while the combined treatment induced apoptosis in MDA-MB-231 cells. Furthermore, cisplatin and *C. nutans* differentially regulated specific genes in which differentiation markers (mono cisplatin and *C. nutans*) and KLF4 (all treatments) were up-regulated while CD49f (mono cisplatin and combination) and KRT18 (combination) were down-regulated. As a whole, these findings altogether suggest that both cisplatin and *C. nutans* are potent anticancer targets especially in combination for the targeted therapy of MDA-MB-231 cells and possibly for other cancers enriched with CSCs. Lastly, the up-regulation of KLF4 correlating to increased differentiation of CSCs can be highlighted as an important cancer prognostic marker for the treatment and management of TNBC.

Keywords: Cisplatin; *Clinacanthus nutans*; Gene expression; Breast cancer

Effects of Combined Retinoic Acid-*Clinacanthus nutans* Treatment in HeLa Cancer Cells

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Abstract: Cervical cancer enriched cancer stem cells (CSCs) constituted as one of the highest cancer death recorded among women in the world. Moreover, the chemo-resistance characteristic of CSCs in cervical cancer increases the tendency of patients to have recurrence cancer after chemotherapeutic treatment which resulted in poor survival rate. Recently, most of studies have focused on targeting CSCs in order to make them susceptible to chemotherapeutic treatment. Therefore, combine treatment with plant-derived anticancer drugs is one of the most promising approach. This study was aimed at identifying the anticancer effects of the combined therapy on HeLa cervical cancer cell line. In order to achieve the objectives, the effects of mono-retinoic acid (RA), mono-*C. nutans* and combined RA and *C. nutans* treatments on HeLa cells were examined via cell viability and apoptosis assays. In this study, various concentration of RA, *C. nutans* and combination of RA (3.0 µg/mL) with *C. nutans* were tested on HeLa cells for cell viability analysis. From the findings, both RA and *C. nutans* especially as combined treatment exhibited significant anticancer effects on HeLa cells in a dose dependent manner. An IC_{50} of 6.0 µg/mL, 17.68 µg/mL and 9.0 µg/mL were observed respectively in RA, *C. nutans* and the combination treatment. Furthermore, a study using apoptosis assay also revealed that mono-RA exhibited negligible apoptosis activity, while combined (RA-*C. nutans*) treatment induced better apoptosis activity in HeLa cells compared to mono-*C. nutans*. Overall, this study suggests that combination treatment (RA-*C. nutans*) gave better anticancer effects in the treatment of cervical cancer compare to their own monotherapeutic treatment.

Keywords: Retinoic acid; *Clinacanthus nutans*; Cervical cancer; HeLa cell line; Anti-cancer agent; Combination drug therapy

Effects of Combined Retinoic Acid-Curcumin Treatment in HeLa Cancer Cells

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Abstract: Cervical cancer is a disease in which malignant cells form in the tissues of the cervix. Additionally, cervical cancer has an abundance of cancer stem cells (CSCs). Conventional therapy usually destroy the bulk of cancer cells, but often not able to exterminate the critical CSCs. Recently, most of the studies are focused on targeting CSCs in order of converting them into a more differentiated phenotype by co-administering drugs that work by different molecular mechanisms such as differentiation and apoptosis. This study was aimed at studying the anticancer effect of combined Retinoic acid-Curcumin in cervical cancer cells and the ability of combined Retinoic acid-Curcumin to induce apoptosis in cervical cancer cells. In order to achieve the objectives, the effect of Retinoic acid, Curcumin and combined Retinoic acid and Curcumin on HeLa cells were examined via cell viability as well as apoptosis assays. Both Retinoic acid and Curcumin especially as combined treatment displayed powerful anticancer effects on HeLa cancer cells. In this research, Retinoic acid concentration at (0, 5, 10, 15, 20, 30 and 50 µM), Curcumin concentration at (0, 5, 7.5, 10, 12.5 and 15 µM) and combination of Retinoic acid (10 µM) with Curcumin concentration at (0, 5, 7.5, 10, 12.5 and 15 µM) were tested on HeLa cell viability. From the findings, both Retinoic acid and Curcumin reduced cell viability in a dose dependent manner by 21.89 – 89.73% (Retinoic acid), 6.63 – 61.07% (Curcumin) and 9.76 – 71.69% (combined Retinoic acid-Curcumin). Based on the study in terms of apoptosis assay, it was revealed that Curcumin and combined treatment induced apoptosis in HeLa cancer cells. In conclusion, these findings altogether proposed that both Retinoic acid and Curcumin exhibit strong anticancer activities especially in combination for the targeted therapy of cervical cancer cells as represented by the HeLa cancer cells and probably for other cancers abundant with CSCs.

Keywords: Cancer stem cells; Cervical cancer; HeLa cells; Combination drug; Retinoic acid; Curcumin

DNA Barcoding Using Chloroplastic and Nuclear Marker in *Ficus deltoidea*

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Abstract: *Ficus deltoidea*, also known locally as MasCotek, is a potential medicinal plant contributed by its traditional belief and scientific research on its pharmacological properties. This give rise to the herbal medical products (HMPs) based on this plant. Lack of law regulation for plant source validation by authorities has lead adulteration, incorrect plant substitution and misidentification in HMPs by unethical suppliers. DNA barcoding offers a simple, effective and reliable method for species discrimination in HMP. In this study, genomic DNA (gDNA) was successfully extracted from *Ficus deltoidea* and HMP. Both were subjected to DNA barcodes (*ITS2* and *rbcL*) amplification and sequencing. The DNA barcodes are then analyzed and verified by using bioinformatic tools. Verified *Ficus deltoidea*'s DNA barcodes served as local reference barcodes for HMP verification. The finding suggested that CTAB based lysis buffer from Nucleospin® Plant II Kit effective in gDNA extraction for both plant and HMP. Next, both primers showed good amplification of *ITS2* (~300bp) and *rbcL* (~1000bp) for *Ficus deltoidea* leaves. However, only *ITS2* successfully amplified in HMP due to degraded HMP's DNA. Alternatively, mini-barcode primers were used in *rbcL* amplification (~500bp). From the analysis, the barcodes imply that HMP tested is authentic with 100% BLAST result to the same *Ficus* genus in Genbank and 100% identity to the DNA barcodes generated in this work (novel). In addition, neighbor-joining phylogenetic tree analysis indicates that the HMP falls into the same clade with *Ficus deltoidea* for both *ITS2* and *rbcL*. Thus, we could conclude that DNA barcoding could be applied for species detection in *Ficus deltoidea* HMPs.

Keywords: DNA Barcoding; *Ficus deltoidea*; HMP; *ITS2*; *rbcL*

Development of Reference DNA Barcodes for *Labisia pumila* Using Chloroplastic and Nuclear Marker

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Abstract: *Labisia pumila*/Kacip fatimah is one of the most popular local herbal plant species available in the form of teas, capsules and tablets and used for women's health. However, the herbal medicinal products (HMP) might be adulterated with other species due to its similar morphologies. DNA barcoding techniques which use short DNA sequences from chloroplastic (*rbcL*) and nuclear (*ITS2*) marker has been used in various HMP species identification. However, to date, there is no references barcode available for *Labisia pumila*. Thus, in this work, *Labisia pumila* chloroplastic (*rbcL*) and nuclear (*ITS2*) DNA barcodes were generated. The leaves genomic DNA was extracted using NucleoSpin® 8 Plant II Kit with the best result yield from the CTAB method and later used for PCR template. Universal primers showed good amplification of *rbcL* (~1000bp) and *ITS2* (~300bp) at annealing temperature; 57 °C. Sequence analysis exhibit 96% *rbcL* and 98% *ITS2* BLAST identity to *Ardisia thyrsiflora* and *Ardisia* sp. respectively, but none to *Labisia* species. *Ardisia* belongs to the same family of *Labisia* species, Primulaceae but from different genus and species. The NJ phylogenetic tree results showed that the *Labisia pumila* falls under the same clade of other Primulaceae family, which suggest that these novel barcodes could serve as reference for authentication of *Labisia*-based HMPs.

Keywords: Herbal plant; *Labisia pumila* var *pumila*; DNA barcoding; *rbcL*; *ITS2*; Universal primer

DNA Barcoding Using *ITS2* for Identification of *Labisia pumila* in Selected Malaysia Herbal Medicinal Product

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Abstract: In Malaysia, the use of *Labisia pumila*/Kacip Fatimah herbal medicine product (HMP) in the form of capsules, tablet or tea is increasing in trend due to their claimed health benefits especially for women. However, the role of local authorities in authentication of this products are not clear thus causing high adulteration, mislabelling and substitution in their ingredients, thus might cause harmful effects to consumers. DNA barcoding approach is able to overcome this problem and authenticate the presence/absence of the correct species in the HMP. In this work, a nuclear DNA barcode, *ITS2* is applied in both *Labisia pumila* plant and HMP. DNA extraction was done by using NucleoSpin® Plant II Kit followed by PCR amplification by using universal primers. Results showed that *ITS2* gene (310bp) best amplified at annealing temperature of 57 ºC for both samples. To verify the DNA barcode sequence, bioinformatics tools such as BIOEDIT, BLAST, JALVIEW, and MEGA software were used for sequencing analysis and multiple sequence alignment. The sequence analysis of *Labisia pumila* plant showed 91% BLAST identity to *Embelia ribes* which belongs to the same family of Primulaceae, but from different genus and species of *Labisia pumila*. No BLAST result was obtained from any *Labisia* species due to lack of reference barcodes in Genbank. The NJ phylogenetic tree results also showed that the *Labisia pumila ITS2* falls under the same clade of other Primulaceae family. Thus, this verified barcode is novel and will serve as reference for HMP authentication. On the other hand, HMP sequence analysis obtained showed 89% BLAST identity to *Cuminum cyminum* which is from a different genus and species from *Labisia pumila*. The NJ analysis also implies that the HMP falls into a different genus which suggest that the HMP analyzed is not authentic and has potentially been adulterated with a different species. This work concludes that the *Labisia pumila ITS2* gene, a novel barcode generated from this work could serve as a good DNA reference barcode for *Labisia pumila* HMP species authentication. However, further analysis with more HMP is needed to test the reproducibility and consistency of this barcode.

Keywords: DNA barcoding; *Labisia pumila*; Herbal medicinal product (HMP); Universal primer; *ITS2*

cDNA Synthesis of Flavanones Biosynthetic Genes of *Citrus* Plant of Local Variety

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Abstract: Flavonoids are plant specific secondary metabolites which are known to have significant properties such as an anti-cancer, anti-inflammatory and anti-oxidant. These potential roles of flavonoids have received attention from researchers to identify the genetic information of the enzymes involved in the flavonoids biosynthetic pathway. The information is important for further application especially to produce flavonoids in large-scale using metabolic engineering approaches. Therefore, this study aims to synthesize cDNA (complementary deoxyribonucleic acid) of 4-Coumarate:CoA ligase (4CL) and Chalcone isomerase (CHI), enzymes that involved in the synthesis of flavanones, from young leaf of *Citrus sinensis* plant. Synthesis of *4CL* cDNA from total RNA extract resulted in the application of approximately 2116 base pair (bp) nucleotide sequence. In addition, approximately 889 base pair (bp) of *CHI* cDNA has been amplified using the similar method. Nucleotide sequence alignment using BLAST showed that the synthesized *CHI* shared 98% identity with *CHI* of both *Citrus sinensis* and *Citrus clementina*. Whereas, the synthesized *4CL* shared 95% identity with *4CL* of both *Citrus sinensis* and *Citrus clementina*. Findings from this study provide insights into the genetic information of enzymes involved in the flavanones biosynthetic pathway in *Citrus* plant, which can be further use for potential genetic manipulation in the future.

Keywords: *Citrus* plant; Flavanones; cDNA synthesis; Chalcone isomerase (CHI); 4-Coumarate:CoA ligase (4CL)

Development of Loop-mediated Isothermal Amplification (LAMP) Technique for Detection of Porcine DNA for Food Quality

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Abstract: Loop-mediated isothermal amplification (LAMP) is a single tube technique used to amplify a DNA fragment. The method required six different primers to amplify the target gene and using *Bacillus stearothermophilus* (*Bst*) DNA polymerase for strand displacement activity. In this study, LAMP technique was used to amplify the porcine DNA of mitochondria D-loop region from *Sus scrofa* due to an issue of adulteration and contamination of food product. Porcine DNA was extracted from raw pork heart muscle. A set of six primers for LAMP technique which are two inner primers (FIP, BIP), two outer primers (F3, B3) and two loop primers (LF, LB) was designed using Primer Explorer V5 to detect the porcine DNA. Effectiveness of LAMP reaction was optimized based on reaction time, annealing temperature, and the concentration of magnesium sulfide ($MgSO_4$). The porcine DNA was amplified in the presence of 1X ThermoPol Reaction Buffer (NEB, USA), 6 mM $MgSO_4$, 1.0 mM of deoxynucleoside triphosphates, 1.6 µM of each the primer (FIP and BIP), 0.2 µM of each of the outer primers (F3 and B3), and 0.8 µM (1 µl) of loop primer, 8 unit of *Bst* DNA polymerase, and 100 ng of genomic DNA as a template. Based on the result, a better LAMP reaction could be achieved at 55 °C for 50 minutes and the concentration for $MgSO_4$ is 6 mM. The result for optimization of reaction time, temperature and concentration of $MgSO_4$ and dNTPs was analyzed with 2% (w/v) agarose gel electrophoresis. To increase the specificity of primers all the LAMP reagent need to be control and optimize to avoid the non-binding of primer to DNA template. In addition, the requirement for primer designs also important to increase the specificity of LAMP reaction. As a conclusion, LAMP reaction can be used to amplify the DNA sequence to detect the porcine DNA.

Keywords: LAMP technique; Mitocondria D-loop; Porcine; *Sus scrofa*; *Bacillus stearothermophilus* (*Bst*)

Development of Loop-mediated Isothermal Amplification (LAMP) Technique for Food Safety: Detection of Porcine Gene

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Abstract: The used of genetic analysis method such as Loop-mediated Isothermal Amplification (LAMP) is known to be more specific, rapid, selective and cost effective compared to the conventional PCR method which required an expensive equipment like thermocycler and extensive reaction period. In this study, the LAMP technique was used for detection of porcine gene which is significant for identification of food adulteration in halal food industry. Porcine DNA was extracted from pork heart using DNA purification kit with concentration of DNA was 64 ng/µL and purity DNA was 1.88. While LAMP primers were designed using specialized software known as primer explorer V5 where the published target sequence of porcine gene with GeneBank accession number AY534296.1 was acquired from NBCI database. The designated primers were used to performed LAMP reaction assay in the mixture of 0.2 µM each of F3 and B3, 1.6 µM of each FIP and BIP and 0.4 µM each of LF and LB. The LAMP reaction was developed and being optimized using different concentration of $MgSO_4$ (2 mM, 4 mM, 6 mM, 8 mM and 10 mM), different concentration of DNTP mix (0.6 mM, 0.8 mM, 1.0 mM, 1.2 mM and 1.4 mM), different incubation temperature (35 °C, 45 °C, 55 °C, and 65 °C) and different incubation period (10 min, 20 min, 30 min, 40 min, 50 min, 60 min and 70min). The LAMP products only showed a one distinct band at approximately at 100bp and further analysis are needed to identify the identity of product amplicon. This method should be suitable to be used in the amplification of nucleic acid for molecular diagnosis without using complex instrumentation and limited personal experienced.

Keywords: Loop-mediated Isothermal Amplification; LAMP; Porcine; Food safety

Sub-cloning of *Cyclin-Dependent Kinase 3* (*CDK3*) Gene into a Mammalian Expression Vector4

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Abstract: Cancer, the world's second leading cause of death, has encountered a 33% increase in incidence worldwide from 2005 to 2015 (Global Burden of Disease Cancer, 2017). In Malaysia, there is an evident escalation in prevalence of cancers such as breast and colorectal cancer (Islam *et al.*, 2015; Veettil *et al.*, 2017). Cancer is generally caused by uncontrolled proliferation due to dysregulation of the cell cycle. *CDK3* (*cyclin-dependent kinase 3*) is one of the regulatory factors that, when overexpressed, could lead to the progression of several cancers, such as breast, colorectal, nasopharyngeal, and skin cancers (Cui *et al.*, 2015; Lu *et al.*, 2016; Wang *et al.*, 2014; Xiao *et al.*, 2017). Since very little is known about it today, *CDK3* was the protein of interest in this study whereby it was sub-cloned into the mammalian expression vector pHLmMBP-10. Primers were designed to amplify *CDK3* from the previously cloned pGEM-*CDK3*. Once amplified, the PCR product *CDK3* and pHLmMBP-10 vector were double digested with the same two enzymes to form sticky ends that could be joined by ligase. After ligation, pHLmMBP-10-*CDK3* plasmids were transformed into competent DH5α cells and screened with colony PCR and restriction enzyme digestion for the successfully transformed clones prior to sequencing. The sequencing results revealed that a DH5α colony had been successfully cloned with pHLmMBP-10-*CDK3* without mutations.

Keywords: Sub-cloning; *Cyclin-dependent kinase 3* (*CDK3*); Cancer; Targeted cell therapy

Sub-cloning of *Brain Expressed X-linked 3* into Mammalian Expression Vector

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Abstract: *Brain Expressed X-linked 3* (*BEX3*) is located on chromosome Xq22 with 3 exons encoding NADE mediate apoptosis in neural cells when responded to Nerve Growth Factor (NGF). NADE from cancer cells are involved in transcriptional regulation and signaling pathways. Overexpression of *BEX3* can suppress tumor formation in breast cancer cell but it enriches the stemness features of nasopharyngeal cancer cell (NPC) to resist cisplatin treatment. *BEX3* was sub-cloned into pHLmMBP-10, a mammalian expression vector by molecular cloning technique to enable for future study of its structure and function. Forward and reverse primers for amplification were designed with two extra restriction enzyme sites, *NotI* and *NheI*. *BEX3* was amplified from pGEM-BEX3 plasmid and inserted into pHLmMBP-10 vector via double digestion and ligation. The optimum PCR condition for amplification of *BEX3* was 60 °C. The recombinant DNA was then introduced into *E. coli* DH5α host cell for replication. There were 4 positive clones validated by DNA sequencing result. This study showed that *BEX3* was successfully amplified and sub-cloned into the mammalian expression vector, pHLmMBP-10.

Keywords: Sub-cloning; *BEX3*; Cancer; Targeted cell therapy

Antibacterial Activity of *Citrus microcarpa* (Limau Kasturi) Leaves Extract

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Abstract: The antibiotic resistance bacteria have become a major concern worldwide as they can lead to infectious diseases in human. In this context, many researchers have focused on natural products for potential antibacterial agents. The aim of this study was to evaluate the antioxidant and antibacterial activities of *Citrus microcarpa* extracts against Gram-positive bacteria which is *Bacillus subtilis*, *Staphlycoccus aureus*, *Staphlycoccus epidermidis*, and Methicillin-resistant *S. aureus* (MRSA) and Gram-negative bacteria *Escherichia coli* and *Pseudomonas putida*. The plant was extracted with different polarities of solvents (hexane, acetone, ethanol and aqueous) by maceration technique. The total phenolic content (TPC) was evaluated and the antioxidant assay by DPPH free radical scavenging assay. For the antibacterial screening, disc diffusion method was used followed by minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. The results showed that the aqueous extract exhibited the highest percentage of yield (15.8%), acetone extract showed the highest TPC, 12.57 ± 0 mg GAE/100 g as well as highest DPPH activity with IC_{50} value, 0.052 ± 0.006 mg/mL. The TPC and antioxidant activity of all plant extracts exhibited a significant negative correlation at $p < 0.01$ with $r = -0.866$. Disc diffusion method indicated that all of extracts showed inhibitory activity against *E. coli*, meanwhile, for MRSA, only acetone extract showed highest zones of inhibition which are 14.3 ± 2.9 and 18.5 ± 1.0 mm respectively at 1.0 and 2.5 mg/mL in concentration. Furthermore, the lowest MIC value, 0.97 ± 0.43 mg/mL was exhibited by acetone extract against *E. coli*. Therefore, the results obtained in this study suggested that acetone extract of *C. microcarpa* leaves can be a good candidate and a source of antibacterial agent against resistance bacteria.

Keywords: *Citrus microcarpa*; Antibacterial; Antioxidant

Antibacterial Activity of *Murraya koenigii* Leaves Extract

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Abstract: Infectious diseases from pathogenic bacteria nowadays are too common and some of the bacteria become resistance towards multi types of antibiotics. Therefore, this study was attempted to investigate the antioxidant and antibacterial activities of *Murraya koenigii* "curry leaves" against several pathogenic bacteria. Hexane, acetone, ethanol and aqueous were used to isolate its leaves extract by using maceration technique. All extracts were analysed for their antioxidant activity (DPPH radical scavenging assay) and total phenolic content (TPC). The extracts were then tested for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* and *Pseudomonas putida* using disc diffusion assay, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) test. From the result, the aqueous extract of *M. koenigii* exhibited the highest percentage of yield at 18.38%, followed by ethanol, acetone and hexane at 12.72%, 11.24% and 5.70% respectively. Ethanol extract give the highest percentage for DPPH inhibition, (79.92% ± 3.68) while aqueous extract have the highest total phenolic content (1421 ± 0.002 mg of GAE/100g of extract). When TPC and DPPH was studied for their correlation through Pearson's correlation test, (r = 0.97) indicate that TPC contribute to the antioxidant activity in *M. koenigii* plant. In antibacterial test, all plants extracts showed to be effective against *E. coli* without any observed for *S. aureus* and *P. putida*. It is worthy to note that ethanol extract is the most effective for antibacterial activity against *B. subtilis*, *S. epidermidis* as well as MRSA. The highest zone of inhibition was at 2.5 mg/mL concentration against *B. subtilis*, indicates by 27.8 ± 0.2 mm at MIC value, 0.21 ± 0.38 mg/mL. In conclusion, the results of this study illustrate that ethanol extract of *M. koenigii* has potential as antibacterial agent especially against pathogenic bacteria.

Keywords: *Murraya koenigii*; Antibacterial activity; DPPH radical scavenging assay; Total phenolic content (TPC)

Antibacterial Activity of *Citrus hystrix* Leaves Extract

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Abstract: According to World Health Organization (WHO), the antibiotic resistance bacteria have currently become a global concern. Many approaches have been made to produce the alternative antibacterial agents, including from medicinal plant. Hence, the purpose of this study was to evaluate the antioxidant and antibacterial activities of hexane, acetone, ethanol and aqueous extract of *Citrus hystrix* leaves extracted using maceration technique. The determination of total phenolic content (TPC) of the extract was using Folin-Ciocalteu method while for antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Meanwhile, for the antibacterial activity, disc diffusion test followed by minimum inhibitory concentration (MIC) test and minimum bactericidal concentration (MBC) test were conducted against Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, Methicillin-resistant *Staphylococcus aureus* (MRSA)) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas putida*). The aqueous extract of *C. hystrix* exhibited the highest percentages of extract yield of 21.43% followed by acetone (17.56%), ethanol (17.56%) and hexane (7.90%). In antioxidant test, the lowest IC50 values based on the DPPH assay were shown by ethanol (0.33 ± 0.001 mg/mL) with highest TPC value of 505.07 ± 0.00 mg of GAE/100g extracts compared to the other extracts. However, not all extract that contain high TPC value also exhibit high antioxidant activity. Thus, there was weak correlation between TPC and antioxidant activity at R^2 = 0.0244. In antibacterial testing, the biggest zone of inhibition was shown by acetone extract against *E. coli* and MRSA with 18.2 ± 0.05 mm and 17.0 ± 0.01 mm. The MIC value ranged the lowest by acetone extract with 0.80 ± 0.78 mg/mL for *E. coli* and 1.90 ± 1.1 mg/mL for MRSA which indicated that less concentration of extract required to inhibit bacterial growth. Therefore, *Citrus hystrix* extract were observed to have antibacterial and antioxidant activity that can be used for medicinal purposes.

Keywords: Antibacterial; Antioxidant; Bacteriostatic; Bactericidal; *Staphylococcus epidermidis*; Methicillin-resistant *Staphylococcus aureus* (MRSA)

Effect of Plant Growth Regulator on *In Vitro* Regeneration of Banana cv. Berangan

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Abstract: Banana is among the important fruits cultivated in Malaysia. Problem of pests and diseases, unorganized production and inadequate provision of suitable land contribute to low productivity of banana. Plant tissue culture appeared as an alternative biotechnology tools that contributed to the growth and development of banana. In this study, effect of explant type (sucker, male buds and shoot-tips) and different combination of BAP (4 – 12 mg/L) and IAA (0.1 – 0.3 mg/L) concentration on shoot induction of banana were investigated. Optimization of surface sterilization for sucker and male buds were optimised using different bleach concentration. All explants were cultured on MS-based medium supplemented with 30 mg/L sucrose, 10 mg/L ascorbic acid, 2.6 mg/L phytagel and PGR tested. For sucker surface sterilization, there was no explants survived at all bleach concentration tested. Meanwhile, all apical dome explants from male bud survived (25 %) with highly blackening and browning around the explants but no male inflorescence explants survived. In shoot-tips culture, medium containing 5 mg/L BAP concentration performed best shoot regeneration with 0.67 shoot/explant after 90 DAI. However, incorporation of 5 mg/L BAP with 0.2 mg/L IAA help to enhance and doubling number of shoot with 1.0 shoots/explant. In conclusion, *in vitro* propagation of banana cv. Berangan were successfully induced and established.

Keywords: Banana; BAP; IAA; Shoot regeneration

Effect of Growth Regulators on Adventitious Root Induction of *Justicia gendarussa*

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Abstract: Induction of adventitious root is influenced by the several growth parameters such as plant growth regulator and type of medium. *Justicia gendarussa* consists several bioactivities including anti-oxidant and anti-inflammatory properties. Phenolic compounds that exist in adventitious root could contribute to antioxidant property. Factors such as auxin, medium and explant type are very important for induction of adventitious root of *Justicia gendarussa*. Moreover, the efficient surface-sterilization method also crucial to provide contamination-free explant in order to initiate adventitious root. Therefore, the study aimed to determine the suitable chlorox concentrations for leaf surface-sterilization and to investigate the effect of plant growth regulator on percentage of adventitious root induction. In this study, leaf explants were cultured on Murashige and Skoog (MS) media plate containing various concentration of indole acetic acid (IAA) (0.5 to 2.5 mg/L) and combination of naphthaleneacetic (NAA) (1 to 3 mg/L) and indole acetic acid (IAA) (0.5 to 1.5 mg/L) concentration for 7 weeks. Results demonstrated that the highest percentage of root induction was achieved at combination of 3 mg/L IAA + 1.0 mg/L NAA (0.44 g ± 0.2). However, there was no adventitious root induced when leaf explants were treated with IAA alone. In conclusion, combination of IAA and NAA promoted adventitious root induction of *Justicia gendarussa*.

Keywords: Adventitious roots; *Justicia gendarussa*; Plant growth regulators; Tissue culture

Degradation of Palm Oil Mill Effluent (POME) at Different pH Using Single Strain *Bacillus* sp. and POME Treatment via Microbial Fuel Cell (MFC)

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Abstract: Palm Oil Mill Effluent (POME) is the effluent produced by the palm oil industry. Malaysia which is one of the largest exporters of palm oil in the world contributes to massive amount of effluents being produced each year, which is hazardous to the ecosystem. In this study, *Bacillus* sp. was used to determine the degradation of pre-treated POME at within a pH range between 6 and 9. The initial pH of pre-treated POME was 4.6 was used as a control pH (without *Bacillus* sp.). Dry Cell Weight (DCW) of POME + *Bacillus* sp. at several pH (4.6, 6, 7, 8, 9) were collected from day 0 to day 7. Other parameters studied include Chemical Oxygen Demand (COD), Total Phenolic Content (TPC) and colour density. The supernatant collected in DCW tests were used in these analyses. Potassium Dichromate ($K_2Cr_2O_7$) colorimetric method was used for COD determination and was tested on all pH from day 0 to day 4. By day 4, pH 9 shows the lowest COD activity compared to that of other pH, while pH 4.6 (control) showed the highest COD activity. Based on the results in COD test, samples at pH 9 were used in TPC analysis. TPC test was conducted using Folin-Ciocalteu reagent method, using Gallic acid as the standard at several concentration (0, 50, 100, 150, 200, 250) mg/mL to obtain Gallic acid standard curve where the straight line equation is, $y = 0.0013x + 0.0459$ with coefficient of determination, R^2 of 0.9928. Results of total phenolic compound content from samples at pH 9 taken from day 0 until day 7 showed that at day 7 the phenolic degradation was at the lowest level. Colour density test performed on samples at different values revealed that the initial dark-brown colour turned into lighter brown shade at day. *Bacillus* sp. was tested to probe ability to harness electricity via Microbial Fuel Cell (MFC) approach. Graphite felt were used as electrodes in the Dual-Chamber MFC. The maximum current density of 2.998104 mA/m^2 and power density of 1.279981 mV/m^2 respectively were produced.

Keywords: Palm Oil Mill Effluent (POME); *Bacillus sp.*; pH; Microbial Fuel Cell (MFC); Graphite felt

Isolation and Partial Characterization of Amylolytic Bacteria during Food Waste Fermentation Using Solid State Fermentation (SSF)

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Abstract: Food waste is one of the elements that humans encounter with every day without knowing that it could bring good to the industry especially food industry. The composition of each food category may vary depending on what food is responsible in starch degradation through enzymatic process of amylolytic enzyme. The main objective of this study is to isolate the amylolytic bacteria during Solid State Fermentation (SSF) of food waste collected from home kitchens. The initial moisture of the sample was 65%. In this study, amylolytic bacteria which it was expected to produce amylolytic enzymes were extracted from the food waste. The amylolytic enzymes which play important role in starch degradation during SSF are amylase and glucoamylase. This experiment took place in 24 days. Amylase and glucoamylase show the highest activity at Day 22 and Day 12 with the enzymes activity of 1.403 U/mL and 2.2341 U/mL, respectively. The highest concentration of starch was seen on Day 20. Isolation and screening of potential amylolytic bacteria from the food waste was conducted using medium supplemented with starch. The starch hydrolysis of each isolated bacteria was observed based on halo zones produced after reaction with iodine solution. From Day 1 to Day 13, 14 colonies were isolated. From Day 14 to Day 24, the bacterial strains started to decrease 2 colonies. 7 of the strains isolated showed positive results of starch hydrolysis test. Partial characterization of the amylolytic bacteria isolated from food waste was conducted using biochemical test. Amylolytic bacterial strains isolated do not feed on glucose since they showed negative result of glucose fermentation test. Most of the amylolytic bacteria are Gram-positive *Streptobacilli*. The kitchen waste is a good source for isolation of amylolytic bacteria and enzymes can be used for industrial purpose.

Keywords: Amylolytic bacteria; Food waste fermentation; Solid State Fermentation (SSF); Isolation; Partial characterization

Isolation of Bacteriophage Infecting Enterobacteriaceae from Wastewater

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Abstract: Phages are viruses that infect bacteria. They are approximately 50 times smaller than the size of bacteria and ubiquitous in nature. The main purpose of this study is to isolate bacteriophage infecting Enterobacteriaceae from wastewater. In this study, bacterial hosts were isolated from four different sources which are oxidation pond at Jalan Peladang, UTM; Tasik Ilmu UTM; and Tasik PKU, UTM. One sample each was taken from oxidation pond (sample 1) and Tasik Ilmu (sample 2), while 2 samples were taken from Tasik PKU (sample 3 and 4). Out of 14 different host colonies isolated from all of the samples, two were selected for identification. Both of the hosts come from sample 1, both are Gram-negative but they have distinct morphology from each other. However, no bacteriophage managed to be isolated from both of the hosts, several limitations were detected and improvements are needed for further study.

Keywords: Bacteriophage; Enterobacteriaceae; Wastewater; Isolation

Identification of Sewage Bacteriophage Using Sequencing Method

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Abstract: Isolation and identification of bacteriophage, commonly depend on morphology characteristic, which limits the understanding on the bacteriophage's true nature, development and infection capability. Since, bacteriophage is species specific and has gain interest to be utilized as biomarker, in this research, we study on a lytic bacteriophage which capable of infecting bacteria host *Klebsiella pneumoniae* from sewage water sample. The bacteriophage was isolated using double layer agar method. The isolated sewage bacteriophages had pin-prick size morphology and exhibit 30 minutes host-phage infection time with large burst size at $2.75x10^6$ pfu/mL. Extraction and purification of the bacteriophage genome was using the RTP® Bacteria DNA Mini Kit with some modification and identification of phage was done by sequencing using specific designed PCR primer. The sewage bacteriophages had genome size of approximately 1000 kb. DNA template sequence was compared with available sequence databases using NCBI and we report the bacteriophage strain belongs to Klebsiella phage variant KP15, a lytic bacteriophage that infect bacteria host *Klebsiella* sp..

Keywords: *Klebsiella* sp.; Klebsiella bacteriophage; Genome sequencing

Isolation of Bacteriophages Infecting Enterobacteriaceae from Sheep Stool

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Abstract: River pollution has become a common issue nowadays. There are so many sources of the pollution which include farm animals' faeces. In these particular stools, there are bacteriophages that can be used as biomarker for the pollution but prior to the identification of the bacteriophages, the bacteria from the stools should be first recognised. In this research, stool from sheep was taken as sample in order to do bacterial isolation. Additionally, horse stool was also taken in order to compare the amount and characteristics of isolated bacteria from both samples. Since the targeted bacteria were from Enterobactericeae family, so MacConkey agar was used as selective medium to get only bacteria from the family. The pure colonies that presence on the MacConkey media were analysed as white and pink, rod-shaped and Gram-negative bacteria. Compared to bacterial colonies on nutrient agar (NA) medium, results showed that the amount of bacteria on the MacConkey agar was much more smaller due to its selectivity. Apart from that, the study showed that there was presence of ampicillin resistance bacteria in the sample. Further research and investigation can be done for the next step which is bacteriophages isolation.

Keywords: Bacteriophages; Enterobacteriaceae; Sheep stool; Isolation; River pollution; Biomarker

Screening and Identification of Bacteria from Copper Ores Effluent

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Abstract: Copper contamination in water systems represent a serious threat to the human population and natural water sources. The present study reported the isolation and characterization of bacteria from copper ores effluent of Mengapur Copper Mine in Pahang. Three isolates were successfully isolated from copper ores effluent and named Copper Tolerant Bacteria 2 (CTB2), Copper Tolerant Bacteria 4 (CTB4) and Copper Tolerant Bacteria 5 (CTB5) via enrichment in nutrient broth that containing 300 mgL^{-1}. The isolated copper tolerant bacteria from copper ores effluent were further characterized by determining the minimum concentration of Cu (II) that can inhibit the growth of bacterial isolates by increasing concentration of Cu^{2+} (200 – 900 ppm). The Minima Inhibitory Concentration (MIC) of Cu (II) for CTB2, CTB4 and CTB5 were determined to be 600 mgL^{-1} Cu (II). Molecular characterization, 16S rRNA analysis revealed that isolates CTB4 shares 97% identity match to *Bacillus cereus* while isolates CTB5 shares 96% identity match to *Staphylococcus* sp. strain. These bacteria could be used as a model microbial strain to study the mechanism of heavy metal resistance with potential application for bioremediation of heavy metals.

Keywords: Copper ores effluent; Copper contamination; Screening; Identification

Screening and Identification of Copper-Tolerant Bacteria from Copper Concentrate

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Abstract: Copper-tolerant bacteria are the bacteria that able to grow in the presence of free copper ions (Cu^{2+}). In this study, copper-tolerant bacteria were isolated from Mengapur Copper Mine in Pahang. Bacteria CO1 and CO2 were obtained by cultivating the crushed copper concentrate which is previously done in sterile salt solution into nutrient broth (NB) medium containing 300 mg/L Cu^{2+} and was further streaked onto nutrient agar (NA) medium containing 300 mg/L Cu^{2+} to obtain pure colonies. Two Gram-negative, rod-shaped bacteria were successfully isolated namely CO1 and CO2. The isolates were found to grow optimally in aerobic condition at 37 °C, 180 rpm. Copper sulphate screening revealed that the isolates exhibited resistance to high concentration of Cu^{2+} as high as 500 mg/L copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) with 50% or higher of the bacterial growth. Based on 16 small subunit of ribosomal ribonucleic acid (16S rRNA) gene sequence analysis, CO1 was identified as *Ochrobactrum haematophilum* and CO2 remain unidentified due to the error derived from the 16S rRNA sequence analysis which led to poor quality reads of CO2. The study showed that this strain could be a potential candidate for bioremediation of copper-contaminated area.

Keywords: Copper-tolerant bacteria; Copper (II) ions (Cu^{2+}); Copper concentrate

Screening and Identification of Copper Tolerant Bacteria

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Abstract: Copper is a trace element that is required for humans, prokaryotic and eukaryotic organisms. Copper is essential at low concentration but toxic to microbial cells when present in high levels in its free ionic form Cu (II). Copper toxicity also results in electron transfer to hydrogen peroxide, producing hydrogen radicals that easily attack and damage cellular biomolecules. Copper toxicity results in accumulation in river water that can cause pollution because it is non-biodegradable and may be hazardous to the environment as well as causing adverse health effect to humans and animals. The main purpose of this project was to isolate and screen for copper tolerant bacteria from copper mine raw water from the Mengapur Copper Mining site in Pahang. Three bacterial strains, CTB01, CTB02, and CTB03 were successfully isolated via enrichment in NB containing 300 mgL^{-1}. The isolated copper tolerant bacteria were further characterized by determining the minimum concentration of Cu (II) that can inhibit the growth of bacterial isolates. The percentages of growth inhibition were calculated to determine the minimum inhibition concentration (MIC) of the bacterial isolates. The MIC of Cu (II) for CTB01 and CTB03 was determined to be 600 mgL^{-1} Cu (II) while that for CTB02 MIC was 500 mgL^{-1} Cu (II). Bacterial isolates were identified via 16S rRNA gene sequence analysis. Phylogenetic analysis was conducted by Molecular Evolutionary Genetics Analysis (MEGA7) to determine the phylogenetic placement of each isolates relative to the type strains. Isolates of CTB01 and CTB02 were identified as *Acinetobacter* sp. while CTB03 as *Raoultella* sp. The Cu (II) tolerant bacteria isolated in this study can potentially be used for bioremediation of copper contaminated effluent.

Keywords: Copper tolerant bacteria; River; Copper mine raw water; Minimum inhibitory concentration

Marine Bacterial Biofilm of *Salinimonas lutimaris* sp. on Paint Surfaces under Hydrodynamic Conditions

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Abstract: Adhesion of biofilm produced by the marine bacteria on the ship hull can caused many problems such as extra energy consumption, increase maintenance of the ship and also surface corrosion. In this study, the painted surface is used because to know or to study more about the biofilm formation on it and since microorganisms on a surface can increase the attachment of other organisms, inhibition of microbial biofilm development might decrease subsequent development of barnacles on the surface. Therefore, the aim of this study was to investigate the marine biofilm formation on paint surfaces. Paint surfaces were identified as hydrophobicity material as investigated by its Gibbs free energy. The Gibbs free energy was measured as -14.01 mJm^{-2}. The *Salinimonas lutimaris* biofilm was allowed to grow on the paint coated surfaces in the marine broth medium for 7 days at temperature of 37 °C and shaken at 150 rpm. The biofilm structure growth on paint surfaces were observed under Scanning Electron Microscope (SEM) and it shown the shape of surface features is of importance in microbiological binding to a surface. As indicated by biofilm assay, the yield of EPS contents in biofilm structure was 0.5414 mg and dry weight of 0.0294 mg. This finding indicated that marine bacterial biofilm are able to grow on the paint surfaces, thus need a further investigation and new formulation of antimicrobial paint.

Keywords: *Salinimonas lutimaris* sp.; Biofilm; Scanning Electron Microscope (SEM); Exopolysaccharides (EPS); Adhesion

Effect of Surface Roughness of Stainless Steel on The Formation of *Salinimonas lutimaris* Biofilm

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Abstract: The adhesion of marine bacteria and biofilm formation on the surface of stainless steels promote deterioration of the material and have a major concern in marine industry. Therefore, the aim of this research is to investigate the effect of marine bacteria adhesion on stainless steel (Type 304) with different surface roughness under hydrodynamic conditions. Since natural seawater nutrient contents were not sufficient for bacterial grow as analysed by using Carbon Hydrogen Nitrogen Sulphur (CHNS) Analyzer, thus commercial marine broth has been used as a medium for bacterial growth. An isolated marine bacteria *Salinimonas lutimaris* was used as a model bacterium for investigation. The growth profile of *Salinimonas lutimaris* was determined by monitoring the absorbance at $OD_{600\ nm}$ and dry cell weight measurement. A range of rougher stainless steel surfaces (Ra values from ≅ 70 µm to ≅ 90 µm) were tested upon the selected marine bacterial biofilm. The *Salinimonas lutimaris* biofilm was allowed to grow on stainless steel for 7 days at 37 °C and 100 rpm. The 7 days grown biofilm of *Salinimonas lutimaris* on stainless steel was visualized using Scanning Electron Microscope (SEM). The results showed that biofilm formation of *Salinimonas lutimaris* on stainless steel coupons were greater on rougher surfaces (Ra of 82.45 $\pm$ 4.73 µm and 91.63 $\pm$ 2.82 µm) than those of the stainless steel coupons with lower surface roughness of 73.18 $\pm$ 1.27 µm. These observations support the hypothesis that the surface roughness is one of the factors that influences biofilm formation on stainless steel surfaces especially when the scratches on the surface are comparable to the size of the bacteria. Therefore, it can be concluded that the rougher surface with wider scratches exhibits a higher fraction of bacteria adhered on the surface.

Keywords: Stainless steel; Surface roughness; Adhesion; Bacteria; Biofilm

Isolation and Screening of Thermophilic Cyanobacteria from Ulu Slim Hot Spring

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Abstract: Thermophilic cyanobacteria are less studied but are proposed to be an important microorganism species for various biotechnology applications. In Malaysia, there are about 45 known hot springs, which is well studied for various thermophilic microorganisms profiling. This study was conducted to screen and isolate thermophilic cyanobacteria strains from Ulu Slim Hot Spring in Perak, Malaysia. Four cyanobacterial isolates were characterized based on their morphological and molecular characteristics. Out of four, two were isolated from sediment samples and the other two were obtained from water samples. Amplification of approximately 700 base pair (bp) nucleotides of 16S rRNA gene were obtained using combination of forward and reverse universal cyanobacterial primers, CYA106F and CYA781R, respectively. Sequencing of the 16S rRNA gene identified the isolates as *Thermosynechococcus* sp.. According to the nucleotide sequence alignment using BLAST, all the isolates shared 100% identity with *Thermosynechococcus* sp.. In addition, the microscope analysis showed that the isolates possess rod shape of unicellular cyanobacteria. This study provides insights into the initial profiling of cyanobacteria in Malaysia hot spring.

Keywords: Thermophilic cyanobacteria; Unicellular cyanobacteria; 16S rRNA gene sequence; Malaysia hot spring

Comparative Study of Fungal Growth Inhibition by Encapsulated Fungicide in MWCNTs and Activated Carbon

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Abstract: Chemical fungicide is often used by farmers to control fungal disease at their black pepper farms. However, the conventional method of applying fungicide is known to cause fungicide leaching especially during rainy seasons. Carbon nanoparticles such as multiwall carbon nanotubes (MWCNTs) and activated carbon possessed a promising property for wide a range uses in agricultural. This study aims to manipulate these two carbon particles to act as fungicide carrier in order to inhibit fungal growth by allowing gradual release of fungicide. Inhibition of two different fungal strains isolated from black pepper plants were observed when encapsulated MWCNTs and activated carbon with different mancozeb concentration (5×10^{-4} M, 10×10^{-4} M and 20×10^{-4} M) were independently applied for 7 days. Reliable inhibition of fungal growth was achieved when 500 and 5 000 spores incubated with encapsulated MWCNTs with 10×10^{-4} M mancozeb. In contrast, complete inhibition of fungal growth was observed when the fungal cultures were treated with encapsulated activated carbon with 5×10^{-4} M, 10×10^{-4} M and 20×10^{-4} M mancozeb. Additionally, nucleotide sequencing analysis has identified that the fungus isolated from leaf of pepper plants shared 98% similarity with *Mucoromycotina* sp.. Whereas, another fungal strain was identified to be *Fusarium incarnatum* due to 99% of nucleotide sequence similarity. Findings from this study suggest that activated carbon can be potentially manipulated to inhibit fungal diseases on black pepper plants.

