Biotechnology Towards Sustainable Development Goals and Circular Bioeconomy

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Khanom Simarani
Sharifah Aminah Syed Mohamad
Zaharah Ibrahim
Koh Soo Peng

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Opening Speech

Prof Datuk Ts Dr Ahmad Fauzi Ismail
Vice Chancellor
Universiti Teknologi Malaysia, Malaysia

Distinguished guests, ladies, and gentlemen, a very good morning and Salam Sejahtera.

First and foremost, I would like to thank the Asian Federation of Biotechnology Malaysia Chapter (AFOBMC) for inviting me to officiate the Asian Federation of Biotechnology Malaysia Chapter International Symposium (**AFOBMCIS 2021**). Today, I am delighted to be part of this highly anticipated event which gathers biotechnology intellectuals comprising of academicians, scientists, private sectors, and students. To all international delegates, I would like to wish "Selamat Datang" and I hope you have a memorable online conference.

I would like to congratulate the Asian Federation of Biotechnology Malaysia Chapter (AFOB) for organizing AFOBMCIS 2021 with the theme **"Biotechnology toward Sustainable Development Goals (SDGs) and Circular Bioeconomy"**.

This theme is timely and in line with the current trend of Biotechnology, which focuses on the sustainable development goals (SDGs) and circular bioeconomy. Biotechnology promises to make a significant contribution in facilitating the sustainable development of, for example, improved health care, enhanced food security, improved supplies of potable water, more efficient industrial development processes for transforming raw materials, support for sustainable methods of afforestation and reforestation, and detoxification of hazardous wastes. It offers new opportunities for global partnerships. In a circular bioeconomy, biological resources are renewable, sustainably managed, recovered and reused as much as possible. This economic model is gaining its momentum to deliver society's need in responding to the current sustainability issues.

I was informed that AFOBMCIS 2021 is jointly organized by Universiti Teknologi Malaysia (UTM) and Universiti Malaysia Pahang (UMP) with several universities (Universiti Putra Malaysia (UPM), Universiti Teknologi Mara (UiTM), Universiti Malaysia Sabah (UMS), Universiti Malaysia Sarawak (UNIMAS), Universiti Islam Antarabangsa Malaysia (UIAM), Universiti Malaya (UM), Universiti Malaysia Perlis (UniMAP), USCI University, Universiti Kuala Lumpur (UniKL) and research institutions (MPOB) and MARDI) in Malaysia as collaborators. These scientist–academia linkages should be encouraged, and I wish to see more collaboration in future for the enhancement of research in biotechnology.

The signing of Memorandum of Understanding (MoU) extension between Asian Federation of Biotechnology Malaysia Chapter (AFOBMC) and Biotechnology and Biochemical Engineering Society of Taiwan (BEST) for another 3 more years will continue the international collaboration between Malaysia and Taiwan researchers in the development of Biotechnology and it enhances research development through global networking. I was told that this collaboration started since 19 August 2018. Finally, this is an opportune time for me to declare the official opening of the "3rd Asian Federation of Biotechnology Malaysia Chapter International Symposium (**AFOBMCIS 2021**)". I hope that you have a pleasant conference in sharing ideas and outcomes for our sustainability and bioeconomy. I wish you to have a fruitful day of interesting and beneficial program.

Thank you.

Welcoming Speech

Prof Ts Dr Suraini Abd-Aziz
President
AFOB Malaysia Chapter
Advisor
AFOB Malaysia Chapter International Symposium 2021

It is my great pleasure to welcome all the participants to the 3rd Asian Federation of Biotechnology Malaysia Chapter International Symposium (**AFOBMCIS 2021**) from 22nd – 23rd September 2021 in which for the first time will be held online. This symposium is jointly organised by AFOB–Malaysia Chapter (AFOB–MC), Universiti Teknologi Malaysia (UTM) and Universiti Malaysia Pahang (UMP). Also, several universities and research institutions in Malaysia as collaborators, which are Universiti Putra Malaysia (UPM), Universiti Teknologi MARA (UiTM), Universiti Malaya (UM), Universiti Malaysia Sarawak (UNIMAS), Universiti Malaysia Sabah (UMS), International Islamic University of Malaysia (IIUM), Universiti Malaysia Perlis (UniMAP), UCSI University, University Kuala Lumpur (UniKL), Malaysia Palm Oil Board (MPOB) and Malaysian Agricultural Research and Development Institute (MARDI). The 3rd AFOBMCIS 2021 was Chaired by Dr Mohd Helmi Sani (UTM) and co–chaired by Dr Rozaimi Abu Samah (UMP).

AFOB Malaysia Chapter (AFOB–MC) is a regional branch of AFOB with headquarters at Incheon, Korea is a non–profit organization, established and registered with the Registrar of Society in 2013. In line with the function of the Asian Federation of Biotechnology (AFOB), AFOB–MC also aims to promote cooperation on scientific grounds, between the scientists from academia and industry in the Asian region, for the general advancement of biotechnology as an interdisciplinary field of research and as a means of bringing the scientific development to the industrial level. As a record, the AFOB–MC had organised the AFOB Regional Symposium 2014 (ARS2014) in Seri Pacific Hotel, Kuala Lumpur, Asian Congress on Biotechnology 2015 (ACB2015) in Hotel Istana, Kuala Lumpur, AFOBMCIS 2018 in Pullman Kuching, Sarawak and AFOBMCIS 2019 at The Everly Hotel, Putrajaya.

The conference theme "**Biotechnology toward Sustainable Development Goals (SDGs) and Circular Bioeconomy**", will contribute to the current biotechnology area with 12 technical sessions that cover vast area of biotechnology. Due to the COVID–19 pandemic, the 3rd AFOBMCIS 2021 will be held virtually on the online platform, I hope that this symposium will be a successful event with the enthusiastic participation of locals and worldwide biotechnologists for the realisation of SDGs and bioeconomy.

I hope that this symposium will help establish collaborative research programs, hence strengthening research relations and networking between universities, industries, and government. I would like to express appreciation to the AFOBMCIS 2021 Organising Committee members for their effort and hard work to ensure a successful and meaningful symposium for all of us.

Welcoming Remarks

Dr Mohd Helmi Sani
Chair
AFOB Malaysia Chapter International Symposium 2021

Dear delegates, it is my great pleasure to welcome you to the 3rd Asian Federation of Biotechnology Malaysia Chapter International Symposium (**AFOBMCIS 2021**).