Keywords: MWCNTs; Activated carbon; Fungicide; Encapsulation; Black pepper

Isolation and Identification of Thermophilic Protease-Producing Bacteria from Kuala Woh Hot Springs, Perak

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Abstract: Thermostable enzymes produced by thermophilic bacteria are active at high temperature as they are widely required for industrial applications. This present study was conducted to isolate thermophilic protease-producing bacteria from Kuala Woh Hot Springs, Perak, Malaysia. Screening of the bacterial isolates on Skim Milk Agar (SMA) from water and sand samples showed positive results based on the transparent zones around the colonies. The formations of colonies on SMA were identified as Gram-positive bacteria and most of them were rod and irregular shapes under magnification (100x). The bacterial isolates were cultivated in nutrient broth with varying temperatures of 40 °C, 50 °C and 55 °C. Among 16 isolates, the best three which are KW40P, KW50P(3) and KW55P(i) from each temperature range revealed 1.457 U/mg, 1.438 U/mg and 1.344 U/mg levels of specific proteolytic activity respectively. The three bacterial isolates (KW40P , KW50P(3), KW55P(i)) were then chosen for further 16S rRNA analysis. Based on the 16S rRNA gene sequence and phylogenetic tree analysis, the strains are from the same genus of *Bacillus* but different species. The data analysis revealed that KW40P (Accession MH424449) is much related to *Bacillus cereus* with 93% identity, KW50P(3) (Accession MH424450) is closely related to *Bacillus sonorensis* with 99% identity and finally KW55P(i) (Accession MH424451) has a close affiliation with *Bacillus licheniformis* showing 99% identity. The thermophilic protease-producing bacteria are great extracellular protease producers which employ higher processing temperature hence a promising thermostable enzyme which can be used in industrial purposes such as bioconversion of poultry wastes into value-added products.

Keywords: Thermophilic bacteria; Thermophilic proteases; Hot springs water; 16S rRNA

Formation of *Salinimonas lutimaris* Biofilm on Wood with Epoxy Coating Surfaces under Hydrodynamic Condition

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Abstract: Marine biofilm is an organized bacterial community that enclosed within matrix of extracellular polymeric substances attached to marine structures especially on wood substrates. This matter has a significant concern in marine industry. In this study, wood substrate surfaces with and without epoxy resins have been examined for the formation of biofilm of marine bacterium *Salinimonas lutimaris*. Since natural sea water nutrient contents were not sufficient for bacterial grow as analyzed by using Carbon, Hydrogen Nitrogen, Sulfur (CHNS) Analyzer, thus commercial marine broth has been used as a medium for bacterial growth. The growth profile of bacteria was determined by monitoring the absorbance at $OD_{600\ nm}$ and the dry cell weight of biomass. The biofilm of bacteria *S. lutimaris* was grown under hydrodynamic condition by constant shaking at 100 rpm and temperature of 37 °C for seven days. The surface roughness and hydrophobicity of each wood surface with and without epoxy coating also had been analyzed. In quantitative results, exopolymeric substances (EPSs) produced by the marine bacteria grown on wood substrate with and without epoxy coating was determined by calculating the dry cell weight of biomass of the biofilm produced after seven days. Wood with epoxy coating showed higher EPSs production compared to wood without epoxy coating with 151.1 ± 13.3 mg and 108.6 ± 5.90 mg of EPSs, respectively. The grown biofilm on both surfaces were observed under Scanning Electron Microscope (SEM). The results show that biofilm of *S. lutimaris* has the potential to grow on wood with and without epoxy coating surfaces. Therefore, it can be concluded that marine bacteria *S. lutimaris* are able to produce biofilm layer on wood substrate surface with and without epoxy coating.

Keywords: *Salinimonas lutimaris*; Marine bacteria; Epoxy; Wood; Biofilm; Hydrophobicity; SEM

Isolation and Identification of Thermophilic Protease-Producing Bacteria from Ulu Slim Hot Spring, Perak

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Abstract: Proteases play a significant role in application of many natural products and nowadays, proteases undeniably have been the most applicable classes in industrial enzyme. Initially, the water samples and sand samples of hot spring were collected from the rural area of Ulu Slim Hot Springs (3°53'58.5''N, 101°29'52''E) Perak, Malaysia and were investigated with the screening activity based on the relatively appearance of halo zone on Skim Milk Agar (SMA) plate to indicate the proteolytic activity. Next, further selection was carried out by quantitative enzyme assay as casein has been used as a substrate in the media and ultimately, 2 samples were chosen and finalized based on higher enzymatic activity. Five potential thermophilic protease producers were successfully isolated. The bacterial species were designed as SUS40 and SUS60. Both strains have been undergoes characterization, extraction, purification and identification via Gram Staining and 16S rRNA gene sequence analysis. Polymerase Chain Reaction (PCR) was performed to amplify the bacterial partial genome using 27F and 1492R primer. Finally, construction of phylogenetic tree has been made to show the evolutionary relationships among bacterial species. The isolates were identified as in *Bacillus mycoides* SUS40 and *Bacillus cereus* SUS60.

Keywords: Protease; Thermophilic; *Bacillus* sp.

Optimisation of Thermophilic *Bacillus licheniformis* strain MEA-01'S Proteolytic Activity Towards Casein Based on Temperature and Incubation Period

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Abstract: Thermostable protease is a proteolytic enzyme that cleaves the peptide bonds in protein molecules to produce smaller peptides and free amino acids that functions at a relatively high temperature. However, the available studies about thermostable protease isolated from hotsprings in Malaysia are limited which led to insufficient information available on the optimum conditions for efficient enzymatic activity and other possible applications of the thermostable protease. In this study, the thermostable protease activity of *Bacillus licheniformis* strain MEA-01 towards casein based on temperature and incubation period were optimised. The *B. licheniformis* strain MEA-01 was cultured and centrifuged to obtain the supernatant containing thermostable protease. The supernatant or sample was first analysed for protein concentration by using the Lowry's method. The protein concentration was 1.377 ± 0.008 mg/mL. The sample then investigated for proteolytic activity by using caseinolytic enzyme assay at 37 °C for 10 minutes. The amount of tyrosine liberated was 0.732 ± 0.058 µmole while the proteolytic activity of sample was 0.805 ± 0.063 U/mL. Meanwhile, the optimisation of proteolytic activity based on temperature and incubation period was also analysed by using the same caseinolytic enzyme assay. The assay for optimisation of temperature was conducted for 30 minutes whereas assay for optimisation of incubation period was carried out at 37 °C. The result showed that the optimum temperature was 40 °C with 0.226 ± 0.047 µmole of tyrosine and 10.824 ± 2.244 U/mL of proteolytic activity; and the optimum incubation period was 10 minutes with 0.212 ± 0.055 µmole of tyrosine and 30.417 ± 7.899 U/mL of proteolytic activity. The casein hydrolysate obtained during the caseinolytic enzyme assay for optimisation of incubation period was further analysed for casein degradation patterns by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The analysis showed that casein was completely degraded after 24 hours.

Keywords: Casein; *Bacillus licheniformis*; Protease activity; Thermostable protease; Optimisation

Biosugar and Biovanillin Productions from Pineapple Crown in Solid State Fermentation by *Phanerochaete chrysosporium*

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Abstract: Pineapple crown is one of agricultural residues which are important to be utilized in generating value-added products. Biosugar and biovanilllin productions were carried out using a solid state fermentation of pineapple crown by *Phanerochaete chrysosporium*. The significant parameters identification by one-factor-at-a-time (OFAT) method and optimization of the production of biosugar and biovanillin by Response Surface Methodology (RSM) were conducted. Quantitative analysis of biosugar was analyzed by Dinitrosalicyclic (DNS) assay. For biovanillin, quantitative analysis was measured by High Performance Liquid Chromatography (HPLC). Medium A showed the best medium used to produce biosugar (2.99697 mg/mL) and biovanillin (2.7942 mg/mL) with the ingredients of 1 g glucose, 0.05 g magnesium sulfate heptahydrate, 0.022 g ammonium sulfate, 0.01 g calcium chloride, 0.2 g potassium dihydrogen phosphate, 0.1 g thiamine, 0.67 g veratryl alcohol, 0.02 g yeast extract and 0.003 g cupric sulfate. The optimum condition for the highest yield of biosugar production were at 1% inoculum size (5.2381 mg/mL); 24 hours incubation period (4.84111 mg/mL); 25 °C incubation temperature (7.37949 mg/mL) and 60% moisture content (4.00508 mg/mL) by using OFAT method. The highest biovanillin yield obtained by using 4% inoculum size (1.15851 mg/mL); after 72 hours of incubation time (1.76031 mg/mL); at 35 °C incubation temperature (0.55383 mg/mL) and 50% moisture content (7.53084 mg/mL) by OFAT method. Optimization by RSM for biovanillin, ferulic acid and biosugar productions gave the highest production with 4.5 mg/mL, 34.208 mg/mL and 4.871 mg/mL, respectively. These findings demonstrate the potential of pineapple crown to produce biosugar and biovanillin.

Keywords: Biosugar; Biovanillin; *Phanerochaete chrysosporium*; Pineapple crown; Solid state fermentation

Optimization of Ferulic Acid Recovery from Lemongrass Leaves for Biovanillin Production by *Phanerochaete chrysosporium*

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Abstract: Lemongrass leaves are one of the major contributors in agricultural biomass for its high lignocellulosic content. Ferulic acid which is recovered from the lemongrass leaves is the main precursor for biovanillin production. In this study, physicochemical pre-treatment was carried out by using autoclaving method 121 °C for 20 minutes per sessions for ferulic acid recovery from lemongrass leaves. Through Central Composite Design (CCD) of Response Surface Methodology (RSM), significant parameters and optimum condition for optimizing the ferulic acid recovery from lemongrass leaves were identified. The quantitative analysis of ferulic acid recovered from the autoclaved hydrolysate was analyzed using High Performance Liquid Chromatography (HPLC). Meanwhile, the quantitative analysis for reducing sugar yield was conducted through 3,5-Dinitrosalicylic acid (DNS) assay. Primary screening supported that maximum ferulic acid was recovered through three times of autoclaving step. The highest concentration of reducing sugar and ferulic acid obtained from the pre-treatment of lemongrass leaves using autoclaving method was 1.258 g/L and 2.038 g/L respectively. The maximum recovery of ferulic acid through One-Factor-At-A-Time (OFAT) analysis and optimization stage of CCD analysis were 1.553 g/L and 2.038 g/L, respectively. By employing *Phanerochaete chrysosparium*, a white-rot fungus, submerged liquid fermentation was performed using the optimized hydrolysate for biovanillin production. The maximum yield of biovanillin produced was 0.63 g/L, and the overall utilization of ferulic acid was 50.07%. In conclusion, this study has demonstrated the effects of physicochemical pre-treatment used which was autoclaving method on the recovery of ferulic acid from lemongrass leaves.

Keywords: Lignocellulosic; Agricultural biomass; Parameters; Precursor; Ferulic acid; Reducing sugar; Biovanillin; *Phanerochaete chrysosporium*; Submerged liquid fermentation

Preparation, Characterization and Antibacterial Activity of Gentamicin Loaded Surfactant-Kaolinite

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Abstract: Kaolinite is a common component of soils. It was found that the negatively charged kaolinite can act as an adsorbent material and it has the ability to adsorb antimicrobial agents. In this study, kaolinite was used to adsorb gentamicin and cationic surfactant molecules as an improved antimicrobial agent. The gentamicin loaded surfactant-kaolinite (GSK) was prepared by the attachment of cationic surfactant hexadecyltrimethyl ammonium (HDTMA) 4.0 mM on raw kaolinite creating surfactant-kaolinite (SK) and then, loaded with gentamicin sulphate (50 and 200 mg/L). Gentamicin loaded kaolinite (GK) was also prepared and compared. All samples were characterized by X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, dispersion behaviour and CHNS elemental analysis. The characterization results proved that the framework structure of kaolinite was not disrupted even undergoes modification with the antimicrobial agents. The antibacterial activity of the samples was tested against *Escherichia coli* (ATCC 11229) and *Enterococcus faecalis* (ATCC 29212) through disc diffusion technique (DDT) and minimum inhibition concentration (MIC) assay. From the DDT and MIC, raw kaolinite did not exhibit antibacterial activity but it exhibits antibacterial activity when HDTMA and/or gentamicin loaded on kaolinite. In addition, GSK showed better antibacterial activity compared to GK and it performed better against Gram-positive bacteria compared to Gram-negative bacteria. As a conclusion, immobilization of HDTMA on kaolinite proved that kaolinite can act as an adsorbent to adsorb antibiotics and it has the potential to be developed as an enhanced antimicrobial agent.

Keywords: HDTMA; GSK; Antibacterial activity; Adsoprtion; Immobilization

Adsorption of Streptomycin on Organo-Kaolinite and Its Antibacterial Activity

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Abstract: Antibiotics or antibacterial agents are medicines used against bacterial infection. In spite of its ability to kill bacteria, it also showed problem with bacterial resistance that makes it less effective in killing the bacteria. Therefore, an improvement of antibacterial agent is needed to inhibit bacterial growth. Organo-kaolinite was selected in this study to act as a carrier system to improve the antibacterial agent immobilization and increase the effectiveness of antibiotics in inhibiting bacteria growth. Organo-kaolinite was prepared using cationic surfactant hexadecyltrimethyl ammonium bromide (HDTMA-Br) and it was adsorbed with different concentrations of streptomycin. The organo-kaolinite and streptomycinorgano-kaolinite were characterized by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and dispersion behaviour. The characterization results showed that the attachment of cationic surfactant molecules and the adsorption of streptomycin on kaolinite surfaces did not affect the original structure and morphology of kaolinite. The antibacterial assay of the samples was carried out against Gram-negative bacteria (*Escherichia coli* ATCC 11229) and Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212) through disk diffusion technique (DDT) and minimum inhibition concentration (MIC) technique. Based on the antibacterial assay result, streptomycin-organo-kaolinite showed better antibacterial activity compared to organo-kaolinite and it performed well in both distilled water and 0.9% saline solution. This study revealed that the adsorption of streptomycin on organo-kaolinite showed a great effect on killing bacteria and significantly increased its antibacterial activity compared to organo-kaolinite. For the conclusion, immobilization of streptomycin on organo-kaolinite has potential to be developed as new and enhanced antibacterial agent.

Keywords: Organo-kaolinite; Antibacterial activity; Streptomycin; Adsorption; Bacterial resistance

Antibiofilm and Antiadherence Activities of *Amaranthus* spp. Leaf Extracts Against *Streptococcus mutans*

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Abstract: Amaranth leaf (*Amaranthus* spp.) has been used by native practitioners in some Asian countries to treat various health problems such as blood disorders, bladder distress, dysentery, and tooth ache. Very little is known on the antibacterial activity of the amaranth leaf in particular against oral bacteria. Therefore, series of experiments were conducted to study the antibacterial activities of green amaranth extract (GAE) and red amaranth extract (RAE) against *Streptococcus mutans*. In this study, the minimum inhibitory concentration (MIC) assay was performed, followed by antiadherence and antibiofilm assays. Determination of phytochemical compound of both extracts was done using the High Performance Liquid Chromatography (HPLC). From the MIC assay, the results demonstrated that the MIC value of GAE and RAE against *S. mutans* was 6.25 mg/mL and 12.50 mg/mL, respectively. GAE was observed to be more effective in inhibiting cell adherence compared to RAE. GAE inhibited 82.4% ± 1.4% of bacterial cell adherence, while RAE inhibited only 66.0% ± 2.0% of bacterial cell adherence. In addition, GAE and RAE showed over 30% reduction in biofilm formed at the extract concentration of 0.78 mg/mL and 25 mg/mL, respectively. As observed in the antiadherence assay, the antibiofilm effect was also found to be in a dose-dependent manner for both studied extracts. The antibiofilm and antiadherence activities possess by *Amaranthus* spp. leaf extracts could be attributed by the presence of bioactive compounds quercetin, kaempferol, and catechin which were identified by using HPLC. These compounds are proven to possess antimicrobial, antiadherence, and antibiofilm effects. To summarize, both extracts exhibited comparable activities, including (i) the ability to inhibit the growth of *S. mutans*; (ii) inhibition of *S. mutans* adherence; and (iii) the inhibition of biofilm formation by *S. mutans*. In conclusion, the results of this study shed new insights on the antibacterial properties of amaranth extracts against *S. mutans*.

Keywords: *Amaranthus* spp.; *Streptococcus mutans*; MIC; Antiadherence; Antibiofilm

Antimicrobial Activity of *Pithecellobium jiringa* Against Oral Bacteria

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Abstract: Dental caries is a major oral health problem in most countries, where the current data from FDI World Dental Federation shows that it has a global prevalence over 40% for all age combined. Research on finding alternative compounds for controlling the diseases are extensively done, with many focusing on plant-derived natural compounds. *Pithecellobium jiringa*, locally known as jering, is a famous plant that can be found abundantly in Malaysia. The locals especially Malays, have eaten *P. jiringa* seed either raw or half boiled with rice and used pounded leaves for skin ailments or toothache as they believe that it has medicinal effects. Therefore, due to the potential of its medicinal values, the antimicrobial activity of *P. jiringa* leaves against oral bacteria was investigated. In this study, the *P. jiringa* leaves were extracted using ethanol and the extract was analyzed by High Performance Liquid Chromatography (HPLC). The minimum inhibitory concentration (MIC) of the extract against *Staphylococcus aureus*, *Streptococcus mutans* and *Streptococcus sobrinus* was determined using the broth microdilution method. The influence of the extract on bacterial adhesion and biofilm development was also investigated. From the phytochemical screening, gallic acid, quercetin and kaempferol were detected in *P. jiringa* extract. It was observed the MIC of the extract against *S. aureus*, *S. mutans* and *S. sobrinus* were 3.13 mg/mL, 1.56 mg/mL and 3.13 mg/mL, respectively. *P. jiringa* extract was found to be most effective against adherent cells of *S. aureus* with more than 50% reduction at 6.25 mg/mL, and moderate effect for *S. sobrinus* and *S. mutans*. Excellent inhibition was shown by the extract towards *S. sobrinus* where at 25 mg/mL, only 5.72 ± 0.14% of biofilms were detected. However, low antibiofilm activity was identified against *S. aureus* and no significant inhibition detected on *S. mutans*. The results from this study provide an interesting preliminary data for the development of *P. jiringa* extract as oral care agents against several bacteria.

Keywords: *Pithecellobium jiringa*; Medicinal plants; Oral bacteria; Antibacterial activity; Dental caries

Antibiofilm and Antiadhesion Activities of *Clinacanthus nutans* (Burm. F.) Lindau Leaves Extract Against Oral

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Abstract: Oral diseases such as periodontal disease and dental caries are one of the major public health problems. A number of antimicrobial agents have been used to prevent oral diseases. However, they may be associated with several side effects such as diarrhea and tooth staining. Nowadays, many studies are done especially in looking for plant's natural compounds to overcome the side effects. *Clinacanthus nutans* (Burm. F.) Lindau has shown various biological activities such as anti-herpes simplex, antioxidant, and antimicrobial activities. In this study, the bioactive compounds from *C. nutans* leaves extract were analyzed by using High-Performance Liquid Chromatography (HPLC). Antibacterial, antiadhesion and antibiofilm activities of *C. nutans* leaves extract were investigated against three different oral bacteria; *Streptococcus mutans*, *Streptococcus sobrinus* and *Staphylococcus aureus*. From HPLC, the highest amount of quercetin was detected in *C. nutans* extract followed by kaempferol and catechin. The minimum inhibitory concentration (MIC) value of *C. nutans* extract against *S. mutans* was 12.5 mg/mL and 25 mg/mL against *S. sobrinus* and *S. aureus*. For antiadhesion activity of *C. nutans*, at the concentration of 25 mg/mL, the reduction percentage of adhered bacteria was 36.65% for *S. mutans*, 45.89% for *S. sobrinus* and 46.60% for *S. aureus*. Around 20% reduction was observed in biofilm formation of *S. sobrinus* and *S. aureus* at 25 mg/mL of *C. nutans* extract and no reduction in biofilm formation of *S. mutans*. In conclusion, this study provides a preliminary data to represent *C. nutans* leaves extract as a potential antiadhesion and antibiofilm agent against oral bacteria.

Keywords: *C. nutans*; *S. mutans*; *S. sobrinus*; *S. aureus*; Dental caries; Antibacterial activities

Optimization of Cassava Wastewater for Efficient Starch Degradation by *Bacillus* sp. strain LFSF-20

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Abstract: The disposal of agricultural waste into water bodies and a poor waste management can contribute to many environmental problems. Agricultural waste such as cassava wastewater contains a high amount of starch that can be used as a substrate for starch-degrading bacteria to produce value-added products via fermentation. The starch-degrading bacteria are capable to utilize starch and convert it into glucose. This process is catalyzed by an enzyme called α-amylase. In this study, a strain of starch-degrading bacteria, *Bacillus* sp. strain LFSF-20 was used to investigate the optimum parameters for starch degradation. The capability of LFSF-20 in utilizing starch was tested at several different parameters; initial starch concentration (0.5 g/L, 1.0 g/L, 1.5 g/L), pH (4.5, 5.0, 5.5) and temperature (30 °C, 35 °C, 40 °C). The starch utilization was recorded the highest at 30 °C, pH 5.5 and initial starch concentration of 1.0 g/L, with 52% maximum starch degradation. The specific growth rate recorded at this condition is 0.235 h^{-1}. While the doubling time recorded at this condition is 2.95 h and the maximum reducing sugar produced is 1.07 g/L, with 20.85 U/mL of maximum amylase activity. This study has successfully determined the optimum condition for maximum starch degradation by *Bacillus* sp. strain LFSF-20, towards the application in the conversion of waste starch into value-added products.

Keywords: *Bacillus* sp. LFSF-20; Starch; Wastewater; α-amylase

Screening of Nigella Sativa-based Herbal Medical Products Using Analytical Methods

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Abstract: *Nigella sativa* seed which is also called "*Habatusauda"*" is known to be used as Herbal Medicinal Products (HMPs). But, the adulteration of HMPs has raised concerns among consumers. This study aims to extract and characterize thymoquinone which is the most important essential oil and bioactive compound from *Nigella sativa* seed and quantify the presence of thymoquinone from *Nigella sativa* based product to identify adulteration in these products. Thymoquinone was isolated by using two different extraction methods of methanolic extract and aqueous and the extracts were subjected to qualitative and quantitative analysis to determine the thymoquinone present in *Nigella sativa* seeds. Qualitative analysis on the extracts showed that *Nigella sativa* seeds contains of thymoquinone compound. The result of tymoquinone analysis was obtained analysed using a high performance liquid chromatography and thin layer chromatography to quantify the contents of thymoquinone in the extracts sample. Thymoquinone concentration in methanolic seed extract is higher than aqueous extraction with 63.4 µg/mL and 15.3 µg/mL. The high performance liquid chromatography (HPLC) method was used in to analyse the 13 samples of *Nigella sativa* based product to study the present of thymoquinone in this products and to aware people in adulteration by using herbal medicinal product (HMPs). The results showed that the Habatusauda' oil had the highest concentration of thymoquinone with 138.21 µg/mL followed by capsule Cap Kurma Ajwa 118.15 µg/mL and the lowest concentration thymoquinone is in Safi toothpaste orange 1.21 µg/mL. In conclusion, the screening of thymoquinone from *Nigella sativa* was successfully done by using HPLC method from both extraction, methanol and aqueous extraction. This also proves that the methanol is better for thymoquinone extraction compared to aqueouse extraction.

Keywords: Herbal Medicinal Products (HMPs); *Nigella sativa* seed*;* Extractions; Thymoquinone; HPLC

Screening of Epigallocatechin-3-gallate (EGCG) Content in Commercialized *Camellia sinensis* Products

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Abstract: Tea is one of the most consumed beverages all over the world. Tea is a major source of epigallocatechin-3-gallate (EGCG) with the pharmacological properties such as anti-obesity, anti-diabetes and anti-inflammatory. The objective of this study is to determine EGCG content in commercialized tea products. This study was divided into two phases. The first phase involved the extraction of EGCG compound from four types of tea namely as white tea (WT), green tea (GT), oolong tea (OT) and black tea (BT) using aqueous and methanol extraction techniques. The presence of EGCG compound was detected using Thin Layer Chromatography (TLC) and the amount of EGCG content in the tea products were analyzed using High Performance Liquid Chromatography (HPLC). The second phase involved the determination of EGCG compound in infusion tea bags (ITB) and ready-to-drink tea beverages (RTD) using HPLC. Fourteen ITB samples and twelve RTD samples were randomly purchased in the market. Results showed that methanolic extracts produced higher EGCG content than aqueous extract. The optimized temperature and extraction time to extract EGCG from WT, GT, OT and BT were also established in this study whereby WT, GT and OT were best extracted at 80 °C for 60 minutes, 20 minutes and 40 minutes respectively. Meanwhile, the optimized condition to extract EGCG from BT is at 29 °C for 40 minutes. Finally, the analysis of commercialized tea drinks products revealed that the infusion tea had the highest level of EGCG content compared to ready-to-drink tea. Among the four types of tea tested, GT contain the highest EGCG concentration compared to WT, OT and BT. Therefore, infusion tea bag especially green tea is recommended to obese people due to high EGCG concentration. On the other hand, ready-to-drink tea should be prevented by obese people due to lower concentration of EGCG.

Keywords: Tea; Commercialized product; EGCG; HPLC

Propagation of Local Pineapple (*Ananas Comosus* L.) in Temporary Immersion System

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Abstract: The demands for high number of pineapple plants increased year by year. Therefore, a good pineapple cultivation method needed to be developed. *In vitro* propagation is an established tissue culture method that performed cell cultivation to obtain large number copies of plantlets. However, conventional and asexual propagation methods produce low multiplication rate of shoots with long period of time. Present studies were conducted using semi-solid system, liquid system and temporary immersion system (TIS) for high shoot induction of Sarawak pineapple plants. The effect of different immersion time for shoot regeneration using temporary immersion bioreactor (TIB) was investigated. TIB was designed to subculture shoots with different immersion time (3 minutes / 2 hours, 9 minutes / 2 hours and 15 minutes / 2 hours) and produced 1.50 ± 1.50 shoots at best immersion time (3 minutes / 2 hours) in 3 weeks. For liquid system, the effect of different volume (5 mL, 25 mL and 50 mL) of liquid medium was investigated and optimum shoot induction was obtained when shoots were cultured at 50 mL volume of MS liquid medium supplemented with 2 mg/L BAP (3.67 ± 0.58) after 4 weeks culture. The effect of different BAP and Kinetin concentration (0, 1, 2 and 3 mg/L) on shoot induction was investigated. The maximum number of shoots produced per explant observed on MS semi-solid supplemented with 2 mg/L BAP (3.50 ± 0.56) and 2 mg/L Kin (1.83 ± 0.31) after 8 weeks culture. In conclusion, the use of the TIB system with the same condition had significantly shorter time to produce shoots than semi-solid system.

Keywords: Pineapple; Semi-solid system; Liquid system; Temporary immersion system; Shoot regeneration

Response of Rice Varieties to Salinity Stress at Seedling Stage

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Abstract: Salinity has shown a big impact on the growth of the rice, especially in Asia. It is one of the main constraints to rice productivity as rice is sensitive to salinity. Seedling stage in rice is the most important plant developmental phase, which has been reported to be a sensitive phase in rice. Soil salinity is caused by a combination of different salts, however NaCl is the most predominant salt which affects the rice productivity severely. Present study was conducted to determine the morphological and physiological response of rice seedlings under several salinity levels. Salinity was created by using NaCl. Five rice varieties (Panderas, KS282, IR36, Firat, and Bas370) were subjected to 0, 100, and 150 mM NaCl at seedling stage in three replications. Eight morphological and two physiological parameters were measured. Physiological parameters (sodium and potassium contents in shoots) were analyzed by flame photometer. Statistical analysis was conducted using Statistical Package for the Social Science (SPSS). The data was subjected to analysis of variance (ANOVA) and correlation. ANOVA reflected that morphological as well as physiological parameters showed significant differences for varieties and stress levels. Seedling injury score was increased as the stress level increased while the rest of the morphological parameters showed decline. All varieties were affected, however minimum effects were observed in KS282. Shoot sodium contents were increased with and increment in stress level while shoot potassium contents were reduced. Shoot sodium and potassium contents showed a negative correlation under high stress level. Seedling injury score is positively correlated with shoot sodium contents in saline condition, therefore it would be a determinant criteria to assess the effects of salinity in rice.

Keywords: Salinity; NaCl; Rice; Morphology; Physiology; Seedlings

Combined Effects of Salinity and Alkalinity on Rice Seedlings

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Abstract: Soil salinity is one of the main limiting factors for crop productivity worldwide. Rice is sensitive to soil salinity. The effects of soil salinity and high soil pH damage physiological functions of roots and root cell structure due to mineral deficiencies e.g. potassium and sodium toxicity. Soil salinity effects on rice have been studied intensively; however limited research is carried out regarding the combined effects of salinity and high pH. Therefore, it was proposed to investigate the morphological and physiological response of rice seedlings under the combined effects of salinity and alkalinity. Five rice varieties (Panderas, KS282, IR36, Firat, Bas370) were grown in seedling trays under salinity, using NaCl (100 mM, 150 mM) and under alkalinity, using Na_2CO_3 (pH 8.5, pH 10.5). Eight morphological parameters and two physiological parameters were measured. Analysis of Variance (ANOVA) and correlation were done to determine the effects on these parameters under salinity and alkalinity stresses. ANOVA reflected that the combined effects of salinity and alkalinity showed significant differences for all parameters for varieties as well stress treatments. Morphological parameters showed a significant decline in all varieties under stress, however minimum effects were observed in KS282. With an increase in stress level, shoot sodium contents were significantly increased while shoot potassium contents showed significant reduction with an increase in the stress level. Seedling injury score showed a positive correlation with shoot sodium contents while a negative correlation with shoot potassium contents. Results reflected that seedling injury score, shoot sodium contents and shoot potassium contents would be used as a seedling respond under saline and alkaline conditions.

Keywords: Salinity; Alkalinity; Combined effects; Rice seedlings

Molecular Modelling and Bioinformatics Analysis of Twin Arginine Translocation (TatD) Deoxyribonuclease

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Abstract: Deoxyribonuclease TatD is a cytoplasmic protein with DNase I activity and highly involved in DNA degradation during apoptosis or cell death of bacteria. To date, only a few 3D structure of Deoxyribonuclease TatD available thus limiting our understanding on the protein's functional mechanisms on the molecular level. In this study, we report two homology-based model of Deoxyribonuclease TatD from *Shewanella psychrophila* (strain WP2) and *Desulfotomaculum australicum* (DSM 11792). The result shows that both of the microorganisms belong to the same superfamily, metal dependent hydrolase. *S. psychrophila* and *D. australicum* show 30.08% and 35.67% identity with their template, respectively. Both of the protein sequences share the same conserved amino acids sequences and can be seen in sequence based analysis. Despite the sequence differences between both of the protein and other Deoxyribonuclease TatD, their structural model share similar fold as they came from the same superfamily and has conserved TIM Barrel fold as the experimentally solved template structures. This promotes theoretical understanding on the 3D structure of the protein with the metal binding interaction and their adaptation on extreme temperature.

Keywords: Twin Arginine Translocation (TatD) Deoxyribonuclease; Molecular modelling; Bioinformatics analysis; Homology-based model; 3D structure

Homology Modeling and Adaptation Study of N-acyl-homoserine Lactonase from Extremophiles

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Abstract: Quorum sensing plays an important role in controlling various biological functions such as virulence and biofilm formation which is mediated by autoinducers such as N-acyl-homoserine lactone (AHL). The most characterized quorum quenching enzymes so far is N-acyl-homoserine lactonase (AHL lactonase) which belong to the metallo-beta-lactamase (MBL) superfamily. Previous study had identified AHL lactonase from *Thermaerobacter nagasakiensis* (thermophile) and *Planococcus versutus* strain L10.15T (psychrophile), however their 3D structures are not being identified yet. Their deduced amino acid sequences share a substantially low similarity (< 50%) with other AHL lactonase sequences that have their structures solved in PDB. This study describes a homology-based model of AHL lactonase from *P. versutus* (AidP) and *T. nagasakiensis* (AiiT). Both of the structures constitute a αβ/βα sandwich fold with the outer helices layer surrounding a core made up of β-sheet. Both enzymes contain the conserved motif sequence 'HXHXDH' which commonly found in MBL superfamily. The sequence and structural analysis shows that AidP has longer loops, fewer arginine residue, fewer charged, aromatic and aliphatic residues, and hydrophobic interactions during the substrate-binding interaction may contribute to the local flexibility of the AidP structure and increase the capability of the enzyme to be active at low temperature. Meanwhile the presence of higher hydrophobic, aliphatic, aromatic, arginine and charged residues, and also higher helical contents in AiiT might contribute to the local stability of the structure and thus enable thermophilic protein to stay stable and avoid denaturation at high temperature. This work should help facilitate the design of more effective quorum-quenching catalysts with enhanced activity and substrate specificity.

Keywords: AHL lactonase; *Planococcus versutus*; *Thermaerobacter nagasakiensis*; Homology modelling

Modelling and Adaptation Study of Alkaline Phosphatase

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Abstract: Alkaline phosphatase (APs) is a nonspecific metalloenzymes that involve hydrolysis of phosphate monoester and it is classified into enzyme class 3 hydrolase with E.C. number 3.1.3.1. This enzyme is found across a wide variety of organisms with same general function but different structural forms. In this study, the structure of APs from psychrophilic organism, *Godus morhua* (Atlantic cod) and hyperthermophilic organism, *Thermatoga neapolitana* were constructed using Swiss Model server and further validated using Verify-3d, ERRAT and PROCHECK server. Based on the result computed from those three servers, both structure of cod AP and TnAP were classified as stable and reliable. Structure superimpose was visualized using Chimera program to identify the exact location of active site residue for cod both AP and TnAP which were identified to be at Ser94 and Ser71, respectively. The result obtained from ProtParam analysis showed that the instability index computed for APs in *Godus morhua* and *Thermotoga neapolitana* were 29.16 and 31.07 which means that both structure considered stable. Adaptation strategy for both structures were studied and showed that high number of Glycine (Gly) residues, longer chain loops, low number of Proline (Pro) residues, low hydrophobicity and present of Ca^{2+} were confirmed to be the main factors contributing in enzyme flexibility for psychrophilic enzyme, *Gadus morhua* to survive at low temperature habitat. Contrary, hypertermophilic enzymes, *Thermotoga neapolitana* was identified to have low number of Glycine (Gly) residues, shorter chain loops, high number of Proline (Pro) residues and high hydrophobicity as the main factors contributing to the enzyme stability and rigidity at high temperature.

Keywords: Alkaline phosphatase; Modelling; Adaptation study

Expression of Recombinant Serine Protease from *Acinetobacter baumannii* in *Escherichia coli*

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Abstract: *Acinetobacter baumannii* is a nosocomial pathogen that has been studied extensively for medical purpose. This microorganism can cause several diseases such as meningitis, pneumonia and urinary tract infection to the patients in the hospital environment. The present study aimed to investigate the expression of serine protease from *Acinetobacter baumannii* in *Escherichia coli* as the host expression system. The recombinant serine protease containing a His-tag was expressed in *Escherichia coli* BL21 (DE3) (Novagen®) cells, using pET22b (Novagen®) as the expression vector. SDS-PAGE and Western blotting analysis were performed to detect expression of recombinant serine protease. The recombinant protease was expressed as a mature form of the proteases with an estimated molecular weight of 30 kDa as verified on SDS-PAGE and Western blot analysis. The overexpression induction conditions are as follows: (optical density before induction is $OD_{600\ nm}$: ~0.6; isopropyl-ß-Dthiogalactopyranoside (IPTG) induction concentration: 0.4 mM; post-induction temperature: 20 °C; post induction time: 15 h), whereupon the resultant expressed-protease in this condition was observed to be in soluble form. The soluble expressed protease showed positive activity on both skim milk and azo-casein agar plate. These findings suggest that the optimum condition to express the recombinant serine protease is by applying lower IPTG induction concentration and lower induction temperature. This study indicated that *Escherichia coli* harboring the plasmid pET22b with a DNA fragment containing the *A.baumannii* protease gene successfully expressed soluble and active mature recombinant protease in the specified expression conditions as stated above.

Keywords: *Acinetobacter baumannii*; Serine protease; Expression; BL21(DE3); pET22b (+)

Purification and Characterisation of an *Anoxybacillus* β-Glucosidase with Lactase Activity

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Abstract: Lactose intolerance is a general health concern for many people. Since milk is the primary source for calcium and vitamin D, lactose intolerance individuals may obtain insufficient amounts of these nutrients which resulted unfavourable health issues. Daily industry generates lactose-free formulations by hydrolysing lactose into glucose and galactose by enzymes such as lactase, β-galactosidase and β-glucosidase. Although β-glucosidase is generally applied on cellulose degradation, some of these enzymes exhibit side-activity in lactose hydrolysis. In this work, β-glucosidase originated form *Anoxybacillus* sp. DT3-1, specified as DT-Bgl was subcloned into pET28a in *Escherichia coli* BL21 (DE3) and being expressed and purified by immobilised metal affinity chromatography (IMAC) using Ni-NTA superflow column. A single band of 53 kDa observed on SDS-PAGE designates that the enzyme was successfully purified. DT-Bgl was active against ortho-Nitrophenyl-β-D-galactopyranoside (*o*NPG), a substrate used for the β-galactosidase activity determination. The highest DT-Bgl enzymatic activity was determined at 60 °C and pH of 8.5. The kinetic parameter, V_{max} and K_m, for DT-Bgl activity towards *o*NPG was 1116.57 U/mg and 26.88 mM respectively. Lactose hydrolysis by DT-Bgl was confirmed by a HPLC analysis as glucose and galactose were yielded after treating the milk samples with DT-Bgl. Under current experimental setup, DT-Bgl was able to hydrolyse approximately 11% of the total lactose amount contained in commercial milk. In conclusion, this study clearly suggested DT-Bgl has a potential usage for lactose hydrolysis in food industry.

Keywords: Lactose intolerance; *Anoxybacillus* sp.; β-galactosidase; Lactose hydrolysis

Expression and Solubility of H368A Human HSP47 Mutant

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Abstract: Heat shock protein 47 (HSP47) is a molecular chaperone specific collagen. It is expressed only in collagen-synthesizing cells. HSP47 assists the folding and assembly of procollagen and prevents the secretion of abnormal collagen from the cell. Histidine residues in HSP47 play a role in pH switch mechanism that controls the collagen binding and release. Site directed mutagenesis in combination with binding assays and biophysical methods have been used to investigate the important histidine residues in human HSP47. However, H368A mutant showed low expression and solubility, affecting the subsequent studies. The project is focused on maximizing soluble mutant, based on two factors: isopropyl-β-D-thiogalactopyranoside (IPTG) concentration during expression, and sonication time during cell lysis. The mutant was expressed in recombinant *E. coli* BL21(DE3) host using pET-21a vector. The expression and solubility were analysed using SDS-PAGE with Coomassie Brilliant Blue staining. The mutant was purified using His-tagged affinity chromatography. The protein concentration was measured using NanoDrop™ 1000 spectrophotometer. H368A mutant expression was found to be optimum at 0.3 mM IPTG and the highest soluble protein content was obtained at sonication time of 1.5 min. The mutant was then purified using His-tagged affinity chromatography. This study paved the way for H368A mutant to be further analysed using collagen binding assay and circular dichroism spectroscopy.

Keywords: H368A mutant; Human HSP47; Expression; Solubility; Collagen

Purification and Characterization of Endoglucanase from *Dictyoglomus* sp.

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Abstract: Generation of biofuel from lignocellulosic biomass is a promising alternative of fossil fuels. The process generating biofuel involves three steps which are biomass pre-treatment, enzymatic saccharification and fermentation of sugar. Extensive research had been done to study and obtain thermostable cellulases with higher activity with pre-treated biomass substrates. In a previous study, an endoglucanase EglDG gene (936bp) from *Dictyoglomus* sp. was directly amplified from a metagenome (environmental) DNA obtained from Ulu Slim hot spring. The gene was cloned and expressed in *Escherichia coli* BL21(DE3). The enzyme, EglDG was added to cocktail enzymes (Cellic Ctec2) (Sigma-Aldrich®) and found to enhance the release of glucose from empty fruit bunch. Phylogenetic analysis indicated that EglDG belonged to Glycosyl Hydrolase 5 subfamily 25. The recombinant EglDG was highly active on cellulosic chains with degree of polymerization (DP) number 3 to 7. The recombinant EglDG was purified to homogeneity using Ni-NTA column, with molecular mass of 41 kDa using an SDS-PAGE analysis. It exhibited optimum activity at 90 °C and pH 5.5 and did not require any co-factors. All tested metal ions (chloride salts of Cu^{2+}, Ba^{2+}, Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, Ni^{2+}, Co^{2+}, NH_4^{+}, Zn^{2+} and Mn^{2+}) were not required to enhance EglDG activity. EDTA, Tween-20, and Tween-80 boosted the enzyme activity to 142.08%, 137.58% and 187.20% respectively. The K_m and V_{max} values for the purified EglDG were 42.5 mg/mL and 1250 U/mg respectively, with carboxymethyl cellulose (CMC) as substrate. In conclusion, EglDG is a thermostable endoglucanase with optimum enzyme activity at high temperature and converts long chain cellulase chains to mainly cellobiose. The finding also suggested that EglDG could be used in saccharification of lignocellulosic biomass.

Keywords: Endoglucanase; *Dictyoglomus* sp.; Purification; Characterization; Biofuel

SELECTED PROCEEDINGS

Isolation and Characterization of Antibiotic Resistant Bacteria from Rivers in Melaka

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Abstract: Contamination of antibiotics in water environment becomes are great worldwide concern. The widespread uses of antibiotics in many fields are contributing to the emergence of pathogenic antibiotics resistance bacteria. The emergence of these antibiotic resistance bacteria become one of the most serious challenges in diseases treatment. The aim of this study is to determine the presence of antibiotic resistance bacteria from rivers in Melaka. A total of 7 bacterial strains were successfully isolated from Melaka River and 8 bacterial strains were successfully isolated from Tuang River, Melaka respectively. The isolated bacteria were tested for resistance against six different types of antibiotics including ampicillin, gentamicin, ciprofoxalin, chloramphenicol, rifamicin and tetracycline using antibiotic susceptibility test by disc diffusion. Most of the isolated bacteria show highest resistance effect toward chloramphenicol and tetracycline, less in rifamicin, gentamicin and ampicilin. None of the isolated bacteria showed resistance to ciprofoxalin. The MAR index was calculated and 6 isolated bacteria have the MAR index more than 50% were selected to be further analyzed by using 16S rRNA sequencing in order to determine their species. The characterization of bacteria has been conducted by Gram staining and biochemical tests. All the bacterial strains from Tuang River were identified as *Escherichia vulneris* sp., *Escherichia fergusonii* sp., *Citrobacter freundii* sp. and *Bacillus thuringiensis* sp.. While, antibiotic resistance bacteria identify from Melaka River were *Burkholderia multivorans* sp. and *Escherichia vulneris* sp..

Keywords: Antibiotic resistance bacteria; River; Melaka

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1. Introduction

Due to widespread use of antibiotics in many fields, antibiotics residues may enter the environment (Hartman, 2011). Environments containing antibiotic residues are particularly worrisome because antibiotics might contribute to the appearance of resistant bacteria. Especially in aquatics environment, the study reported there is an existent of antibiotics resistance bacteria in aquatic environment (Koczura *et al*., 2012) including rivers, ocean and drinking water (Kümmerer, 2009) surface water (Pereira *et al*., 2013), the hospital effluents (Fuentefria *et al*., 2011) and sewage treatment plants (Ferreira da Silva *et al*., 2007).

Antibiotic resistant bacteria are bacteria that are not killed or controlled by antibiotics and even multiply in the presence of an antibiotic. These bacteria has an ability to adjust in environmental conditions and serve as vectors for the dispersing of antibiotic resistance (Kruse, 1999). Bacteria easily adapting into a new environment by transferring genes between bacterial strains, that could be facilitated by mobile genetic elements, such as plasmids, transposons, bacteriophages, integrons, insertion elements (IS), and genomic islands (Li *et al*., 2010). The potential to adapt quickly and reproduce rapidly is a key factor in the evolution of antibiotic resistant bacteria to adapt readily in a changing environment and colonize new habitat.

Majority antibiotics resistance bacteria have an ability to cause the severe infections of diseases. It is because it becomes resistant to at least some antibiotics. In other word, one species of these microorganisms can either resist to more than one type of antibiotics. Bacteria that are resistant to many antibiotics are called as multi-resistant organism (MRO). Among of the infectious, the one from this category, it become one of the most serious challenges in clinical therapy. Some MRO pathogens, such as Klebsiella pneumoniae and Acinetobacter baumannii, are now virtually untreatable with current antibiotics (Li *et al*., 2010). This could be the unpleasant situation because these microorganisms could be potential sources of novel resistance genes in clinical pathogens (Li *et al*., 2010).

There are several studies has been conducted on the emergence of resistant microorganisms in ecology either because antibiotic resistance mechanisms in the bacteria itself or because of human and animal commensalism and pathogens (Vaz-Moreira *et al*., 2014). Besides, to acknowledge the importance of

antimicrobial resistance in nature, and to identify resistance of environmental reservoirs and the contribution towards understanding the propagation routes of these resistant bacteria. Thus, this proposal aims to isolate and characterize the antibiotic resistance bacteria in rivers in Melaka.

2. Materials and Methods

2.1. Water sample collection

Sampling process was taken from two different rivers; Melaka River and Tuang River, Melaka. Sampling was performed by using the sterile glass bottle. After sampling, bottle was placed into the ice box to maintain 4 °C temperature while transferring to laboratory and store in cold room until further analysis.

2.2. Isolation of bacteria

Isolation of bacteria was performed by using nutrient agar. The spread plate has been performed by pipette out 100 µL of seven-fold serial dilution of water sample onto the center of the surface of nutrient agar plate. To obtain the pure colonies, five times of subculture has been performed based on colony morphology characteristic.

2.3. Antibiotics susceptibility testing

The isolated bacteria were tested for resistance against six different types of antibiotics including Ampicillin, Gentamicin, Ciprofoxalin, Chloramphenicol, Rifamicin and Tetracycline with two different concentrations; 10 µg/mL and 30 µg/mL using antibiotic susceptibility test by disc diffusion. The size of inhibition zones were interpreted by referring to Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints (CLSI, 2012). The organisms were identified as susceptible, intermediate or resistant.

2.4. Multiple Antibiotic Resistance (MAR) Index Analysis

The MAR index was calculated according to the equation below:

$$MAR\ Index = \frac{a}{b} \times 100$$

Where (a) is the total number of antibiotics that the isolates resistant to and (b) is the total number antibiotics that been tested with. If the index value is more than 20%, it shows that the area of isolation is in high possibly risk of contamination by several antibiotics (Matyar, 2012).

2.5. Characterization of bacteria

Gram staining are use to identify whether the bacteria isolates Gram-positive or Gram-negative bacteria. Meanwhile, the biochemical test are use to determine the biochemical characteristics of bacteria isolates including oxidase test, catalase test, urease test, indole test, catalase production test, MacConkey agar test, phenol red test, Mannitol Salt Agar, Oxidative Fermentative test and citrate utilization test.

3. Results

3.1. Isolation of antibiotic resistance bacteria

A total of 7 bacterial strains were successfully isolated from Tuang River. While 8 bacterial strains were successfully isolated from Melaka River.

3.2. Antibiotic Susceptibility Test

The results are qualitative assessments using the categories susceptible, intermediate, or resistant (Reller *et al.*, 2009). Table 1 shows the overall qualitative result of antibiotic resistance of bacterial strains isolated from Tuang River and Melaka River against tested antibiotics; Ampicillin, Gentamicin, Ciprofoxalin, Chloramphenicol, Rifamicin and Tetracycline with two different concentration of antibiotic.

Table 1: Antibiotic resistance of bacterial strains isolated from Tuang River and Melaka River against tested antibiotics

Isolated Point		Tuang River (Point 1)				Tuang River (Point 2)			Melaka River (Point A)					Melaka River (Point B)		
Strain		T1	T2	R1	R2	T3	T4	T5	M1	M2	M3	M4	M5	M6	M7	M8
Antibiotic	**Concentration**															
Ampicilin	10µg/mL	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**S**	**S**	**S**	**R**	**S**
	30 µg/mL	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**S**
Gentamicin	10µg/mL	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
	30 µg/mL	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
Ciprofloxalin	10µg/mL	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**
	30 µg/mL	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**
Chloramphenicol	10µg/mL	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**
	30 µg/mL	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**S**
Rifampicin	10µg/mL	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**
	30 µg/mL	**R**	**R**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**
Tetracycline	10µg/mL	**R**	**R**	**S**	**S**	**R**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**R**
	30 µg/mL	**R**	**R**	**S**	**S**	**R**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**R**

Figure 1 a) show all isolated bacteria from Tuang river show highest resistance effect toward Chloramphenicol (100%), Tetracycline (100%) and Rifampicin (100%), less often in Ampicilin (75%) and Gentamicin (50%). None of the isolated bacteria showed resistance to Ciprofoxalin (0%). While, Figure 1 b) show all isolated bacteria from Melaka river show highest resistance effect toward Chloramphenicol (100%), Tetracycline (100%) and Gentamicin (100%), less often in Rifampicin (50%). None of the isolated bacteria showed resistance to Ampicilin (0%) and Ciprofoxalin (0%).

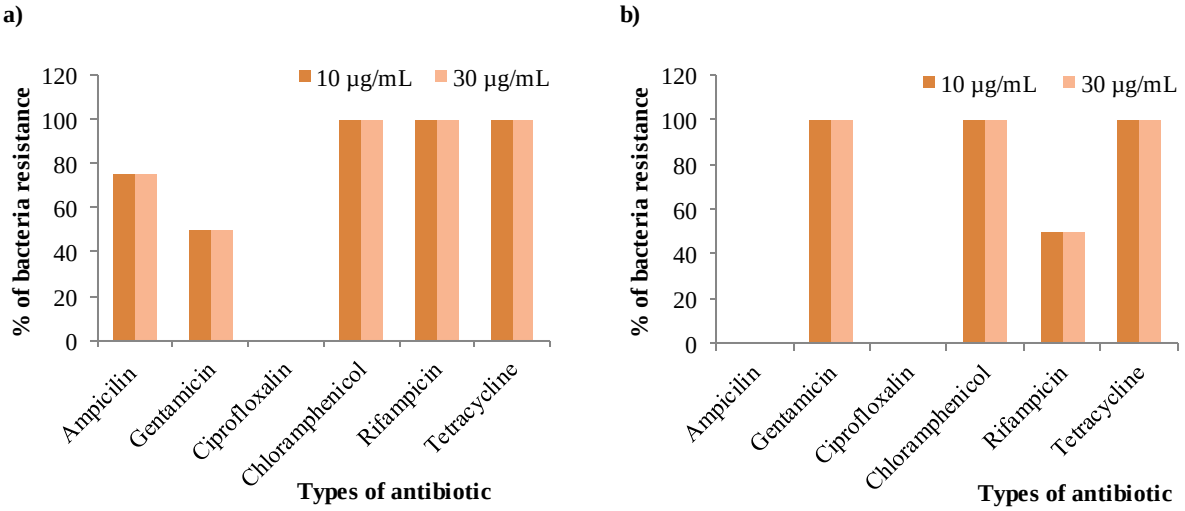

Figure 1: Percentage of bacteria isolated resistant towards the tested antibiotics a) Tuang river and b) Melaka river

3.3. Multiple Antibiotic Resistance (MAR) Index Analysis

Figure 2 shows the MAR index for all of the isolated bacteria. The results show that 12 of the isolates have MAR index value more than 20%.

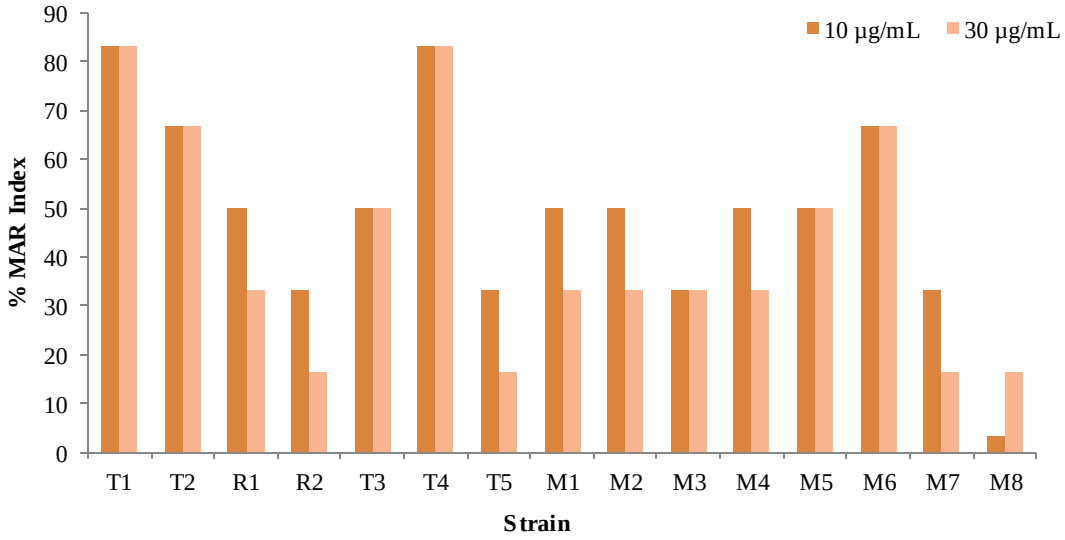

Figure 2: Percentage of MAR index value for each antibiotic resistant bacteria (ARB)

3.4. Biochemical Test

Table 2 shows the summary of biochemical test result for the bacterial strains from Tuang River, while Table 3 shows the summary of biochemical test result for the bacterial strains from Melaka River; '+' indicate as a positive result, '-' indicate as a negative result, 'K/A' indicate as alkaline slant/acid butt, 'A/A' indicate as acid slant/acid butt, 'K/K' indicate as alkaline slant/alkaline butt, 'NC' indicate no chance, 'O' as a oxidative, 'F' as a fermentative and 'N' as a non-saccharolytic.

4. Discussion

4.1. Antibiotic Susceptibility Test

An important task of this study is the performance of antimicrobial susceptibility testing for all isolates from Tuang and Melaka rivers. The aims of performing the antibiotic susceptibility test are to detect possible antibiotic resistance in common pathogens and to assure susceptibility to antibiotics. Disk diffusion method has been choosing due to the methods provide flexibility and possible cost savings. The results are qualitative assessments using the categories susceptible, intermediate, or resistant (Reller *et al.*, 2009).

Figure 1 a) shows all isolated bacteria from Tuang river demonstrate highest resistance effect toward chloramphenicol (100%), tetracycline (100%) and rifampicin (100%), less often in ampicilin (75%) and gentamicin (50%). None of the isolated bacteria shows resistance to ciprofoxalin (0%). All the isolated bacteria are resistance to chloramphenicol (100%), tetracycline (100%) and rifampicin (100%). It can be assumed that chloramphenicol, tetracycline and rifampicin are highly used in the area of Tuang River, thus conclude that the three antibiotics are the most often used antibacterial agents in the community. The results also show the bacteria resistance towards ampicillin and gentamicin is 75% and 50%, respectively. Thus, this can be concluded that these two antibiotics are also use in the community area. Meanwhile, no bacteria shows resistance to ciprofoxalin thus it can be concluded that no application of ciprofoxalin in Tuang river area.

Figure 1 b) shows isolated bacteria from Melaka river exhibit the highest resistance effect towards chloramphenicol (100%), tetracycline (100%) and gentamicin (100%) and rifampicin (50%). None of the isolated bacteria shows resistance towards ampicilin (0%) and ciprofoxalin (0%). Based on the results, all the bacteria are resistance to chloramphenicol (100%), tetracycline (100%) and gentamicin (100%). It is assumed that high used of chloramphenicol, tetracycline and gentamicin in the area of Melaka River. It also can be concluded that that rifamipicin are also being used in the community area. As for ciprofoxalin and ampicillin, it can be concluded that there is no application of ciprofoxalin and ampicilin in Melaka river area.

4.2. MAR Index Analysis

Figure 2 shows all the isolated bacteria from both rivers were tested for resistance against six different types of antibiotics including ampicillin, gentamicin, ciprofoxalin, chloramphenicol, rifamicin and tetracycline with two different concentrations; 10µg/mL and 30 µg/mL, using antibiotic susceptibility test by disc diffusion. From 13 isolates, 12 of them show MAR Index more than 20%.

The MAR index analysis has been conducted in order to determine whether there is the present of Multiple-resistant organism (MRO). Multiple-resistant organism (MRO) is bacteria that are resistant to many antibiotics. Most infection causing bacteria can become resistant to at least some antibiotics. Too much application and dosage of antibiotics used is factor for the emergence of multi antibiotic resistance bacteria in water environment. Multi-resistant organism is the species of bacteria that can be resistance to more than three type of antibiotics agent.

Table 2: Summary of biochemical test result for Tuang river

Isolation point			Tuang River (Point 1)				Tuang River (Point 2)		
Strains			T1	T2	R1	R2	T3	T4	T5
Biochemical tests	Simmon citrate		+	+	+	-	+	-	+
	Phenol red broth-durham tube	Acid production	+	-	+	+	+	+	+
		Gas production	+	-	+	+	-	+	+
	Indole test		+	+	-	-	+	-	-
	MacConkey agar		+	+	-	+	+	-	+
	Catalase		+	+	+	-	+	+	-
	Oxidase		-	-	+	-	-	+	-
	Triple sugar iron TSI		**A/A**	**A/A**	**K/K**	**K/K**	**A/NC**	**A/A**	**A/A**
	H_2S gas production		+	+	-	-	+	-	+
	Oxidative fermentative test		**F**	**F**	**O**	**O**	**N**	**F**	**F**
	Mannitol salt agar		-	-	+	-	-	-	+
	Urease test		+	+	-	-	+	-	-
Predicted family of strain based on biochemical test			*Enterobacteriaceae* - *Escherichia* sp. - *Klebsiella* sp.	*Enterobacteriaceae* - *Escherichia* sp. - *Vibro* sp.	*Lactobacillaceae* - *Lactobacillus* sp.	*Pseudomonadaceae* - *Pseudomonas* sp.	*Enterobacteriaceae* - *Citrobacter* sp. - *Escherichia* sp. - *Klebsiella* sp.	*Bacillaceae* - *Bacillus* sp.	*Enterobacteriaceae* - *Citrobacter* - *Klebsiella* sp.

Table 3: Summary of biochemical test result for Melaka river

Isolation point			Melaka river (Point A)					Melaka river (Point B)		
Strains			M1	M2	M3	M4	M5	M6	M7	M8
Biochemical test	Simmon citrate		-	-	+	-	-	+	-	-
	Phenol red broth-durham tube	Acid Production	+	-	+	+	+	+	+	-
		Gas production	+	-	+	+	+	+	+	-
	Indole test		-	-	+	-	-	+	-	-
	MacConkey agar		+	-	+	-	+	+	-	+
	Catalase		-	+	+	+	-	+	+	+
	Oxidase		-	+	-	+	+	-	+	+
	Triple sugar iron		**K/K**	**K/K**	**A/A**	**A/A**	**A/A**	**A/A**	**A/A**	**K/K**
	H_2S gas production		-	+	+	-	-	+	-	-
	Oxidative fermentative test		**O**	**N**	**F**	**F**	**O**	**F**	**F**	**N**
	Mannitol salt agar		-	-	-	-	+	-	-	+
	Urease test		-	+	+	-	-	+	-	-
Predicted family of strain based on biochemical test			*Lactobacillaceae* - *Lactobacillus* sp.	*Brucellaceae* - *Brucella* sp.	*Enterobacteriaceae* - *Escherichi* sp. - *Klebsiella* sp.	*Bacillaceae* - *Bacillus* sp.	*Enterobacteriaceae* - *Escherichia* sp. - *Vibro* sp.	*Burkholderiaceae* - *Burkholderia* sp.	*Bacillaceae* - *Bacillus* sp.	*Bacteroidaceae* - *Bacteriod* sp.

4.3. Biochemical Test

Based on the conducted biochemical tests, it can be concluded that the isolation area for Tuang river (Point 1), the isolates are belongs to *Enterobacteriaceae* family which involving three types of bacteria species which are *Escherichia* sp., *Vibro* sp. and *Klebsiella* sp.; *Pseudomonadaceae* and *Lactobacillaceae* family. As for point 2 for Tuang river, the isolated strains can be assumed from the *Enterobacteriaceae* family which involving three types of bacteria species*; Klebsiella* sp., *Citobacter* sp. and *Escherichia* sp. and *Bacillaceae* family.

Meanwhile for Melaka river (Point A), The strains belong to *Enterobacteriaceae, Bacillaceae, Lactobacillaceae* and *Brucellaceae* family. For Melaka river (Point B), the isolated bacteria belongs to *Bacillaceae* family (such as *Bacillus* sp.), *Burkholderiaceae, Lactobacillaceae* and *Bacteriodaceae* family (such as *Bacteriod* sp.).

5. Conclusions

The emergences of antibiotic resistance study indicate there is antibiotic contamination in Tuang River and Melaka River. Out of 13 isolates, 12 of them show MAR Index more that 20% indicate there are antibiotic resistance bacteria in both area.

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Isolation and Characterization of Antibiotic Resistant Bacteria from Rivers in Negeri Sembilan

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Abstract: The antibiotic utilization in the environment may have escalated the occurrence of antibiotic resistant bacteria in the aquatic environment. The emergence of antibiotic resistant bacteria in the aquatic environment especially rivers increases the health risks associated with the waterborne bacteria in animals and humans that have becoming a worldwide concern nowadays. The mostly considered hot spot for becoming the widespread of the antibiotic resistance is the aquatic environment due to pollution with emerging antibiotics as the contaminants that were derived from the human activities. In this study, a total of 35 bacterial isolates were isolated from the water samples collected from point sources (near to residential areas) and from the non-point sources (soil leaching waters from agricultural and poultry farms) from Linggi and Jempol River, Negeri Sembilan. The bacterial isolates were tested for their susceptibility towards 6 antibiotics; gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin respectively by using disk diffusion method. From the test, 45.7% of the bacterial isolates were antibiotic resistant (AR) that were resistant to at least one antibiotic and another 54.3% were multiple antibiotic resistant (MAR) that were resistant to at least two antibiotics. Hence, a multiple antibiotic resistant (MAR) index was calculated for each water samples and 5 bacterial isolates that have the MAR index for more than 50% were selected for further identification by 16S rRNA sequencing. The bacteria identified were *Ralstonia pickettii*, *Ochrobactrum ciceri* and *Staphylococcus kloosii*. Based on this study, the existence of antibiotic resistant bacteria in the rivers revealed that the antibiotic contamination was indeed occurred in the Linggi River and Jempol River, Negeri Sembilan and their dispersion in the nature may elevate serious threats toward both public and environmental health.

Keywords: Antibiotic resistance bacteria; Multiple antibiotics resistant; River waters; Disk diffusion method

1. Introduction

The occurrence of antibiotic resistance in the worldwide has been caused by the over usage of the antibiotic agents (Al-Bahry *et al*., 2015). In few years back, the issue of antibiotic contamination has been recognized as a threatening environmental pollution towards the aquatic environments like the rivers due to their possible negative effects on the human health and also the ecosystem (Dada *et al*., 2012). The continuous exposure of the bacteria that present in the aquatic environment towards the antibiotic agents will potentially causes the bacteria to become resistant towards the antibiotics (Marti *et al*., 2014). Most of the antibiotics used for treating any infections caused by bacteria in humans, plants, and livestock animals are being released into the surrounding environment via numerous pathways, including human waste, discharge from the wastewater effluent, and the agricultural runoff from farming land or the livestock animal farming site (Tahrani *et al*., 2015).

The massive outburst of the antibiotic resistant phenotypes in animals and human pathogens, from the evolutionary perspective, it has become a very recent occasion that resulting in the usage of antibiotics in the veterinary and clinical medicine, aquaculture, horticulture, agriculture, and other human activities that followed by the large scale production of antibiotics for these purposes (Aminov, 2009). However, the uncontrolled use of antibiotics for human and animals are resulting in the increase in antibiotic resistance bacteria in the environment especially the rivers (Ho *et al*., 2014). This shows that human greatly contribute to the increasing proportion of resistance bacteria in the environment that lead to higher number of resistance bacteria occur in polluted habitat rather than in unpolluted habitat (Garcia-Armisen *et al*., 2013).

Humans do greatly contribute to the increase in the antimicrobial resistance, but the main factor of the risk for the problem is the result of the extensive usage of antibiotics which lead to the presence and spreading of the antibiotic resistant bacteria in humans and animals (Cantón, 2009). One of the most commonly used antibiotic is the β-lactam antibiotics due to the various positive attributes that it possesses as it shows low effects in toxicity and effective to the consumers (Marta Tacão, 2012). It was discovered that the antibiotics that ended up in the

wastewater stream are coming from the urine and feces. This is because the main route for the excretion of the antibiotics consumed by the human and also animal is by the secretion into the wastes (Páll *et al*., 2013). These wastes will end up in the sewage and the effluents from the sewage treatment plants that flows into the rivers can be considered as the most crucial source of the emergence of the antibiotic resistant bacteria in the environment (Al-Gheethi *et al*., 2013).

2. Materials and Methods

2.1. Study site and sampling

The sampling process took place at the two selected rivers in Negeri Sembilan which were Linggi River and Jempol River. The sampling sites chosen from the Linggi River was located near to a palm plantation area, a poultry farm and a recreational area in Bukit Putus at the coordinate of (2.720111, 102.022564) (Point 1) and the other site was at the residential and industrial area near to Taman Pinggiran Senawang at the coordinate of (2.657238, 101.994087) (Point 2). The Linggi River flows to Seremban and ended up at the Selat Melaka.

In a meanwhile, another river that had been selected was Jempol River that was located at Batu Kikir and Bahau, Jempol. The first sampling site selected from the Jempol River was located near to the residential area in Kampung Lonek in Bahau, Jempol at the coordinate of (2.825212, 102.331082) (Point 3) while the second sampling site from Jempol River was located at Batu Kikir, Jempol. That sampling site was located near to the palm plantation area at the coordinate of (2.851562, 102.302156) (Point 4). The Jempol River eventually met with the Muar River and ended up at Tanjung Emas, Muar, Johor. An amount of 500 mL of water sample were taken from surface water of both rivers at two different places each. The water samples collected in sterile glass bottles and the samples then were put in an ice box to maintaining its temperature at 4 °C while transferring to laboratory and stored in the cold room until further analysis.

2.2. Isolation of pure bacterial isolates

From the water samples collected, 1 mL from each samples were diluted with 9 mL of distilled water. A series of serial dilution of water sample were prepared. Then, 1 mL of the diluted water samples were spread on the nutrient agar. The plates then were incubated at 37 °C for 24 hours. The single colonies of different morphology from the spread plates were streaked for subcultures for obtaining the pore colonies of the bacterial isolates. The subcultures of the bacterial pure colonies isolated were characterized based on their morphological characteristics and Gram staining was performed to further characterize them into Gram-positive and Gram-negative bacteria (Barile, 2012).

2.3. Antibiotic susceptibility testing

The bacterial isolates were tested for the antibiotic susceptibility testing by using the standard Kirby-Bauer disk diffusion method in order to determine the antibiotic susceptibility profiles of the bacterial isolates. The antibiotic susceptibility testing was performed by using Mueller-Hinton agar against six antibiotics; gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloaxin. Each of the antibiotics disk had two different concentrations which were 10 and 30 µg/mL per disk. The bacterial inoculums were prepared by suspending the freshly grow bacterial isolates in 4 to 5 ml of sterile nutrient broth. The turbidity of the broth was adjusted to 0.5 McFarland standards to be spread on Mueller-Hinton agar plates using sterile cotton swab. The different antibiotic impregnated disks were placed over the freshly prepared agar that already had bacterial cultures on it. All the plates were the incubated at 37 °C for 24 hours (Hudzicki, 2009). The zone of inhibition were measured and compared with Clinical and Laboratory Standard Institutes (CLSI) guidelines in order to be classify as resistant, intermediate or sensitive to the antibiotics (Institute, 2018). A multiple antibiotic resistant (MAR) index was calculated for each of the bacteria and the antibiotic resistant bacteria that have the MAR index for more than 50% were selected to be further analyzed.

2.4. Characterization of the bacterial isolates by biochemical test

The antibiotic resistant bacteria were further characterized by the biochemical tests since that bacterial physiology are differs from one species to another. The biochemical tests used to determine the biochemical characteristics of bacteria were catalase, oxidase, indol, MacConkey agar, urease, mannitol salt agar, and oxidase test. The biochemical test results were compared to Bergey's Manual of Determinative Bacteriology to predict the possible bacteria species based on the biochemical activities of the bacteria (Holt *et al*., 1989; Vos *et al*., 2011).

2.5. MAR Index

The Multiple Index Resistance (MAR) index values for the isolates were determined by dividing the numbers of antibiotics to which the isolates is resistant by the total of antibiotics tested (Nurhazlin *et al*., 2014).

$$MAR\ Index\ value = a/b \times 100 \qquad \text{Equation 1}$$

If the MAR index value of isolate is more than 50%, it indicates high risk of contamination by antibiotics in area of selected rivers in the area of sampling.

3. Results

3.1. Isolation and identification of the isolated bacteria

From the samples, 35 pure colonies were managed to be isolated from the samples from both of the rivers. From Linggi river, 7 and 12 pure colonies were isolated at Point 1 and Point 2, respectively. Meanwhile, from Jempol river, 10 and 6 pure colonies were successfully isolated from Point 3 and Point 4, respectively. Table 1 shows the morphology characteristics of the bacterial colonies isolated from Linggi and Jempol River and Table 2 shows the bacterial colonies isolated from both Linggi and Jempol River with the Gram stains viewed under 100X magnification.

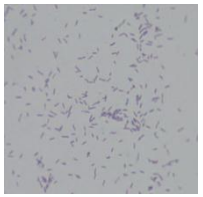
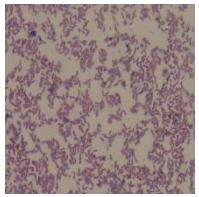
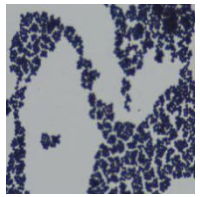
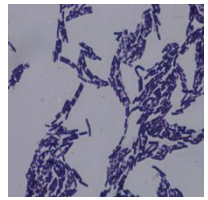
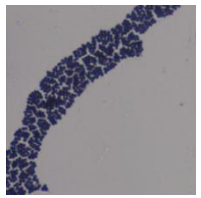
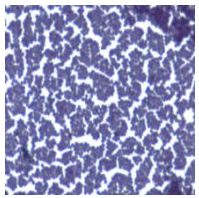
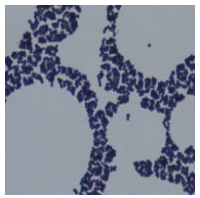
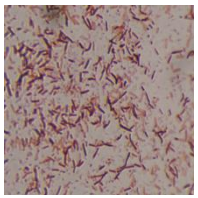
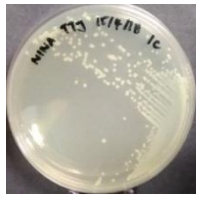
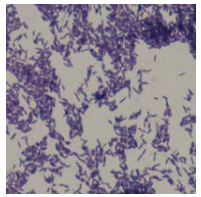
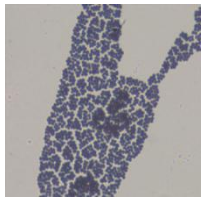
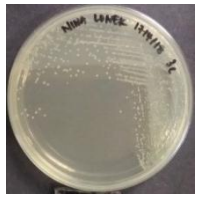
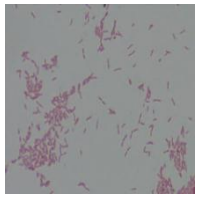
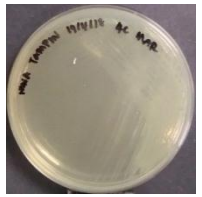
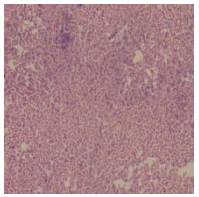
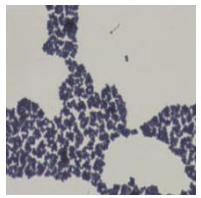
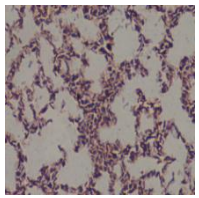
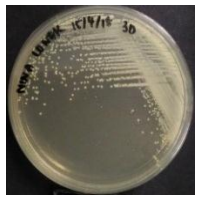
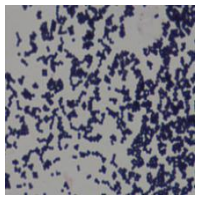
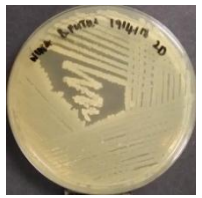
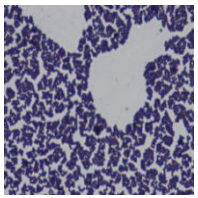
3.2. Antibiotic Susceptibility Testing by using Disk Diffusion Method

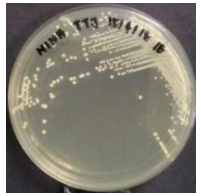
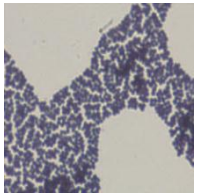
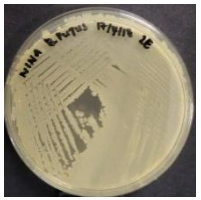
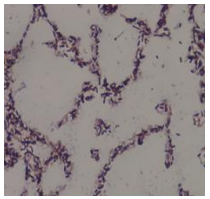
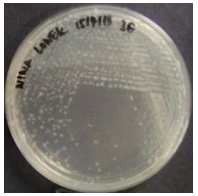
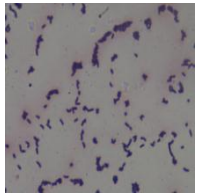
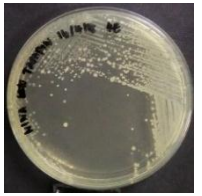
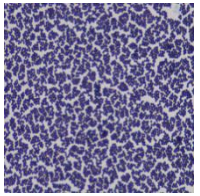
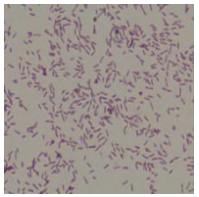
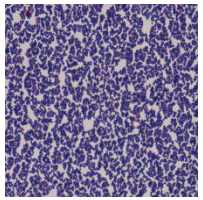
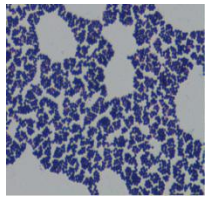
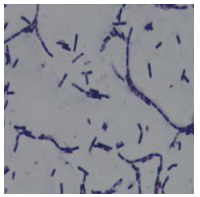
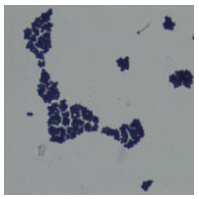
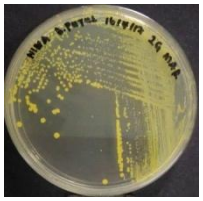
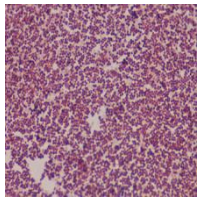
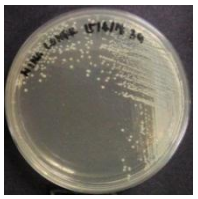
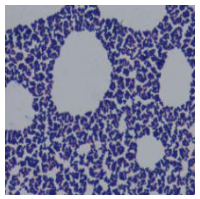
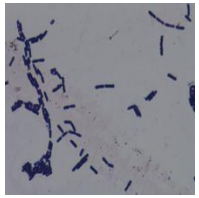
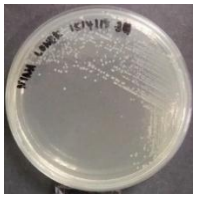
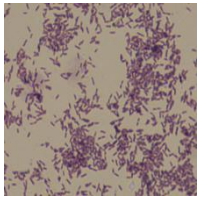
The result for the antibiotic susceptibility tests were as listed in Table 3 and Table 4. From the results, the most antibiotics that the isolated bacteria show resistance at least once are gentamicin, ampicillin, chloramphenicol, and tetracycline used. However, for rifampicin, only a few of the bacterial isolates from Linggi River showed resistance towards at least one of the concentration used whereas none of the bacterial isolates from Jempol River were resistant towards rifampicin. Meanwhile, none of the bacterial colonies isolated from both Linggi and Jempol River showed any resistance towards both of the concentrations of ciprofloxacin used. It shows that no contamination caused by the ciprofloxacin from both rivers.

Table 1: The morphology characteristics of bacterial colonies isolated from Linggi and Jempol River

Linggi River						Jempol River					
	Opacity	**Form**	**Elevation**	**Surface**	**Color**		**Opacity**	**Form**	**Elevation**	**Surface**	**Color**
Point 1						**Point 3**					
1A	Translucent	Circular	Flat	Smooth, glistening	Cream	**3A**	Opaque	Circular	Convex	Smooth, glistening	White
1B	Opaque	Circular	Raised	Smooth, glistening	White	**3B**	Opaque	Circular	Raised	Smooth, glistening	White
1C	Translucent	Circular	Flat	Smooth, glistening	Cream	**3C**	Translucent	Circular	Raised	Smooth, glistening	Cream
1D	Opaque	Circular	Raised	Smooth, glistening	White	**3D**	Opaque	Circular	Raised	Smooth, glistening	White
1E	Opaque	Circular	Raised	Smooth	White	**3E**	Translucent	Circular	Flat	Smooth	Cream
1F	Translucent	Circular	Flat	Smooth, glistening	Cream	**3F**	Opaque	Circular	Flat	Smooth	White
1G	Opaque	Circular	Raised	Smooth, glistening	White	**3G**	Opaque	Circular	Flat	Smooth, glistening	White
						3H	Translucent	Circular	Raised	Smooth, glistening	Cream
Point 2						**Point 4**					
2A	Opaque	Irregular	Flat	Rough	Whitish	**4A**	Translucent	Circular	Raised	Smooth, glistening	Cream
2B	Opaque	Small, circular	Raised	Smooth	Whitish	**4B**	Opaque	Circular	Raised	Smooth	Yellow
2C	Opaque	Circular	Convex	Smooth	White	**4C**	Translucent	Circular	Raised	Smooth, glistening	Cream
2D	Opaque	Circular	Umbonate	Rough	Whitish	**4D**	Opaque	Irregular	Umbonate	Rough	Whitish
2E	Opaque	Irregular	Crateriform	Rough	Whitish	**4E**	Translucent	Circular	Flat	Smooth	Whitish
2F	Opaque	Circular	Flat	Smooth	Whitish yellow	**4F**	Opaque	Irregular	Umbonate	Rough	Whitish
2G	Opaque	Circular	Convex	Smooth	Bright yellow						
2H	Translucent	Irregular	Flat	Rough	Whitish						
2I	Opaque	Circular	Convex	Smooth, glistening	White						
2J	Opaque	Irregular	Umbonate	Glistening	Whitish						
2K	Opaque	Circular	Convex	Smooth, glistening	White						
2L	Opaque	Circular	Flat	Smooth, glistening	Yellowish						

Table 2: The bacterial colonies isolated from both Linggi and Jempol River with the Gram stains viewed under 100X magnification

	Linggi River					Jempol River				
	Point 1			Point 2			Point 3			Point 4	
	Strain	Gram Stain		Strain	Gram Stain		Strain	Gram Stain		Strain	Gram Stain
1A			2A			3A			4A		
1B			2B			3B			4B		
1C			2C			3C			4C		
1D			2D			3D			4D		

1E			**2E**			**3E**			**4E**		
1F			**2F**			**3F**			**4F**		
1G			**2G**			**3G**					
			2H			**3H**					

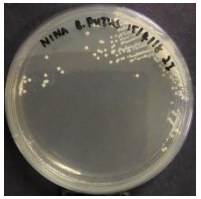
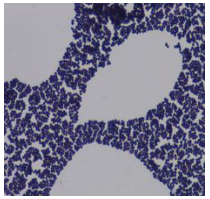
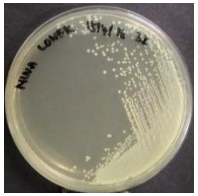
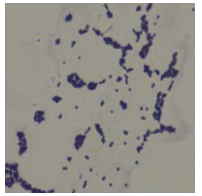
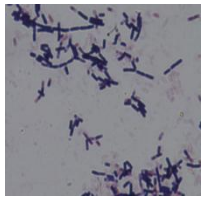
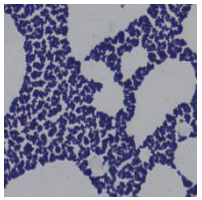
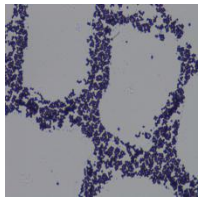
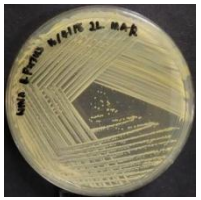
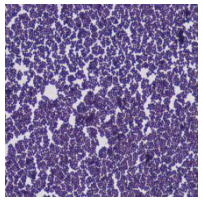
		2I			**3I**			
		2J			**3J**			
		2K						
		2L						

Table 3: Antibiotic susceptibility tests for the bacterial colonies isolated from Point 1 and Point 2 at Linggi River

		Antibiotics	Gentamicin		Ampicillin		Rifampicin		Chloramphenicol		Tetracycline		Ciprofloxacin	
		Bacterial Colonies	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Linggi River	**Point 1**	1A	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		1B	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		1C	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
		1D	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**
		1E	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		1F	**R**	**R**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**S**
		1G	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
	Point 2	2A	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**
		2B	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		2C	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**
		2D	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**
		2E	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		2F	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**
		2G	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**S**	**S**
		2H	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**
		2I	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		2J	**R**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		2K	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
		2L	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**S**	**S**

Table 4: Antibiotic susceptibility tests for the bacterial colonies isolated from Point 3 and Point 4 at Jempol River

		Antibiotics	Gentamicin		Ampicillin		Rifampicin		Chloramphenicol		Tetracycline		Ciprofloxacin	
		Bacterial Colonies	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Jempol River	**Point 3**	3A	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		3B	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		3C	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		3D	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**
		3E	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		3F	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**S**
		3G	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
		3H	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**
		3I	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		3J	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
	Point 4	4A	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		4B	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		4C	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		4D	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
		4E	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
		4F	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**

3.3. MAR Index Analysis

From Figure 1 a), 16 isolates from Linggi river show MAR Index more that 20%. Meanwhile for Jempol river, 11 isolated out 16 isolates show MAR Index more that 20%. Based on the results, 45.7% of the bacterial isolates are antibiotic resistant (AR) in which they are resistant to at least one antibiotic and another 54.3% were multiple antibiotic resistant (MAR) that are resistant to at least two antibiotics.

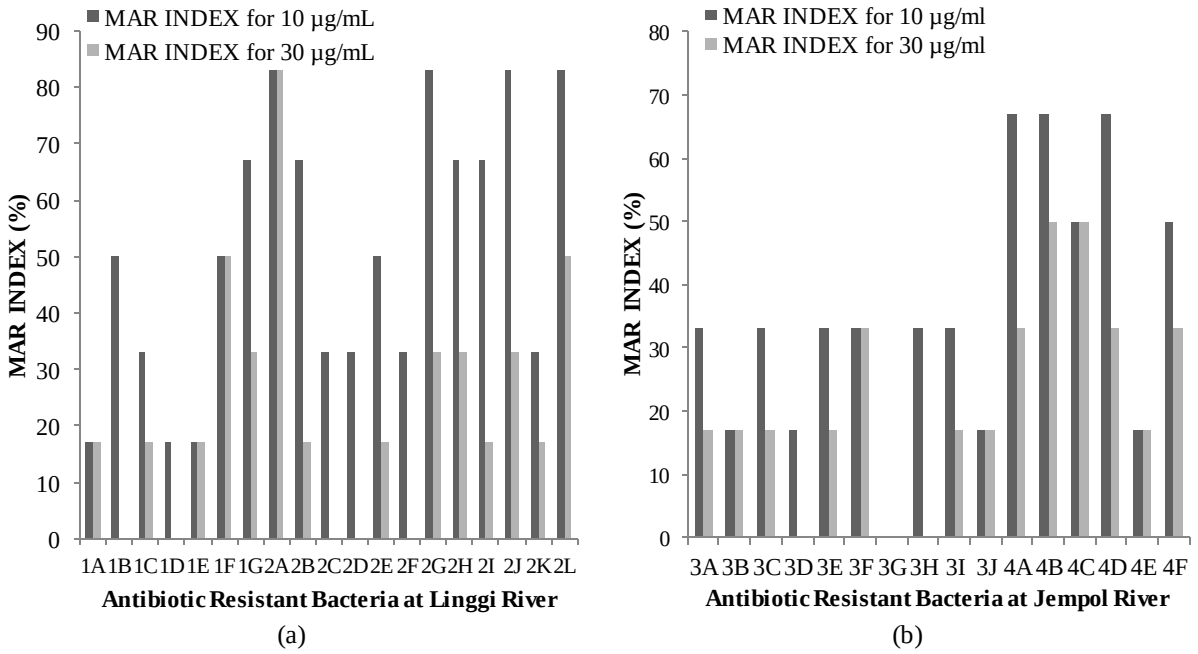


Figure 1: MAR Index for bacteria isolated from (a) Linggi river and (b) Jempol river

3.4. Biochemical test

Table 5 tabulates the biochemical tests conducted for all the isolates collected from Linggi and Jempol rivers. Based on the table, it can be concluded that the population for different point in the same river can be different. This may be due to the human activities at the around the sampling areas.

4. Discussion

Linggi River and Jempol River were chosen in this study because Linggi River is a major river in Negeri Sembilan and it serves as the most important water resources in the west coast area of Peninsular Malaysia (Daneshmand *et al*., 2011) while Jempol River was near to the urban and agricultural areas. The combination of rapid growth in human population added by the blooming of the industrial and urban development in the last few decades had resulted in a severe downturn in the quality of water in the downstream area.

From Point 1 (Linggi River), 2 colonies are Gram-positive rod bacteria, 4 colonies are Gram-positive cocci, and only 1 colony is Gram-negative rod bacteria. As for Point 2 (Linggi River), 2 colonies are Gram-negative rod bacteria, 1 colony is Gram-negative coccus, 6 colonies are Gram-positive cocci, and 3 colonies are Gram-positive rod bacteria. For Jempol river, Point 3 Jempol River, 7 of them are Gram-positive cocci, 2 colonies are Gram-negative rod bacteria, and only 1 colony is Gram-positive rod bacteria. Whereas in Point 4 (Jempol River), there 2 colonies are Gram-positive rod bacteria, 2 colonies are Gram-negative rod bacteria, and another 2 colonies are Gram-positive cocci.