AFOBMC International Symposium (AFOBMCIS) is one of the annual events of the Asian Federation of Biotechnology Malaysia Chapter (AFOB–MC). This year, AFOBMCIS 2021 is jointly organised by Universiti Teknologi Malaysia (UTM) and Universiti Malaysia Pahang (UMP) with several universities (Universiti Putra Malaysia (UPM), Universiti Teknologi Mara (UiTM), Universiti Malaysia Sabah (UMS), Universiti Malaysia Sarawak (UNIMAS), Universiti Islam Antarabangsa Malaysia (UIAM), Universiti Malaya (UM), Universiti Malaysia Perlis (UniMAP), USCI University, Universiti Kuala Lumpur (UniKL) and research institutions (MPOB) and (MARDI) in Malaysia as collaborators.

The 3rd AFOBMCIS 2021 highlights the multidisciplinary focus, emerging scientific and technological developments areas related to biotechnology. AFOBMCIS 2021 is aimed to provide a platform for local and international scientists, academia, and industries to share their knowledge and expertise, ideas and opinions and showcase research outcomes in biotechnology.

The theme of the symposium is "**Biotechnology toward Sustainable Development Goals (SDGs) and Circular Bioeconomy**" covers various fields Agricultural and Food Biotechnology; Applied Microbiology; Biopharmaceutical and Medical Biotechnology; Biocatalysis and Protein Engineering; Bioprocess and Bioseparation Engineering; Bioenergy and Biorefinery; Environmental Biotechnology; Marine Biotechnology; Nanobiotechnology, Biosensors and Biochips; Systems and Synthetic Biotechnology; Tissue Engineering and Biomaterials and Bioindustry Promotion and Bioindustry.

This symposium also invites prestigious speakers in the biotechnology and bioeconomy to share their knowledge and expertise. We would like to thank our Plenary, Keynote and Invited Speakers from various countries and institutions for accepting our invitation. We also would like to express our gratitude to all the oral presenters for sharing your findings and ideas. This symposium also organizing Young Researcher Sessions and 3 Minutes Poster Sessions that will be evaluated by our appointed judges. The best Young Researchers and 3 Minutes Poster Presenters will be awarded.

Along with this symposium, a signing MOU ceremony between AFOB–MC and Biotechnology and Biochemical Engineering Society of Taiwan (BEST) will also be held after the opening ceremony. Besides, suitable topics presented in this symposium will be invited for publication in Special Issues by Frontiers Microbiology, Food Research Journal and Malaysian Applied Biology (MAB).

I would like to extend my gratitude to Prof. Ts. Dr. Suraini Abd-Aziz (President of AFOB–MC and advisor of AFOBMCIS 2021), Dr. Rozaimi Abu Samah (Co–chair), Dr. Lisa Ong Gaik Ai (Secretary), Assoc. Prof. Dr. Phang Lai Yee (Treasurer) and all the organising committee and event team members of the AFOBMCIS 2021 for their efforts and supports in developing such an inspiring and remarkable symposium programme.

On behalf of all organizers, I would like to express appreciation for the sponsorship given by the respective organizations towards the success of this AFOBMCIS 2021. I hope that this symposium will help establish collaborative research programs, hence strengthening research relations and networking between universities, industries, and government.

Best wishes.

Anti–inflammation, Anti–cancer Mechanism and Microarray Gene Expression of Soft Coral Derived Secondary Metabolites

Charles Santhanaraju Vairappan*

Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, 88450, Kota Kinabalu, Sabah, Malaysia

*csv@ums.edu.my

ABSTRACT

Borneo marine ecosystem is rich with a wide array of flora and fauna at a density of close to 1000 species per m^2. Number of secondary metabolites produced by these marine organisms are believed to be directly related to its biodiversity. We studied the diversity of secondary metabolites in soft corals (*Alcyonacea*). A total of 200 compounds were isolated and their diversity analysed, in addition their anti–inflammation and anti–cancer activities were investigated. Inflammation of a powerful innate immune system defence that is an orchestrated maneuver designed to eliminate cellular treats. Chronic inflammatory response plays an important role in cancer development and resistance to chemotherapy. Molecular mediators that regulate inflammation and cancer are promising targets for preventing and treating these diseases. In this study, we have identified several soft corals derived novel secondary metabolites with potent anti–inflammation and anti–cancer activities. Their inflammatory potential and mechanism of action was evaluated using RAW 264.7 macrophages, their PGE_2, TNF–α, IL–1β, IL–6, iNOS, and COX_2, were evaluated. Cancer cell bioassay was concluded using HL60 and MCF 7 cell lines, with control normal cells. Apoptosis mechanism was evaluated using Sub–G1 proportion, microscopic technique, Bax, Bcl–xl, Cleaved Capcase 3 and β–actin. In addition, we also investigated the microarray gene expression on the cells when these compounds were tested against human primary breast cancer cells that were derived from primary lobular carcinoma. Data was analysed using two–dimensional clustering of top genes–expression intensities, AKR1C1/2, TPD52, SPRY2, PLK1, KIF11genes were identified as highly expressed in the influence of these compounds.

Keywords: Anti–inflammation; Anti–cancer; Microarray–gene–expression; Soft corals; Borneo

1.0 INTRODUCTION

Marine natural products are important source of secondary metabolites and potential lead pharmaceuticals [1]. Soft corals in particular have produced many new chemical scaffolds with interesting chemical structures [2]. Recently, we have collected and reported structurally interesting terpenoids from soft corals from Tun Mustapha Marine Park, Borneo [3]. Among soft corals, genus *Sinularia* has produced many structurally and biologically interesting compounds. In an effort to better understand their importance as anti-inflammation and anti-cancer lead pharmaceutical candidates, we investigated a new population of soft coral genus *Sinularia*, and it resulted in 3 terpenoids that exhibited interesting anti-inflammation, anti-cancer and lead to the identification of genes that regulates these activities.

2.0 MATERIALS AND METHODS

2.1 Biological Specimens

A specimen of *Sinularia* sp. Was collected from Tun Mustapha Marine Park, North Borneo (7.338711'N, 117.35017'E), in April 2017. The voucher specimen (BORMI0058) was deposited in the BORNEENSIS Collection of Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah.

2.2 Extraction and Isolation

Fresh soft coral (4.0 kg wet wt) extracted in methanol (MeOH) for 3 days, subsequently filtered, concentrated, and partitioned between ethyl acetate (EtOAc)/water (H_2O), EtOAc crude was partitioned with *n*-hexane/90% MeOH. The resulting crude extracts were subjected to column chromatography eluting with a gradient of *n*-hexane and EtOAc with increasing polarity. Repetitive Thick Layer Chromatography resulted in the isolation of 3 diterpenes, identified as **1**, **2** and **3**.

2.3 Anti-Inflammation and Anti-Cancer Assay

The cytotoxicity of compounds on RAW264.7 macrophage cells was evaluated using the standard 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (1.0×10^5 cells/mL) were seeded in 96-well plate followed by treatment of compounds at various gradient concentrations (3.125, 6.25, 12.5, and 25 µg/mL) respectively in triplicate. The cells were maintained in 5% CO_2 at 37 °C for 24 hours. The determination of NO production was performed by pre-cultured RAW 264.7 cells (1.0×10^5 cells/mL) for 24 hours, subsequently pre-incubated with compounds for 1 hour, followed by treatment with LPS (1 µg/mL) and incubated for 24 hours. Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) was used to quantify NO production as described in (). Anti-cancer assay was conducted using human promyelocytic (HL60), and human breast cancer cell lines (MCF-7). The cancer cells each were cultured as monolayers in RPMI-1640 (Roswell Park Memorial Institute) media while B16F10 cell line required DMEM (Dulbecco's Modified Eagle Medium) media supplemented with 10.0% (v/v) heat-inactivated FBS, 100 U/mL penicillin and 100.0 µg/mL streptomycin. All cell cultures were grown in a humidified incubator at 37 °C in 5.0 % CO_2 and 95 % O_2 [4].

2.4 Structure Elucidation

^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded on a JEOL ECA 600 NMR spectrometer (Japan) using $CDCl_3$ with TMS as an internal standard. High-resolution mass spectrum was acquired via Shimadzu LCMS-ESI-IT-TOF (Japan). Rudolph Research Analytical AUTOPOL IV automatic polarimeter (USA) was used to measure the optical rotation. Infrared spectra were recorded on a Perkin Elmer Spectrum Two Universal Attenuated Total Reflection Spectrometer (USA). Preparative TLC was performed with Merck Kieselgel 60 F_{254} silica gel glass plates (USA), and column chromatography (CC) with Merck Kieselgel 60, 70-230 mesh silica gel (USA) [4].

2.5 Microarray Gene Expression

Human primary breast cancer cells derived from primary lobular carcinoma with comedo-structures removed at tumor pT1. Cells were positive for oestogen receptor, EpCAM, CD24, CD66 and negative for CD133. Assay was done using Flouresence Resazurin Assay Kit, prior to assay, cells were glucose-starved for 24 hours. RNA extracted using QIAzol Kit, purified RNAs processed using Affymetrix WT Expression Kit and GeneChip 3000 system. Claroim S Human Array gene level whole transcriptome was performed.

3.0 RESULTS AND DISCUSSION

Population of *Sinularia* sp, collected from Tun Mustapha Marine Park yielded a total of 3 cembrane-type secondary metabolites ent-Sinuflexibilin D (**1**), 11,12-epoxy-13,14-dihydroxycembrene-C (**2**) and Sinularin (**3**), as shown in Figure 1. Compound 1 was reported as new enantiomer by Phan et al. (2017) [5], while 2 and 3 were reported by Gray et al. (2000) and Duh et al (1998), respectively. In addition, all these three compounds were also isolated and before as part of a doctoral dissertation from populations of soft corals from Mantanani Island, Pulau Tiga, and Mangallum Island by Phan (2018)[8]. These compounds were also reported by Kashman et al 1980 [9], and Winheimer et al. 1977 [10]. These compounds were isolated based on their absorption of UV light at 215 nm, and was not isolated using a bioassay-guided separation approach. This approach was part of our effort to isolate ecological chemicals as biomarkers in soft corals in Borneo.

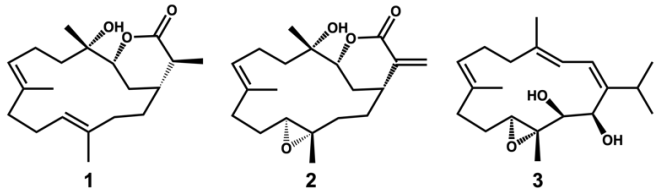

Figure 1: Cembrane-type secondary metabolites from a single population of *Sinularia* sp., from Tun Mustapha Marine Park, North Borneo

In vitro assay of cytotoxicity of isolated compounds toward RAW 264.7 cells and preliminary screening of inhibitory compound against accumulation of NO production were conducted to select potential compound for further experiments. Selected compound was subjected to several experiments to investigate the inhibitory effect of respective compound on NO, PGE2 and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) productions. In addition, the inhibitory ability of respective compound on iNOS and COX-2 protein expressions by Western blot was recorded. All the three compounds did not show any cytotoxicity towards tested macrophages but exhibited protective nature by reducing the release of NO when the macrophages were treated with the compounds before LPS was administered. All compounds showed activities from 40 μg/ml, 20 μg/ml and 10 μg/ml, compounds showed a concentration dependent response in the NO release, TNF-α, IL-1β and IL-6. Compounds also responded well towards MCF-7 cell lines, but only compound 3 was most prominent with a response at 15.0 μg/ml. It also showed clear apoptosis activity towards the MCF-7 cell lines with concentration dependent response in the titter of Bax, Bcl–xl, Cleaved Capcase 3 and β–actin. Further, microarray gene expression experiments resulted in the identification of genes from cluster AKR1C1/2 and TPD52 as prominent genes that were upregulated in MCF-7 cells when tested with Sinularin (**3**).

4.0 CONCLUSION

All three cembrane-types terpenoids isolated from this population of *Sinularia* sp., exhibited very potent and interesting biological activities against RAW267.4 macrophage and MCF-7 cell lines with almost negligible level of cytotoxicity. Secondary metabolites form marine invertebrates will continue to offer a wide array of secondary metabolites as important ecological chemicals for the development of lead pharmaceuticals. To date, we have isolated and reported almost a total of 1000 compounds from marine organisms, but this is just as very small fraction of the resources that is available in the sulu Sulawesi Marine Ecoregion in Borneo.