The major pollutants included industrial wastewater, domestic sewage, agricultural and urban runoff, and livestock discharge that flowed into the river (Daneshmand *et al*., 2011; Nather Khan and Begham, 2012). In this study, the samples obtained from this river were collected near to a chicken poultry farm, a palm plantation estates and also from the location that were close to the residential area. Our findings show clear evidence of antibiotic resistant bacteria (ARB) emergence at the sampling locations.

Table 5: Biochemical test conducted for samples from Linggi and Jempol river

Linggi River – Point 1

Strain	Catalase	Citrate	MacConkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar	Conclusion (Family Type)
1A	-	-	+	-	-	+	-	*Enterobacter* sp. *Vibrio* sp. *Klebsiella* sp.
1B	+	-	+	-	+	-	-	*Enterobacter* sp. *Pseudomonas* sp. *Klebsiella* sp.
1C	-	+	+	-	-	+	-	*Proteus* sp. *Klebsiella* sp. *Vibrio* sp.
1D	+	-	+	-	+	-	+	*Staphylococcus* sp. *Proteus* sp. *Klebsiella* sp.

Linggi River – Point 2

Strain	Catalase	Citrate	MacConkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar	Conclusion (Family Type)
2A	+	+	-	-	+	+	-	*Enterobacter* sp. *Vibrio* sp. *Proteus* sp.
2B	+	-	+	-	+	-	+	*Enterobacter* sp. *Staphylococcus* sp. *Proteus* sp.
2C	+	-	+	-	+	-	+	*Staphylococcus* sp. *Klebsiella* sp. *Proteus* sp.
2D	+	+	-	-	-	+	+	*Bacillus* sp. *Vibrio* sp. *Klebsiella* sp.

Jempol River – Point 3

Strain	Catalase	Citrate	MacConkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar	Conclusion (Family Type)
3A	+	-	+	-	+	-	+	*Staphylococcus* sp. *Proteus* sp. *Klebsiella* sp.
3B	+	-	-	-	+	-	+	*Staphylococcus* sp. *Proteus* sp. *Klebsiella* sp.
3C	-	-	+	-	-	+	-	*Enterobacter* sp. *Vibrio* sp. *Klebsiella* sp.
3D	+	-	+	-	+	-	-	*Enterobacter* sp. *Proteus* sp. *Klebsiella* sp.

Jempol River – Point 4

Strain	Catalase	Citrate	MacConkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar	Conclusion (Family Type)
4A	+	+	-	-	-	-	+	*Klebsiella* sp. *Enterobacter* sp. *Pseudo-monas* sp.
4B	+	-	+	-	+	+	-	*Klebsiella* sp. *Proteus* sp. *Vibrio* sp.
4C	+	+	+	-	-	+	-	*Pseudomonas* sp. *Vibrio* sp. *Proteus* sp.
4D	+	-	-	-	-	+	+	*Staphylococcus* sp. *Klebsiella* sp. *Pseudomonas* sp.

1E	+	-	+	-	+	-	+	*Enterobacter* sp. *Proteus* sp. *Klebsiella* sp.	2E	+	+	-	-	-	+	+	*Enterobacter* sp. *Klebsiella* sp. *Bacillus* sp.	3E	+	-	+	-	-	+	-	*Pseudomonas* sp. *Klebsiella* sp. *Bacillus* sp.	4E	+	-	-	-	-	-	+	*Staphylococcus* sp. *Klebsiella* sp. *Pseudomonas* sp.
1F	-	+	+	-	-	+	-	*Bacillus* sp. *Klebsiella* sp. *Vibrio* sp.	2F	+	-	+	-	+	-	+	*Staphylococcus* sp. *Proteus* sp. *Klebsiella* sp.	3F	+	-	-	-	-	-	-	*Pseudomonas* sp. *Klebsiella* sp. *Staphylococcus* sp.	4F	+	+	+	-	-	-	-	*Klebsiella* sp. *Enterobacter* sp. *Proteus* sp.
1G	-	-	+	-	+	-	+	*Enterobacter* sp. *Klebsiella* sp. *Staphylococcus* sp.	2G	+	-	+	-	+	+	-	*Klebsiella* sp. *Proteus* sp. *Vibrio* sp.	3G	+	-	-	-	-	-	+	*Staphylococcus* sp. *Klebsiella* sp.								
									2H	+	-	-	-	+	+	-	*Enterobacter* sp. *Pseudomonas* sp. *Proteus* sp.	3H	+	-	+	-	+	+	+	*Bacillus* sp. *Enterobacter* sp. *Proteus* sp.								
									2I	+	-	-	-	+	-	-	*Staphylococcus* sp. *Klebsiella* sp. *Proteus* sp.	3I	+	-	-	-	-	-	+	*Staphylococcus* sp. *Klebsiella* sp. *Pseudomonas* sp.								
									2J	-	+	+	-	-	+	-	*Salmonella* sp. *Vibrio* sp. *Bacillus* sp.	3J	+	-	+	-	-	-	+	*Enterobacter* sp. *Staphylococcus* sp. *Klebsiella* sp.								
									2K	+	-	+	-	+	-	+	*Staphylococcus* sp. *Klebsiella* sp. *Proteus* sp.																			

5. Conclusions

The study was conducted in 2 rivers; Linggi and Jempol river in Negeri Sembilan. Samples were taken from two points from each rivers. From Linggi river, 7 and 12 isolates were successfully isolated from Point 1 and Point 2, respectively. As for Jempol river, 10 and 6 isolates were obtained from Point 3 and Point 4, respectively. The isolates were tested for its susceptibility towards 6 antibiotics; gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin. MAR Index revealed, 16 isolates from Linggi river show MAR Index more that 20%. Meanwhile for Jempol river, 11 isolated out 16 isolates show MAR Index more that 20%.

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Study on the Emergence of Antibiotic Resistance Bacteria from Rivers in Kuala Lumpur

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Abstract: Currently dispersion of antibiotic resistance bacteria has elevated in the environment which set-up a serious worldwide impact in both environment and public health. The purpose of this study was to determine the presence of antibiotic resistance bacteria from rivers in Kuala Lumpur, Malaysia which is Batu River and Gombak River. Water samples were collected from two different points of both rivers respectively. Isolated bacteria were tested for resistance against six different antibiotics (ampicillin, chloramphenicol, ciproflaxin, gentamicin, tetracycline and rifampicin) using antibiotic susceptible testing by disc diffusion method. From both of the rivers, the greatest frequency of resistances in isolates was against chloramphenicol and ampicillin but less often in rifampicin, gentamicin and tetracycline. However, there is zero resistance of the bacteria isolates against ciproflaxin. Hence, most of the bacteria show Multiple Antibiotic Resistance (MAR) index value more than 20% which indicates the sampling areas are in high risk with antibiotic resistant bacteria contamination. Seven over forty-one isolates that shows MAR index value more than 50% were further identified by performing 16S rRNA polymerase chain reaction, PCR. Those isolates that showed resistance to most antibiotics were identified as *Pseudomonas* sp., *Acinetobacter baumannii* sp., *Pseudomonas putida* sp. and *Uncultured Ralstonia* sp.. From this study, it reveals the existence of antibiotic resistance bacteria which also proves the antibiotic contamination in the rivers (Batu River and Gombak River), Kuala Lumpur and dispersion of these kinds of bacteria in environment could lead to serious threat to agriculture, human and animal population.

Keywords: Antibiotic resistance bacteria; River; Kuala Lumpur

1. Introduction

Spreading and accumulation of antibiotic resistance bacteria in the ecosystem such as rivers are becoming major concern of most human nowadays. Independent evolution of bacteria with very short generation time and wide diversity, able to segregate within the natural environment widely. Previous cases show that rivers are mainly contaminated either by antibiotic resistance coliform groups of bacteria, *E. coli* and also treated wastewater effluent. In addition, the antibiotic resistant bacteria are emerged from soil organisms, run-off from animal feed or crops, and even waste products from treated animals or human (Hershberg, 2017).

Either antibiotic resistance bacteria formed naturally or anthropogenic origin. Its presence in the water surfaces will definitely bring harm to human health. Humans become infected by antibiotic resistance bacteria through aquatic products, drinking water supply or either by direct connection with the water environment (Lu *et al*., 2010). Regarding the issue on emergence of antibiotic resistant bacteria (ARB) in developing areas, and there is no any previous research related to the condition of the rivers in Kuala Lumpur particularly. Information's that will be obtained from the current study about ARB in rivers of Kuala Lumpur would be really useful for future references. This study focused on the identification and characterization of the isolated bacterial samples from two rivers, Batu river and Gombak river in Kuala Lumpur by performing Gram staining and few biochemical test. Antibiotic susceptibility tests were carried out using six; antibiotics ampicillin, chloramphenicol, ciproflaxin, gentamicin, tetracycline and rifampicin in search for multi-drug resistance bacteria.

2. Materials and Methods

2.1. Study site and sampling

In this study, two sampling sites were selected within the city of Kuala Lumpur. First site is Batu River, a river that flows along Kuala Lumpur city by passing through several former mining and residential area. Two sampling points from the river were selected. The first point was located beside AEON Big Riverwalk Village,

with coordinate (point A) GPS: 3.178345, 101.682919, while the other sampling area was at Kem Batu Kentonmen, (point B) GPS: 3.197522, 101.678123. Second sampling river was along the Gombak River; GPS: 3.170147, 101.695182 (point C) and GPS: 3.170836, 101.686803 (point D). About 500 mL of the river water samples were taken at two different points along both of the rivers. Sterile glass bottles with different labels had been used to collect the water sample and placed in an ice box for a longer storage. This is to maintain the samples at 4 °C while transferring in to laboratory and store it in the cold room until further usage (Al-Badaii and Shuhaimi-Othman, 2015).

2.2. Isolation of pure bacterial strains

Water samples from both of the rivers were diluted by performing ten fold serial dilution. Serial dilution was done by diluting 1ml of the water sample with 9 mL of sterile distilled water. 1 mL of each of the diluted water sample with the dilution factor 10^{-3}, 10^{-5} and 10^{-7} was spread plated on the nutrient agar (NA). Each dilution factors were being triplicated to minimize error. The plates, then was incubated at 37 °C for 24 hours. After the incubation period, from each plate just one or two single colony with similar morphology was picked. This was done in order prevent redundancy of the similar strain and sub-cultured few times in order to obtain pure colony. The pure colony obtained from sub-culture, was stored as stock culture in glycerol at -80 °C (Tissera and Lee, 2013).

2.3. Antibiotic susceptible test

Isolated pure bacteria colony was tested for their sensitivity and resistance pattern against several antibiotic by using the Kirby-Bauer method. A single pure colony obtained was re-dissolved in normal saline solution until its turbidity reaches 0.5 standards of McFarland. The bacteria suspension was then plated on the Muller Hinton agar by using sterile cotton swab. On the Muller Hinton agar plate, different types of antibiotic discs with two different concentrations which is 10 µg and 30 µg for each antibiotic were placed a part away from each other and followed by incubating it at 37 °C/aerobic condition for about 18 – 24 hours. The zone inhibition of the antibiotics around the discs was observed after the incubation period. The bacteria was classified as antibiotic resistance or not by referring to standards of National Committee for Clinical and Laboratory (NCCLS 1999) (Al-Badaii and Shuhaimi-Othman, 2015). Following were the antibiotics and their concentration respectively that was used in this test, ampicillin (10 µg and 30 µg), chloramphenicol (10 µg and 30 µg), ciproflaxin (10 µg and 30 µg), gentamycin (10 µg and 30 µg), rifampicin (10 µg and 30 µg) and tetracycline (10 µg and 30 µg). These particular antibiotics were chosen based on the importance of it in treating disease and infections of animal and human caused bacteria as well as their crucial usage in animal farms as additives in animal feeds to enhance growth and also their usage as representative of different classes of antibiotic in agricultural areas (Literak *et al*., 2010).

2.4. Multiple Antibiotic Resistance (MAR)

Multiple Antibiotic Resistance index values of the susceptible isolates were calculated by dividing the total number of antibiotics which the bacteria isolates were resistant to with the total number of antibiotics that been tested (Riaz *et al*., 2011). The respective formula is as below:

$$\textbf{\textit{Multiple Antibiotic Resistance Index Value}} = \frac{\boldsymbol{a}}{\boldsymbol{b}} \times \mathbf{100}\%$$

Where (a) indicates the total number of antibiotics that the isolates resistant to, while (b) indicates the total number antibiotics that been tested with. If the index value is more than 20%, it shows that the area of isolation is in high possibly risk of contamination by several antibiotics (Matyar, 2012).

2.5. Characterization of bacteria

Antibiotic resistant bacteria with more than 50% MAR index value were proceeded into characterization. Characterization of the isolated antibiotic resistance bacteria colony was being determined by performing several tests, specifically Gram staining and biochemical tests such as urease test, oxidase test, MacConkey, citrate test, mannitol salt agar, indol production and catalase test. Pure colony obtained from the isolation was Gram stained in order to determine whether the respective strain is Gram-positive or negative bacteria. Further, seven biochemical test was carried out to determine the differential biochemical characteristics of the isolated bacteria. All of the biochemical tests that was been carried out according to the method provided by Bergeys Manual of Determinative Bacteriology (Buchanan *et al*., 1994).

3. Results and Discussion

3.1. Isolation of the antibiotic resistance bacteria from rivers (Batu River and Gombak River) in Kuala Lumpur

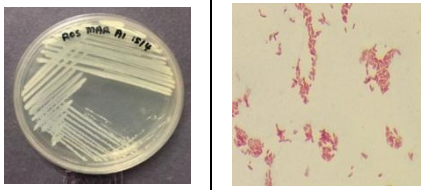
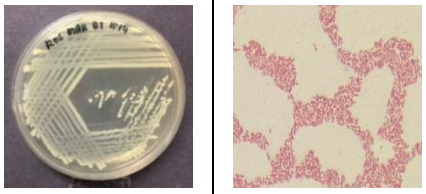
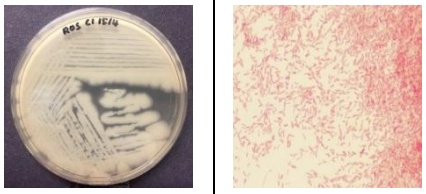
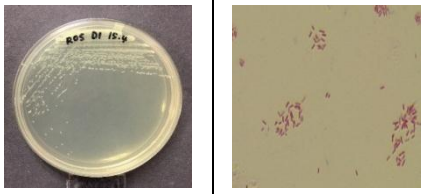
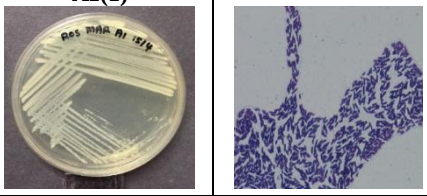
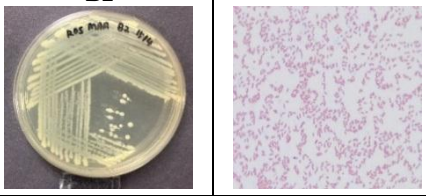
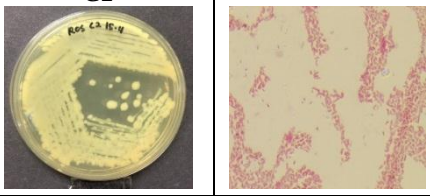
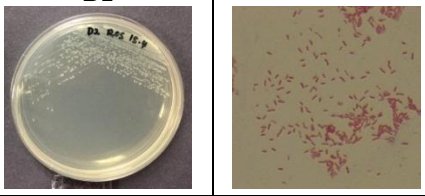
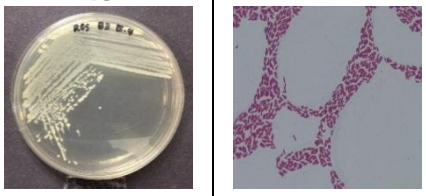
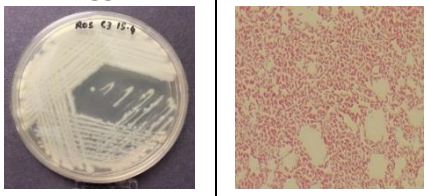
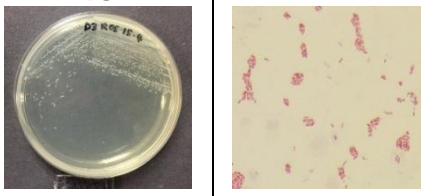
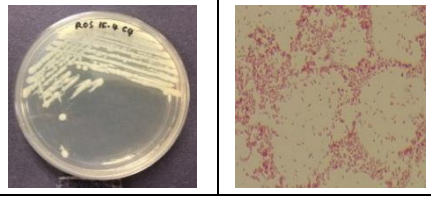
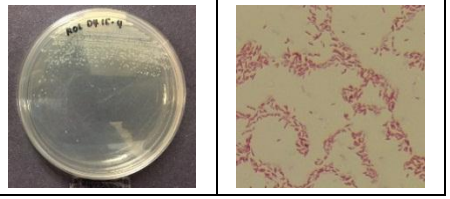
Water samples from both rivers were diluted with three different dilution factors. After dilution of each factor, a loop of bacteria suspension been spread as triplicate on nutrient agar plates, this is to determine the total amount of bacteria number per sample and minimize error that may arises (Tao *et al.*, 2010). From the triplicates, only one plate which has the best morphological characteristic was chosen to be sub-cultured. The bacteria colony has been sub-cultured repeatedly to obtain pure single colony of specific bacteria.

Table 1 shows the results of some pure bacteria isolates with variety in their morphological characteristics including different shapes and colors. 68% from the overall isolates which is 28/41 isolates from both rivers shows the property of the isolates as Gram-negative and bacilli shaped. Least of the isolates, which is only 1% are Gram-negative and cocci shaped. From total 41 isolates, only 9 of them were Gram-positive and cocci shaped. As majority of the isolates are Gram-negative, it indicates that the sampling area were contaminated with pathogenic bacteria that could harm human (Wang and Quinn, 2010).

Detailed morphological characteristics of each isolates from both rivers were characterized based on the aspects of different shape, size, elevation and also appearances as tabulated in the Table 2 (Cowan and Steel, 2003). This shows that there might be chances that those isolates with similar properties are similar strain. In order to avoid redundancy of similar bacterial isolate, few biochemical tests were carried out. Those isolates with similar characteristics will be eliminated, which is necessary to avoid any wastage of chemicals (Tao *et al.*, 2010). The strains were characterized based on their different opacity, form, elevation, surface and colony color.

The results from the characterization based on the morphology show the isolates opacity are opaque and translucent only. Most of the isolates form both rivers are circular in shape and raised in elevation. The identification also shows that most of the bacteria are smooth, glistering and yellow in colour. From all of the bacterial isolates that been obtained, it is expected that most of them are Gram-negative or even from *Enterobacteriaceae* sp.. This is because previous studies show that majority of the bacteria that been isolated from those kind of rivers that located in the urban area mostly contains *Enterobacteriaceae* sp. and shows negative property in Gram staining, which are toxic and pathogenic (Tao *et al.*, 2010; Tissera and Lee, 2013).

Table 1: Some of the isolated bacteria strains from Sungai Batu and Sungai Gombak with Gram stains viewed under 100X magnification

Gombak River				**Batu River**			
Point A		**Point B**		**Point A**		**Point B**	
Strain	**Gram Stain**	**Strain**	**Gram Stain**	**Strain**	**Gram Stain**	**Strain**	**Gram Stain**
A1		**B1**		**C1**		**D1**	
A1(1)		**B2**		**C2**		**D2**	
A2		**B3**		**C3**		**D3**	
A3		**B4**		**C4**		**D4**	

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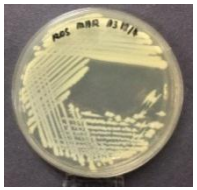
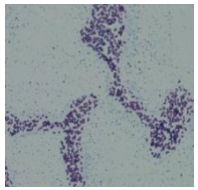
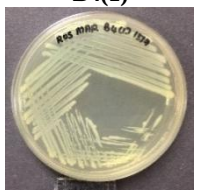
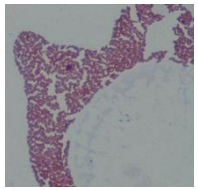
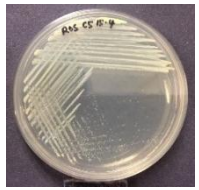
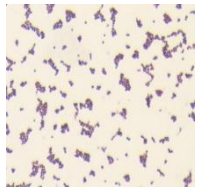
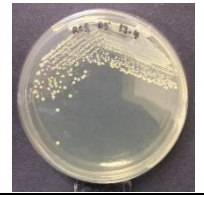
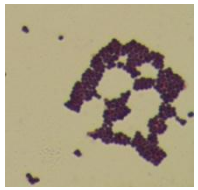
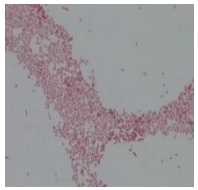
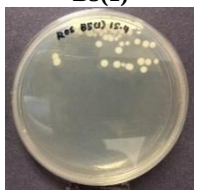
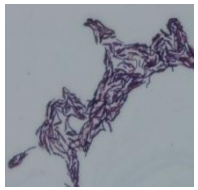
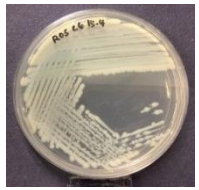
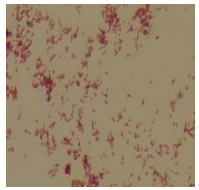
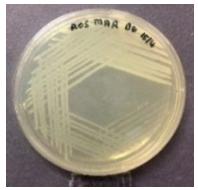
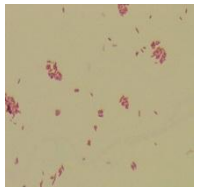
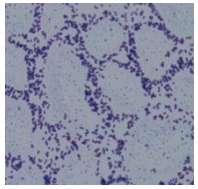
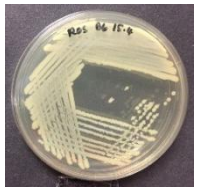
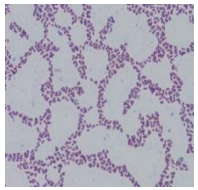
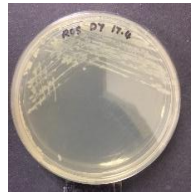
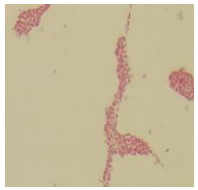
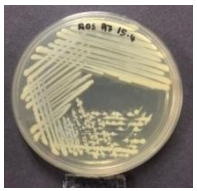
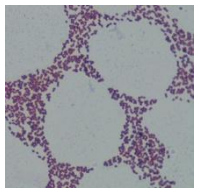
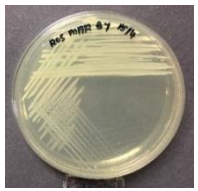
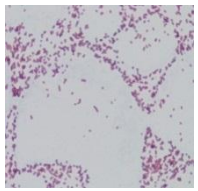
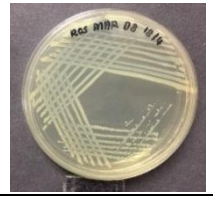
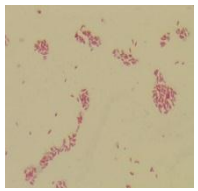
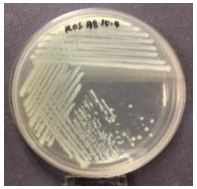
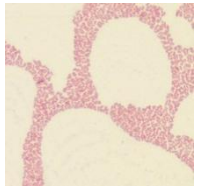
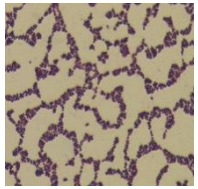
							
A5		**B5(1)**		**C6**		**D6**	
							
A6		**B6**				**D7**	
							
A7		**B7**				**D8**	
							
A8						**D9**	
							

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			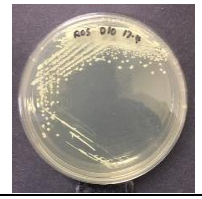	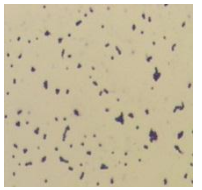
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			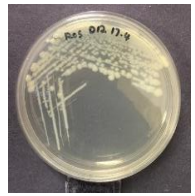	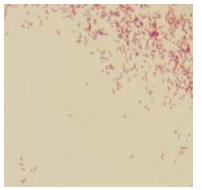
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				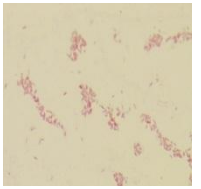
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			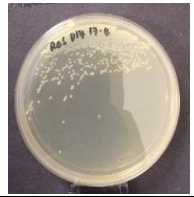	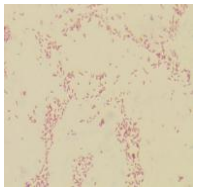
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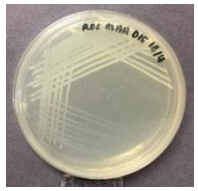
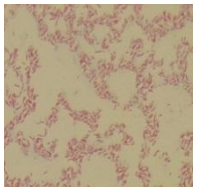
				

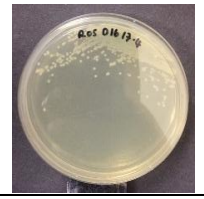
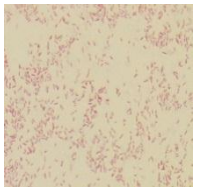
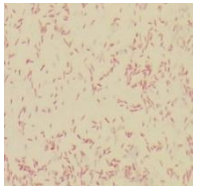
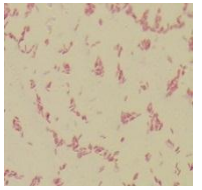
			D16 	
			D17 	
			D18 	

Table 2: The morphology characteristics of the bacteria isolates

Sungai Gombak						**Sungai Batu**					
Point A						**Point C**					
Colonies	**Opacity**	**Form**	**Elevation**	**Surface**	**Colour**	**Colonies**	**Opacity**	**Form**	**Elevation**	**Surface**	**Colour**
A1	Opaque	Circular	Crateriform	Rough	White	**C1**	Translucent	Circular	Raised	Glistering& smooth	Colour of the agar
A1(1)	Opaque	Circular	Raised	Glistering& smooth	Whitish yellow	**C2**	Translucent	Circular	Raised	Glistering& smooth	White
A2	Translucent	Small circle	Convex	Glistering& smooth	Colour of the agar	**C3**	Translucent	Circular	Flat	Glistering& smooth	White
A3	Opaque	Irregular	Flat	Rough	Whitish yellow	**C4**	Opaque	Irregular	Crateriform	Glistering& smooth	Yellow
A4	Opaque	Irregular	Raised	Rough	Whitish yellow	**C5**	Opaque	Small circle	Convex	Glistering& smooth	White
A5	Opaque	Irregular	Flat	Rough	White	**C6**	Opaque	Circular	Crateriform	Glistering& smooth	Yellow
A6	Opaque	Circular	Umbonate	Rough	Whitish yellow	**Point D**					
A7	Opaque	Irregular	Umbonate	Rough	Yellow	**D1**	Translucent	Small circle	Convex	Glistering& smooth	Colour of the agar
A8	Opaque	Irregular	Flat	Rough	Yellow	**D2**	Translucent	Irregular	Flat	Glistering& smooth	Colour of the agar
Point B						**D3**	Translucent	Small circle	Raised	Smooth	Light yellow
B1	Opaque	Irregular	Raised	Glistering& smooth	White	**D4**	Translucent	Irregular	Raised	Glistering& smooth	Yellow
B2	Opaque	Circular	Raised	Glistering& smooth	Yellow	**D5**	Opaque	Circular	Flat	Smooth	White
B3	Opaque	Irregular	Raised	Rough	Yellow	**D6**	Translucent	Small circle	Convex	Glistering& smooth	Colour of the agar
B3(1)	Opaque	Irregular	Raised	Rough	Yellow	**D7**	Translucent	Small circle	Umbonate	Glistering& smooth	Colour of the agar
B4	Translucent	Irregular	Raised	Glistering& smooth	Yellow	**D8**	Opaque	Circular	Convex	Glistering& smooth	Yellow
B4(1)	Opaque	Irregular	Flat	Rough	White	**D9**	Opaque	Small circle	Raised	Smooth	White
B5	Opaque	Filamentous	Raised	Glistering& smooth	Dark yellow	**D10**	Opaque	Small circle	Convex	Rough	White
B5(1)	Opaque	Circular	Unbonate	Rough	Yellow	**D11**	Opaque	Circular	Raised	Smooth	White

B6	Opaque	Circular	Raised	Glistering& smooth	White	**D12**	Opaque	Irregular	Raised	Rough	Yellow
B7	Opaque	Circular	Raised	Glistering& smooth	White	**D13**	Translucent	Circular	Flat	Glistering& smooth	White
						D14	Translucent	Circle	Flat	Glistering& smooth	Colour of the agar
						D15	Opaque	Irregular	Flat	Glistering& smooth	Light yellow
						D16	Translucent	Small circle	Convex	Glistering& smooth	Colour of the agar
						D17	Translucent	Irregular	Flat	Glistering& smooth	Colour of the agar
						D18	Opaque	Circular	Raised	Glistering& smooth	Light yellow

3.2. Antibiotic Susceptibility Testing for isolated bacteria strains using different antibiotics

Table 3 shows the results of the antibiotic susceptibility testing using disc diffusion method of bacteria strains isolated from Sungai Gombak against different antibiotics such as ampicillin, chloramphenicol, ciproflaxin, tetracycline, gentamycin and rifampicin. All of the antibiotics tested by using two different concentrations which is 10 µg/mL and 30 µg/mL.

The Table 3 shows the difference in susceptibility of the isolated strains from Gombak river against the different antibiotics that been tested. Antibiotic resistance frequency of the isolates from Gombak river is great in chloramphenicol and gentamycin, while least in ciproflaxin. The dispersion pattern of antibiotic resistance bacteria there were due to some activities related with livestock or animal farming, because antibiotics been added in animal food that used to feed them and it can indirectly have flown into the environment by two ways, either by direct leaching from the feed into the river during rain or through livestock waste water from the particular area.

Table 4 shows the results of the antibiotic susceptibility testing using disc diffusion method of bacteria strains isolated from Sungai Batu against different antibiotics such as ampicillin, chloramphenicol, ciproflaxin, tetracycline, gentamycin and also rifampicin. All of the antibiotics tested by using two different concentrations which is 10 µg/mL and 30 µg/mL.

Table 4 shows the various pattern of bacteria resistance towards the six different antibiotics that have been used to be tested. The resistance activity in Batu River also shows the highest percentage against antibiotic rifampicin and least against ciproflaxin. Bacteria isolated from this river evolved into antibiotic resistant due to the influence by washing, bathing and excess waste disposal from the nearby residential area (Ryan *et al.*, 2006).

Figure 1 shows the percentage of bacteria strains isolated from rivers in Kuala Lumpur that resistance towards different antibiotics. All antibiotics that were used are broad-spectrum antibiotics.

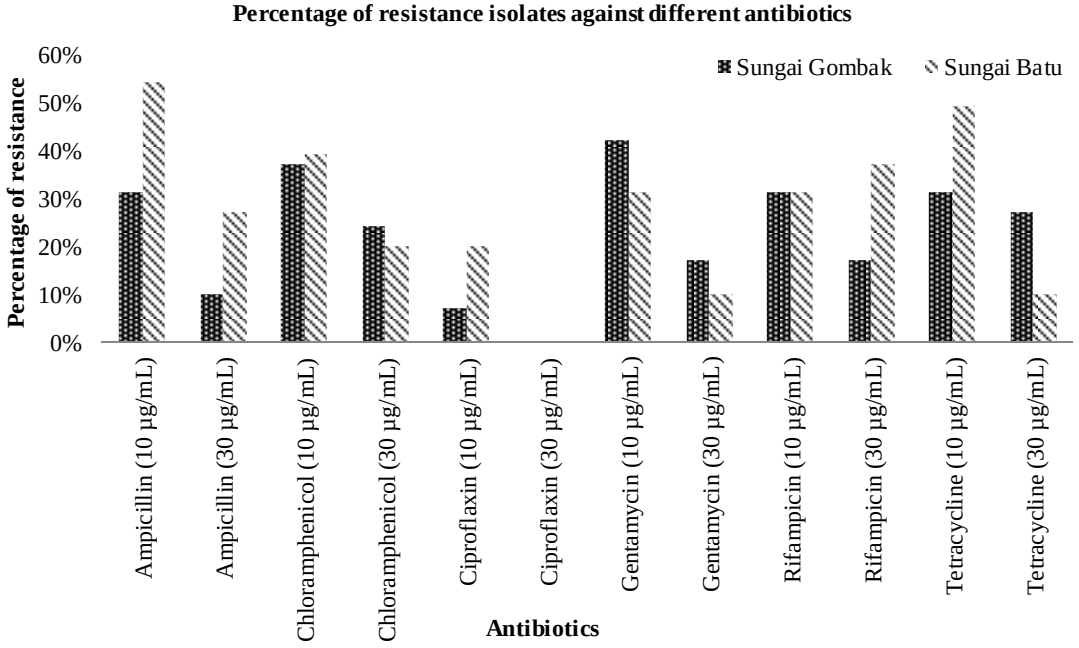


Figure 1: Percentage of bacteria resistance towards different antibiotics from Sungai Batu and Sungai Gombak

The results from the figure above clearly shows that most of the isolated bacteria strains have highest resistance against antibiotic, ampicillin at both of the study area. Interestingly, all of the isolates show low resistance against antibiotic ciproflaxin. Which means antibiotic, ciproflaxin associated application are still rare in the study area. Existence of resistant bacteria in both rivers was related to rubbish dumb, wastewater and also untreated waste water discharge to the river from the nearby housing area (Al-Badaii and Shuhaimi-Othman, 2015).

Table 3: Antibiotic susceptibility test of isolated bacteria strains from Sungai Gombak ('R' indicates 'Resistant' and 'S' indicates 'Susceptible')

Antibiotics/ Strain	Ampicillin		Chloramphenicol		Ciproflaxin		Gentamycin		Rifampicin		Tetracycline	
	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Point A												
A1	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**
A1(1)	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
A2	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**R**
A3	**S**	**S**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**
A4	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**R**
A5	**R**	**S**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
A6	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
A7	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
A8	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
Point B												
B1	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**
B2	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**S**	**S**	**S**	**S**
B3	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**
B4	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**S**
B4(1)	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**
B5	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
B6	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**R**
B7	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**

Table 4: Antibiotic resistance bacteria strains isolated from Sungai Batu ('R' indicates 'Resistant' and 'S' indicates 'Susceptible')

Antibiotics/ Strain	Ampicillin		Chloramphenicol		Ciproflaxin		Gentamycin		Rifampicin		Tetracycline	
	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Point C												
C1	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
C2	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**R**
C3	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**S**	**S**	**R**
C4	**R**	**S**	**R**	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**R**
C5	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
C6	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
Point D												
D1	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
D2	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
D3	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**S**
D4	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**S**
D5	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**S**	**S**
D6	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**
D7	**R**	**R**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**
D8	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**S**
D9	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
D10	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
D11	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
D12	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**
D13	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**S**	**R**	**R**
D14	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**S**
D15	**R**	**R**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
D16	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**
D17	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**
D18	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**

3.3. Multiple Antibiotic Resistance (MAR) Index Value Analysis

Multiple Antibiotic Resistance (MAR) Index Value was calculated after completing susceptibility test on the isolates. MAR index value of the isolated strains was calculated into order to determine the exact resistance rate of each bacteria towards multiple antibiotics that been used (Palaniappan and Holley, 2010). Figure 2 shows the result of MAR index values of the bacteria strains that was isolated from Sungai Gombak.

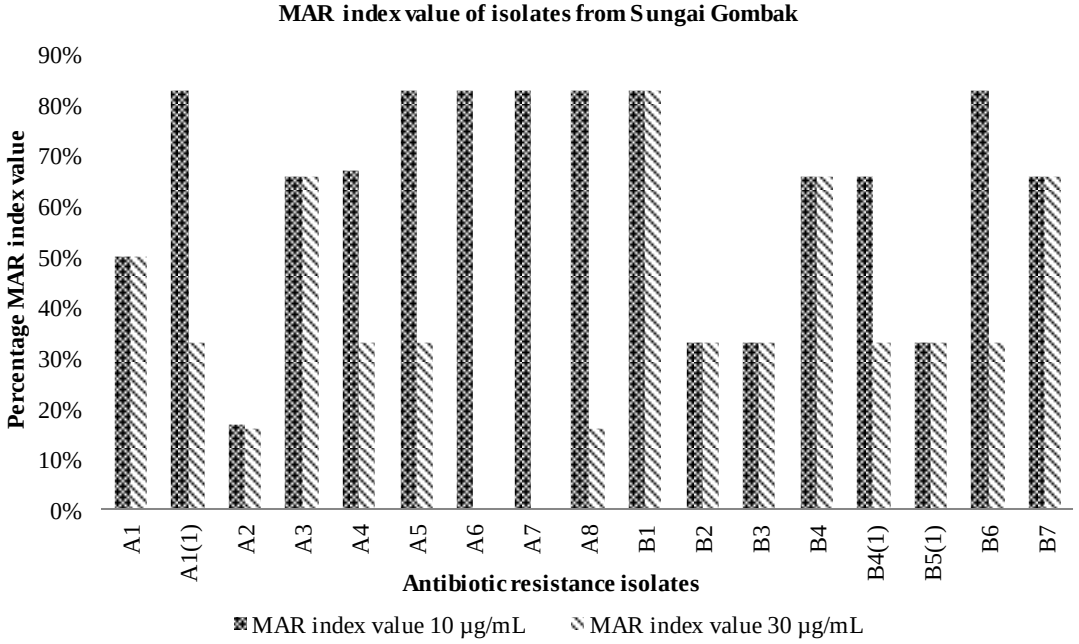


Figure 2: Multiple Antibiotic Resistance Index value of the isolates from Sungai Gombak

The results of the Multiple Antibiotic Resistance (MAR) index value for the isolates from Sungai Gombak shows ranges from 0% to 90%. Higher the percentage of MAR indicates that the strain is resistance towards higher number of antibiotics. Majority of the bacteria isolated from Gombak river shows high resistant activity towards antibiotic, ampicillin, tetracycline and rifampicin as compared to the other antibiotics. Those strains that marked in boxes are the selected strains for further characterization and identification. Strains A1, A3, B1, B4 and B7 show MAR value index more than 50% for both concentrations used.

Similar to the isolates from Sungai Gombak, isolates from Sungai Batu also have MAR value index ranges from 0% to 90%. Most bacteria isolated from Batu river, also shows its highest resistance activity towards antibiotic ampicillin, tetracycline and rifampicin. Strain D6 and D8 show MAR index value more than 50%. As Sungai Batu located near hospitals, resistance bacteria presents might due to the waste water released from it. Thus, poor management of the medical waste water that released into the water bodies, would bring serious consequences to the ecosystem in future.

The finding of this study shows that all of the isolates from both rivers are classified as Multiple Antibiotic Resistance. As most of the isolates from the study area was Gram-negative, it could presume higher resistance rate against the antibiotics that normally been used and they could easily become resistant towards the antibiotics which makes it harder to prevent any bacteria related infections (Zhang *et al*., 2011).

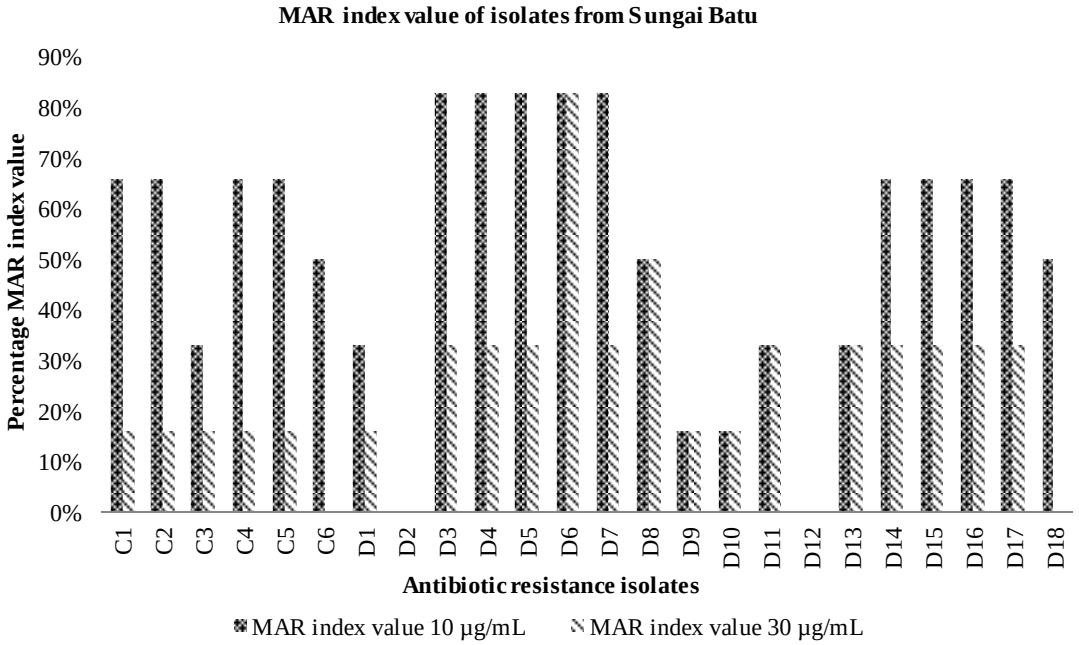


Figure 3: Multiple Antibiotic Resistance Index value of the isolates from Sungai Batu

3.4. Characterization of Antibiotic Resistance Bacteria isolated from rivers in Kuala Lumpur

3.4.1. Gram Staining

Performing Gram staining is a crucial method for identification of bacteria isolates especially medically important bacteria. Gram staining method assists to differentiate between Gram-positive and negative bacteria based on the differential staining by using safranin, iodine and crystal violet complexes (Bhattacharyya *et al.*, 2015). Gram staining was being carried out for all of the isolates (Table 1).

From the overall isolated bacteria, thirty isolates were Gram-negative and bacilli shaped. Nine of the isolates are Gram-positive and cocci shaped, followed by one strain which is bacillus-shaped and Gram-positive and another one strain is Gram-negative-cocci shaped. It can be concluded that majority of the bacteria isolated from rivers in Kuala Lumpur are Gram-negative. Previous study states that Gram-negative bacteria are pathogenic and harmful to human due to the plasmids that contain antibiotic resistance genes which able to transfer among pathogenic and non-pathogenic Gram-negative bacteria that exists in nature (Zhang *et al.*, 2011).

3.4.2. Biochemical tests

Seven different biochemical tests were carried out with different purposes. Figure 4 and Figure 5 show the different family of the bacteria that dominate the rivers in Kuala Lumpur based on the conducted biochemical tests.

Figure 4 shows that *Proteus* sp. was the most commonly predicted strain from Sungai Gombak. It is the most commonly founded bacteria in human body, mainly in the intestine and infection caused by it is hard to be treated because of its susceptibility against most of the antibiotics (Holt *et al.*, 2015). Based on Figure 5, isolates from Sungai Batu was predicted dominated by *Proteus* sp. which is one of the Enterobacteriaceae family similar as *Klebsiella* sp.. *Proteus* sp. also could be found in human intestine and may cause urinary tract infection (Ithoi *et al.*, 2011).