ACKNOWLEDGEMENT

The author would like to declare that the soft coral specimens were collected during the Taman Tun Mustapha Expedition organized by Sabah Parks (Sabah, Malaysia) in 2017. Author also would like to knowledge the logistic support given to us during specimen collection and facilitating the access permits from Sabah Biodiversity Centre. This work was financially supported by the Sabah Biodiversity Centre [GL-0070] research grant, Universiti Malaysia Sabah [GUG 0090-STWN-2/2016] and Ministry of Higher Education [FRG 0464-2017]. The author would also like to express his appreciations to Dr. Phan Chin Soon (2018) and Dr. Kazuki Tani (2020) for their assistance during the course of this investigation.

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A Preliminary Study on The Efficacy of Spinetoram Against Melon Thrips (*Thrips palmi*) in Malaysia

Jiunn Luh, Tan[1,2*], Peter Aun Chuan, Ooi[3], Gideon Khoo[4]

[1]*Department of Zoology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic*
[2]*Department of Biochemistry and Physiology, Institute of Entomology, Biology Centre CAS, České Budějovice, Czech Republic*
[3]*Department of Agricultural and Food Science, Faculty of Science, Universiti Tunku Abdul Rahman, Perak, Malaysia*
[4]*Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, Perak, Malaysia*

*jiunnluh@gmail.com

ABSTRACT

Thrips palmi Karny is one of the major pests for eggplants, chilli and bell pepper in Cameron Highlands, Malaysia. Most of the farmers depends on chemical insecticides to suppress the population of *T. palmi*. However, chemical control is typically not recommended in the control of *T. palmi* due to the nature of this species which will easily develop resistance against a wide range of insecticides. Spinetoram was currently the most popular insecticide used to control *T. palmi* in Cameron Highlands. Leaf-dipped bioassay was carried out and the mortality results obtained were used to determine the lethal dosages at 50% mortality (LD_{50}) using Probit Analysis. The resistance ratio (RR) of the *T. palmi* populations against spinetoram was found to be 1.69. This indicates low resistance of *T. palmi* against this insecticide. However, the RR value of more than 1.5 can signify a decrease in the effectiveness of spinetoram in suppressing *T. palmi*. Therefore, it is suggested to continue monitoring the RR for *T. palmi* in the area as well as expanding to other vegetable growing regions in Malaysia. In addition, the feasibility of alternative strategies, such as integrated pest management, should be studied to manage the *T. palmi* populations more sustainably.

Keywords: Cameron Highlands; Insecticide resistance; Solanaceae; *Thrips palmi*; Vegetable thrips pest management

1.0 INTRODUCTION

Thrips palmi Karny, also known as the melon thrips, are widely distributed in three regions, namely, South Asia, Southeast Asia and Oceania [1]. Besides these three regions, they are also found in South America (Brazil, Colombia, French Guiana and Venezeula), North America (Florida and Hawaii), Africa (Mauritius, Nigeria, Reunion and Sudan) and throughout the Caribbean [2]. Since 1978, *T. palmi* has been one of the most important vegetable pests in Japan and the host plant includes eggplant, cucumber, muskmelon, green pepper and watermelon [3]. *T. palmi* does not only damage the crops but also acts as a vector for the tospoviruses [4]. Fauziah and Saharan [5] reported that *T. palmi* is the most important pest of vegetable cultivation in Malaysia and it is a common thrips species found on chilli (*Capsicum annuum*), cucumber (*Cucumis sativus*) and eggplant (*Solanum melongena*). Besides Asia, *T. palmi* has been observed to heavily infest eggplants, bell peppers and other vegetable crops in South Florida [6]. The adult and larvae of *T. palmi* usually feed on the foliage by sucking the plant sap. The damage symptoms of *T. palmi* are leaf crinkling and discoloration. Slightly infested leaves exhibit silvery feeding scars on the abaxial surface, while heavily infested leaves turn brown or yellow and appear dry. Infested fruits become scarred and deformed [1,7]. These damages have led to shorter crop life cycle and fruits are not marketable which cause a loss of income to farmers in Cameron Highlands [8].

Chemical control is usually not recommended in the management of *T. palmi* as it is ineffective but farmers in Cameron Highlands still depend on insecticides for the suppression of thrips [9,10]. Certain strains of *T. palmi* have been found to be very resistant to the insecticide spinosad, which is about 1938-fold more resistant as compared to the susceptible strains. It is noted that resistant strains may possess genes that allow them to be insensitive towards Spinosad [11]. Spinetoram and Spinosad belong to the same chemical class, spinosyns [12], therefore, cross-resistance may happen. *Thrips palmi* is also resistant to imidacloprid, where certain strains are 12.2-fold more resistant as compared to susceptible strains [13]. According to Cannon *et al.* [14], *T. palmi* is resistant towards many insecticides under the chemical classes of organophosphate, carbamate and pyrethroid. Therefore, this study aims to investigate the efficacy of commercially formulated spinetoram against *T. palmi* in Cameron Highlands.

2.0 MATERIALS AND METHODS

2.1 Sampling Sites

Thrips palmi samples were collected from both conventional farms and organic farms in Cameron Highlands, Malaysia. The most common insecticides used to suppress *T. palmi* in conventional farms were imidacloprid, abamectin, spinetoram and emamectin benzoate. Among these insecticides, commercially formulated spinetoram (Endure® 58.7g/l SC; Dow AgroSciences (M) Sdn Bhd) was the most effective. Insecticide applications were carried out once a week. The organic farms did not apply any chemical pest management approaches and depended only on natural control. The location of the sampling sites is shown in Table 1.

2.2 Leaf-dip Bioassay

Eggplant, *Solanum melongena* L., leaves were used because it is easily available. These eggplant leaves were collected from organic farm to ensure that they were not contaminated with insecticides from the field. The eggplant leaves were washed thoroughly with distilled water and air-dried. They were cut into disc shapes with a diameter of approximately 5 cm using a scalpel blade [15]. Six different concentrations (5X serial dilutions) of commercially formulated spinetoram (Endure® 58.7g/l SC; Dow AgroSciences (M) Sdn Bhd) were prepared and leaf discs were dipped into different concentrations respectively [16]. The control leaf-discs were dipped in distilled water only [16,17]. The leaf-discs were placed in petri dishes on semi-solid water agar (10g/L agar) with the abaxial surface of the leaf-disc facing upward. Ten to fifteen adult thrips were placed on each leaf-disc [15,18]. There were five replicates for each concentration. The mortality of *T. palmi* was determined after 48 hours of incubation under room temperature [18,19]. The adult thrips were considered dead if they did not move after gentle stimulation with the paint brush (size 0/2).

2.3 Probit Analysis and Resistance Ratio

The lethal dosage at 50% mortality (LD_{50}) was evaluated using the mortality results obtained from the leaf-dip bioassay and the Probit analysis function in the Statistical Analysis System (SAS) Enterprise Guide 7.11. The LD_{50} for *Thrips palmi* from organic and conventional farms were assessed. With the LD_{50} value, the resistance ratio was calculated using the following formula [20]:

$$\text{Resistance Ratio} = \frac{LD_{50} \text{ of } T.\ palmi \text{ from conventional farms}}{LD_{50} \text{ of } T.\ palmi \text{ from organic farm}}$$

Table 1: Farming areas where the thrips sampling was carried out

The type of farms	Location	Estimated Coordinates	
Conventional Farms	Kampung Raja	4°34'03"N	101°24'06"E
	Habu and Boh Road Area	4°35'06"N	101°25'05"E
Organic Farms	Blue Valley	4°35'06"N	101°25'05"E

3.0 RESULTS AND DISCUSSION

The resistance ratio (RR) is obtained by comparing the LD_{50} of the *T. palmi* collected from conventional farms against those collected from the organic farm. It is used as an indicator to determine the effectiveness of spinetoram against *T. palmi* populations in Cameron Highlands. The level of insecticide resistance is categorised as susceptible (RR = 1.0), low resistance (RR = 2–10), moderate resistance (RR = 11–30), high resistance (RR = 31–100) and very high resistance (RR > 100) [17]. The resistance ratio (RR) was 1.69-fold, indicating low resistance against commercially formulated spinetoram. However, the RR value of more than 1.5 can be an indication that the effectiveness of spinetoram in suppressing *T. palmi* population is slowly decreasing. In addition, the low RR may be due to spinetoram which is a newly introduced insecticide in the market for use against *T. palmi* populations in Cameron Highlands. According to the Pesticide Control Division of the Department of Agriculture Malaysia, spinetoram was first registered in Malaysia in 2008. According to the information provided by local pesticide dealers and sales representatives, spinetoram was first marketed in Cameron Highlands in 2011. Furthermore, Cameron Highlands farmers practise rotation of insecticides with different modes of action in the control of thrips which might result in delaying the development of insecticide resistance [21]. This is, however, not a sustainable thrips management approach and will eventually lead to pest outbreaks.

The RR obtained by comparing the *T. palmi* population collected from organic and conventional farms gives an early indication that the thrips populations have started to develop resistance against spinetoram. This is because even though the *T. palmi* population collected from organic farm might comprise a mixture of some resistant thrips that migrated from neighbouring conventional, non-organic farms, the RR is still above 1.5. According to Bao *et al.* [11], certain strains of *T. palmi* may possess a gene that enables them to resist the effect of Spinosad, achieving an RR as high as 1938-fold. The development of spinetoram resistance may be attributed to the fact that they are from the same chemical group (group 5, nicotinic acetylcholine receptor (NACHR) allosteric modulators – site 1) as Spinosad [22]. Therefore, a similar gene possessed by resistant strains of *T. palmi* may allow the population to be insensitive to the effect of spinetoram.

In addition, evidence shows that previously effective insecticides, such as, imidacloprid and abamectin, have also become ineffective due to the development of insecticide resistance among the *T. palmi* populations in Tokushima, Japan with resistance ratio ranging from 2.5 to 12.2 [13]. A similar incident was observed in *T. palmi* populations collected from Nangoku, Japan, where resistance had developed against imidacloprid (RR = 16.6) [4]. Seal [23] also noted that imidacloprid was ineffective in reducing the *T. palmi* population in Florida possibly due to the development of insecticide resistance. Commercially formulated abamectin was not effective against *T. palmi* even when it was mixed into a cocktail with zeta-cypermethrin and bifenthrin [24]. Local farmers in Cameron Highlands also noted the decrease in effectiveness of imidacloprid and abamectin in the suppression of thrips populations, and recently spinetoram was also ineffective in certain farms. Most farmers will increase the dosage and frequency of insecticide application in respond to the decrease in effectiveness to suppress the resistant thrips population. Such practices will result in high RR and pest outbreaks. Moreover, local farmers who

practiced calendar spraying and the use of cocktail pesticides will promote the development of insecticide resistance among thrips [25]. If such practices are continued, a similar scenario of high RR against imidacloprid and abamectin will happen in the case of spinetoram or any newer insecticides in future. Therefore, further monitoring and studies should be carried on the resistance of *T. palmi* against existing and new insecticides, so that quick strategies can be implemented before an outbreak.

In addition, the infestation of *T. palmi* was less serious in unsprayed gardens [25]. Therefore, it can be inferred that *T. palmi* outbreaks are mostly due to the development of insecticide resistance among thrips, and the chemical control method is not a suitable thrips management approach. It is suggested that the feasibility of alternative thrips management strategies, such as integrated pest management and biological control, should be studied as a better thrips management approach. For instance, phytoseiid mites and multiple *Orius* spp. could be employed as effective biological control agents against pest thrips.

4.0 CONCLUSION

The resistance ratio (RR) obtained by comparing *T. palmi* collected from organic and conventional farms was found to be 1.69-fold against commercially formulated spinetoram. The RR value indicated a decrease in the effectiveness of commercially formulated spinetoram against *T. palmi* population. Further studies and monitoring of the insecticide resistance of *T. palmi* population are highly recommended. Subsequent insecticide resistance studies of *T. palmi* should be expanded to other vegetable cultivation regions in Malaysia to provide a better understanding on the status of insecticide resistance of this pest. It is also recommended to investigate the feasibility of alternative strategies to sustainably manage the *T. palmi* population.