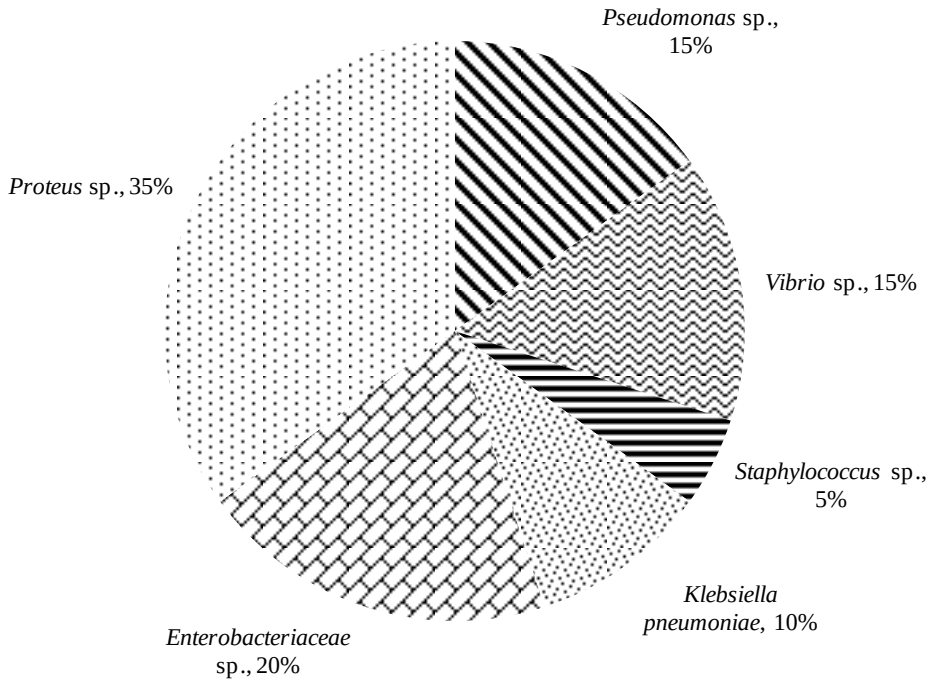


Figure 4: Different species of bacteria that dominate in Sungai Gombak (Point A and B) as mentioned in this study

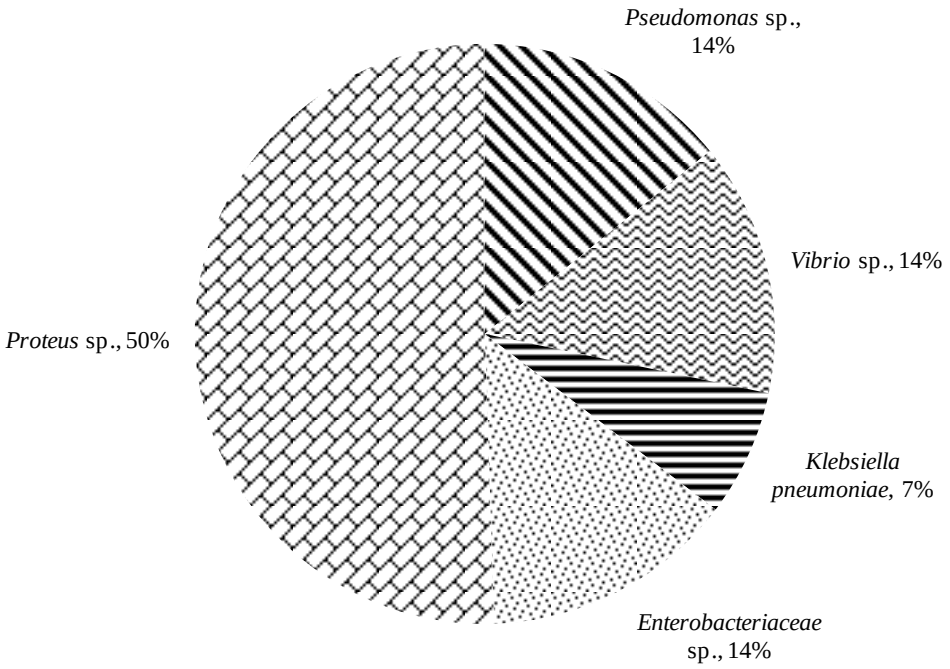


Figure 5: Different species of bacteria that dominate in Sungai Batu (Point D) as mentioned in this study

The results for the oxidase test shows variation among the strains. Strain A1, B1, B4, D6 and D8 show positive results while strain A3 and B7 show negative results. Basically, purpose of this test was to identify the bacteria strain that able to produce cytochrome C oxidase enzyme. When the enzyme presents, it able to oxidize the tetramethyl-p-phenylenediamine reagent into indophenols, this could be identified by observing the color changes into purple. While, if the result is negative, it will appear as colorless. Those strains with positive results were identified as aerobics which uses oxygen as an important component in respiration process. Bacteria with negative results can be deduced as anaerobic strains, which mean it does not have cytochrome C oxidase enzyme but uses other enzyme for respiration. Mostly, bacteria in Enterobacteriaceae family are classified as oxidase negative (Aslanzadeh, 2006).

Catalase test shows positive results for strain A3 only. The other strains, A1, B1, B4, B7, D6 and D8 show negative results. Basically this test was carried out to differentiate the Gram-positive bacteria which is in Enterobacteriaceae family (Taylor and Achanzar, 1972). Oxygen bubbles can be observed in the bacteria with positive catalase test which determines that the particular bacteria use oxygen in their respiration and protects themselves from toxic end product from oxygen metabolism. Those catalase negative strains were predicted as anaerobes or facultative anaerobes (Magnani *et al*., 2010).

The results for Indole production test shows negative results for all of the bacteria strain; A1, A3, B1, B4, B7, D6 and D8. Generally, the purpose of Indol test was to identify the Enterobacter family and those bacteria strain that able to dissociate tryptophan into indole component. As all of the isolated strains show negative results, which means it could not produce indole. Normally, if indole was produced by the bacteria it could react with the Kovac's reagent that been added. This Kovac's reagent contains 4-dimethylamino benzaldehyde component which causes the presence of red layer if indole was produced (Hemraj *et al*., 2013).

For Citrate utilization test, three bacteria strains; A1, B1 and D6 show positive results while A3, B4, B7 and D8 show negative results. This test was conducted to identify the strains that able to utilize citrate as the main source of energy. When the strain able to utilize citrate, ammonium salt in it will be broken into ammonia which will increases the pH thus, makes the slant agar's color changes from green to blue. If the agar changes into blue, it shows positive result. But if it remains the same, it is citrate negative (Hemraj *et al*., 2013).

Mannitol Salt Agar (MSA) test also shows negative results for all of the bacteria strains. Basically, this test was conducted to identify the *Staphylococcus* sp. from the isolates. As MSA is differential and selective agar with high concentration of salt, it only allows the growth of those organisms that able to tolerate high saline content. If the bacteria show positive results, it means the bacteria able to ferment mannitol and changes the color of phenol red into yellow. Organism from other family may grow, but at very slow rate (Kateete *et al*., 2010).

Overall results revealed that the biochemical tests result is consistent with the identification results of the isolates. Strain A1, A3, B1, and B4 were isolated from Sungai Gombak which been extremely effected due to wastewater discharge from nearby hospitals which consists of higher antibiotics content. On other hand, the strain of D6 and D8, indicated that they are contaminated with antibiotic resistance bacteria from rubbish dumb, wastewater, and also untreated waste water discharge to the river. It can relate to the source of the strain that had been isolated, which is Sungai Batu. As mention by (Ithoi *et al*., 2011), Sungai Batu is one of the main river in Kuala Lumpur that prone to the contamination and flood risk due to the daily activity of local peoples.

4. Conclusions

This study proves the antibiotic contamination in the selected rivers of Kuala Lumpur, which was indicated by the presence of antibiotic resistance bacteria isolated from the river water sample. Majority of the isolates exhibits multiple antibiotic resistance properties which strongly prove that the rivers of Kuala Lumpur as a whole at risk of being exposed to severe antibiotic contamination in the future if there is no any actions or measures taken to prevent this contamination from occur. Moreover, the results of this study also determine the importance of controlled release of contaminants into local surface water. This study will be a useful basic guidance for further extended studies related to understanding the status of antibiotic contamination in water bodies of urban area.

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Study on Emergence of Antibiotic Resistance Bacteria from Rivers in Selangor

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Abstract: The emergence of antibiotic resistant is primarily due to excessive and often unnecessary use of antibiotic in animals and humans especially from clinical and agriculture. The widespread uses of antibiotic will contribute the development of antibiotic resistant bacteria (ARB). This study was conducted to determine the presence of ARB strains present in the Chemubong River and Langat River in Selangor. It was performed by collecting the water samples from both rivers. The bacteria were isolated and their morphological and biochemical analysis was conducted. Morphological structure and Gram staining study were conducted to differentiate between Gram-negative bacteria and Gram-positive bacteria. Antibiotic susceptibility test was also performed to indicate their resistances by using disk diffusion method. Identification of the bacterial isolates were determined from their 16S rRNA gene sequencing. A total of 38 bacteria were isolated and tested against six different antibiotic; chloramphenicol, ciprofloxacin, rifampicin, gentamycin, tetracycline and ampicillin. In this study, nine ARB strains were successfully isolated based on their susceptibility and MAR Index for more than 50% toward different antibiotics. It was identified that Chemubong River and Langat River waters are contaminated with *Burkholderia* sp., *Pseudomonas* sp., *Bacillus* sp., *Vogesella* sp. and *Staphylococcus hominis*, which are human opportunistic pathogens and may affect human health. Thus, this research can be useful to bring awareness to the Malaysian Government and public about the safety precaution and measures to be taken into contamination of ARB prevention in water sources in Selangor.

Keywords: Antibiotic resistance bacteria; Antibiotic; River; Selangor

1. Introduction

Over the years, the study of antibiotic resistance bacteria has increased drastically due to excessive disposer of antibiotic agents such as kanamycin, tetracycline, penicillin, and ampicillin into the natural environment especially into river and sea water. When these antibiotics constantly exposed to the bacteria at sub-inhibitory concentrations in the environment helps them adapted to certain antibiotic. Thus, causes many bacteria develop resistance to multiple antibiotics and difficult to be exterminated (James and Wong, 2015). The development of antibiotics resistance bacteria had been existing in nature a long time ago before they we used in medical and veterinary purpose (Garcia-Armisen, 2013). In addition, this antibiotic resistance bacteria (ARB) is not only exist in medical and veterinary area but also can be found in aquatic environment (Al-Bahry *et al*., 2015), agriculture (Khachatourians, 1998), sewage treatment plants and hospital effluents (Ferreira *et al*., 2011; Fuentefria *et al*., 2011; Spindler *et al*., 2012).

The used of antibiotic actually is to cure disease that was caused by microbial infection such as diarrhea, pneumonia, and tuberculosis (James and Wong, 2015). In order to survive the ARB has the ability to multiply and adapt to the condition, hence this disease able to remain until today (Al-Bahry *et al*., 2015). The main contribute to the development of ARB strain are due to the incomplete metabolism in human and released of unused antibiotic (Bouki *et al*., 2013). This will attributed to the difficulty in treating some life threatening diseases to both humans and animals (Ferreira da Silva *et al*., 2007). The presence of pathogenic bacteria in aquatic environment may result in chronic disease when the bacteria are antibiotic resistance (Managaki *et al*., 2007). Besides, agricultural activities also showed higher concentration of ARB that may impact environmental ecosystem (Munir *et al*., 2011). According to Al-Bahry (2015), there are presence of ARB in mammals, birds, turtles and fish. As example, in Oman, the fish that feeding near to the sewage effluent was isolated and confirmed infected by ARB (Al-bahry, 2015).

Multiple antibiotic resistance bacteria (MARB) are commonly distributed in aquatic and terrestrial habitat (Al-bahry, 2015). Furthermore, it also affects many environmental ecosystems to change (Martinez, 2008). ARB have ability to transfer to human and occur naturally in environment and will result chronic disease (Martinez,

2008). Despite these, there are limited studies regarding the presence of ARB in aquatic such as river or sea water. Hence, this project was studies in order to determine the presence of ARB in river in Selangor.

2. Materials and Methods

2.1. Sampling site

This study has been conducted in a state of Selangor, Malaysia. Chemubong River located at Sungai Buloh and Langat River were selected as a site for sampling due to its geographical factors that may cause development of antibiotic resistance bacteria (ARB). This site was located nearer to agriculture area, hospital and community surrounding it, thus enhance the effluent has high potential of containing antibiotic. Two sampling sites of water sample starting at the upstream and end at the downstream of the river nearer to human activities. The sampling occurs at two different site was to study the effect of different human activities will affect the growing of different bacteria at same river.

2.2. Selection of antibiotic

In this study, 6 antibiotics were used in this study; ciprofloxacin, chloramphenicol, rifampicin, tetracycline, gentamicin and ampicillin. This antibiotic was selected due to the broad spectrum toward organisms and due to the possibility of widely distributed microorganism.

2.3. Antibiotic screening

The bacterial isolates were first grown on NB medium for overnight at 37 °C and then spread on the Muller Hinton agar by using a sterilized cotton bud to make a bacterial lawn. Then, sterilized punch filter paper will be immersed into the antibiotic and placed on the bacteria lawn with correct labelled. The final concentrations of the antibiotics used in the plates were 10 and 30 µg/mL for all antibiotics: chloramphenicol, ciprofloxacin, rifampicin, gentamicin and tetracycline. The bacterial isolates were also streaked onto NA and growth was recorded for all the bacterial isolated. Bacterial isolated which grew on the plates containing different antibiotics were scored as resistant and those which did not grow were described as sensitive.

2.4. Characterization of bacteria

Two tests can be used to identify the characterization of bacteria by Gram staining and biochemical test. Besides, the effect of temperature and pH on the growth of bacteria will be identified. Gram staining is used to identify either the bacteria are Gram-positive or Gram-negative.

2.5. Determination of MAR Index

The Multiple Index Resistance (MAR) index values of each isolates were determined by dividing the numbers of antibiotics to which the isolates is resistant by the total of antibiotics tested (Nurhazlin *et al*., 2014).

$$MAR\ Index\ value = a/b \times 100 \qquad \text{Equation 1}$$

Where *a* represents the number of antibiotics that isolate bacteria for both concentration (10 and 30 µg/mL) show resistant to and *b* represents the total number of antibiotic were tested. If the MAR index value of isolate is more than 50%, it indicates high risk of contamination by antibiotics in area of selected rivers in Selangor.

3. Results

3.1. Bacterial isolates characterization

A total of 36 bacteria were successfully isolated from Langat and Chemubong river, 16 isolates and 20 isolates, respectively. From Langat river, nine bacteria were isolated from point A and seven bacteria were isolated from point B. Eight out of nine isolates from point A are Gram-negative and all isolates are Gram-negative form point B. As for Chemubong river, nine out of ten are Gram-negative for both sampling area (point A and B). Table 1 represents morphological characteristic of the isolates from Langat and Chemubong river.

3.2. Antibiotic susceptibility tests

All isolates were tested for its susceptibility towards 6 different antibiotics (chloramphenicol, rifampicin, tetracycline, ampicillin, ciprofloxacin and gentamycin). Table 2 shows the antibiotic susceptibility test for all isolates from both rivers.

3.3. MAR Index

The multiple antibiotics resistance (MAR) index value of each isolate was calculated to determine the resistant of bacteria to multiple antibiotics. Figure 1 concludes the MAR index value for all the isolates. From these findings, multiple antibiotics resistance were observed in isolates for each point of both rivers (Table 2).

3.4. Biocehmical tests

All these isolates were characterized biochemically. Table 3 shows the detailed results on the bacteria isolates from Langat and Chemubong river using different biochemical tests.

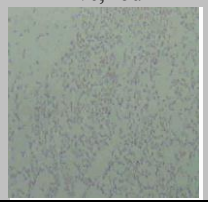
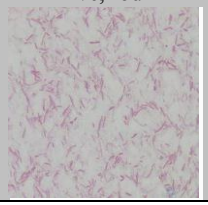
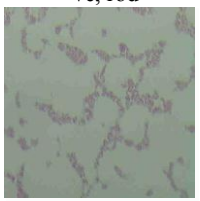
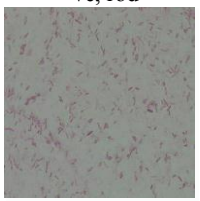
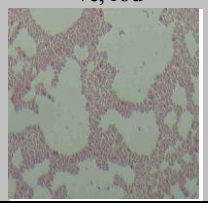
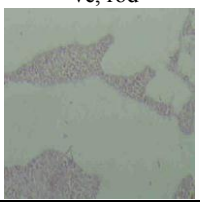
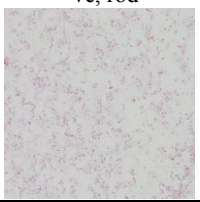
4. Discussion

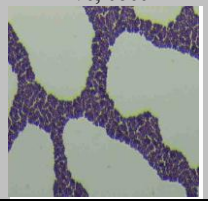
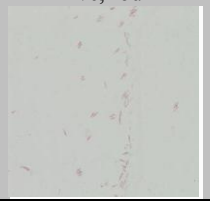
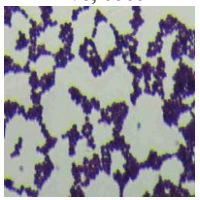
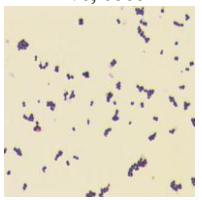
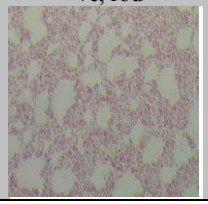
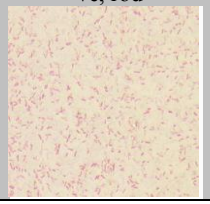
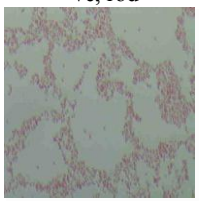
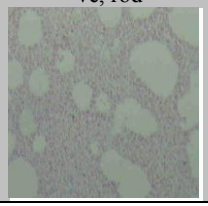
Based on this study, we can conclude that there is an emergence of antibiotic resistance bacteria (ARB) in Langat and Chemubong river, Selangor, Malaysia. Based on Figure 1 a) and b) (Langat river), six out of 9 and six out of seven isolates possess more that 20% of MAR index value, respectively. Meanwhile in Figure 1 c) and d), ten out of ten and eight out of ten bacterial isolates have more that 20% MAR Index value, respectively.

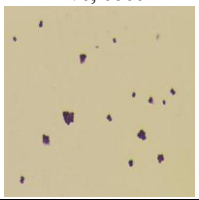
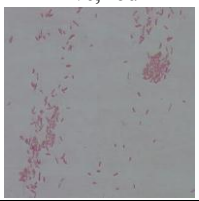
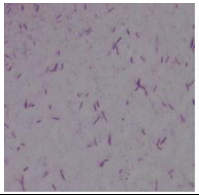
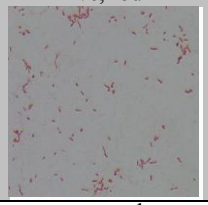
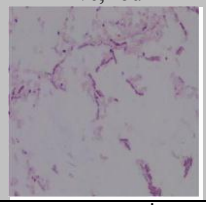
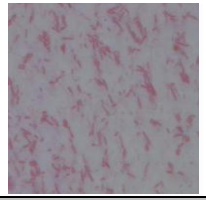
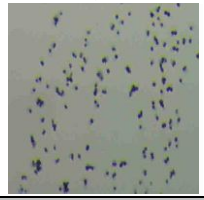
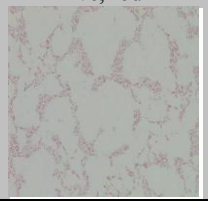
The emergence of ARB can be an indicator showing that the selected sampling area is highly contaminated with different kind of antibiotics particularly the antibiotics that have been chosen in this study. Besides, the discharge of effluent and waste water from the hospital and clinic, fertilizer from agriculture activities, human urban and restaurant which located along Langat and Chemubong river might also contribute to antibiotic contamination of the areas. Moreover, the lack monitoring waste water discharge and overuse of antibiotic into the environment especially river might lead to development of ARB.

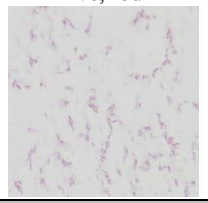
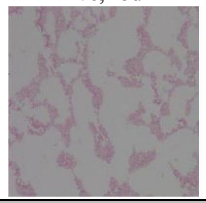
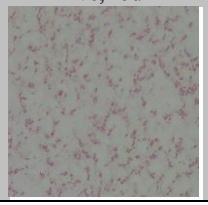
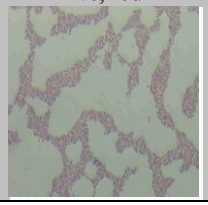
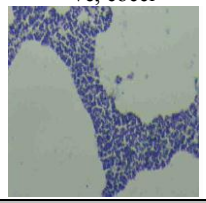
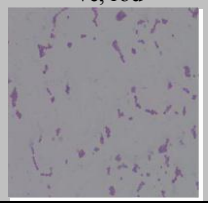
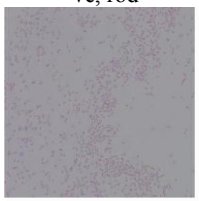
Besides that, majority of the isolates were Gram-negative bacteria and rod in shape, they showed stronger resistance towards the antibiotics tested than Gram-positive bacteria which similar patterns as in the literatures (Nurhazlin *et al*., 2014). Previous study reported that antibiotic resistant gene was transferred between pathogenic and non-pathogenic Gram-negative in the environment which exhibited high resistance toward the antibiotics (Nurhazlin *et al*., 2014). These finding of pathogenic bacteria had high opportunities to become resistant to antibiotics. Thus, making it more difficult to prevent bacterial disease.

Table 1: Morphological characteristic of the isolates from Langat and Chemubong river

Langat River				Chemubong River			
	Color	**Nature of colony**	**Gram staining**		**Color**	**Nature of colony**	**Gram-staining**
Point A				**Point A**			
LA1	Yellow	Circular, entire, flat	-ve, rod 	**CA1**	White	Smooth, circular	-ve, rod 
LA2	Cream	Circular, convex	-ve, rod 	**CA2**	Yellow	Circular, smooth	-ve, rod 
LA3	White	Circular, entire, flat	-ve, rod 	**CA3**	Cream	Flat, irregular, shiny	-ve, rod 
LA4	Yellow	Circular, smooth, entire	-ve, rod 	**CA4**	Yellow	Shiny, circular	-ve, rod 

LA5	White	Circular, smooth, entire	+ve, cocci 	**CA5**	White	Entire, shiny	-ve, rod 
LA6	Cream	Circular, entire	+ve, cocci 	**CA6**	White	Shiny, circular, entire	+ve, cocci 
LA7	Shiny white	Circular, convex	-ve, rod 	**CA7**	White	Smooth, circular	-ve, rod 
LA8	Cream	Irregular, sticky, convex	-ve, rod 	**CA8**	White	Shiny, entire	-ve, rod 
LA9	Yellow	Circular, entire	-ve, rod 	**CA9**	Cream	Smoothe, shiny	-ve, rod 

				CA10	White	Shiny, circular, raised	+ve, cocci 
Point B				**Point B**			
LB1	Colorless	Circular, entire, flat	-ve, rod 	**CB1**	Shiny yellow	Irregular, raised	-ve, rod 
LB2	Colorless	Irregular, smooth, convex	-ve, rod 	**CB2**	White	Smooth, flat, irregular	-ve, rod 
LB3	Yellow	Circular, entire, flat	-ve, rod 	**CB3**	Yellow	Irregular, convex, entire	+ve, cocci 
LB4	Yellow shiny	Irregular, undulate, flat	-ve, rod 	**CB4**	Cream	Irregular, entire, flat	-ve, rod 

LB5	White	Circular, convex	-ve, rod 	**CB5**	Shiny colorless	Circular, entire, convex	-ve, rod 
LB6	White	Circular, entire, flat	-ve, rod 	**CB6**	Shiny yellow	Irregular, convex	-ve, rod 
LB7	Yellow shiny	Circular, entire	-ve, rod 	**CB7**	White	Circular, convex	+ve, cocci 
				CB8	White	Circular, flat	-ve, rod 
				CB9	Colorless	Smooth, entire	-ve, rod 

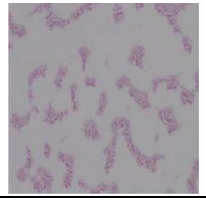
[redacted]	[redacted]	**CB10**	Colorless	Smooth, entire, flat	-ve, rod 

Table 2: Antibiotic susceptibility test for Langat and Chemubong river (S: Sensitive, R: Resistant)

		Antibiotics	Chloramphenicol		Ciprofloxacin		Rifampicin		Gentamycin		Tetracycline		Ampicillin	
		Bacterial Colonies	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Langat river	**Point A**	LA1	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
		LA2	**R**	**R**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
		LA3	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**S**	**S**
		LA4	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
		LA5	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
		LA6	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		LA7	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		LA8	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**
		LA9	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
	Point B	LB1	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
		LB2	**S**	**R**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**R**
		LB3	**S**	**R**	**S**	**S**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**
		LB4	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		LB5	**S**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
		LB6	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
		LB7	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**R**
Chemubong river	**Point A**	CA1	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**R**
		CA2	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**
		CA3	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**
		CA4	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		CA5	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**R**
		CA6	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
		CA7	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		CA8	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**S**
		CA9	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		CA10	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
	Point B	CB1	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**
		CB2	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**R**
		CB3	**R**	**R**	**R**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**R**
		CB4	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
		CB5	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		CB6	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
		CB7	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
		CB8	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**
		CB9	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**
		CB10	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

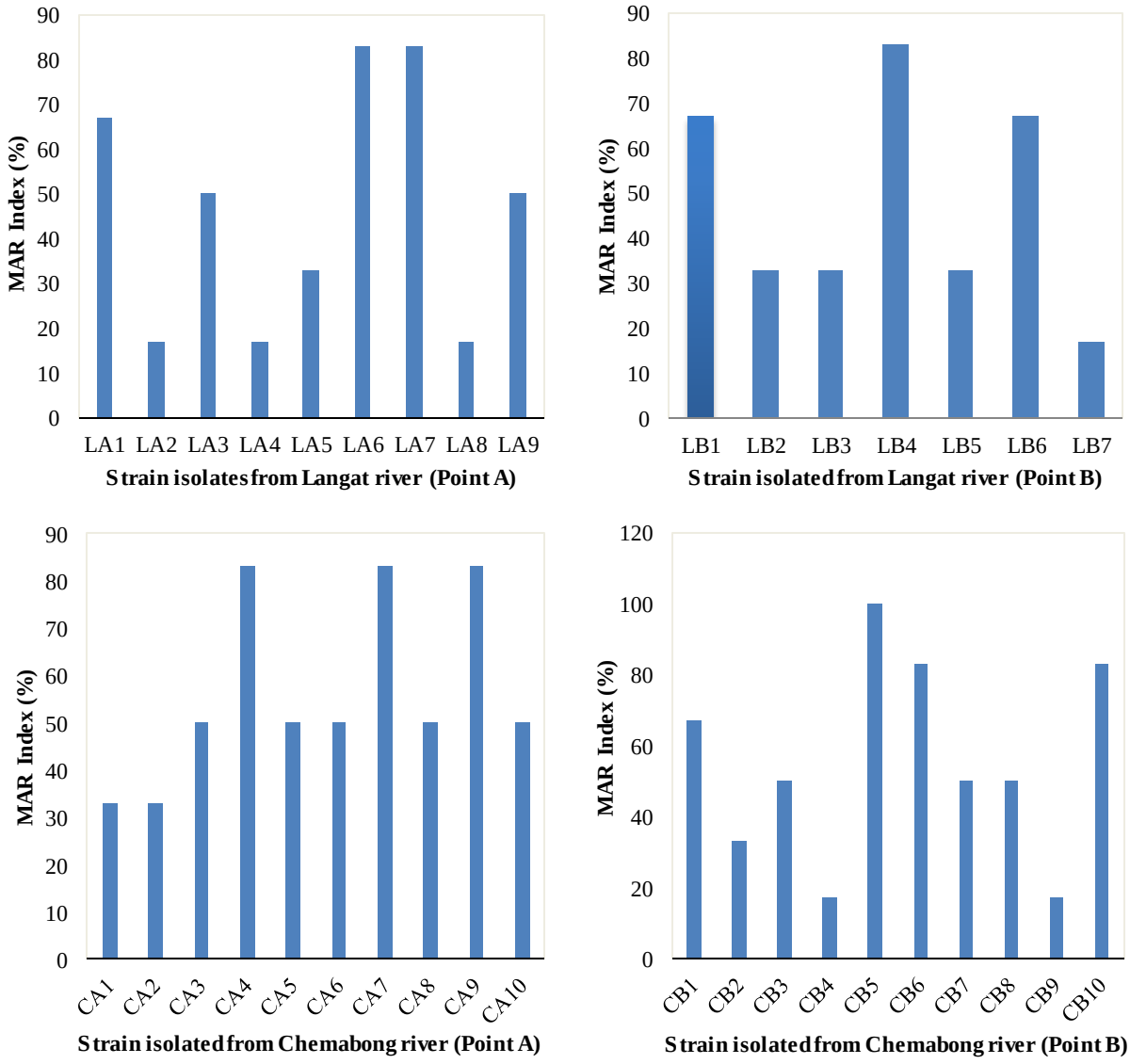


Figure 1: MAR Index for isolated strain from Langat and Chemabong river

Table 3: Biochemical characteristics of the isolates for Chemubong River

Langat River														Chemubong River											
Point A							Point B							Point A							Point B						
Strain	Biochemical Test					Conclusion (Family Type)	Strain	Biochemical Test					Conclusion (Family Type)	Strain	Biochemical Test					Conclusion (Family Type)	Strain	Biochemical Test					Conclusion (Family Type)
	Citrate	Oxidase	Catalase	Indol	MacConkey Agar			Citrate	Oxidase	Catalase	Indol	MacConkey Agar			Citrate	Oxidase	Catalase	Indol	MacConkey Agar			Citrate	Oxidase	Catalase	Indol	MacConkey Agar	
LA1	-	+	+	-	+	*Enterobacter* sp. *Vibrio* sp.	LB1	-	+	+	-	+	*Vibrio* sp. *Klebsiella* sp.	CA1	+	+	+	-	+	*Enterobacter* sp. *Klebsiella* sp. *Vibrio* sp.	CB1	-	+	+	+	-	*Enterobacter* sp. *Vibrio* sp. *Staphylococcus* sp.
LA2	-	+	+	-	-	*Enterobacter* sp. *Klebsiella* sp. *Vibrio* sp.	LB2	-	+	+	+	-	*Enterobacter* sp. *Vibrio* sp. *Staphylococcus* sp.	CA2	-	+	+	-	-	*Enterobacter* sp. *Vibrio* sp.	CB2	-	-	+	-	+	*Pseudomonas* sp. *Salmonella* sp.
LA3	-	-	+	-	+	*Vibrio* sp. *Klebsiella* sp. *Pseudomonas* sp.	LB3	-	-	+	-	+	*Pseudomonas* sp. *Enterobacter* sp.	CA3	-	-	+	-	+	*Pseudomonas* sp. *Enterobacter* sp.	CB3	-	+	+	-	+	*Staphylococcus* sp. *Vibrio* sp.
LA4	-	+	+	-	-	*Enterobacter* sp. *Salmonella* sp. *Klebsiella* sp	LB4	-	+	+	-	+	*Klebsiella* sp. *Vibrio* sp.	CA4	-	+	+	-	-	*Enterobacter* sp. *Klebsiella* sp. *Vibrio* sp.	CB4	-	-	+	-	+	*Vibrio* sp. *Klebsiella* sp.

LA5	-	-	+	-	-	*Staphylococcus* sp. *Salmonella* sp. *Vibrio* sp.	LB5	-	-	+	-	+	*Pseudomonas* sp. *Enterobacter* sp.	CA5	-	-	+	-	-	*Vibrio* sp. *Klebsiella* sp.	CB5	-	+	+	-	+	*Enterobacter* sp. *Vibrio* sp.
LA6	-	-	+	-	-	*Staphylococcus* sp. *Vibrio* sp.	LB6	+	+	+	-	+	*Enterobacter* sp. *Klebsiella* sp. *Vibrio* sp.	CA6	-	-	+	-	-	*Pseudomonas* sp. *Salmonella* sp.	CB6	-	+	+	-	-	*Pseudomonas* sp. *Salmonella* sp.
LA7	-	+	+	-	-	*Vibrio* sp. *Salmonella* sp.	LB7	+	-	+	-	+	*Vibrio* sp. *Salmonella* sp.	CA7	+	-	+	-	+	*Enterobacter* sp. *Salmonella* sp. *Klebsiella* sp.	CB7	-	+	+	-	+	*Vibrio* sp. *Klebsiella* sp.
LA8	-	-	+	-	-	*Pseudomonas* sp. *Enterobacter* sp.								CA8	+	-	+	-	+	*Vibrio* sp. *Salmonella* sp.	CB8	+	+	+	-	+	*Staphylococcus* sp. *Vibrio* sp.
LA9	-	+	+	-	+	*Pseudomonas* sp. *Salmonella* sp.								CA9	-	+	+	-	+	*Vibrio* sp. *Klebsiella* sp.	CB9	+	-	+	-	+	*Vibrio* sp. *Klebsiella* sp.
														CA10	-	+	+	-	+	*Enterobacter* sp. *Vibrio* sp.	CB10	-	+	+	-	-	*Pseudomonas* sp. *Enterobacter* sp.

5. Conclusions

In brief, this study had successfully proofed that there is an emergence of antibiotic resistance bacteria from rivers in Selangor. Based on MAR index for both rivers, it shows that most bacteria isolated are resistance toward chloramphenicol, rifampicin, tetracycline and ampicillin. Majority of the bacteria exhibited multiple antibiotic resistances which possess risk to the community in the areas. Thus, finding from this study would provide further understanding on the antibiotic contamination in rivers in Selangor.

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The Effect of Silver Nanoparticles-Multiwalled Carbon Nanotubes (AgNP-MWCNT) on Bioaerosol Bacteria Contamination of Air-conditioner Filter

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Abstract: Hybrid nanostructure has been widely studied due to its high potential in biotechnology. This study reports the development of nanohybrid composed of silver nanoparticles (AgNP) and oxidized multi-walled carbon nanotubes (ox-MWCNT) for its antibacterial effect on bioaerosol bacteria from air-conditioner filter. It was first prepared by treating pristine multi-walled carbon nanotubes (p-MWCNT) with concentrated sulphuric and nitric acid mixture (3:1) to generate ox-MWCNT. Afterwards, the AgNP, produced from silver ion reduction method was attached on ox-MWCNT by sonication in ethanol. The successful attachment was verified using Fourier transform infrared spectroscopy (FTIR), energy dispersive X-ray spectroscopy (EDX), transmission electron microscopy (TEM), zeta potential and dispersion test. The antibacterial effect of AgNP-MWCNT was confirmed by measuring the inhibition zone against isolated bacteria culture, and was compared to ox-MWCNT. From the results, AgNP showed successful attachment to ox-MWCNT, confirmed by the changes of C=O intensity peak at 1720 cm^{-1} in AgNP-MWCNT, as compared to ox-MWCNT. AgNP-MWCNT also showed noticable peaks at 1401, 1065 and 6707 cm^{-1} that indicates the interaction of silver with functional group. Silver element in AgNP-MWCNT was at high percentage (69.5%) in EDX results, proved by TEM analysis that showed AgNP embedded on surface of ox-MWCNT. AgNP-MWCNT also showed good stability in water as ox-MWCNT, confirmed by zeta potential results with value of -31.23 mV and -29.99 mV, respectively. In the antibacterial test, 100 µg/mL AgNP-MWCNT yielded the most efficient inhibitory zone against the Gram-positive Bacillus bacteria. The results are promising to show synergistic activity of AgNP-MWCNT compared to ox-MWCNT alone. This would be beneficial for researchers who interested to study requirement of AgNP-MWCNT needed to hinder growth of bacteria. Further studies are required to determine bacterial strain inhibited by 100 µg/mL AgNP-MWCNT as lower concentration is not effective for antibacterial treatment modality.

Keywords: Antibacterial; MWCNT; Silver nanoparticles; Hybrid nanostructure; Bioaerosol

1. Introduction

A good indoor air quality (IAQ) is one of important indoor environmental characteristic to ensure the good health of its occupants, (Al-Mijalli, 2016). The air-conditioning system is most commonly used to achieve a more comfortable interior environment. However, it was found that such environment will easily trap pathogens such as fungi, bacteria, protozoans and mites growth. The exposure to these microorganisms may bring health risks to the users, ranging from hypersensitivity to serious illness as such pneumonia and conjunctivitis (Al-Mijalli, 2016).

These pathogens contributed to 5 – 35% of the indoor air pollution due to the lack of cleaning and ventilation checking of the air conditioning systems. Several approaches have been identified to remove such biofilms that further enhance the growth of bacteria in the bioaerosol, for instance the use chemicals and antibiotic compounds (Kim *et al*., 2017). These steps however, were too toxic and have led to the occurrence of antibiotic resistance among bacteria (Seo *et al*., 2014).

Silver nanoparticles have been one of the promising antimicrobial but its synergistic antibacterial and antifungal properties by combination with carbon nanotubes has scarcely reported. This study was carried out to develop silver nanoparticles-multi-walled carbon nanotubes (AgNP-MWCNT) for its efficiency to prevent bacterial growth using bacteria samples isolated straight from the air conditioning system.

2. Materials and Methods

2.1. Functionalization of MWCNT

The oxidation of MWCNT method was carried out to purify and modify the surface of MWCNT with functional groups. One gram of pristine MWCNT was treated with (3:1) mixture of 65% sulphuric acid (H_2SO_4) and 98% nitric acid (HNO_3). Subsequently, the mixture solution was stirred for 24 hour. The resulting ox-MWCNTs were washed with deionized water until reached nearly neutral pH and filtered with nylon filter membrane. The residue, ox-MWCNT were dried in the oven at 70 °C for 24 hour.

2.2. The attachment of silver nanoparticles (AgNP) onto MWCNT

The AgNP produced from silver ion reduction method was attached on ox-MWCNT by sonication in ethanol. Initially, 1 M of silver nitrate solution was prepared by dissolving 1.7 g of silver nitrate in 10 mL of deionized water. The AgNP was produced by dispersing 4 mL of 1 M silver nitrate solutions in 12 mL of ethanol and sonicated for 1 hour to let the silver ion reduction process occur. Subsequently, 20 mg of ox-MWCNT was also dispersed in ethanol for 1 hour. Both solutions were mixed and sonicated to load the silver nanoparticles onto MWCNT. The resulting AgNP-MWCNT samples was then washed with deionized water, filtered using nylon membrane and freeze-dried.

2.3. Characterization of AgNP-MWCNT

The characterization of AgNP-MWCNT was examined using Fourier transformed infrared spectroscopy (FTIR), energy dispersive X-ray (EDX) and dispersion behaviour analysis.

2.4. Bacteria isolation

Bacteria samples in this research project was collected from the filter of air-conditioner at T02 Building, Faculty of Bioscience and Medical Engineering (FBME), Universiti Teknologi Malaysia, Johor by using a sterilized cotton swab. Subsequently, the cotton swab was soaked into the nutrient broth (NB) and incubated for overnight at temperature 37 °C, 150 rpm. The bacterial growth in the NB was monitored the next day by using UV-vis spectrophotometer. Serial dilutions were also made and the bacteria were grown on NA plate, and were kept in an incubator overnight at 37 °C. The morphology of the bacterial colonies was identified.

2.5. Antibacterial test

The antibacterial analysis was performed by determining the CFU/mL in the NA culture medium, where the CFU is the colony forming unit. The bacterial count was evaluated following treatment with AgNP-MWCNT at concentration of 30, 50 and 100 µg/mL. The content of the bacteria was compared to those treated with ox-MWCNT and AgNP at 100 µg/mL. The ethanol solution was used as positive control and untreated NB with bacteria culture was the negative control. These culture media were incubated overnight at 37 °C and counted for their CFU/mL in the following day.

3. Results and Discussion

3.1. FTIR characterization

Fourier Transform Infrared Spectroscopy (FTIR) analysis was conducted in the spectral range of 500 – 4000 cm^{-1} to reveal information with regard to functional groups interacting at the nanotube surface. Figure 1 (i) and (ii) shows the IR spectra of the AgNP-MWCNT and ox-MWCNT after following oxidation with acid mixture (ox-MWCNT).

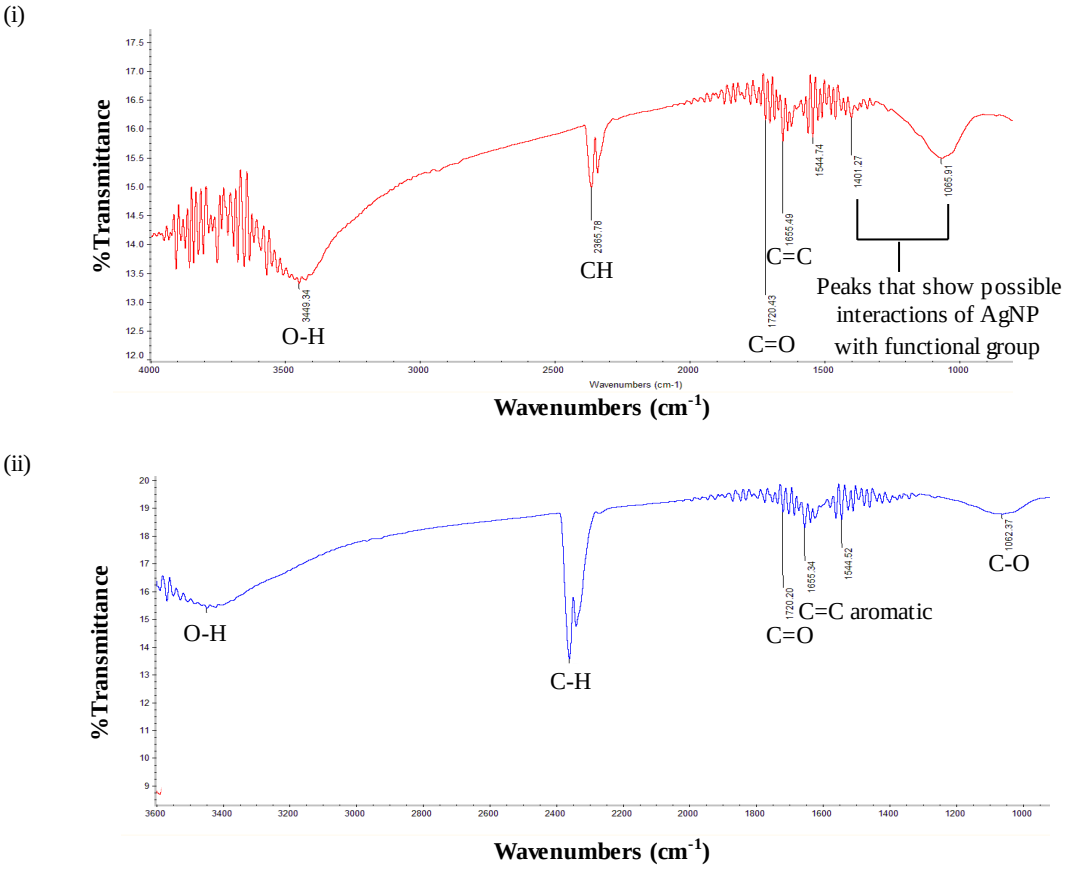


Figure 1: FTIR spectra of (i) ox-MWCNT, (ii) AgNP-MWCNT

The IR spectra of AgNP-MWCNT showed the presence of several new peaks at 1401.27 and 1065.91 cm^{-1} when compared with ox-MWCNT sample. According to Dinh *et al.* (2015) these new peaks are attributed to the possible interactions of silver ion (Ag+) from AgNP with functional group from the ox-MWCNT. Other noticable change observed in the AgNP-MWCNT sample is includes the change of O-H stretching absorption intensity at 3449.34 cm^{-1}. This demonstrated the interaction between Ag+ with the –OH groups of ox-MWCNT. The FTIR results in this study is in agreement to a study by Dinh *et al.* (2015) that functionalize AgNP on MWCNT surface via modified Tollen process, where they observed a shifted peak of –OH. According to the authors, this indicates the interactions between the silver ion and functional group via electrostatic attraction. The C=O group on the ox-MWCNT is also possible to anchor the AgNP, however, it was not seen clearly in the spectra.