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Effect of Plant Growth Regulator on Antioxidant Activity from Shoot Biomass of *Orthosiphon stamineus*

Nur Kamilah Mohd Nordin[1], Sayang Baba[1], Azman Abd. Samad[1]*, Nik Ahmad Nizam Nik Malek[1,2], Wan Rosmiza Zana Wan Dagang[1]

[1]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor
[2]Centre for Sustainable Nanomaterials (CSNano), Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor

*azmansamad@utm.my

ABSTRACT

Orthosiphon stamineus (*O. stamineus*) or locally known as 'misai kucing' exhibits high antioxidant activities. The relationship between shoot biomass and antioxidant activities of *O. stamineus* extracts was not yet investigated. Therefore, a study was conducted to evaluate the effect of plant growth regulator (PGR) on shoot regeneration frequency, shoot biomass and antioxidant activity of *in vitro*-grown *O. stamineus*. Nodal explants (1 cm long) were cultured on MS plates supplemented with 1 – 4 mg L^{-1} BAP. All cultures were incubated and subcultured fortnightly for 6 weeks. Antioxidant activities were determined using FRAP and DPPH assays. Results demonstrated that nodal explants treated with 4 mg L^{-1} BAP produced the highest shoot number (15.80 ± 0.76) and shoot length 6.63 ± 2.32 cm). As for antioxidant activities, the highest FRAP value (7200.00 ± 103.02 µM Fe (II)) and lower EC_{50} (56.65 ± 0.17 µgmL^{-1}) were obtained for 4 mgL-1 BAP as compared to other treatments. In conclusion, PGR did enhance antioxidant activities in *vitro O. stamineus* shoots.

Keywords: *Orthosiphon stamineus* shoot; Regeneration; Plant growth regulators; FRAP and DPPH

1.0 INTRODUCTION

Orthosiphon stamineus is commonly found in China and Eastern Tropical Asia. *O. stamineus* is locally known as 'misai kucing' in Malaysia. It has two varieties: purple-flowered plant and white-flowered plant. The tea extract can treat kidney stones and uncomplicated urinary tract infections (UTI) [1]. Many companies have been producing tea that is based on *O. stamineus*. The benefits may come from the antioxidant compounds such as diterpenes, flavonoids and phenolic acids. Different flower colours are influenced phenolic contents. For instance, the flavonoids content in red flowers of *Fagopyrum tataricum* (L.) Gaertn was higher than the green-flowered plant, *F. esculentum* Moench [2].

The presence of phenolic compounds such as danshensu, caffeic acid, rosmarinic acid, sinensetin and eupatorin in *O. stamineus* has contributed to the antioxidant potential of this species. Antioxidant compounds can neutralize radical compounds as they hold H^+ in the hydroxyl group. The biomass of field-derived *O. stamineus* is usually produced by stem cuttings. However, Yaacob *et al*. [3] reported that the conventional propagation would not provide sufficient supply for large market demand. Plant tissue culture and plant growth regulators (PGR) serve as an alternative strategy to enhance the production of *O. stamineus* biomass. Some articles reported that PGR affected both biomass and antioxidant content such as flavonoid was found to be the highest in callus of *Ipomoea batatas* when 2 mg/L of kinetin (Kin) was applied [4]. Flavonoids and phenolic acids were also detected in the callus when a leaf was treated with Kin [5]. In this study, the effect of BAP of *O. stamineus* on the antioxidant activity and shoot biomass of *O. stamineus* was investigated.

2.0 MATERIALS AND METHODS

O. stamineus plants were grown and maintained at a greenhouse, Faculty of Science, UTM. *In vitro* plants were maintained in a tissue culture room at 25°C for a 16-hour photoperiod and under 1000 lux of light intensity. The six-week-old *in vitro* plants were used as starting materials. Nodal segments of field-grown *O. stamineus* were surface-sterilized at 70 % (v/v) ethanol for 30 seconds, agitated for 45 minutes in 30 % (v/v) Clorox + Tween 20. All explants were then rinsed six times with sterilized distilled water. For shoot induction, nodal segments (1 cm) from six-week-old *in vitro* plants were cultured on Murashige and Skoog (MS) plates supplemented with different BAP concentrations (1.0, 2.0, 3.0, and 4.0 mg L^{-1}). Each treatment was conducted in five replicates. All cultures were incubated at growth conditions (25±2°C; 16-h photoperiod; the light intensity of 1000 lux). Data were collected after six weeks culture.

For sample extraction preparation, plant samples were washed several times with deionized water to remove dust and dried in the oven at 60°C. Dried samples were weighed every hour until a constant weight was achieved. The samples were ground into a powder and then mixed with deionized water in the ratio of 1:50 (w/v). The mixture was poured into a Falcon tube and centrifuged at 6000 rpm for 20 minutes to remove the debris. The mixture was filtered using Whatman filter paper. The filtrate was poured into a Falcon tube to be freeze-dried in a freeze dryer for 24 h. The filtrate was then diluted to 2 mg mL^{-1} with deionized water to be used for further analyses [1].

For antioxidant assays, a DPPH assay was conducted as described by Ojha *et al*. [1]. A 100 µL of ethanol absolute was pipetted into a 96-well microplate. 100 µL of the 2mg/mL sample was added into column 2 before serial dilution was made from column 2 to column 10. For every transfer from one column to the next, 100 µL was taken until the last 100 µL was pipetted out. 100 µL of 0.04% DPPH was added into row A-E, from column 2 to column 12. Absorbance was taken at 517 nm [1]. The FRAP assay was conducted as described by Ojha *et al*. [1]. Dilution for 2 mg mL^{-1} sample was made by adding 900 µL of deionized water to 100 µL of the sample. 100 µL of the diluted sample was put into each column of a 96-well microplate before 200 µL of FRAP reagent was added. Absorbance was taken at 593 nm. Fresh cultures of *O. stamineus* were harvested and weighed. Then, the plant samples were placed on a Petri dish that was labeled and dried in the oven at 40°C for 48h. After that, the dried culture was subjected to two assays; total phenolic content (TPC), total flavonoid content (TFC), DPPH and FRAP assays.

The data were statistically analysed using IBM SPSS Statistics 20. For the DPPH assay, EC_{50} values were carried out using one-way ANOVA, using post-hoc tests; Dunnett and Tukey HSD. Dunnett's test was used to compare each of the treated samples with L-ascorbic acid that acts as a single control, where the values of other groups should be less than the control value.

3.0 RESULTS AND DISCUSSION

All nodal segments from six-week-old of *in vitro O. stamineus* plants were treated with different BAP concentrations (1 – 4 mg L^{-1}). BAP is a cytokinin that was used to induce and proliferate shoots from the nodal segments as well as to increase *O. stamineus* shoot biomass. The morphology of BAP treated leaves and stems was small, smooth, and green in colour. As BAP concentration increased from 1 – 4 mg L^{-1}, shoot morphology changed from green to yellowish green and less bushy. Nodal segments treated with 1 mg L^{-1} BAP and 2 mg L^{-1} BAP produced larger true leaves and leaf sizes than other BAP treatments. The size of the leaves became smaller and tapered when treated with 3 mg L^{-1} and 4 mg L^{-1}. The small and tapered shoots could be due to vitrification which was a result of increasing cytokinin concentrations [6].

Figure 1 shows the induction of *O. stamineus* shoots from the nodal segments treated with 1-4 mg L^{-1} BAP. The control shoots had the darkest green shoots and grew loosely to each other as compared to

the BAP-treated cultures. Contrarily, shoots cultured at 1 mg L^{-1} BAP were bushy than the control. The serrate leaf margin in 1 mg L^{-1} BAP also appeared more significant than the control culture which had an even margin. The 2 mg L^{-1} BAP treatment produced light green and yellowish-green leaves. As BAP concentration increased, the green colour became lighter. For 3 and 4 mg L^{-1} BAP, the leaf appeared as needle-like morphology and grew very close to each other. As the concentration of BAP increased, the leaves' size became smaller and less bushy. The shoots became more translucent, shortened and brittle. This phenomenon is known as hyperhydricity when the water uptake by the plant culture is very high. Hyperhydricity in the shoot culture led to fresh weight inconsistency.

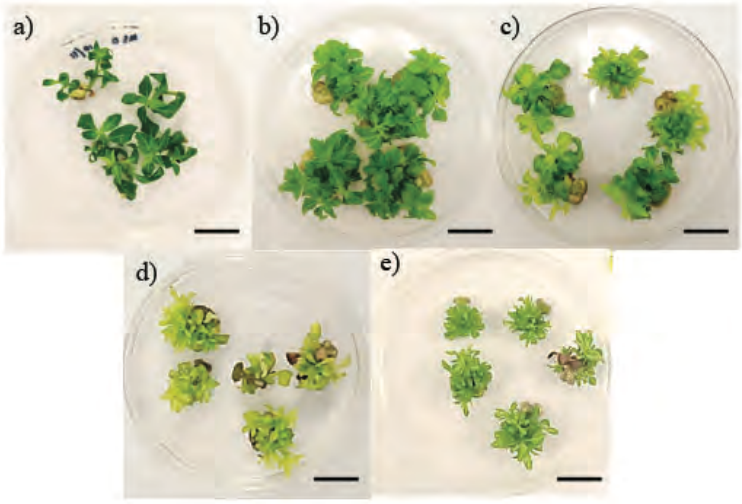

Figure 1: Shoots regenerated from nodal segments of *O. stamineus* a) control, b) 1 mg L^{-1} BAP, c) 2 mg L^{-1} BAP, d) 3 mg L^{-1} BAP, e) 4 mg L^{-1} BAP. Scale bars: 1cm = 2.25 cm.

Table 1 shows the effect of BAP (1-4 mg L^{-1}) on the number of shoots, percentage of shoot induction (%), shoot length (cm) and fresh weight (g). The increment of BAP concentrations from 0 to 4 mgL^{-1} BAP resulted in an increasing number of shoots. The highest number of shoots (15.80 ± 0.76) was achieved when the explants treated with 4 mg L^{-1} BAP, as compared to the control (2.24 ± 0.19). A report by Kousalya and Narmatha Bai [4] revealed the optimum concentration of BAP on *Canscora decussata* was 2 mg L^{-1} with several shoots produced of 30.20 ± 6.53. However, when BAP concentration was increased to 3 mg L^{-1}, the number of shoots dropped significantly to 19.80 ± 6.45. A 1 mg L^{-1} BAP produced a higher shoot length (6.70 ± 0.09 cm) than the control (2.70 ± 0.35 cm). However, there was no significant difference in shoot length above 1 mg L^{-1} BAP. An experiment conducted by Ashraf *et al*. [7] also supported that the shoot length and number of shoots were not proportional to the increment of cytokinin concentrations. For shoot fresh weight, the optimum was achieved at 3 mg L^{-1} BAP (0.64 ± 0.20 g) and the lowest was produced by the control.

Table 1: Effect of BAP on shoot regeneration of *O. stamineus*

BAP Concentration (mg L^{-1})	No. of shoots produced per explant	Percentage of shoot induction (%)	Shoot length (cm)	Shoot Fresh weight (g)
0 (Control)	2.24 ± 0.19^{a}	100^{a}	2.70 ± 0.35^{a}	0.17 ± 0.02^{a}
1	6.40 ± 0.07^{b}	100^{a}	6.70 ± 0.09^{b}	0.41 ± 0.03^{c}
2	10.2 ± 1.73^{b}	100^{a}	5.40 ± 0.38^{b}	0.38 ± 0.03^{c}
3	$13.32 \pm 1.04^{b,c}$	100^{a}	5.51 ± 0.39^{b}	0.64 ± 0.20^{d}
4	$15.80 \pm 0.76^{b,c}$	100^{a}	$6.63 \pm 2.32^{b,c}$	0.31 ± 0.02^{c}

Data were expressed as mean ± standard error mean (SEM) of analysis (N=15). Different letters (a,b,c,d) shown significant level between means at *p<0.05. The same letter indicates no significant difference between the means (p>0.05). The data analyzed is from a one-ANOVA sample test; post-Hoc test (Bonferroni).

DPPH assay measures the ability of antioxidant molecules in quenching DPPH molecules, which acts as free radicals that change the colour of DPPH from deep purple to yellow (DPPH-H). The antioxidant activity of both field-grown and *in vitro* culture extract was evaluated. Figure 2 and Table 2 show EC_{50} values of field-grown and BAP treated *O. stamineus* with ascorbic acid as the standard compound.