3.2. EDX analysis

To prove the successful oxidation of ox-MWCNT and attachment of AgNP on ox-MWCNT, the element content of AgNP-MWCNT, ox-MWCNT and p-MWCNT were compared in the EDX analysis in Table 1.

Table 1: EDX analysis of MWCNT samples

Element	Weight (%)		
	p-MWCNT	ox-MWCNT	AgNP-MWCNT
Carbon	72.3	57.3	29.5
Oxygen	26.9	42.7	9.9
Silicon	0.07	0.008	0.07
Nickel	0.7	0.0	0.0
Silver	0.0	0.0	69.5

It can be seen that carbon and oxygen appeared most abundantly in all MWCNT samples. Following oxidation, the percentage of carbon decreases about 15%, while the oxygen content was found to increase about 15.8%. The increment of oxygen element in the sample occurred as the graphitic network of the CNT was broken and was decorated with –OH and –COOH groups following acid oxidation (Stobinski *et al*., 2010). This result was in agreement with the previous FTIR results that showed presence of both oxygenated functional groups. When ox-MWCNT was functionalized with AgNP, the oxygen content was found to decrease from 42.7 to 9.9% in AgNP-MWCNT sample. This corresponds to the attachment of AgNP on the oxygenated functional groups, which act as nucleation sites. It also caused free oxygen element on AgNP-MWCNT surface to deplete. Other observation includes the presence of silver element in AgNP-MWCNT sample that was very high (69.5%) as compared p-MWCNT and ox-MWCNT samples. This inferred to the presence of silver on the surface of AgNP-MWCNT.

3.3. Dispersion behaviour analysis

Dispersibility test is among common techniques that provides fast information on colloidal dispersion state. Figure 2 below shows dispersion behaviour of p-MWCNT (a), ox-MWCNT (b), AgNP-MWCNT (c) and AgNP (d) alone in deionized water at the moment of dispersion and after 5 days incubation.

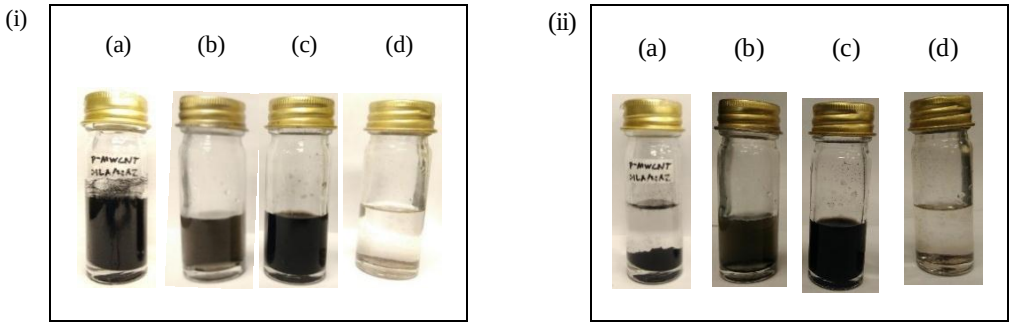
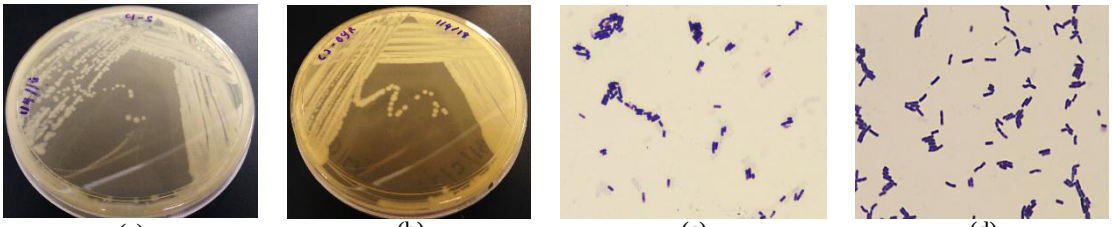


Figure 2: Dispersion behaviour analysis of (a) p-MWCNT, (b) AgNP-MWCNT, (c) ox-MWCNT, (d) AgNP that (i) just dispersed and (ii) after 5 days incubation

It can be seen that only ox-MWCNT and AgNP-MWCNT showed stable colloidal suspension after 5 days incubation. This indicates the presence of the functional groups on both samples that form hydrogen bond with water molecules. The p-MWCNT is poorly stable, indicated by and precipitation formed in the solution. This was due to the hydrophobicity of the material surface and absence of functional groups to form hydrogen bond with water (Skwarek *et al*., 2016). The AgNP-MWCNT showed good stability as ox-MWCNT suggests its stable dispersion state. It can be speculated that the combination of AgNP with MWCNT has helped to increase their stability in the water, but also may led to an improved biocompatibility and effect against bacteria cell walls (Seo *et al*., 2014). The AgNP that failed to disperse in the deionized water was in agreement to previous report for its limited antibacterial efficacy (Roy *et al*., 2017).

3.4. Bacteria isolation

The isolated bacteria obtained from the mix culture were identified as two types of colonies, Colony 1 and Colony 2, based on its morphology and Gram staining results. Figure 3 (a) showed Colony 1 morphology that was small in size, circular shape, white and flat compared to Colony 2 (Figure 3(b)) that was slightly bigger size compared to Colony 1. They also are in circular shape, creamy yellow colour and raised elevation with smooth surface. The Gram staining result (Figure 3c and 3d) of both colonies were identified as Gram-positive *Bacillus*, indicated by the rod shape and purple colour when viewed under light microscope with 1000x magnification.



(a) (b) (c) (d)

Figure 3: Streak plate of isolated bacteria from air-conditioner filter of (a) Colony 1 and (b) Colony 2 and Gram staining result of (c) Colony 1 and (d) Colony 2 viewed under 1000x magnification in light microscope

3.5. Antibacterial test

Figure 4 below showed the growth of bacteria colonies (CFU/mL) following treatment with AgNP-MWCNT, ox-MWCNT and p-MWCNT samples. It can be seen that Colony 2 was most efficient to be killed by the AgNP-MWCNT compared to Colony 1. AgNP-MWCNT was more effective against Colony 2 at lower concentration (30 and 50 µg/mL) compared to higher concentration (100 µg/mL). In Colony 1, it gave a different result whereby 100 µg/mL AgNP-MWCNT was more potent to kill the bacteria cells compared to lower concentration of AgNP-MWCNT.

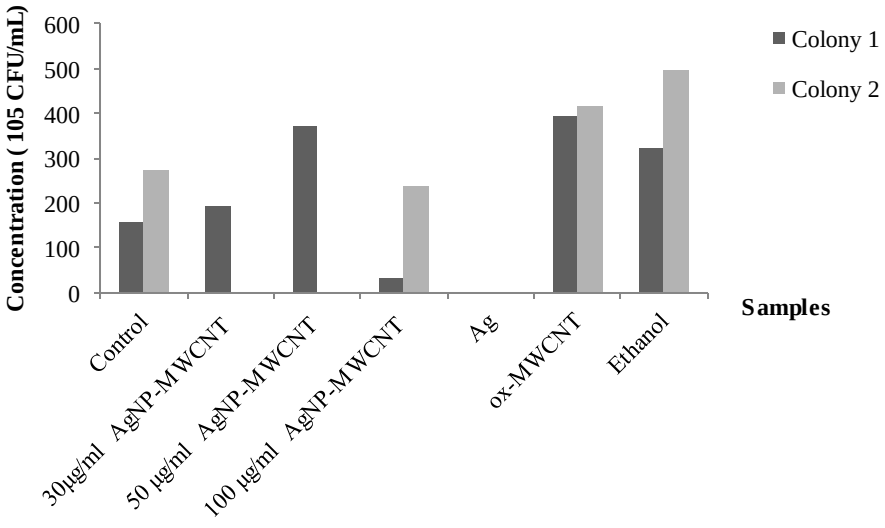


Figure 4: Bacterial count (CFU/mL) for antibacterial analysis of Colony 1 and Colony 2 bacteria

This preliminary result showed promising usage of AgNP-MWCNT, as the use of ox-MWCNT alone was not effective to kill the cells. Although the silver alone 100% eradicates both Colony 1 and 2, their promotion for use was in raising issues in an effort to address uncontrolled usage of nanoparticles in specific forms. Furthermore, their use at high amount was reported as toxic to the ecosystem and aggregated in aqueous solution (Roy *et al.*, 2017) While the combination of AgNP with ox-MWCNT showed good dispersibility profile in water, their application hence is encouraging leaving its as less toxic materials. However, a full understanding of the exact mechanism of action is required, as well as determining the bacterial type (Colony 1 and 2) in future.

4. Conclusions

The present study represents some initial work for the development of AgNP-MWCNT for antibacterial properties. The results are promising to indicate synergistic activity of AgNP-MWCNT for killing bioaerosol bacteria compared to ox-MWCNT alone in both Colony 1 and 2. Future studies are recommended to recognize the types of strain effectively killed by the AgNP-MWCNT developed in this study.

Acknowledgement

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Effects of Combined Retinoic Acid-*Clinacanthus nutans* Treatment in HeLa Cancer Cells

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Abstract: Cervical cancer enriched cancer stem cells (CSCs) constituted as one of the highest cancer death recorded among women in the world. Moreover, the chemo-resistance characteristic of CSCs in cervical cancer increases the tendency of patients to have recurrence cancer after chemotherapeutic treatment which resulted in poor survival rate. Recently, most of studies have focused on targeting CSCs in order to make them susceptible to chemotherapeutic treatment. Therefore, combine treatment with plant-derived anticancer drugs is one of the most promising approach. This study was aimed at identifying the anticancer effects of the combined therapy on HeLa cervical cancer cell line. In order to achieve the objectives, the effects of mono-retinoic acid (RA), mono-*C. nutans* and combined RA and *C. nutans* treatments on HeLa cells were examined via cell viability and apoptosis assays. In this study, various concentration of RA, *C. nutans* and combination of RA (3.0 µg/mL) with *C. nutans* were tested on HeLa cells for cell viability analysis. From the findings, both RA and *C. nutans* especially as combined treatment exhibited significant anticancer effects on HeLa cells in a dose dependent manner. An IC_{50} of 6.0 µg/mL, 17.68 µg/mL and 9.0 µg/mL were observed respectively in RA, *C. nutans* and the combination treatment. Furthermore, a study using apoptosis assay also revealed that mono-RA exhibited negligible apoptosis activity, while combined (RA-*C. nutans*) treatment induced better apoptosis activity in HeLa cells compared to mono-*C. nutans*. Overall, this study suggests that combination treatment (RA-*C. nutans*) gave better anticancer effects in the treatment of cervical cancer compare to their own monotherapeutic treatment.

Keywords: Retinoic acid; *Clinacanthus nutans*; Cervical cancer; HeLa cell line; Anti-cancer agent; Combination drug therapy

1. Introduction

Cancer-related death constituted as one of the main cause of morbidity in the world with reported an estimation of 8.2 million deaths and 14 million new cases in 2012 (Torre *et al*., 2015). This proportion is expected to grow worldwide particularly in low and middle-income countries (LMIC), with over 20 million new cancer cases expected annually as early as 2025 (Ferlay *et al*., 2015). According to the GLOBOCAN 2012, cervical cancer is the second most common cancer in less developed regions (445,000 cases) while ranks only 11th in more developed regions (83,000 cases) (Ferlay *et al*., 2015). Cervical cancer is an abnormal growth of cells arising from the cervical lining area which located at the lower end of the uterus in the human female reproductive system (Schiffman and Wentzensen, 2013). There are many treatments available to treat cervical cancer such as surgery, chemotherapy, radiotherapy, chemoradiotherapy and associated biological therapy (Wang *et al*., 2014). Despite that, many patients developed progressive or recurrent tumour after primary treatment (Poolkerd *et al*., 2006). From clinical studies, it showed that approximately 35% of women diagnosed with cervical cancer have recurrent tumour incidence, with 90% of them occurs within 3 years after the initial treatment (Gadducci *et al*., 2010; López *et al*., 2012). Therefore, it demonstrated that certain cervical cancer cells are not able to be eliminated by current therapeutics means.

CD133 is one of key biomarkers that has been used for characterization of cancer stem cells in solid tumours by which its expression is associated with regeneration, differentiation, and metabolism of the cancer cells (Li, 2013). Based on previous study, higher expression of CD133 was observed in isolated HeLa human cervical carcinoma cell line which exhibits a high degree of chemoresistance (López *et al*., 2012). This finding indicates that, HeLa cells which represent an invasive form of cervical cancer enriched in cancer stem cells, which explained the recurrence tumour incidence. One of the possible ways to target the self-renewal ability of cancer stem cells is by employing the concept of differentiation therapy (Schenk *et al*., 2014). Differentiation therapy offers less aggressive treatment by forcing the cancer stem cells to undergo differentiation to a more differentiated phenotype, making them more susceptible toward secondary treatment (Chumsri *et al*., 2007).

Retinoic acid (RA) as differentiating agent have been used for cancer stem cell-targeted therapy predominantly because of their ability to induce cellular differentiation in cancer stem cells (CSCs), cell cycle arrest and exerting cytotoxic effects in cancerous cells (Bushue and Wan, 2010; Sun and Lotan, 2002). In reference to previous studies, RA particularly all-trans retinoic acid (ATRA) has been successfully eliminated leukaemia-initiating cells (LICs), which are a type of cancer stem cell in Acute Promyelocytic Leukaemia (APL), resulted in cure rate of more than 70% (Chumsri *et al*., 2007). Recently, combination therapy with RA is highly demanded in order to quest for novel combination drugs that yield significant anticancer effects in cancer treatment compared to the treatment with RA alone (Schenk *et al*., 2014). Another alternative treatment is by incorporating with medicinal plants that have potential antioxidants, cytotoxic agents as well as generally safe that can give rise to better cancer treatment (Pratheeshkumar *et al*., 2012). Moreover, combination of RA with potential plant-derived anticancer drugs treatment may give another advantage such as reducing the RA dosage which in turn minimizing the adverse effects of RA toward patients which previously reported to cause respiratory distress, fever, and acute renal failure (Patatanian and Thompson, 2008).

One of the medicinal plants that have been acknowledged to possess anticancer agents is *Clinacanthus nutans* or also known as Sabah Snake Grass (Huang *et al*., 2015). *C. nutans* is a medicinal herb belongs to the family Acanthaceae and comprises of two species *Clinacanthus nutans* (Burm. f.) *Lindau* and *Clinacanthus siamensis Bremek* which proven to contains cytotoxic, antiviral, anti-inflammatory and antioxidant agents, thus, have become an important plant of research in the recent years (Shim *et al*., 2013; Yahaya *et al*., 2015). Most of the phytochemical constituents extracted from leaves, roots, stems, flowers and bulbs of the plant had demonstrated anticancer properties (Fong *et al*., 2016). Several bioactive components that can be found from *C. nutans* extract are flavonoids, C-glycosyl flavones, terpenoids, glycoglycerolipids, and sulphur containing glucosides (Yahaya *et al*., 2015). Among the various natural products, flavonoids have gain enormous amount of interest among medical researcher due to their remarkable spectrum of pharmacological activities such as antioxidant, antimutagenic, and anti-cancer activity (Arullappan *et al*., 2014; Ghasemzadeh *et al*., 2014). However, the mechanism underlying this anticancer activity is yet to be understood (Kamarudin *et al*., 2017). Overall, this study involves incorporation of retinoic acid with *Clinacanthus nutans* that both have potential anticancer properties to study the combined effects of these chemical and natural-based drugs in the inhibition of growth of HeLa, cervical cancer cells enriched in cancer stem cells (CSCs) rather than focus solely on single anticancer drug.

2. Materials and Methods

2.1. Drug preparation

The stock solution of retinoic acid (RA) by Tocris Bioscience (trans-retinoic acid) was prepared in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and further diluted with culture medium. The stock solution of *C. nutans* was prepared in 10% Tween 20 and further diluted with incomplete media.

2.2. Cell culture

Human cervical cancer cell line (HeLa cells) were cultured in 25 cm^3 flasks and maintained at 37 °C and 5% of CO_2 in DMEM/F12+GlutamaxTM1 (Life Technologies, USA), supplemented with 10% of Fetal Calf Serum (FCS), and 1% of antibiotic contained (100 U/mL penicillin, 100 µg of streptomycin/0.25 µg/mL) (Invitrogen). Cervical cancer cell line (HeLa cells) was passaged three times a week at 60 – 70% cell confluency.

2.3. Cell viability test

For cell viability assay, 3×10^3 of cells were seeded and incubated for 24 hours. After 24 hours, mono-RA and mono-*C. nutans* drugs at different concentrations (0, 1.5, 3.0, 4.5, 6.0, 9.0 and 15.0 µg/mL) and (0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL) were added respectively. As for the combined (RA + *C. nutans*) treatment, cells were incubated first with 3.0 µg/mL of RA for 24 hours prior to *C. nutans* treatment at various concentrations (0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL) for a further 24 hours. Next, the effect of all treatments on the cell viability of HeLa cells were assessed using CellTiter-Glo® 2.0 Assay (Promega) according to the manufacturer's instructions. The CellTiter-Glo® 2.0 assay provides a homogenous method to determine the number of viable cells in culture by measuring the amount of ATP present, which indicate the presence of metabolically active cells present in culture. Plates and its content were equilibrated to room temperature for 15 minutes. Then 100 µL of CellTiter-Glo® 2.0 reagent were added per well (100 µL of culture medium) and the contents were mixed for 2 minutes on a vortex mixer to induce cell lysis. The plates were

incubated for 10 minutes to stabilize luminescent signal. Finally, the assay activity was read at 490 nm by using GloMax®-Multi Microplate Multimode Reader (Promega GloMax®-Multi Detection System). Experiments were done in triplicate in three independent experiments and the results were averaged.

2.4. Cell apoptosis assay

In this assay, 3×10^3 cells per well were seeded in a 96-well plate. The cells were incubated for 24 hours. After 24 hours, the RA (3.0 µg/mL), *C. nutans* (20 µg/mL) and combined RA-*C. nutans* (10 µg/mL) along with 20 nM Taxol (positive control drug for apoptosis) were added to the respective wells. Then the cells were incubated for another 24 hours. After 24 hours of incubation, caspase-3/7 activities was assessed by Caspase-Glo®3/7 (Promega) according to the manufacturer's instructions. Before performing the assay, Caspase-Glo®3/7 reagent was prepared. The reagent was equilibrated to room temperature and mixed well. The 96-well plates that containing cultured cells were equilibrated to room temperature. 100 µL of Caspase-Glo®3/7 reagent was added to each well that contain control, RA, *C. nutans*, combined RA-*C. nutans* drug and Taxol. The plate is covered and the contents in the well were mixed gently for 30 seconds and incubated at room temperature for 2 hours. Finally, the luminescence of the sample was measured by using GloMax®-Multi Microplate Multimode Reader (Promega GloMax®-Multi Detection System). Experiments were done in triplicate in three independent experiment and the results were averaged.

2.5. Statistical analysis

Statistical and graphical analysis of the data were done in Microsoft Excel and GraphPad Prism-7. Student's paired t-test with two-tailed distribution was used to compare RA-treated, *C. nutans*-treated, combined RA-*C. nutans* and untreated cervical cancer cells. The results were presented in a mean ± SEM for (cytotoxicity, cell apoptosis (caspase activity)). The significance is shown as follows: ** $p < 0.01$, *** $p < 0.001$,**** $p < 0.0001$.

3. Results and Discussion

3.1. Cell viability assay

Figure 1 (A) shows the effect of retinoic acid (RA) on HeLa cells. Overall, the cytotoxic activity of RA in HeLa cells was exhibited in a dose-dependence manner, ranging from doses of 0 µg/mL (denoted as non-treated (NT)) to 15 µg/mL. As seen in Figure 1 (A), RA at all doses showed significant reduction in HeLa cells viability by 21.89% ($p < 0.001$), followed by RA at 1.5 µg/mL by 31.32% ($p < 0.001$), 3.0 µg/mL by 41.21% ($p < 0.001$), the IC_{50} of mono RA treatment: 6.0 µg/mL reduced by 49.06% ($p < 0.001$), 9.0 µg/mL by 69.36% ($p < 0.0001$) and at final dose which is 15.0 µg/mL was reduced by 89.73% ($p < 0.001$) when compared to non-treated (NT) HeLa cells which have 100% of viable cells. Figure 2 (A) shows the morphological changes of HeLa cells after 24 hours of treatment with RA. As shown in Figure 2 (A), non-treated HeLa cells (control) were adherent and displayed epithelial like-shaped structure with intact plasma membrane. RA treated HeLa cells on the other hand showed increase reduction in cell density along with increase in RA concentration. Moreover, RA at higher doses (6.0, 9.0, and 15.0 µg/mL) seem to exert sufficient anticancer effects via inducing significant cytotoxicity towards HeLa cells caused the cells to loss their membrane integrity. This finding suggests that RA induced significant anticancer effect at higher concentration.

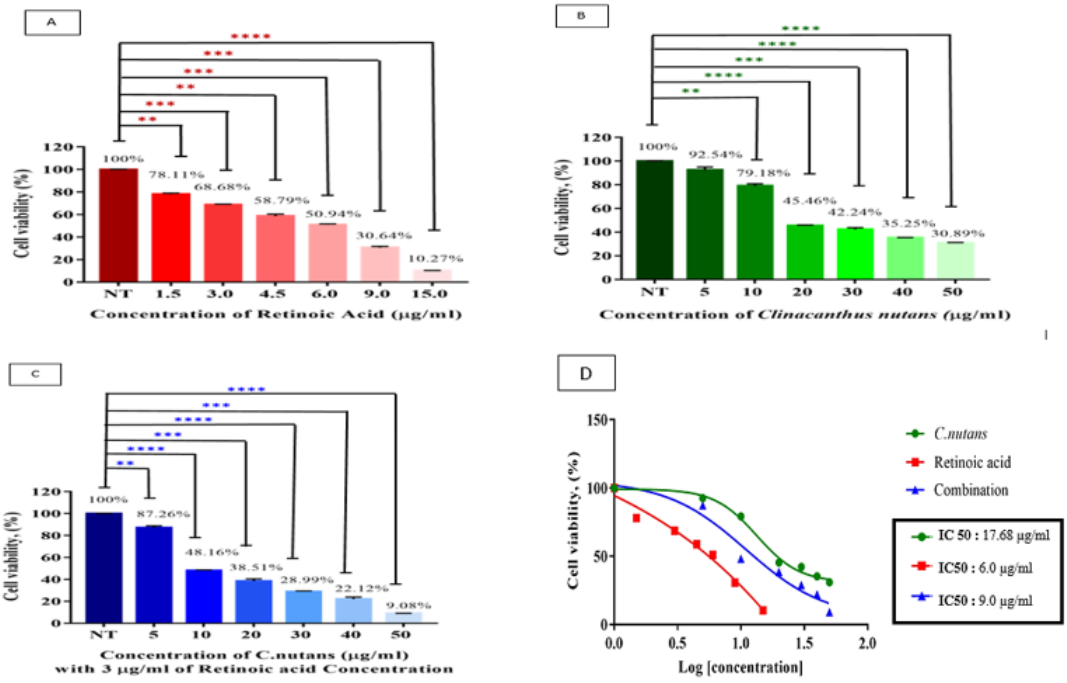

Figure 1: Cell viability assay of Retinoic Acid (RA), *C. nutans* and combined RA-*C. nutans* treatment on HeLa cell. (A) Effect of RA on HeLa cells viability before and after treatment at increasing concentration (0, 1.5, 3.0, 4.5, 6.0, 9.0 and 15.0 µg/mL) (B) Effect of *C. nutans* on HeLa cells viability before and after *C. nutans* treatment at increasing concentration (0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL). (C) Effect of combined therapy: RA (3.0 µg/mL) and *C. nutans* at increasing concentration (0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL) on HeLa cells viability before and after treatment. (D) Comparison between RA, *C. nutans* and combined RA-*C. nutans* treatments based on cell viability assay. Data were expressed as mean ± SEM from 3 number of experiment. ** $p < 0.01$, *** $p < 0.001$,**** $p < 0.0001$.

Based on Figure 1 (B), reduction in cell viability was observed after 24 hours of *C. nutans* treatment in a dose dependent manner ranging from 5.0 µg/mL to 50.0 µg/mL. *C. nutans* treated HeLa cells showed small inhibition of cell viability at initial dose which is 5.0 µg/mL by 7.46%. However, a drastic reduction of cell viability was observed at 10 µg/mL by 20.82% ($p < 0.01$), followed by IC_{50}: 17.68 µg/mL was reduced by 50% ($p < 0.001$). Nevertheless, gradual reduction at the subsequent doses of 20, 30, 40 and 50 µg/mL, where the viable cells were reduced by 57.76% ($p < 0.0001$), 64.75% ($p < 0.0001$) and 69.11% ($p < 0.0001$), respectively when compared to non-treated (NT) HeLa cells. This result showed that, *C. nutans* cytotoxicity effect in HeLa cells was not as potent as RA even at higher dosage. In this study, the 50% reduction in cell viability of HeLa cells treated with ethanolic extract of *C. nutans* was 17.68 µg/mL. Though, the IC_{50} dose exhibited by *C. nutans* on HeLa cells is within the range fixed by the National Cancer Institute (NCI), which states that any crude extract can be considered as active if its IC_{50} value is less than 20 µg/mL (Ghasemzadeh *et al*., 2014). Hence *C. nutans* can be considered as a potential anticancer therapeutic agent. The morphological changes of HeLa cells treated with *C. nutans* can be seen in Figure 2 (B). When compared to the non-treated (NT) HeLa cells which showed intact epithelial-shaped structure, however, after 24 hours of treatment with *C. nutans*, the cells in all the tested concentrations displayed reduction in cell density. At early doses, most of the cells still retained their epithelial-shaped appearance and only few of them exhibited cells shrinkage although reduction in cell density were observed. In contrast, dramatic changes in cell density was observed at the dose of 20 µg/mL, where formation of apoptotic bodies were more visible and kept increasing up to 50 µg/mL. These changes in cell density and morphology correlated with the finding shown in Figure 3, in which prominent apoptosis activity was recorded via increased apoptosis activity in HeLa cells treated with the IC_{50} *C. nutans* concentration compared to the non-treated (NT) HeLa cells.

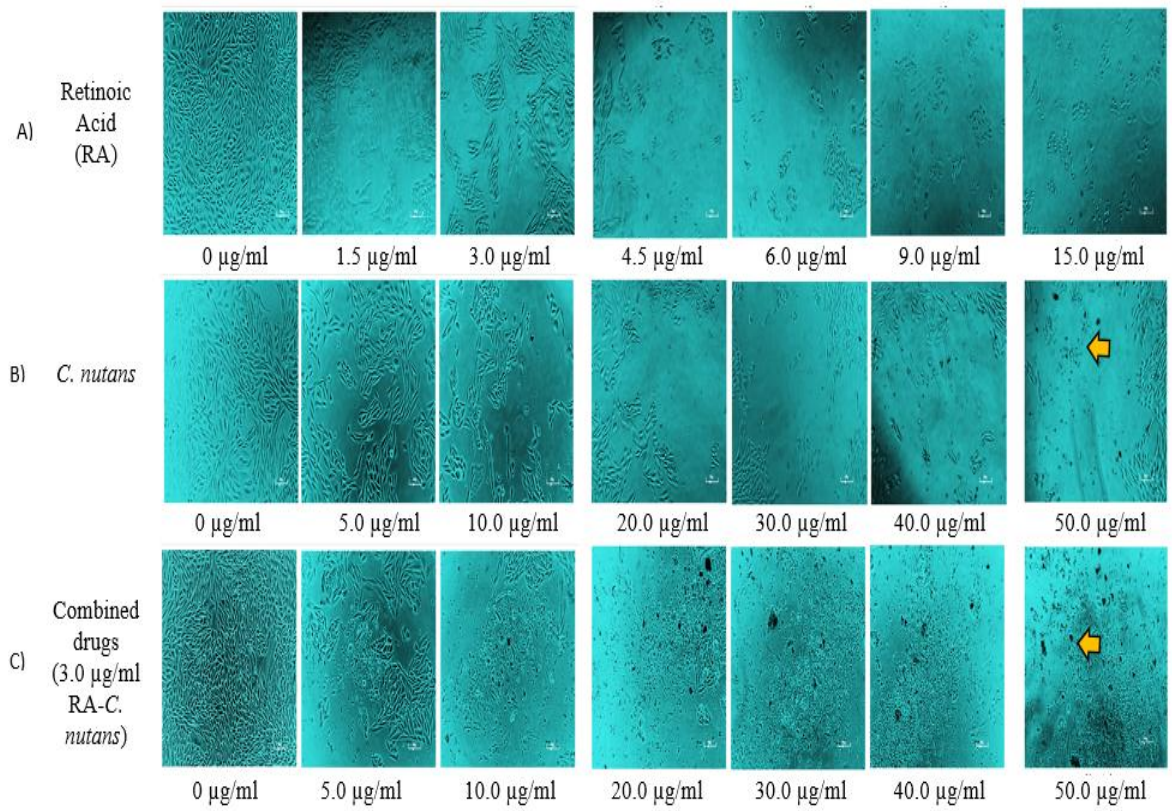

Figure 2: Microscopy images of HeLa cells before and after Retinoic Acid (RA), *C. nutans* and combined RA-*C. nutans* treatment. (A) Morphological changes in HeLa cells induced by RA at increasing doses (0 (NT), 1.5, 3.0, 4.5, 6.0, 9.0 and 15.0 µg/mL). (B) Changes in HeLa cells morphology after treated with *C. nutans* at increasing doses (0 (NT), 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL). (C) The morphological changes of HeLa cells after treated with 3.0 µg/mL RA and *C. nutans* at increasing doses (0 (NT), 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL). The images were obtained using Nikon eclipse TS 100 inverted microscope at 10X magnification. Yellow arrows show the formation of apoptotic bodies. Scale bar: 100 um.

The combined treatment of retinoic acid (RA) and *C. nutans* on HeLa cells was conducted to see whether the combination of both treatments resulted in better anticancer effect in cervical cancer cells. In this study, dose of RA was fixed at 3.0 µg/mL, which is slightly lower than IC_{50} of mono treatment of RA which is 6.0 µg/mL (Refer to Figure 1 (A). The step was done as an attempt to minimizing the dosage which often is presumed to reduce the possible toxic effects manifested by retinoic acid. To support this attempt, RA has been reported to cause oxidative stress in human fibroblast at a dose of 20 µM or at 6.0 µg/mL which result in cell damaged (Gimeno *et al.*, 2004). As shown in Figure 1 (C), the cytotoxic activity of combined RA-*C. nutans* in HeLa cells was exhibited in a dose-dependence manner ranging from 0 µg/mL (denoted as non-treated (NT)) to 50 µg/mL *C. nutans* with fixed dose of RA (3.0 µg/mL). Combined treatment of (3.0 µg/mL RA + 5.0 to 50 µg/mL *C. nutans*) reduced the cell viability of HeLa cells at 5.0 µg/mL by 12.74% ($p < 0.01$), followed by a dramatic decrease at IC_{50} 9.0 µg/mL by 50%, $p < 0.0001$), and at subsequent doses of 20.0 µg/mL by 61.49% ($p < 0.001$), 30 µg/mL by 71% ($p < 0.0001$), 40 µg/mL by 77.88% ($p < 0.001$) and at final dose; 50.0 µg/mL by more than 90% reduction in cell viability. The cytotoxic effect of combination treatment is comparable to mono treatment of RA and far greater than mono-*C. nutans* treatment. This finding may suggest that addition of RA drug prior to *C. nutans* treatment capable of inducing better anticancer effects by sensitize CSCs enriched in HeLa cell culture to *C. nutans* treatment. However, more study is needed to prove this claim.

According to Figure 2 (C), the combined treatment of (3.0 µg/mL RA + *C. nutans*) in HeLa cells displayed great reduction in cell density in all the tested *C. nutans* concentrations in which the cell viability were significantly reduced compared of non-treated (NT) cells as well as the mono *C. nutans* (10 – 50 µg/mL) treated HeLa cells. The cell density in combination treatment of 5 µg/mL *C. nutans*, showed reduction in cell viability although some of them still retained their epithelial-shaped appearance. However, obvious changes in cell morphology and reduction is cell viability was observed between (3.0 µg/mL RA + 10 to 50 µg/mL *C. nutans*) treated HeLa cells represented by the loss of cell adhesion, flooding of apoptotic bodies (Figure 2 (C)). This finding can be correlated well with the finding in Figure 3 in which an increased in caspase 3/7 activity (0.54%) was observed at (3.0 µg/mL RA + 10 µg/mL *C. nutans*) compared to mono-RA treatment (no apoptosis) and

mono-*C. nutans* (0.37%) treated HeLa cells indicating an up-regulated apoptosis activity in action. Furthermore, combined treatment at (3.0 µg/mL RA + 20 to 50 µg/mL *C. nutans*), clearly showed that almost all cells underwent apoptosis as viable cells were almost invisible. From this observation, it can be indicated that the reduction in cell viability are more significant in RA-*C. nutans* treatment compared to mono treatment of *C. nutans*. This can be explained based on the IC_{50} concentrations achieved by the respective treatments. The new IC_{50} concentration of the combined (3.0 µg/mL RA + 0 to 50 µg/mL *C. nutans*) treatment is 9.0 µg/mL compared to mono-*C. nutans* treatment (17.68 µg/mL). Therefore, this result suggested that the addition of secondary anticancer drug can potentially result in better killing effects toward susceptible CSCs and cancerous cells population which overall showed improvement in cancer treatment.

3.2. Apoptosis assay

In this study, IC_{50} of mono-*C. nutans* and combined RA–*C. nutans* treatments were used to examine and confirm its ability to induce apoptosis. Meanwhile, 3.0 µg/mL of RA was chosen for apoptosis assay instead of its IC_{50} value (6.0 µg/mL) due to toxic concentration level which is not adequate to be used in cancer treatment. Figure 3 showed the caspase activity in non-treated (NT), mono treatments of *C. nutans* and RA, Taxol (positive control), and combined RA–*C. nutans* treatments in HeLa cells. All treatments were normalized to 1% which is presumed to be natural apoptosis in cells. Any increase above 1% in caspase 3/7 activity is correlated to positive apoptosis activity induced by the respective drug treatment. Figure 3 shows a visible increase in caspase 3/7 activity upon treatments of 3.0 µg/mL RA – 10.0 µg/mL *C. nutans* treatment (0.54%) apoptosis activity, followed by 20 µg/mL of mono *C. nutans* treatment (0.37%) and non-treated HeLa cells (0%) respectively. In contrast, mono-RA treatment at 3.0 µg/mL showed negligible caspase activity which indicated that the apoptosis activity induced by RA was not significant.

Although *C. nutans* showed apoptosis activity in HeLa cancer cells, its potential may be limited as can only targeting the non-CSCs population (cancerous cells) and not the CSC sub-population within the tumour. However, the combined treatment of (RA + *C. nutans*) showed the second highest apoptosis activity after Taxol treatment when compared to the non-treated (NT) HeLa cells. This finding may suggest that the addition of RA prior to *C. nutans* treatment resulted in HeLa cells being more susceptible to *C. nutans* treatment. It is presumed that the 3.0 µg/mL RA treated HeLa cells (especially the CSCs) were differentiated which in turn sensitised these cells towards *C. nutans* treatment and became more susceptible to apoptosis induced cell death exhibited by the *C. nutans* as seen in Figure 2 (C). The overall findings on the effect of RA and *C. nutans*, both mono- and combination treatment (Figures 1 (D) and 3) suggest that RA sensitised HeLa cells towards *C. nutans* treatment. As a whole, mono-RA treatment (IC_{50}: 6.0 µg/mL) induced better anticancer effect than mono-*C. nutans* treatment even at lower concentration although not by apoptosis mechanism while mono-*C. nutans* treatment (IC_{50}: 17.68 µg/mL) induced apoptosis although the inhibitory effect was not as potent as mono RA. However interestingly, addition of 3 µg/mL prior to *C. nutans* treatment for the combined therapy (IC_{50}: 9.0 µg/mL) suggest that RA capable of sensitising HeLa cells towards *C. nutans* treatment presumably via differentiation of CSCs within cancer niche and significantly inhibiting the cell growth via increased number of apoptotic bodies at 10 µg/mL onwards in comparison to mono-*C. nutans* treatment (reduced IC_{50} of mono-*C. nutans*: 17.68 µg/mL to IC_{50} of 3.0 µg/mL RA + *C. nutans*: 9.0 µg/mL). However many studies is required to validate and confirm this effects.

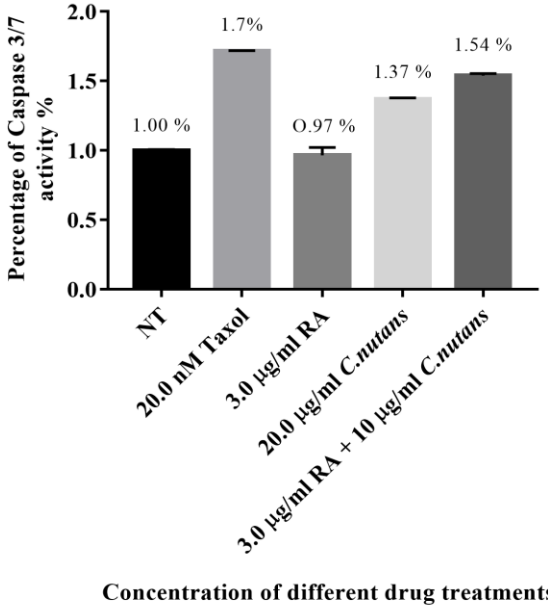

Figure 3: *C. nutans* and combined RA-*C. nutans* treatment induced apoptosis in HeLa cells. The chart shows the effect of Taxol (20 nM), RA (3.0 µg/mL), *C. nutans* (20.0 µg/mL) and combined RA (3.0 µg/mL) + *C. nutans* (10.0 µg/mL) treatment on cell apoptosis in HeLa cells. Taxol was used as a positive control for apoptosis in this assay. All treatments were compared to the non-treated HeLa cells which were normalized to 1% of apoptosis. *** $p < 0.001$.

4. Conclusions

The failure to eradicate chemo-resistance cancer stem cells (CSCs) which is the root cause of recurrence cancer pressing the need for novel and effective forms of cancer therapy. Therefore, concurrent treatment of cancer stem cell-targeted therapy, using retinoic acid (RA) drug with *Clinacanthus nutans* (*C. nutans*), a plant-derived anticancer agent may be a good option to kill the cancer stem cells sub-population enriched in cervical cancer cells instead of single drug use. Current findings showed that cancer cell inhibition in both RA and *C. nutans* only happen significantly at higher doses, where RA (6 µg/mL and above) and *C. nutans* (20 µg/mL). In terms of combination treatment, it was clear that the combination treatment of RA-*C. nutans* drugs resulted in a better anticancer effects than mono-*C. nutans* drug but comparable to the mono-RA drug. Besides, the IC_{50} concentration of the combined treatment was also reduced drastically to 9 µg/mL from IC_{50} of mono-*C. nutans* (17.68 µg/mL) although RA dose in combination treatment was used at 3 µg/mL lower than IC_{50} of mono-RA (6 µg/mL). Achieving 50% cell inhibition upon treatment using lower concentration of drugs is a breakthrough because it can reduce the drug dose without affecting the potency of the drug in inducing its anticancer effects. Furthermore, the finding of this study has also confirms the mode of action of a drug and its efficiency to induce apoptosis through Caspase-Glo® 3/7 assay which used Taxol as a control. The result indicated that both combination RA-*C. nutans* and mono-*C. nutans* induces apoptosis while retinoic acid gave negligible apoptosis activity. It is highly suggested that the use of combined retinoic acid-*C. nutans* treatment on HeLa may be further studied as a way to kill the cervical cancer cells in the future.

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Effects of Combined Retinoic Acid-Curcumin Treatment in HeLa Cancer Cells

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Abstract: Cervical cancer is a disease in which malignant cells form in the tissues of the cervix. Additionally, cervical cancer has an abundance of cancer stem cells (CSCs). Conventional therapy usually destroy the bulk of cancer cells, but often not able to exterminate the critical CSCs. Recently, most of the studies are focused on targeting CSCs in order of converting them into a more differentiated phenotype by co-administering drugs that work by different molecular mechanisms such as differentiation and apoptosis. This study was aimed at studying the anticancer effect of combined Retinoic acid-Curcumin in cervical cancer cells and the ability of combined Retinoic acid-Curcumin to induce apoptosis in cervical cancer cells. In order to achieve the objectives, the effect of Retinoic acid, Curcumin and combined Retinoic acid and Curcumin on HeLa cells were examined via cell viability as well as apoptosis assays. Both Retinoic acid and Curcumin especially as combined treatment displayed powerful anticancer effects on HeLa cancer cells. In this research, Retinoic acid concentration at (0, 5, 10, 15, 20, 30 and 50 µM), Curcumin concentration at (0, 5, 7.5, 10, 12.5 and 15 µM) and combination of Retinoic acid (10 µM) with Curcumin concentration at (0, 5, 7.5, 10, 12.5 and 15 µM) were tested on HeLa cell viability. From the findings, both Retinoic acid and Curcumin reduced cell viability in a dose dependent manner by 21.89 – 89.73% (Retinoic acid), 6.63 – 61.07% (Curcumin) and 9.76 – 71.69% (combined Retinoic acid-Curcumin). Based on the study in terms of apoptosis assay, it was revealed that Curcumin and combined treatment induced apoptosis in HeLa cancer cells. In conclusion, these findings altogether proposed that both Retinoic acid and Curcumin exhibit strong anticancer activities especially in combination for the targeted therapy of cervical cancer cells as represented by the HeLa cancer cells and probably for other cancers abundant with CSCs.

Keywords: Cancer stem cells; Cervical cancer; HeLa cells; Combination drug; Retinoic acid; Curcumin

1. Introduction

Ranked as the second most frequently diagnosed cancer, cervical cancer is the third top reason of cancer death among females in less developed countries (Torre *et al*., 2015). Surgery, radiation therapy, chemotherapy and targeted therapy are some traditional types of cervical cancer treatment (Siegel *et al*., 2012). The HeLa cell line is a commonly studied cervical adenocarcinoma cell line with a high capacity for malignancy (Wang *et al*., 2013). HeLa cell line also have abundance of cancer stem cells (CSCs) which interfere with any therapy given to the patients (Chang *et al*., 2016). During the earliest stages of cervical cancer, combined treatment may be used. For later stages, the main treatment is normally radiation combined with chemotherapy (Secord *et al*., 2007).

However, most of the patients with cervical cancer show tumor recurrence after therapy and this is presumed to be caused by the presence of Cancer Stem Cells (CSC) within the tumor by stimulating and supporting tumor growth (Ortiz-Sánchez *et al*., 2016). The main reason for tumor recurrent is the survival and accumulation of drug-resistant CSCs following chemo or radiotherapy which cause the recurrence of increasingly invasive and malignant tumours (Vinogradov and Wei, 2012). CSCs that survive will undergoes the reactivation of proliferation (Vinogradov and Wei, 2012). As a result, to prevent cancer relapse, CSCs need to be specifically aimed and destroyed because it can help to improve the survival of cancer patients. Another problem is the use of single drug in targeted therapy is not enough to induce apoptosis to cancer cell. Therefore, discovery for novel drug approaches which may potentially target the CSCs are needed in order to tackle the recurrence of cervical cancer.

Combination therapy is defined as disease treatment with two or more drugs to achieve efficacy with lower doses or lower toxicity drugs, chemo-sensitize cells so that an additional compound can be more potent, gain additive or synergistic effects or minimize the possibility for development of drug resistance (Karjalainen and

Repasky, 2016). One combination treatment approach is to co-administer drugs that work by different molecular mechanisms such as differentiation and apoptosis (Florea and Büsselberg, 2011).

In terms of chemical structure, Retinoic acid fall under the same family as vitamin A (retinol) (Duester, 2000). It is known to have direct action on inhibition of cancer cell proliferation and cytotoxicity in HeLa cells in vitro (Vihari and Siddikuzzaman, 2014). In contrast to retinoic acid, curcumin is frequently used in Asia for several things such as health care, food preservation and yellow dye for textiles. Study showed that curcumin is found in turmeric which was derived from *Curcuma longa plant*, a gold-colored spice (Prasad and Aggarwal, 2011). Previous work has proposed that curcumin may have the ability as a cancer chemo preventive and chemotherapeutic agent (Jiao *et al*., 2009) with the mechanism of action through apoptosis.

The objectives of this research are to study the anticancer effect of combined Retinoic acid-Curcumin in cervical cancer cells and to investigate the ability of combined Retinoic acid-Curcumin to induce apoptosis in cervical cancer cells. The work study about the anticancer effects of retinoic acid, curcumin and combination of retinoic acid and curcumin to kill HeLa cancer cells. A range of retinoic acid and curcumin concentration will be tested on cervical cancer cell to examine their cytotoxicity via cell growth inhibition and apoptosis assays. The outcomes of this study revealed the anticancer effects exerted by the combined retinoic-curcumin treatment in cancer therapy and tumor prevention. This study will lead to the initial knowledge on the mode of action of retinoic acid-curcumin in cervical cancer cells. The combined retinoic acid-curcumin treatment can potentially be a potent chemotherapeutic treatment and offer a targeted therapy for cervical cancer cells. The findings in this study will also present a new understanding for adjuvant treatment in treating cancers, especially cancers which are abundant with CSCs. Thus, this study will set the foundation for future researcher to investigate further, optimize and finally apply this method in battling cancer.

This study was carried out to find out whether the combination treatment of retinoic acid and curcumin have anticancer effects and induce apoptosis in cervical cancer cells.

2. Materials and Methods

2.1. Drug preparation

The stock solution of retinoic acid by Tocris Bioscience (trans-retinoic acid) was prepared in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and further diluted with culture medium. All-trans-retinoic acid (ATRA) was purchased from Tocris Bioscience, United Kingdom. The stock solution of curcumin was prepared in serum free-media (incomplete media) and further diluted with culture medium.

2.2. Cell culture

Human cervical cancer cell line (HeLa cells) was cultured in T25 flasks and maintained at 37 °C and 5% of CO_2 in DMEM/F12+GlutamaxTM1 (Life Technologies, USA), supplemented with 10% fetal calf serum (FCS), and 1% of antibiotic ant mycotic (100 U/mL penicillin, 100 µg of streptomycin/0.25 µg/mL) (Invitrogen). Cervical cancer cell line (HeLa cells) was passaged three times a week at 60 – 70% cell confluency.

2.3. Cell viability test

60 – 70% confluency of cell cultures were used to test cell viability (cytotoxicity). About 3 × 103 cells were incubated for 24 hours. After 24 hours, two different cell cultures were added with retinoic acid and curcumin with different concentration (0, 5, 10, 15, 20 and 30 µM) for retinoic acid and (0, 5, 7.5, 10, 12.5 and 15 µM) for curcumin. Another cell culture for combination treatment was added with 10 µM of retinoic acid on all well. After 24 hours of incubation, cell viability on different concentration of mono retinoic acid and curcumin treatment were assessed by CellTiter-Glo® 2.0 Assay (Promega) according to the manufacturer's instructions. Meanwhile, the combination drugs cell culture were added with the second drug which was different concentration of curcumin (0, 5, 7.5, 10, 12.5 and 15 µM) and allowed to be incubated for another 24 hours before assessed by CellTiter-Glo® 2.0 Assay (Promega). Plates and its content were equilibrated to room temperature for 15 minutes. Then 100 µL of CellTiter-Glo® 2.0 reagent were added per well (100 µL of culture medium) and the contents were mixed for 2 minutes on a vortex mixer to induce cell lysis. The plates were incubated for 10 minutes to stabilize luminescent signal. Finally, assay activity was read at 490 nm by using GloMax®-Multi Microplate Multimode Reader (Promega GloMax®-Multi Detection System). Experiments were done in triplicate in three independent experiments and the results were averaged.

2.4. Cell apoptosis assay

In this assay, cell cultures of 60 – 70% confluency were used. 4 × 103 cells per 100 µL were counted by using hemocytometer and the cells were seeded on 96-well plate. The cells were incubated for 24 hours. After 24 hours, retinoic acid, curcumin and combined retinoic acid-curcumin drug were added and after that, Taxol was used as a positive control drug for this assay. The cells were incubated for another 24 hours. IC_{50} of Retinoic acid and curcumin were selected as it shows 50% reduction in cell proliferation assay. After 24 hours of incubation, caspase-3/7 activities was assessed by Caspase-Glo®3/7 (Promega) according to the manufacturer's instructions. Before performing the assay, Caspase-Glo®3/7 reagent was prepared. The reagent was equilibrated to room temperature and mixed well. The 96-well plates that containing cultured cells were equilibrated to room temperature. 100µL of Caspase-Glo®3/7 reagent was added to each well that contain control, retinoic acid, curcumin, combined retinoic acid-curcumin drug and 20 nM of Taxol. The plate wells were covered and the contents in the well were mixed gently for 30 seconds and incubated at room temperature for 2 hours. Finally, the fluorescence intensity of sample was measured at 490 nm by installing a blue filter using a GloMax®-Multi Microplate Multimode Reader (Promega GloMax®-Multi Detection System). Experiments were done in triplicate in three independent experiment and the results were averaged.

2.5. Statistical analysis

Statistical and graphical analysis of the data were done in Microsoft Excel and GraphPad Prism-7. Student's paired *t-test* with two-tailed distribution was used to compare retinoic acid-treated, curcumin-treated, combined retinoic acid-curcumin and untreated cervical cancer cells. The results were presented in a mean ± SEM for (cytotoxicity, cell apoptosis (caspase activity)). The significance is shown as follows: * $p < 0.05$; ** $p<0.005$; *** $p<0.0005$.

3. Results

3.1. Cell viability assay

Figure 1A shows the effect of retinoic acid (RA) on HeLa cells. It can be seen that Retinoic Acid significantly reduced the cell viability when treated with a range of low to high Retinoic Acid concentrations. Based on the above figure, it is observed that Retinoic Acid at its lowest concentration of 5 µM reduced cell viability by 21.89% while at 10 µM by 31.32%, followed by 15 µM (41.21%), 18.7 µM the IC_{50} (50%) and 20 µM (49.06%), and respectively. At 30 µM and 50 µM of Retinoic Acid, the viability of cells decreased more drastically which is by 69.36% and 89.73% respectively.

Figure 1B shows the effect of curcumin on HeLa cells. It can be seen that curcumin significantly reduced the cell viability when treated with a range of low to high curcumin concentrations. Based on the figure, it was observed that curcumin at its lowest concentration of 5 µM reduced cell viability by 6.63% while at 7.5 µM by 9.96%, followed by 10 µM (21.42%), 12.5 µM (48.72%) and 12.61 µM the IC_{50} (50%), and respectively. At 15 µM of curcumin, the viability of cells decreased more drastically which is by 61.07%.

Figure 1C shows the effect of combined Retinoic acid and curcumin on HeLa cells. A fixed Retinoic Acid concentration (10 µM) was chosen as the first drug which was then tested with a range of curcumin concentrations ranging from (0 – 15 µM) as the second drug. It can be seen that the combination drug significantly reduced the cell viability when treated with a range of low to high curcumin concentrations. Based on the above figure, it was observed that curcumin at its lowest concentration of 5 µM reduced cell viability by 9.74% while at 7.5 µM by 28.13%, followed by 10 µM (44.26%), 12.5 µM (57.41%) and 11.06 µM the IC_{50} (50%), and respectively. At 15 µM of curcumin, the viability of cells decreased more drastically which is by 71.69%.

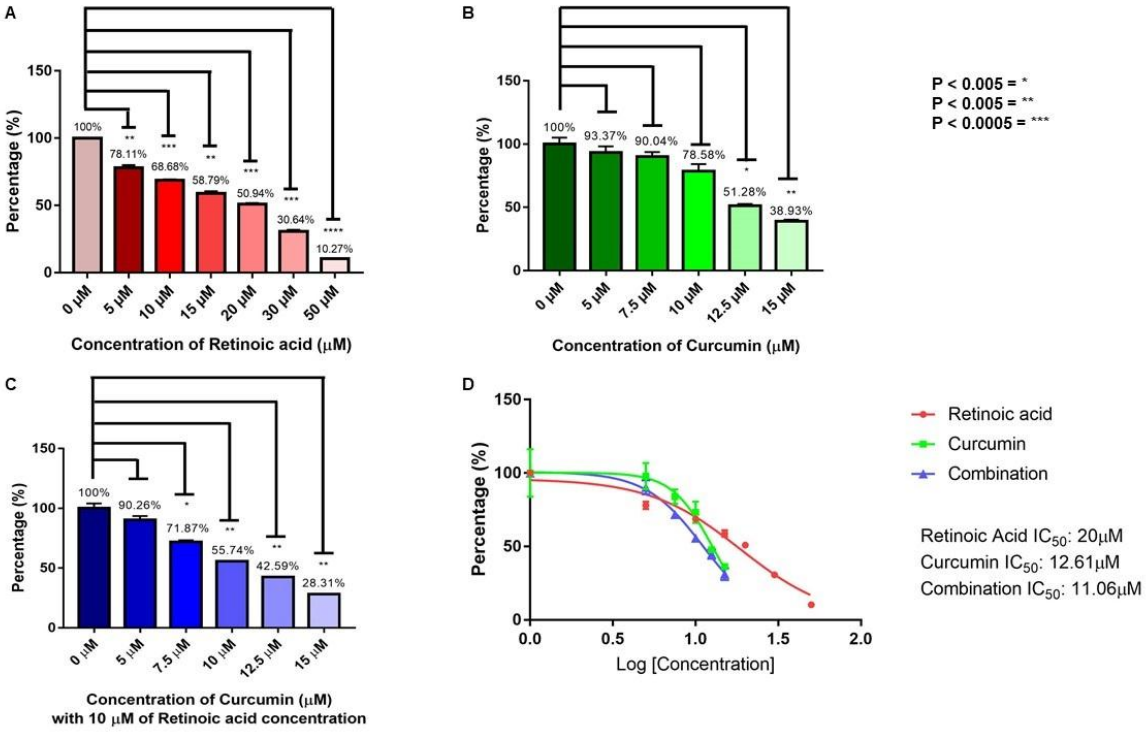

Figure 1: Cell viability Assay of Retinoic Acid, Curcumin and combined Retinoic Acid-Curcumin treatment on HeLa cell. (A) Effect of retinoic acid on HeLa cells viability before and after treatment at increasing concentration (0, 5, 10, 15, 20, 30 and 50 μM) (B) Effect of curcumin on HeLa cells viability before and after curcumin treatment at increasing concentration (0, 5, 7.5, 10, 12.5 and 15 μM). (C) Effect of combined therapy: retinoic acid (10 μM) and curcumin at increasing concentration (0, 5, 7.5, 10, 12.5 and 15 μM) on HeLa cells viability before and after treatment. (D) Comparison between Retinoic Acid, Curcumin and combined Retinoic Acid-Curcumin treatment based on cell viability assay. ($P < 0.005$ = *, $P < 0.005$ = **, $P < 0.0005$ = ***). Data are expressed as mean ± SEM from 3 number of experiment.

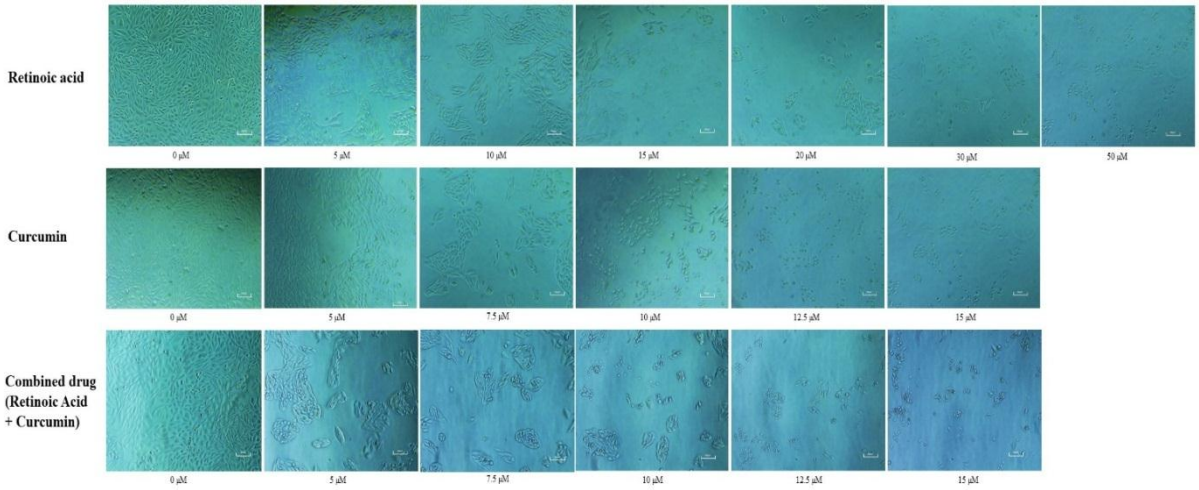

Figure 2: Microscopy image of HeLa cells before and after Retinoic Acid, Curcumin and combined Retinoic Acid-Curcumin treatment. (A) Effect of retinoic acid on HeLa cells viability before and after treatment at increasing concentration (0, 5, 10, 15, 20, 30 and 50 μM) (B) Effect of curcumin on HeLa cells viability before and after curcumin treatment at increasing concentration (0, 5, 7.5, 10, 12.5 and 15 μM). (C) Effect of combined therapy: retinoic acid (10 μM) and curcumin at increasing concentration (0, 5, 7.5, 10, 12.5 and 15 μM) on HeLa cells viability before and after treatment.

3.2. Apoptosis assay

Figure 3 shows a comparison between retinoic acid (10 μM), curcumin (12.5 μM), combined retinoic acid (10 μM) and curcumin (11 μM), as well as Taxol (20 nM), a well-known apoptosis inducing drug treated and untreated HeLa cells. Taxol functions as positive control in the study. The percentage of apoptosis for non-

treated cells was normalized to 1% which indicates the natural apoptosis and treated (20 nM of Taxol, 10 µM of retinoic acid, 12.5 µM of curcumin, combination of 10 µM retinoic acid and 11µM curcumin) is 1.07%, 0.97%, 1.30% and 1.66% respectively. The luminescent percentage different between control (non-treated) and treated (20 nM of Taxol, 12.5 µM of curcumin, combination of 10 µM retinoic acid and 11 µM curcumin) are 0.07%, 0.30% and 0.66% respectively.

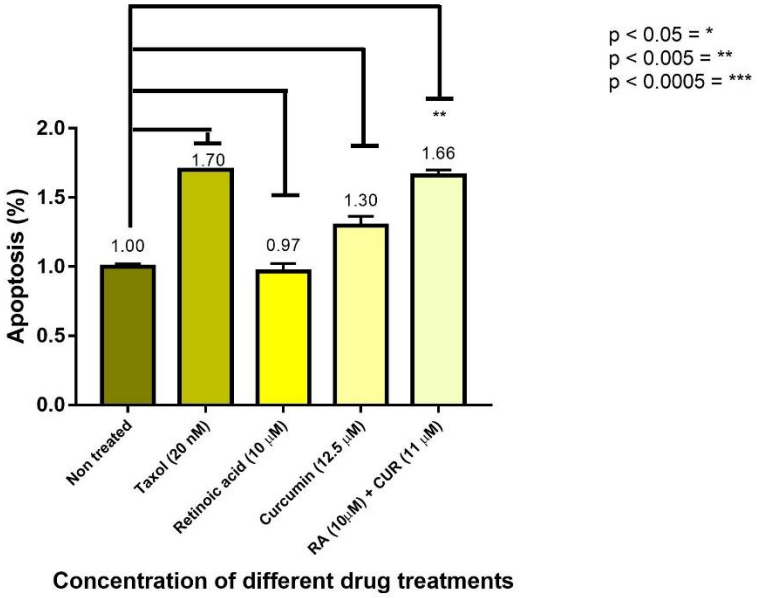

Figure 3: Cell viability Assay of Retinoic Acid, Curcumin and combined Retinoic Acid-Curcumin treatment on HeLa cell. (A) Effect of retinoic acid on HeLa cells viability before and after treatment at increasing concentration (0, 5, 10, 15, 20, 30 and 50 µM) (B) Effect of curcumin on HeLa cells viability before and after curcumin treatment at increasing concentration (0, 5, 7.5, 10, 12.5 and 15 µM). (C) Effect of combined therapy: retinoic acid (10 µM) and curcumin at increasing concentration (0, 5, 7.5, 10, 12.5 and 15 µM) on HeLa cells viability before and after treatment. (D) Comparison between Retinoic Acid, Curcumin and combined Retinoic Acid-Curcumin treatment based on cell viability assay. ($P < 0.005$ = *, $P < 0.005$ = **, $P < 0.0005$ = ***). Data are expressed as mean ± SEM from 3 number of experiment.

4. Discussion

4.1. Cell viability assay

Retinoic acid demonstrated the ability to significant inhibit cell viability of HeLa cells in a dose dependent manner beginning from 5 µM up to 50 µM. The high IC_{50} Retinoic Acid concentration (20 µM) and remaining number of viable HeLa cells even after the treatment of Retinoic Acid at high doses 20 – 50 µM can be correlated with the presence of cancer stem cells (CSCs) which are known to be highly resistant to most drugs. Curcumin demonstrated the ability to significant inhibit cell viability of HeLa cells in a dose dependent manner beginning from 5 µM up to 15 µM. The high IC_{50} curcumin concentration (12.61 µM) and remaining number of viable HeLa cells even after the treatment of curcumin at higher doses 10 – 15 µM can be correlated with the presence of cancer stem cells (CSCs) which are known to be highly resistant to most drugs.

For Figure 1C, 10 µM of retinoic acid was chosen for the combined treatment based on two reasoning; 1) a significant cytotoxicity effect on HeLa cells was observed and 2) 10 µM Retinoic Acid is much lower than the IC_{50} concentration. Based on these reasoning and the fact that high dose of any drug lead to various side effects in patients, hence reducing the drug dose without affecting the potency of the drug to still induce its anticancer effect is very crucial here. In addition to that, the second drug (curcumin) is believed to sensitize the cancer cells to Retinoic Acid in the treatment.

Figure 2C helps us to understand further the morphological changes in cells post combination treatment and correlating it to the cytotoxicity effect of Retinoic Acid and curcumin. Based on Figure 1A-B and 2A-B, it is presumed that both Retinoic Acid and curcumin induced different anticancer mechanism on HeLa cells which are differentiation and apoptosis respectively. Changes in the morphology of the remaining viable cells which are presumed to be cancer stem cells (CSC), suggests that Retinoic Acid may possibly induce differentiation of the CSC sub population. However, more work is needed to confirm this claim.

As the HeLa cell line is known to be enriched in CSCs and previous findings suggest that Retinoic Acid induces differentiation. Retinoic Acid was used as the first drug to differentiate the CSCs first. The aim of combination drug is to target the recurring cancer stem cells. Firstly step is to induce differentiation on cancer stem cells (CSC) into more differentiated cell type (Taylor and Jabbarzadeh, 2017). The differentiated cells then can be killed by administering another drug which induces apoptosis (Taylor and Jabbarzadeh, 2017). Based on this principle, HeLa cells were treated with Retinoic Acid followed by Curcumin. The combined treatment in this study showed that there is a slight improvement where HeLa cells were sensitized to Retinoic Acid treatment by reducing the IC_{50} concentration from Retinoic Acid (20 µM) and curcumin (12.61 µM) to Retinoic Acid (10 µM) + curcumin (11.06 µM). In order to confirm these findings on the possible mode of action induced by Retinoic Acid and curcumin in HeLa cells, the ability of these drugs to induce apoptosis was studied (Figure 3).

4.2. Apoptosis assay

The data suggested that all three treatment (20 nM of Taxol, 12.6 µM of curcumin, combination of 10 µM retinoic acid and 11 µM curcumin) induced caspase -3 and -7 activity in HeLa cells when compared to the non-treated cells. Based on this finding, it can be confirmed that the mode of action for curcumin is through apoptosis because its reading is higher than natural apoptosis by untreated cells. From the observed data, combined retinoic acid-curcumin treatment also has a higher reading compared to natural apoptosis (untreated). This strongly correlates with the cell viability findings of the study that curcumin and combined treatment induced apoptosis in HeLa cells. A T-test between the curcumin treatment and combined treatment shows that they are significantly different where the p value is equal to 0.0418. Based on this study, it shows that combined treatment has a higher apoptosis activity when compared with curcumin. However, it is still lower than Taxol which is one of the drugs that being commercially used these days. Based on the morphological observation, it is suggested that curcumin treatment on HeLa cells induces apoptosis. It can be suggest that by administering retinoic acid followed by curcumin, the cancer stem cells were differentiated first and a larger group of cells were more susceptible to apoptosis resulting in increased cell death and reduced drug.

5. Conclusions

The abundance of cancer stem cells in HeLa cells causes the recurrent of cancer cells to happen even after therapy. Therefore a targeted drug therapy that combines retinoic acid and curcumin maybe a good option as the use of single drug may not be sufficient to kill the invasive cancer stem cells population. Current findings showed that both curcumin and retinoic acid inhibition only happen significantly at higher doses of curcumin (12.5 µM) and retinoic acid (10 µM and above). In terms of combination treatment, it was clear that the combination of 10 µM retinoic acid and 11 µM curcumin shows 50% cell inhibition respectively. To achieve 50% cell inhibition using lower concentration of drugs is a breakthrough because it will reduce the drug dose without affecting the potency of the drug to still induce its anticancer effect. Another study that confirms the mode of action of a drug and the efficiency of a drug to induce apoptosis is apoptosis assay. Apoptosis assay which was carried by using Caspase-Glo® 3/7 Assay while Taxol was used as a control, indicate that both combination treatment and curcumin induces apoptosis. It is highly suggested that the use of combined retinoic acid-curcumin treatment on HeLa may be further studied as a way to kill the cervical cancer cells in the future.

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Identification of Sewage Bacteriophage Using Sequencing Method

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Abstract: Isolation and identification of bacteriophage, commonly depend on morphology characteristic, which limits the understanding on the bacteriophage's true nature, development and infection capability. Since, bacteriophage is species specific and has gain interest to be utilized as biomarker, in this research, we study on a lytic bacteriophage which capable of infecting bacteria host *Klebsiella pneumoniae* from sewage water sample. The bacteriophage was isolated using double layer agar method. The isolated sewage bacteriophages had pin-prick size morphology and exhibit 30 minutes host-phage infection time with large burst size at $2.75x10^6$ pfu/mL. Extraction and purification of the bacteriophage genome was using the RTP® Bacteria DNA Mini Kit with some modification and identification of phage was done by sequencing using specific designed PCR primer. The sewage bacteriophages had genome size of approximately 1000 kb. DNA template sequence was compared with available sequence databases using NCBI and we report the bacteriophage strain belongs to Klebsiella phage variant KP15, a lytic bacteriophage that infect bacteria host *Klebsiella* sp..

Keywords: *Klebsiella* sp.; Klebsiella bacteriophage; Genome sequencing

1. Introduction

Klebsiella sp. is a member of the coliform; Gram-negative, lactose-fermenting bacilli, normal commensal of human intestine. It is the common cause of nosocomial infections especially to the immunocompromised, causing upper urinary tract infection, bacteremia, and pneumonia. Although studies of sewage bacteriophages (phages) are available (Jurczak-Kurek *et al*., 2016), the information on Klebsiella phage is limited. The present study determined the physicochemical property of locally isolated myovirus infecting *K. pneumoniae* for insights towards the development of phage application in therapy or biomarker.

2. Materials and Methods

2.1. Bacterial host and phage

Previously, a Gram-negative and bile-resistant bacterium was isolated from the sewage wastewater in Kolej 9, Universiti Teknologi Malaysia. 16S rRNA sequencing identified the bacterium as *Klebsiella pneumoniae*. Subsequently, phage producing pin-prick-sized plaques, referred herein as ϕKpK9, was isolated from the same K9 sewage wastewater using this bacterium (Ramli, 2017).

2.2. Double-layer agar

Briefly, 0.1 mL phage, 0.1 mL log-phase bacterial host and 2 mM $CaCl_2$ were mixed in sterile tube. The mixture was set aside at RT for 5 minutes to allow absorption of phages onto hosts. Next, the mixture was added into 3 mL of sterile 40 °C molten soft agar (0.6% bacteriological agar; Oxoid, LP0001, in nutrient broth; Oxoid, CM0001), gently mixed by inversion and poured onto solid NA agar base plate (Oxoid, CM0003). A few minutes were allowed for the soft agar to set. Subsequently the plate was incubated at 37 °C overnight. Presence of phage infections was proven with the formation of clear circular zones (phage plaques) on opaque bacterial host lawn.

2.3. Physicochemical tests

The stability of phage was tested in different temperature and pH as described by Lal *et al*. (2016), with modification. Briefly, phage suspension (2.8×10^8 PFU/mL) was placed in sterile tubes at 0.5 mL per tube. Next, each tube was incubated at different temperatures (37 °C, 60 °C, 80 °C and 100 °C) for 1 hour. After the incubation, survived phage was plaque-titred using DLA method. For test of stability in different pH, phage

buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 68 mM NaCl) was adjusted to pH 3, 5, 7, and 9 using 1N HCl or 1N NaOH followed by filter-sterilization through 0.2 µm PES-membrane filter (Sartorius 16532---K). Next, 0.5 mL of phage suspension (2.8×10^8 PFU/mL) was added to each 0.5 mL of pH-adjusted phage buffer, gently mixed, and kept at 4 °C overnight. Next day, survived phage was plaque-titred using DLA method.

3. Results and Discussion

Phage infection of bacteria is species specific. This makes phage appealing in applications that target bacteria, including treatment of bacterial infection or as biomarker for the presence of specific bacteria (Lu and Koeris, 2011). To date, out of 31 reported *Klebsiella* sp. phages, only eight are Myovirus infecting *K. pneumoniae* (Phage database, 2018). Application against multi-drug resistant *K. pneumoniae* was directly assessed for majority of these phages (Kęsik-Szeloch *et al.*, 2013). Yet, the stability of phages against elements it would have encountered in applications, such as the low pH of gastrointestinal tract (GI), was not reported. Here, temperature and pH stability ϕKpK9, a tail-phage (Myovirus) infecting *K. pneumonia*, was determined.

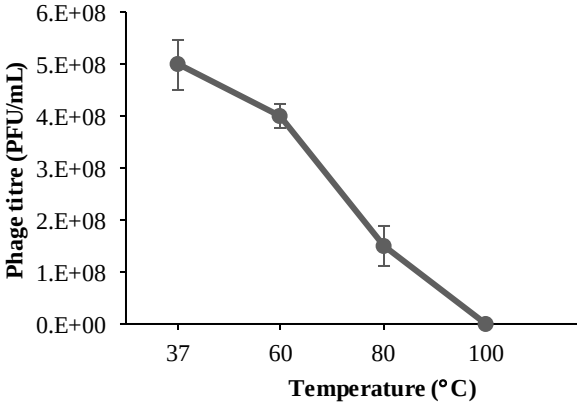

Figure 1: ϕKpK9 is thermostable. ϕKpK9 withstand 1 hr exposure to temperature up to 60° C (reduced by 20%), but not 80 °C and above (n=2, ± SD).

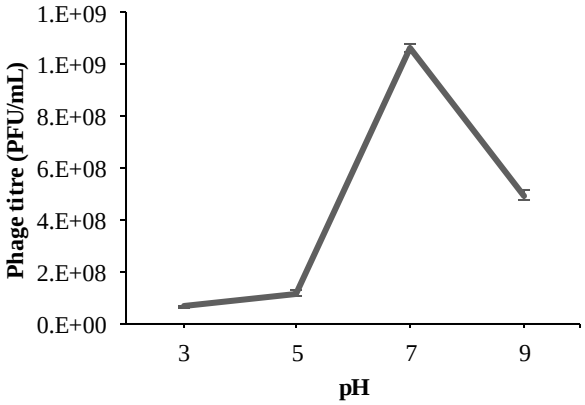

Figure 2: ϕKpK9 is alkali-tolerant. Strong acids (pH 3-5) destroyed the phage, whereas high alkali only reduced phage titre by 50%. (n=2, ± SD).

ϕKpK9 was found to tolerate heat of up to 60 °C resulting with only 20% decreased of infectivity. Viability of phage decreased by 70% and 100% when temperature was increased to 80 °C and 100 °C, respectively (Figure 1). The thermostability of up-to 60 °C resembles phages of the thermophiles *Geobacillus* reported in the range of 55 °C to 60 °C (Marks and Hamilton, 2014; Saunders and Campbell, 1966). On the other hand, ϕKpK9 was labile to strong acid and strong alkali. Strong acids, pH 5 and pH 3, reduced phage infectivity by 89% to 94%, respectively, whereas strong alkali (pH 9) only partially reduced the infectivity by 53% (Figure 2). Sensitivity towards low pH indicates absence of lipid layer enveloping major capsid proteins of the phage (Nobrega *et al.*, 2016). The low pH-lability of ϕKpK9 means the phage would not be suitable for application through the GI tract. However, its thermostability means the phage better suits as biomarker of coliforms, fecal bacteria that are thermostable and selectively grown at 45 °C.

4. Conclusions

The present study reports a locally isolated *K. pneumonia* myovirus phage, ϕKpK9 with the potential to be developed as fecal coliform biomarker. Tests for the fitness of this phage as fecal indicator are underway.

Acknowledgement

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Screening and Identification of Copper-Tolerant Bacteria from Copper Concentrate

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Abstract: Copper-tolerant bacteria are the bacteria that able to grow in the presence of free copper ions (Cu^{2+}). In this study, copper-tolerant bacteria were isolated from Mengapur Copper Mine in Pahang. Bacteria CO1 and CO2 were obtained by cultivating the crushed copper concentrate which is previously done in sterile salt solution into nutrient broth (NB) medium containing 300 mg/L Cu^{2+} and was further streaked onto nutrient agar (NA) medium containing 300 mg/L Cu^{2+} to obtain pure colonies. Two Gram-negative, rod-shaped bacteria were successfully isolated namely CO1 and CO2. The isolates were found to grow optimally in aerobic condition at 37 °C, 180 rpm. Copper sulphate screening revealed that the isolates exhibited resistance to high concentration of Cu^{2+} as high as 500 mg/L copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) with 50% or higher of the bacterial growth. Based on 16 small subunit of ribosomal ribonucleic acid (16S rRNA) gene sequence analysis, CO1 was identified as *Ochrobactrum haematophilum* and CO2 remain unidentified due to the error derived from the 16S rRNA sequence analysis which led to poor quality reads of CO2. The study showed that this strain could be a potential candidate for bioremediation of copper-contaminated area.

Keywords: Copper-tolerant bacteria; Copper (II) ions (Cu^{2+}); Copper concentrate

1. Introduction

Copper is widely distributed in nature and commonly encountered in the environment; however toxic concentrations of copper are only found associated with the industrial or agricultural discharges or mining (Cervantes and Gutierrez-Corona, 1994). This condition caused water and soil pollution which affect many living organisms and has become a major concern worldwide. Copper at elevated concentrations is very toxic to most forms of life (Andreazza *et al*., 2011). Copper intake by living things varies greatly depending on type of organisms, food choices and diet customs (Gaetke and Chow, 2003). Copper exist as copper ions (Cu^{2+}) in its free ionic form within aqueous solution. Cu^{2+} requirements by microorganisms are usually satisfied at a very low concentration (1 – 10 µg/L). In contrast, a high level of Cu^{2+} is toxic to microbial cells (Domek *et al*., 1984; MacLeod *et al*., 1967). According to WHO (1998), acute lethal dose of Cu^{2+} for adults is 4 to 400 mg/kg of body weight based on the data from accidental ingestion and suicide case.

The normal concentration of copper of unpolluted soils may range between 1.6 and 7.5 mg/kg of soil (Shaheen *et al*., 2009) while mean concentration of Cu^{2+} of natural water is 4 – 10 µg/L. 6 to 13% of the average daily intake of copper is contributed by drinking water and according to the US EPA, maximum contaminant level for Cu^{2+} in the drinking water is 1.3 mg/L (Gaetke and Chow, 2003). High concentration of copper which enters soils and sediments is contributed by anthropogenic activities such as smelting, mining, industrial (such as metal plating, steelworks and refineries), waste emission, application of fertilizers, sewage sludge and antimicrobial agents (such as algicides, fungicides and molluscicides) (Flemming and Trevors, 1989). Copper is also released into the air in the form of natural sources such as windblown dust, volcanoes and forest fires (Barceloux, 1999) which may also contributed to the high level of copper in the environment. As the result, this condition cause environmental pollution and damage the ecosystem as it cannot be destroyed, may be consumed by any-living organisms and can interact with their biological system which then causes toxic effect.

Toxic effect of copper in plants, animals and human is of greater concern as it is non-biodegradable in nature (Rathi and Nandabalan, 2017) and could enter the food chain via any trophic levels through ingestion or any uptake systems. Factors affecting the absorption of copper in the human body includes types of the chemical form and presence of other dietary components (Gaetke and Chow, 2003). In the US, the average daily intake of copper is about 1 mg of copper which primarily originates from food and the biological half-life of copper from the diet is within 13 to 33 days at which the main route of elimination is via bilary excretion (Barceloux, 1999).

Copper sulphate is known as a gastric irritant that cause erosion of the lining of the gastrointestinal tract (Barceloux, 1999). At lower dose, copper ions cause symptoms typical of food poisoning such as headache, nausea, vomiting and diarrhoea. Chronic effect or disease arising from excessive copper intake in human varies as it accumulates in the different parts of organs primarily liver. An experimental study of the effect of toxic level of copper showed liver damage in rat (Gaetke and Chow, 2003). In human, chronic effect of copper intoxication would be gastrointestinal bleeding, intravascular haemolysis and hepatocellular toxicity (Agarwal *et al*., 1993) while the disease that could arise from the excessive copper intake would be Indian childhood cirrhosis (Nayak and Chitale, 2013) and Alzheimer's disease (Brewer, 2009). In aquatic life, toxic concentration of copper cause damage to gills, alter growth, reproduction and behaviour (Flemming and Trevors, 1989) while in plant, a study on the effect of elevated level of copper on sweet potato show the reduction in growth, chlorosis on mature leaves and wilting due to severe root damage (O'Sullivan *et al*., 1997).

High levels of copper also exert selective pressure on microorganisms resulting in the appearance of resistant variants possessing copper resistance genetic determinants (Cervantes and Gutierrez-Corona, 1994). Copper resistance microorganisms could be utilized in remediation to treat contaminated soils or water. Ecological remediation of polluted sites has received many attentions around the world because it provides an ecologically sound and safe method for restoration and remediation (He *et al*., 2010).

2. Materials and Methods

2.1. Sample source and sample preparation

Copper concentrate was obtained from Mengapur Copper Mine in Pahang which is located approximately at 3° 51' 0" North and 102° 51' 59" East. It contains a historical copper, sulphur, gold, silver oxide and sulphide. Soil suspension was made from 0.5 g portion of copper concentrate (wet weight) in 25 mL of 0.9% sterile saline water. It was then vortexed and the particulate matter was allowed to settle before aliquoting the solution into medium for bacterial enrichment to allow the separation of soil particles with the bacterial cells.

2.2. Isolation of copper-tolerant bacterial strains

Enrichment of copper-tolerant bacteria was done in 25 mL NB in 250 mL Erlenmeyer flasks. 300 mg/L $CuSO_4.5H_2O$ was filter-sterilized and supplemented into the nutrient broth (Hart *et al*., 1987). 10% v/v inoculum was added to the NB and incubated with shaking at 37 °C and 180 rpm for overnight. Isolation of copper-tolerant bacteria was done using streak plate methods on nutrient agar (NA) containing 300 mg/L filter-sterilized $CuSO_4.5H_2O$. Pure colonies were obtained by repeated streaking on the NA medium containing $Cu^{2+}$and were characterized morphologically by Gram staining.

2.3. Bacterial growth profile

Enrichment of copper-tolerant bacteria was done in 25 mL NB in 250 mL Erlenmeyer flasks. 300 mg/L $CuSO_4.5H_2O$ was filter-sterilized and supplemented into the nutrient broth (Hart *et al*.). 10% v/v inoculum was added to the NB and incubated with shaking at 37 °C, 180 rpm. Optical density (OD) of the bacterial suspension was taken at time interval of two hours.

2.4. Determination of Minimal Inhibitory Concentration (MIC)

To determine the lowest concentration of copper that inhibits 50% or more of the bacterial growth, copper ion screening was performed. The isolated bacteria were enriched using NB medium containing 300 mg/L $CuSO_4.5H_2O$ incubated at 37 °C, 180 rpm for 12 hours. 10% v/v bacterial suspension was inoculated into NB containing different ranges of Cu^{2+} concentrations (200 mg/L to 900 mg/L). Bacterial cultures were incubated at 37 °C, 180 rpm for 12 hours. Medium without copper was set as control. Each of tests was tested in duplicates.

$$\text{Percentage Growth Inhibition} = \frac{OD_{600}\text{ (without copper)} - OD_{600}\text{ (with copper)}}{OD_{600}\text{ (without copper)}} \times 100\%$$

2.5. Identification of copper-tolerant bacterial strains

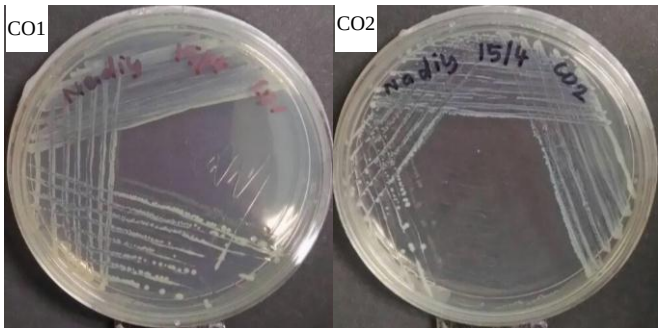
For 16S rRNA gene sequence analysis, genomic DNA was extracted by using Wizard® Genomic DNA Kit (Promega). 16S rRNA genomic DNA was amplified in polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-

AAGGAGGTGATCCAGCC-3'). The PCR mixture (50 µL) containing 1 µL template, 5 µL primers (each), 14 µL Nuclease free water, 25 µL Go-Taq green Master Mix. The PCR was performed with a hot starting performed at 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 1 minute, 52 °C for 1 minute, and 72 °C for 1.3 minutes, followed by a final extension performed at 72 °C for 5 minutes carried out in MJ Mini thermal cycler from Bio-Rad. The purification of 16S rRNA gene sequence was done using Wizard® SV and PCR Clean-Up System (Promega).

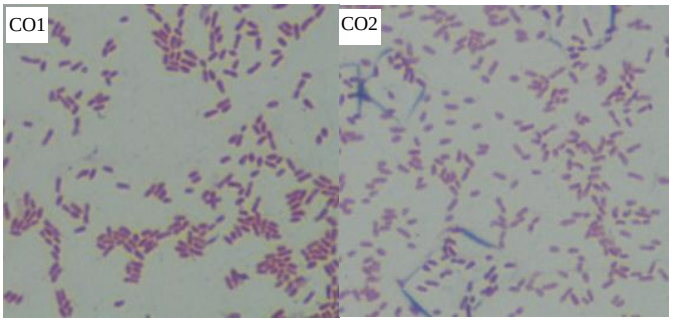
3. Results and Discussion

3.1. Isolation of copper-tolerant bacterial strains

Two colonies were obtained from the streak plate method. The colony morphology of CO1 and CO2 are shown in Figure 1. Both of the bacterial isolates grew chemoheterotrophically on NB containing 300 mg/L copper. The morphology of CO1 and CO2 are summarized in Table 1. In this study, both CO1 and CO2 were found to be round, small (0.5 – 1.0 mm), moist and light beige in colour. Based on Gram-staining, the cells appeared to be Gram-negative rods.



(a)



(b)

Figure 1: Colony morphology of bacterial isolates: (a) CO1 and CO2 grown on NA supplemented with 300 mg/L copper; (b) The image of Gram stained of CO1 and CO2 viewed under E200 Nikon light microscope with 1000X magnification

Table 1: Morphological characteristics of CO1 and CO2

Species	Form					Gram stain
	Form	Diameter	Surface	Texture	Color	
CO1	Round	0.5 – 1.0 mm	Shiny and smooth	Moist	Light beige	Negative
CO2	Round	0.5 – 1.0 mm	Shiny and smooth	Moist	Light beige	Negative

3.2. Bacterial growth profile

Based on Figure 2(a), there was a slightly longer lag phase for CO1 grown in the presence of 300 mg/L Cu^{2+} which may result from the stressful condition for the bacteria due to the presence of high concentration of

Cu^{2+} in the medium creating a stressful condition for the bacteria. Exponential phase for CO1 started at 2 hours of growth in the medium without Cu^{2+} while at 4 hours of growth in the medium with Cu^{2+} and growth was maintained for a further 4 to 6 hours before maximum growth of CO1 reached the end of the exponential.

Based on Figure 2(b), there was also a slightly longer lag phase for CO2 grown in the presence of 300 mg/L Cu^{2+} which may result from the stressful condition created by the presence of high concentration of Cu^{2+} in the medium. Exponential phase for CO2 in both medium conditions also started at 4 hours of growth in the medium with Cu^{2+} and growth was maintained for a further 6 hours before maximum growth of CO2 reached the end of the exponential phase.

The growth rate of bacteria (Table 2) was determined by the doubling time which is the time required for cell replication. From the curve shown in Figure 2(a), CO1 appeared to grow faster in the medium without Cu^{2+} with the growth rate (μ) at 0.3325 h^{-1} compared to medium with Cu^{2+} (0.192 h^{-1}) while for CO2, the isolate also appeared to grow faster as shown in Figure 2(b) in the absence of copper (0.2148 h^{-1}) compared to medium with the presence of copper (0.2103 h^{-1}). CO1 took a shorter time to double in medium without Cu^{2+} (2.08 h) compared to that in the presence of Cu^{2+} (3.61 h) and CO2 also took a shorter time to double in medium with the absence of copper (3.23 h) compared to that in the absence of copper (3.3 h). The shorter the doubling time, the faster the bacterial replication speed. This might happen due to the adaptation of microbial in the medium supplemented with 300 mg/L Cu^{2+}. This might happen due to the adaptation of microbial in the medium supplemented with 300 mg/L Cu^{2+}.

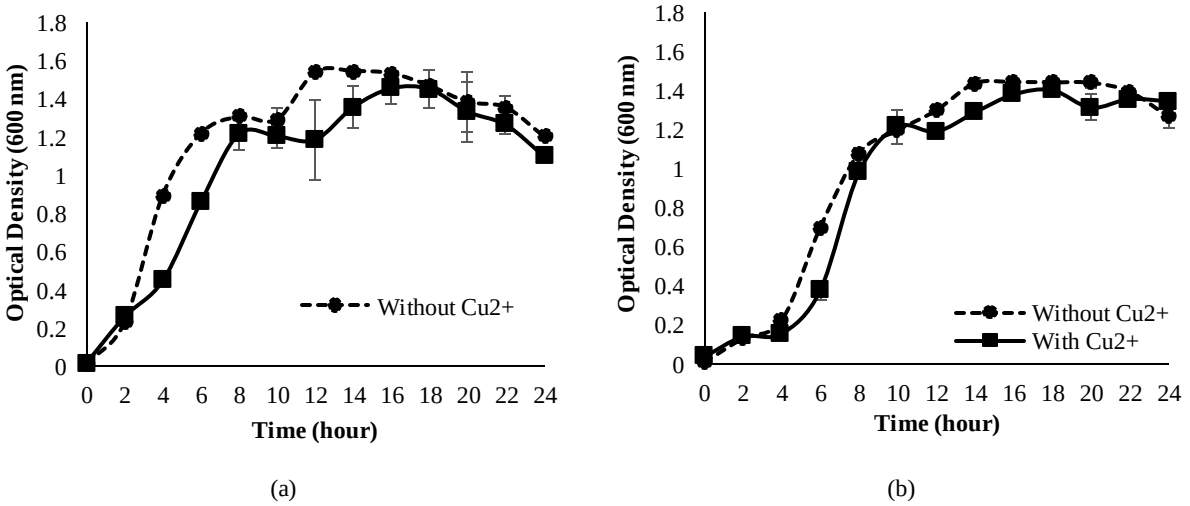


Figure 2: Growth profile of the isolates in the absence and presence of 300 mg/L Cu^{2+} at 37 °C: (a) CO1 isolate; (b) CO2 isolate

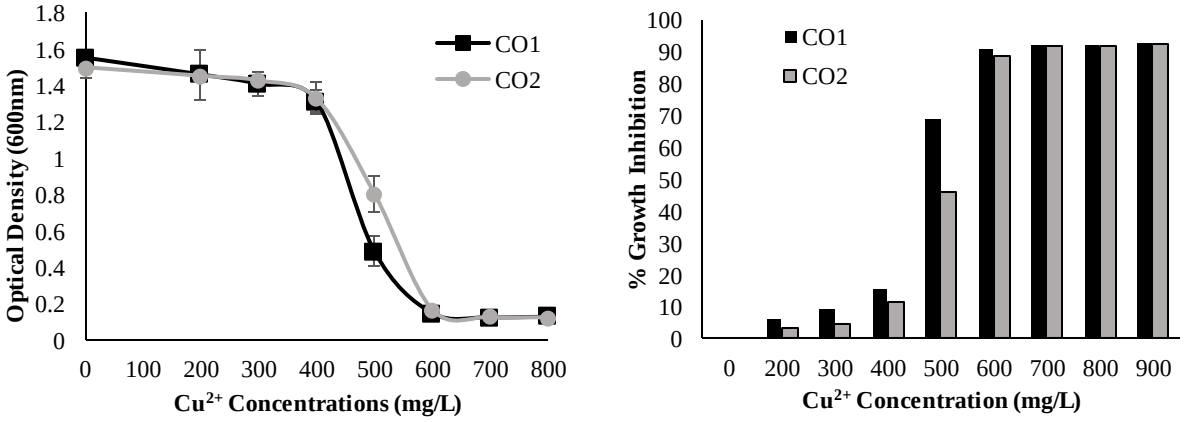
Table 2: The growth rate and doubling time of CO1 and CO2 in medium supplemented with and without of Cu^{2+}

Medium	CO1		CO2	
	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}
Growth rate, μ (h^{-1})	0.192	0.3325	0.2103	0.2148
Doubling time (h)	3.61	2.08	3.30	3.23

3.3. Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration (MIC) represents the lowest concentration of heavy metal ion that inhibits 50% or higher of the microbial growth. Bacteria isolated from the copper-rich environment can tolerate the elevated level of copper in the environment due to the adaptation. The adapted bacteria possess a metal resistance mechanism to protect them from the harsh condition. In this study, the resistance of the isolates towards copper were analysed to determine its potential use in bioremediation of the copper-contaminated area. Figure 3(a) shows that the growth of CO1 and CO2 decreased as copper concentrations increased. Growth of cell for CO1 and CO2 was still observed at concentration of up to 400 mg/L after which growth significantly

decreased exponentially. Therefore, the tolerance level of CO1 and CO2 was at 500 mg/L copper. Copper concentration at 600 mg/L inhibited growth of CO1 and CO2 by more than 60% and 70%, respectively (Figure 3(b)).



(a) (b)

Figure 3: Screening of Cu^{2+}: (a) The effect of increasing Cu^{2+} concentrations on CO1 and CO2; (b) Percentage of growth inhibition of CO1 and CO2 with increasing Cu^{2+} concentrations

3.4. Identification of copper-tolerant bacterial strains

DNA of the isolates were extracted using Wizard Genomic DNA Extraction kit (Promega). fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used as the universal primer to amplify 1.5 kb fragment of isolate DNA through Polymerase Chain Reaction. The extracted genomic DNA (Figure 4 (a)) and PCR genomic product (Figure 4(b)) was then analysed on DNA gel electrophoresis.

The purified PCR products were sequenced by Apical Scientific Sdn. Bhd. DNA sequencing facility. The sequence obtained was uploaded to online analysis tool EzBioCloud.net to determine the similarity of known sequence and identified the particular species (Yoon *et al*., 2017). The query results displayed that the gene of isolate is 78.33% similar to *Ochrobactrum haematophilum* (which is a close-match with *Brucella* sp.) for CO1 and 95.45% similar to *Bacillus cereus* for CO2. Other close matches of bacteria obtained from the EzBioCloud.net were chosen in construction of phylogenetic tree (Figure 5). Phylogram of the bacterial isolates were constructed using Mega7 software (Kumar *et al*., 2016).

The result showed the evolutionary relationship of related *Ochrobactrum* and *Brucella* sp. for CO1 and *Bacillus* sp. for CO2. The phylogenetic tree showed that CO1 strain was 63% similar to *Ochrobactrum haematophilum* and CO2 was 100% similar to *Bacillus cereus*. However, the constitutive bacteria species identified for CO2 mismatches the result obtained from Gram stain. The error might derive from the 16S rRNA gene sequence analysis due to insufficient amount of DNA template after dsDNA quantification which results in poor quality reads of CO2 isolate and the identity cannot be verified. For CO1 isolate, there is still no report on the copper-tolerant bacteria of *Ochrobactrum haematophilum* yet. This study reports for the first time the isolation of *Ochrobactrum haematophilum* from copper concentrate with potential use in bioremediation.

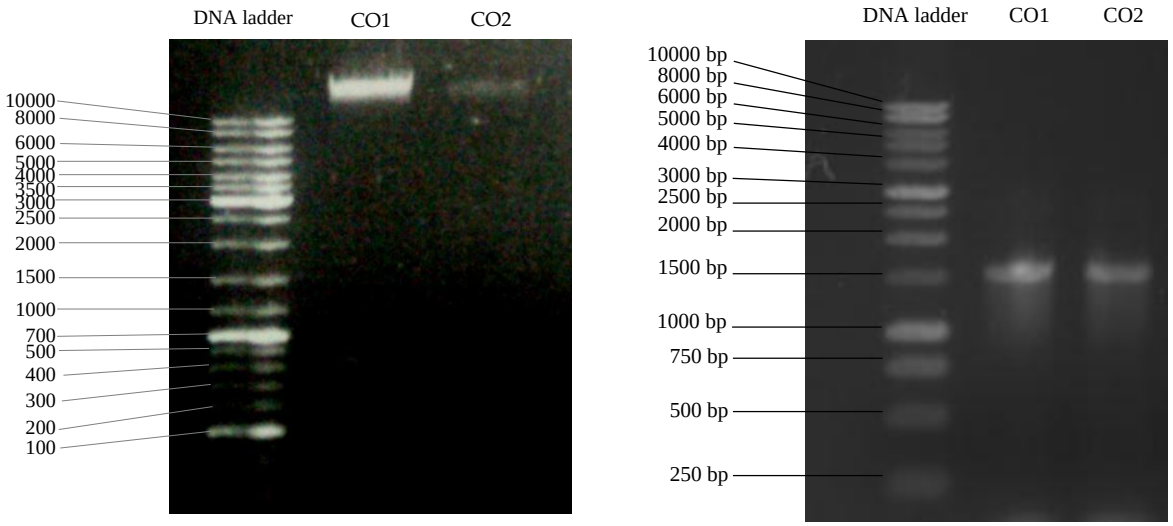


Figure 4: Running DNA gel electrophoresis: (a) Extracted genomic DNA of CO1 and CO2 using Promega kit and run on 1% agarose gel. Concentration of DNA samples; CO1: 208.2 ng/µL; CO2: 163.1 ng/µL based on NanoDrop® ND-1000.; (b) Amplified 16S rRNA gene of isolate (CO1 and CO2) extracted using Promega kit and run on 1% agarose gel. The size of partial amplified DNA from CO1 and CO2 were approximately 1.5 kb (1500 bp).

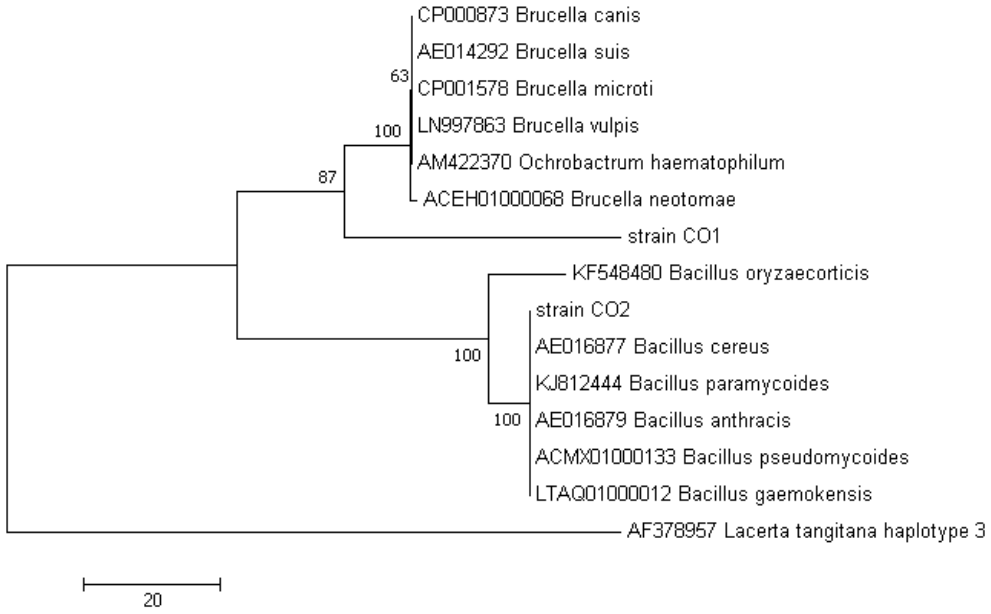


Figure 5: Phylogram showing the relationship between CO1 and other *Brucella* related species (closely related with *Ochrobactrum* sp.), and CO2 and other *Bacillus* related species

4. Conclusions

Two copper-tolerant bacteria were successfully isolated from copper concentrate. The isolated bacteria (CO1 and CO2) are chemoheterotrophic bacteria and can grow optimally in NB medium at 37 °C. Both of the isolates can tolerate copper concentration as high as 500 mg/L copper. Based on 16S rRNA gene sequence analysis, CO1 was identified as *Ochrobactrum haematophilum* and CO2 as *Bacillus cereus*. However, the constitutive bacteria species identified for CO2 mismatches the result obtained from Gram stain. The error might derive from the 16S rRNA gene sequence analysis due to insufficient amount of DNA template after dsDNA quantification. This result in poor quality reads of CO2 isolate making it difficult to identify. Further research can be carried out to have a deeper understanding about the effect of copper on copper-tolerant bacteria. CO1 and CO2 were proven to tolerate Cu^{2+}. Therefore, mechanism of resistance towards Cu^{2+} of both bacteria can be studied in detail for a better understanding. Test on the biosorption capacity of the bacteria towards Cu^{2+} also should be conducted to analyse its capabilities for use in bioremediation. Since the identity of

CO2 could not be verified due to the poor quality sequence reads of the isolate, it is recommended that 16S rRNA gene sequence analysis be repeated.

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Isolation and Identification of Thermophilic Protease-Producing Bacteria from Ulu Slim Hot Spring, Perak

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Abstract: Proteases play a significant role in application of many natural products and nowadays, proteases undeniably have been the most applicable classes in industrial enzyme. Initially, the water samples and sand samples of hot spring were collected from the rural area of Ulu Slim Hot Springs (3°53'58.5''N, 101°29'52''E) Perak, Malaysia and were investigated with the screening activity based on the relatively appearance of halo zone on Skim Milk Agar (SMA) plate to indicate the proteolytic activity. Next, further selection was carried out by quantitative enzyme assay as casein has been used as a substrate in the media and ultimately, 2 samples were chosen and finalized based on higher enzymatic activity. Five potential thermophilic protease producers were successfully isolated. The bacterial species were designed as SUS40 and SUS60. Both strains have been undergoes characterization, extraction, purification and identification via Gram Staining and 16S rRNA gene sequence analysis. Polymerase Chain Reaction (PCR) was performed to amplify the bacterial partial genome using 27F and 1492R primer. Finally, construction of phylogenetic tree has been made to show the evolutionary relationships among bacterial species. The isolates were identified as in *Bacillus mycoides* SUS40 and *Bacillus cereus* SUS60.

Keywords: Protease; Thermophilic; *Bacillus* sp.

1. Introduction

Protease is widely used in variety of industrial applications. For example, proteolysis is a powerful element in the alteration properties of proteins in food area. Basically, it includes solubility changes, gelation, emulsifying and characteristics of foam, minimising of protein allergy, offer a various taste, or bioactive peptides liberation. Moreover, it is undeniable to claim processing at high range of temperatures can bring the advantages, supporting with this method can steadily rise up the reaction and prevents microorganism growth. Adding to the point of stabilization, the use of immobilized enzymes encourage the inactivation of enzyme is not needed at the final process regarding with the catalyst can be easily replaced from the reaction medium, especially important in food protein limited hydrolysis (Tavano, 2013). However, most of the industrial processes require a high temperature. It indicates the enzyme is preferable to have thermo stability characteristics. In this case, this study will be a significant contribution in discovering the potential of this type of bacteria in Ulu Slim Hot Spring, Malaysia.

Therefore, the primary goals of this research were to isolate and screening the thermophilic protease producing bacteria sample from Ulu Slim Hot Springs, Perak. Screening system was used to determine the halo zone appearing indicates the proteolytic activity. The 16S rRNA analysis was applied based on the some taxonomical studies to identify the selected species of the isolated thermophilic protease-producing bacteria. The DNA extraction method was conducted and the DNA result from the extraction was undergo Polymerase Chain Reaction (PCR) and following with the DNA sequencing.

2. Materials and Methods

2.1. Sampling of microorganisms

Isolation of thermophilic bacteria was conducted in Ulu Slim Hot Springs,Perak and the samples was collected with the temperature 89 °C at the specific sites. The water samples was collected by using a 100 mL sterilized serum bottle at each site. Later, the samples were maintained at 4 °C. The temperature of the sampling sites were recorded for references.

2.2. Screening of thermophilic protease-producing bacteria

The spread plate technique was applied in order to determine the proteolytic activity. The samples taken from Ulu Slim Hot Springs was inoculated in 50 mL nutrient broth medium and was incubated at 40 °C with 200 rpm in incubator shaker for 24 hours. Then, the culture was spreaded on skim milk agar and being incubated overnight at 37 °C. Samples with transparent zone on skim milk agar will indicate protease activity therefore select them for further isolation and morphological characterization.

2.3. Isolation of thermophilic protease-producing bacteria

Streak plate method was applied to give single colonies upon isolation on the skim milk agar. Plates were incubated at 37 °C for 24 hours. Distinctive colonies were selected based on their morphologies through repeated streaking until pure colonies were obtained.

2.4. Gram staining

Identification of isolated bacteria was done using Gram staining standardized procedure. This technique distinguishes Gram-positive and Gram-negative groups of bacteria based on their different cell wall constituents. The steps include preparation of slide smear, staining, decolorization and microscopic visualization.

2.5. Enzyme assay and Lowry assay

The sample was inoculated onto protease medium production. The ratio for the medium inoculum is 1:10. Then, 5% of overnight bacterial culture was inoculated into nutrient broth medium by maintaining at 50 °C for 24 hours in a 200 rpm shaker incubator. The supernatant was collected by centrifugation at 4000 rpm for 15 minutes to determine the protein concentration and proteolytic activity. Lowry method can be used to estimate the protein concentration of the bacterial isolates while measuring the proteolytic activity by using casein as substrate and tyrosine as standard.Two best isolates with maximum activity were chosen for 16S rRNA analysis.

2.6. PCR amplification and 16S rRNA identification

By using Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions, the genomic DNA from a 12 h culture cells was extracted. Amplification of DNA was conducted using Perkin Elmer Gene AmpR PCR System 9700 thermal cycle (Perkin-Elmer Corp., Emeryville, Calif.). The components of the PCR mixture were 50 µL of 2X PCR Master Mix, (Promega, USA), 100 ng of forward and reverse primer, 100 ng of DNA template and nuclease free water. The PCR reaction was performed in 100 µL for 25 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. Carry out the additional extension for 10 min at 72 °C. The amplified PCR products was analyzed on 1% (w/v) agarose gel electrophoresis and visualize them by using GeneFlash gel doc (Syngene). Purify the fragments by using the Promega PCR purification kit. Lastly, the services of 1st BASE Pte., Ltd was used to perform DNA sequencing from the amplified DNA fragments.

2.7. Homology search and phylogenetic analysis

A set of sequences were collected undergoes homology and phylogenetic analyses then it was aligned with the 16S rRNA sequences available in the public databases from BLAST. Phylogenetic tree was constructed by using MEGA6 software by using the neighbour-joining method with a bootstrap value of 100 replicates.

3. Results and Discussion

3.1. Isolation and screening of thermophilic protease-producing bacteria

Halo zone were formed at different five plates after being incubated at different temperatures. The formation of halo zone indicates the proteolytic activity of enzyme protease where casein as a substrate has been broken down. To identify the single colony, it was conducted using colonies obtained from skim milk agar by streaking method. Several colonies were streaked repeatedly based on their morphologies on skim milk agar until pure colonies were obtained. Total several morphological distinct bacterial colonies were grown on SMA plate with 24 hours incubation at 40 °C. Physical parameters of the growth medium such as incubation condition (temperature) and pH value have been identified to be the major influence to the microbial culture growth rate

(Cahk, 2010). Limited oxygen supply potentially result the lower production of protease (Moon and Parulekar, 1991).

Table 1: Bacterial growths respond at different incubation temperature. Five from the colonies were chosen and were designed as S1, S2, S3 and S4 and S5. These 5 colonies were then grown on nutrient broth for further experimental studies.

Temp (°C)	Strains	Growth Respond	Indicator	Parameter Setup
40	S1	√	Liquid nutrient broth turns cloudy	24 hours
	S2	√	Liquid nutrient broth turns cloudy	43 hours
50	S3	√	Liquid nutrient broth turns cloudy	5 days
	S4	√	Liquid nutrient broth turns cloudy	21 hours
60	S5	√	Liquid nutrient broth turns cloudy	24 hours

3.2. Initial identification through Gram staining

Gram staining is commonly used microbiological staining techniques that greatly contribute in the morphological identification and characterization of bacteria. Gram staining method was developed by Hans Christian Gram which classified bacteria into Gram-positive and Gram-negative (Coico, 2005).

Table 2: Results for characterizations of the 5 isolates under compound microscope according to Gram reaction, its color and cellular morphology. Based on the initial morphology of the isolates, all the strains were identified as a *Bacillus* species.

Strain	Gram Reaction	Colour	Cellular Morphology
1	Negative	Red	Rod-Shape
2	Negative	Red	Rod-Shape
3	Negative	Red	Rod-Shape
4	Positive	Blue	Rod-Shape
5	Positive	Blue	Rod-Shape

3.3. Determination of protein concentration and proteolytic activity

It has been so common that Lowry protein estimations are a favorable alternative to a rigorous absolute determination in almost all circumstances where protein mixtures or crude extracts are demanded (Walker, 1984). Enzyme assays are performed to offer two different purposes which is firstly a special enzyme can be identified or to prove its presence or absence in a clear specimen, for example like organism or a tissue and the second one will be focusing on the determination of the amount of the enzyme in the sample (Bisswanger, 2014).

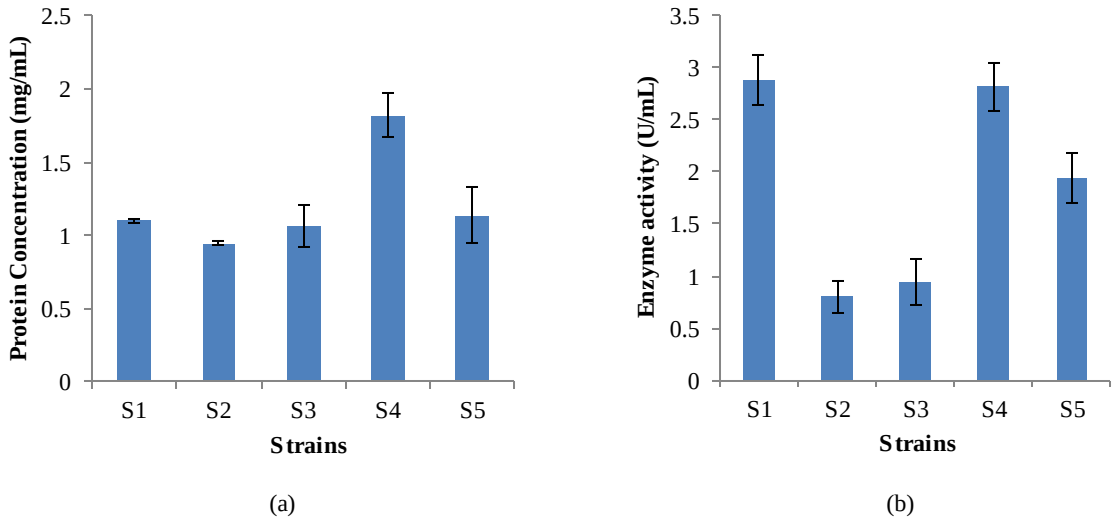

Figure 1: The result of Lowry Assay and Enzyme Assay. (a) Protein concentration resulted from Lowry Assay, (b) The result for enzyme activity. From the Figure 1(a), strain 4 (S4) has the highest value of protein concentration while Figure 1(b), strain 1 (S1) reveals the highest amount of proteolytic activity presence in the sample

Lastly, the study suggests on the correlation between Lowry assay and enzyme assay, hence, by calculating the protease specific activity by dividing the enzyme activity with protein concentration has yield a reliable result indicating the higher proteolytic activity, the faster rate of reaction can happen (Thakur, 2013).

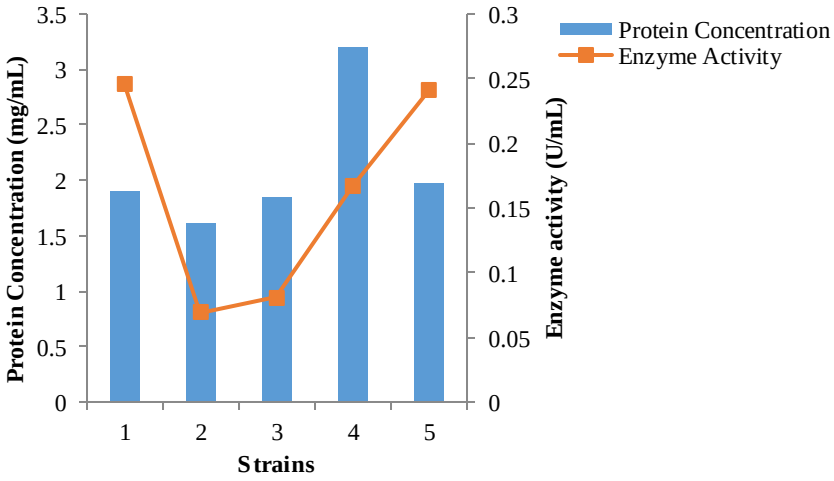

Figure 2: The comparison between Lowry Assay and Enzyme Assay of the five isolates. Among the 5 strains, Strain 1 and Strain 5 were selected for further identification. The selection is made due to the achieving higher enzymatic activity which are S1 (17.595 U/mg) and S5 (16.653 U/mg) amongst the isolates. The strains were then designed as SUS40 and SUS60.

3.4. 16S rRNA identification

DNA was successfully extracted by using boiling treatment method. The isolated DNA samples were securely measured spectrophotometrically by using Nanodrop® instrument. Both strains have achieved the required A260/A280 purity standards. Furthermore, the value of both DNA concentration also fall in optimum range and the amplification of the DNA were then proceeds without the need of dilution.

After DNA genomic isolation, PCR amplifications of 16S rRNA gene was done using the extracted genomic DNA as template. An intense single band appeared for both samples. Both PCR samples were 1500 kb in size. The 16S rRNA conserved region in extracted isolates DNA was successfully amplified by using the universal primer 27F and 1492R. The amplified products were then sent to be sequenced.

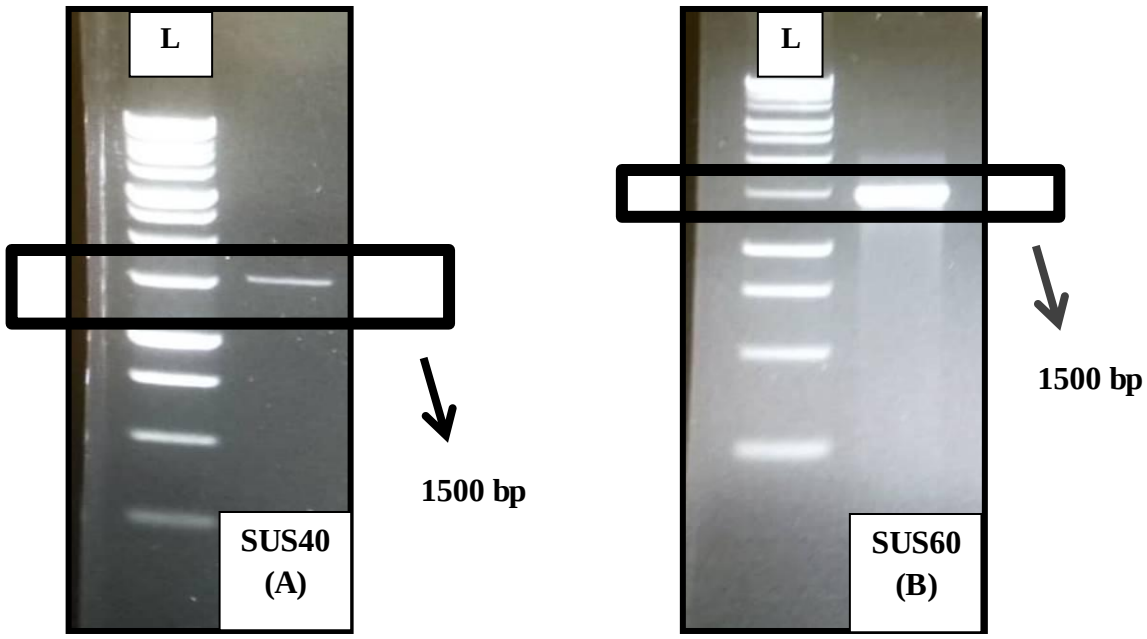

Figure 3: Gel electrophoresis showing approximately 1500 bp PCR amplified genomic DNA extract of isolates SUS40 (A) and SUS60 (B). Lane L represents as 1 kb DNA ladder.

The PCR products of isolates which are SUS40 AND SUS60 are further sequenced by the First Base Laboratory Sdn Bhd. The purification has been done by the company to remove impurities such as nucleotides, primers and protein. The homology findings for strains SUS40 and SUS60 were made using BLAST databases.

Phylogenetic tree was constructed by assembling the sequence of SUS40 and SUS60 gene sequence into the software Mega6 together with the sequence from BLAST. Bootstrap test was used to determine how reliable a multiple sequence alignment is. The percentage of replicate trees in which the associated taxa clumped together in the bootstrap test is shown next to the branches (Tamura *et al*., 2004).

Through the result of NJ tree, phylogenetic tree were divided into cluster. Some of the cluster was divided into sub cluster. From the cluster pattern, the *Bacillus* sp. SUS60 found to possess a high homology (relationship) with the other *Bacillus cereus* strains. As with *Bacillus* sp. SUS40, it can be claimed to fall in *Bacillus mycoides* family as it cluster together with the other *B. mycoides* strains.

Several literature findings reveal some of the *Bacillus* sp. is categorized as specific thermo stable producer of extracellular protease. This includes *Bacillus cereus* (Jabeen and Qazi, 2011). So, the results where the newly discovered bacteria *Bacillus* sp. SUS40 and *Bacillus* sp. SUS60 could produce proteases are agreement with the literature works.

4. Conclusions

In conclusion, generally, all the three objectives manage to succesfully achieved. Five isolates were screened and 2 strains possess higher enzyme activity which are S1 (17.595 U/mg) and S5 (16.653 U/mg). The isolates were identified as in *Bacillus mycoides* SUS40 and *Bacillus cereus* SUS60. *Bacillus* sp. is well known for their ability to produce enzyme proteases (Moon, 1991). Proteolytic enzyme from both strains has a potential to be commercialized in industrial application. Therefore, it can be concluded that the bacterial that can adapt extremely high temperature is a good source of protease-producing bacteria, thus could be utilized in industrial applications.

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Screening of Epigallocatechin-3-gallate (EGCG) Content in Commercialized *Camellia sinensis* Products

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Abstract: Tea is one of the most consumed beverages all over the world. Tea is a major source of epigallocatechin-3-gallate (EGCG) with the pharmacological properties such as anti-obesity, anti-diabetes and anti-inflammatory. The objective of this study is to determine EGCG content in commercialized tea products. This study was divided into two phases. The first phase involved the extraction of EGCG compound from four types of tea namely as white tea (WT), green tea (GT), oolong tea (OT) and black tea (BT) using aqueous and methanol extraction techniques. The presence of EGCG compound was detected using Thin Layer Chromatography (TLC) and the amount of EGCG content in the tea products were analyzed using High Performance Liquid Chromatography (HPLC). The second phase involved the determination of EGCG compound in infusion tea bags (ITB) and ready-to-drink tea beverages (RTD) using HPLC. Fourteen ITB samples and twelve RTD samples were randomly purchased in the market. Results showed that methanolic extracts produced higher EGCG content than aqueous extract. The optimized temperature and extraction time to extract EGCG from WT, GT, OT and BT were also established in this study whereby WT, GT and OT were best extracted at 80 °C for 60 minutes, 20 minutes and 40 minutes respectively. Meanwhile, the optimized condition to extract EGCG from BT is at 29 °C for 40 minutes. Finally, the analysis of commercialized tea drinks products revealed that the infusion tea had the highest level of EGCG content compared to ready-to-drink tea. Among the four types of tea tested, GT contain the highest EGCG concentration compared to WT, OT and BT. Therefore, infusion tea bag especially green tea is recommended to obese people due to high EGCG concentration. On the other hand, ready-to-drink tea should be prevented by obese people due to lower concentration of EGCG.

Keywords: Tea; Commercialized product; EGCG; HPLC

1. Introduction

Tea is one of the most preferable beverages among people throughout the world due to its beneficial effect towards human health. There are four major types of tea has been derived from *Camellia sinensis* leaves known as white tea (WT), green tea (GT), oolong tea (OT) and black tea (BT) which differ in processing step. The compound responsible to provide positive effects of tea is known as EGCG, a tea catechin (Chen *et al.*, 2008; Forester and Lambert, 2011). Different types of tea have different level of tea catechin (Jain *et al.*, 2013). The pharmacological properties of EGCG such as antioxidant, anti-obesity, anti-diabetes and anti-inflammatory makes tea as potential alternative for disease control, weight management and regulating cholesterol level (Othman *et al.*, 2017; Oyama *et al.*, 2017; Sampath *et al.*, 2017).

In the market, there have been an increased number of health supplements and food products desired from tea claims to help in reducing body weight, lowering the blood cholesterol level and blood glucose level. However, the level of EGCG content may differ in different types of tea products hence, the pharmacological effect might be different. Therefore, this study presents a method to characterize the presence of EGCG in several commercialize tea-based product in order to validate its effectiveness as food supplement. This study also reveal the optimize condition to extract EGCG compound from different types of tea samples. Results from this study provide insights in empowering consumer rights and ensure the food security.

2. Materials and Methods

2.1. Material and reagent

Fourteen infusion tea bag samples and twelve ready-to-drink samples were purchased randomly from the market. ECGC standard were purchased from Calbiochem with HPLC purity not less than 99.5%. HPLC grade of Acetonitrile, methanol, acetic acid were used during extraction and HPLC analysis.

2.2. Extraction procedure

5 g of the tea sample was brewed in 250 mL methanol and 250 mL distilled water at temperature 37 °C for 60 minutes with continuous stirring. The tea solution was filtered using Whatman No.1 filter paper twice. Next, the extract solution was centrifuged at 4500 rpm for 3 minutes. The supernatant was collected into conical flask and the residue was discarded. After that, the solution was dried using freeze dryer until powder is obtained. The dried extract was stored in -20 °C refrigerator. This method was repeated for GT and BT samples. The process was repeated twice.

2.3. Optimization of aqueous condition

For optimization of temperature, 5 g of tea sample was brewed in 250 mL distilled water at different temperature (29 °C, 50 °C, 60 °C and 80 °C) for 60 minutes with continuous stirring. The procedure was similar as mention in section 2.2. The optimum temperature determined from each types of tea is used as temperature for optimization of extraction time, 5 g of tea sample was brewed in 250 mL distilled water at different extraction time (20 min, 30 min, 40 min and 60 min). The temperature was set based on the optimum temperature of the desired tea samples. The similar procedure as mentioned in section 2.2 was conducted. This method was repeated for WT, GT, OT and BT samples. The process was repeated twice.

2.4. HPLC analysis

The determination of EGCG was performed by High Performance Liquid Chromatography (HPLC). The chromatographic separations were carried out using Agilent C-18 column (4.6 × 150 mm, 5 micron). The combination of 20% buffer A (water with 0.05% Trifluroacetic Acid (TFA)) and 80% buffer B (acetonitrile with 0.05% TFA) were used as the mobile phase and filtered using 0.20 µm. The flow rate was set at 1.0 mL/min with oven temperature maintained at 25 °C. Detection with diode array detector was used at wavelength 280 nm. The samples were prepared by diluting 5 mg of freeze-dried extract into 1 mL of buffer A and filtered with 0.20 µm nylon filter. For infusion tea and ready-to-drink tea, 1 mL of the tea solutions from each sample is taken and filtered using 0.20 µm before the analysis. The presence of EGCG in tea extracts and tea samples were analyzed by comparing the chromatogram of the samples to the chromatograms of the standard EGCG. The results were presented in mean with standard deviation.

3. Results and Discussion

3.1. Extraction of EGCG from GT and BT using methanol extraction and aqueous extraction method

Methanol extraction yield a higher EGCG concentration compared to aqueous extraction in both GT and BT samples. The similar result was obtained in previous study by Vuong *et al*. (2010), extraction with methanol provide a better efficiency for catechin extraction compared to extraction with hot water only (aqueous extraction). Since EGCG is water soluble compound, it has higher solubility in water compares to organic solvent such as methanol, but it has higher polarity towards methanol which influenced it to be soluble in methanol (Lee and Lee, 2008).

Table 1: Comparison of EGCG extraction process using methanol extraction and aqueous extraction method. Methanol extraction (ME) yield higher EGCG concentration compare to aqueous extraction (AQ) at the same extraction condition, 37 °C and 60 minutes extraction continuous with continuous stirring. The result was presented with mean, n=2 and standard deviation.

Extraction method	Tea type	Extraction yield (%)	EGCG Concentration (mg/mL)
Methanol Extraction	GT	8.21	0.9347 ± 0.08
	BT	1.84	0.5385 ± 0.12
Aqueous Extraction	GT	26.87	0.6705 ± 0.09
	BT	20.46	0.4410 ± 0.12

3.2. HPLC analysis

The presence of EGCG in tea samples were analyzed by comparing the retention time of tea samples to EGCG standard through HPLC results. The EGCG standard showed one sharp and high peak at retention time 2.762. The tea samples showed 2 single peaks at retention time 2.740 and 3.091. Based on the EGCG standard reference, the peak formed at retention time 2.740 minutes was indicated as EGCG while peak second peak obtained at 3.091 minutes was referred as caffeine according to Vasisht *et al*. (2003).

EGCG standard curve is plotted with $R^2 > 0.9882$ to quantify the amount of EGCG is the tea samples. High level of EGCG concentration is recorded in GT samples (1.0958 ± 0.14) followed by WT (0.9604 ± 0.00), OT (0.7747 ± 0.05) and BT (0.7747 ± 0.05). This result is in accordance with Chen *et al*., (2008), Yi *et al*., (2015) and Demir *et al*., (2015) which reported EGCG is abundantly found in GT.

3.3. Optimization of temperature and extraction time in extraction of EGCG from WT, GT, OT and BT using aqueous extraction method

Higher EGCG concentration is obtained using methanol extraction, however, in this study, aqueous extraction method is chosen to be optimized since water is the solvent usually used to prepare a cups of tea for daily consumption.

Extraction of EGCG by aqueous extraction is influenced by the temperature and extraction time (Amra *et al*., 2006; Lee and Lee, 2008; Vuong *et al*., 2011). From the experiment, the extraction of EGCG from WT, GT and OT are influenced by the temperature. As illustrated in Figure 1(a), there are a significant different between the EGCG concentration for WT, GT and OT at different temperature and showed that 80 °C yield the highest EGCG concentration for WT (0.9301 ± 0.04), GT (0.8835 ± 0.09) and OT (0.6949 ± 0.04). However, the highest EGCG concentration for BT samples is obtained at lower temperature with EGCG concentration value of (0.4949 ± 0.02). This finding mimics previous reports by Vasisht *et al*. (2003), Pastoriza *et al*. (2017) and Vuong *et al*. (2011) which 80 °C is the best temperature to yield higher concentration of EGCG content in tea sample especially for green tea and black tea is not affected by temperature (Lee and Lee, 2008).

WT and GT samples were affected by the extraction time. Based on Figure 1(b), the concentration of EGCG increased with time for WT but inversely proportional for GT. This finding mimics previous reports where the yield of EGCG from tea increases as the extraction time increases (Lee and Lee, 2008). Meanwhile, the results for GT is in synchrony with the results obtained by Pastoriza *et al*. (2017) which indicates that the yield of EGCG from GT decrease with time. However, the amount of EGCG obtained from OT and BT are almost similar with highest EGCG concentration was extracted at 40 minutes.

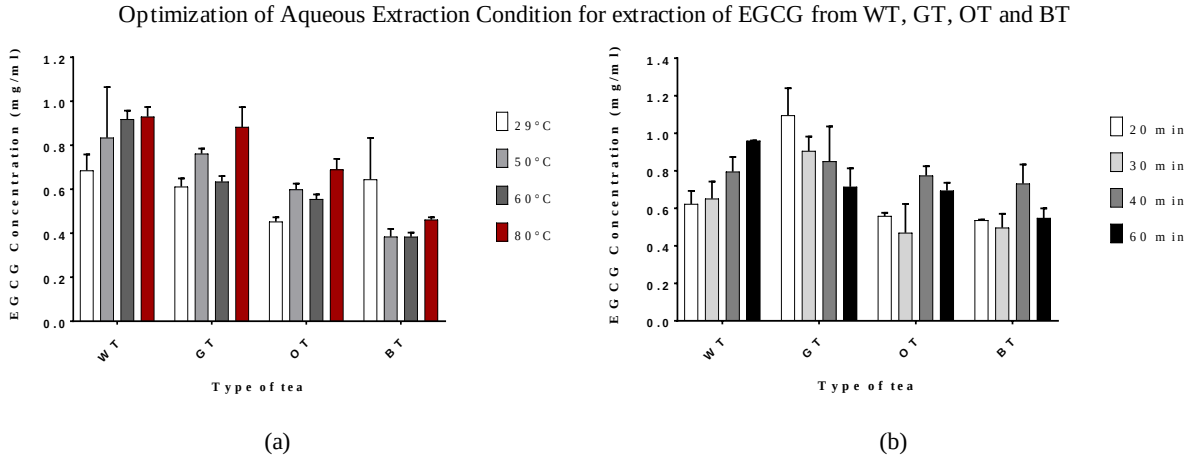

Figure 1: Optimization of aqueous extraction condition. (a) Temperature optimization, (b) extraction time optimization. The result is presented with mean (n=3) and standard deviation.

3.4. Characterization of EGCG content in commercialized infusion tea products and ready-to-drink tea beverages

When comparing the level of EGCG in infusion tea bag (ITB) and ready-to-drink tea beverages (RTD), the EGCG concentration in ITB is much higher compared to RTD. EGCG and other catechin counterparts are easily disrupted by heat, light and chemicals which makes EGCG content in RTD beverages is lesser along the manufacturing process rather than ITB which undergo simple fermentation process. The preparation of infusion tea suggested by manufacturer is brewing the tea in boiling water for 3 minutes may facilitate the extraction of more catechin. The result is in agreement with Oliveira (2012) where by EGCG content in ITB is higher compared to RTD beverages.

Besides, different brands of tea products contain different level of EGCG content which is depends on the processing methods hence the price reflect the quality of the tea brand in the market. Based on Table 2, there are a significant different between the level of EGCG in different brands of WT, GT, OT and BT products which reflects its value for health. Same results were obtained in Table 3 where the difference between the brands is significant in GT and BT products.

Table 2: EGCG concentration in ready-to-drink tea beverages. The result is presented with with mean (n=3) and standard deviation.

Types of tea	Brands	Number of sachets/mg	Price/number of sachets (RM)	EGCG concentration (mg/mL)
WT	A	1 / 2 mg	29.90/25	0.2246 ± 0.034
	B	1 / 1.8 mg	24.50/20	0.4715 ± 0.057
GT	C	1 / 2 mg	5.40/25	0.7222 ± 0.082
	D	1 / 2 mg	11.60/25	0.8741 ± 0.249
	E	1 / 2 mg	8.90/25	0.4619 ± 0.252
	F	1 / 2 mg	9.75/25	0.7106 ± 0.076
OT	G	1 / 5 mg	24.00/52	0.3516 ± 0.057
	H	1 / 2 mg	15.30/25	0.3737 ± 0.020
	F	1 / 2 mg	9.75/25	0.7496 ± 0.004
BT	I	1 / 2 mg	3.10/20	0.2626 ± 0.097
	J	1 / 2 mg	6.50/25	0.3750 ± 0.087
	K	1 / 2 mg	2.30/20	0.2668 ± 0.034
	D	1 / 2 mg	2.70/25	0.2845 ± 0.025
	E	1 / 2 mg	10.55	0.3719 ± 0.049

Table 3: EGCG concentration in ready-to-drink tea beverages. The result is presented with with mean (n=3) and standard deviation.

Types of tea	Brands	Price per 250 mL (RM)	EGCG concentration (mg/mL)
WT	N	2.50	0.0762 ± 0.007
GT	L	2.50	0.1534 ± 0.005
	M	1.80	0.3196 ± 0.010
	N	2.50	0.2967 ± 0.004
	O	1.90	0.1825 ± 0.002
	P	2.20	0.1564 ± 0.040
OT	N	2.50	0.1182 ± 0.008
BT	L	2.50	0.0503 ± 0.004
	M	1.80	0.2626 ± 0.008
	N	2.50	0.1982 ± 0.031
	O	1.90	0.1048 ± 0.001
	P	2.20	0.1358 ± 0.006

4. Conclusions

In conclusion, the optimum extraction condition of white tea, green tea, oolong tea and black tea is different from each other. The different was influenced by the fermentation step during tea processing. GT contain higher EGCG content compare to the other types of tea, WT, OT and BT. The extraction of EGCG compound from WT, GT and OT were influenced by temperature but not for BT. 80 °C is the best temperature for to extract EGCG from WT, GT and OT using aqueous extraction. Apart from that, higher EGCG content is extracted at shorter time for GT but longer time is needed for WT. Both EGCG from OT and BT samples were best extracted at 40 minutes.

The level of EGCG content per ml is much lower compare to infusion tea hence, it provide less benefit for health. Besides, different brands of tea products contain different EGCG content depends on the processing methods hence the price reflect the quality of the tea brand in the market. Ready-to-drink tea beverage is an unhealthy option for drinks. Therefore, it is recommended to drink infusion tea especially GT at least three cups per day to get the benefits of EGCG towards health. However, further study and research are needed to evaluate the effectiveness of tea consumption in treatment and prevention of obesity for normal, overweight and obese people.

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