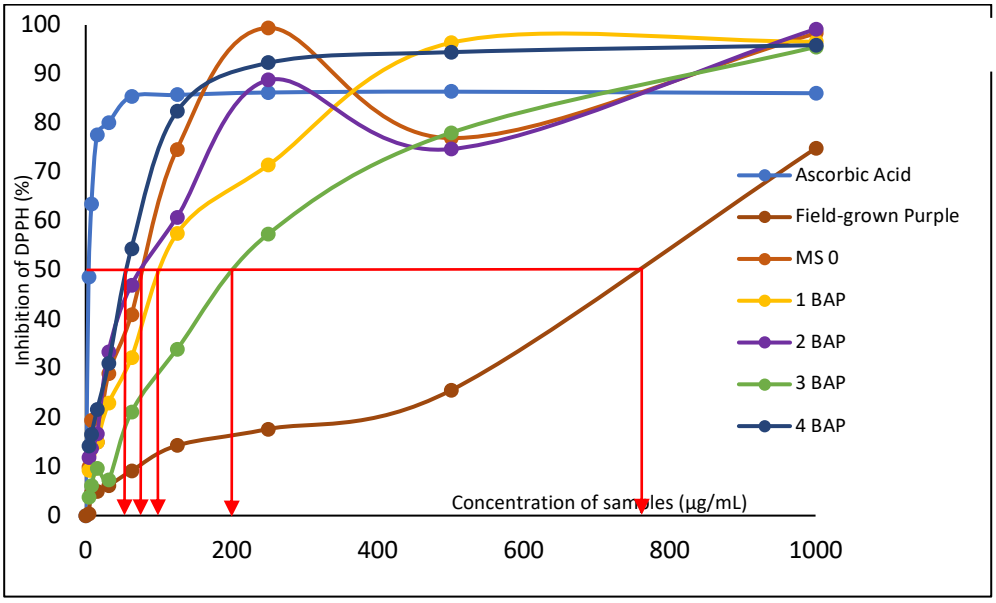

Figure 2: A comparison of inhibition of DPPH (%) between field-grown and *in vitro* of *O. stamineus* (1 – 4 mg L^{-1} BAP). (Red arrow represents EC_{50} values for all samples)

EC_{50} is an effective concentration of any sample for 50% inhibition of DPPH by hydrogen ions from antioxidants. Figure 2 indicates *in vitro* shoot biomass had higher antioxidant activity than field-grown plants at 100% inhibition of DPPH. According to Kedare and Singh [8], antioxidant compounds were soluble in water and fat. In addition, insoluble ones are bound to the cell wall of the plant extract. Hence, they may not be free to react with DPPH and completion of the reaction will not reach in a reasonable time. Therefore, instead of 100% reaction, the activity of 50% scavenging activity is chosen for measuring the antioxidant potential. Both 2 BAP and the control showed a decrease in inhibition of DPPH (%) at 500 µg mL^{-1}. However, the activity increased at 1000 µg mL^{-1} upon reaching 100% reaction. The side-chain functional groups in phenolic compounds gave their antioxidant property. Varying conformations of an antioxidant compound result in different stability to scavenge DPPH molecules due to the variety of interactions between the side chain and the OH phenolic group. This could explain the discrepancies in MS 0 and 2 mg L^{-1} BAP treatments. The exact EC_{50} values of field-

grown and BAP-treated shoot extracts, including ascorbic acid (standard compound) were tabulated in Table 2.

Table 2: One-ANOVA sample test; comparison of DPPH against samples of control, field-grown of white and purple varieties of *O. stamineus* and *in vitro O. stamineus* (control, 1 mg L^{-1} BAP – 4 mg L^{-1} BAP)

Sample	DPPH (EC_{50}) (µg/ mL)
L-Ascorbic acid	2.97 ± 1.50#
Field-grown extract	751.00 ± 20.50a
MS 0	69.33 ± 7.31c
1 mg L^{-1} BAP	107.13 ± 3.96b
2 mg L^{-1} BAP	76.33 ± 2.67c
3 mg L^{-1} BAP	211.70 ± 3.37b
4 mg L^{-1} BAP	56.65 ± 0.17c

Dunnett t-tests (<control)# treat one group as a control and compare all other groups against it at $p<0.05$ significance level. Tukey-HSD comparison subset group between all groups, different alphabets (a, b, c) indicate significantly different at $p < 0.05$ level. Same alphabet indicates non-significant different ($p > 0.05$).

High antioxidant activity is determined by the low value of EC_{50}. Overall, the EC_{50} of BAP-treated samples had significantly lower than the field-grown sample. L-ascorbic acid, a standard compound, had the lowest value of EC_{50}. At 50% reaction, increasing BAP treated culture did not simultaneously increase the antioxidant activity. Moreover, ascorbic acid is a good reducing agent and is usually involved in both metabolic and repair processes. This could be suggested that plant tissue culture influenced the antioxidant potential of the plant extract greatly. However, there might be an optimal EC_{50} value if the concentration was increased over 4 mg L^{-1} BAP.

FRAP assay is another test to determine the antioxidant potential of field-grown and *in vitro* shoot biomass of purple *O. stamineus*. This assay reduces Fe^{3+} - TPTZ complex to Fe^{2+} - TPTZ complex, turning the colourless solution into blue. The FRAP values of the purple-flowered *O. stamineus* plant cultivated in the field and BAP treated samples were shown in Table 3. Table 3 shows the FRAP values of the extract from field-grown and *in vitro* shoot of *O. stamineus* (purple variety) determined at 593 nm. The Field-cultivated sample had the lowest FRAP value, which was 1733.33 ± 101.98 µM Fe (II)/g. In contrast, BAP treated samples showed higher FRAP values compared to the field-cultivated sample and the control, free-BAP medium. The greatest FRAP value was found in 4 mg L^{-1} BAP-treated sample, which was 7200.00 ± 103.02 M Fe (II)/g. However, both field-grown and BAP-treated samples revealed exceptionally strong reduction power. According to Abdullah *et al*. [9], a Fe (II)/g concentration of more than 625 M is regarded as exceptionally high. The FRAP values gradually increased as BAP concentrations increased. Shoot biomass declined when 3 mg L^{-1} BAP was applied.

The use of elicitors i.e., BAP had a positive effect on the antioxidant potentials of shoot biomass from nodal segments of *O. stamineus*. When compared to the field-grown sample, FRAP values of the *in vitro* shoot biomass were higher. When the explants were treated with 4 mg L^{-1} BAP, the FRAP values increased dramatically. The higher the FRAP values, the more intense the blue coloured complex, thus a higher concentration of antioxidants caused Fe^{3+} to be reduced to Fe^{2+}.

Table 3: FRAP values for field-grown and BAP treated *in vitro* shoots of purple variety

Sample	FRAP µM Fe (II)/g
Field-grown extract	1733.33 ± 101.98[a]
MS 0	3019.05 ± 109.83[b]
1 mg L^{-1} BAP	4085.71 ± 75.59[c]
2 mg L^{-1} BAP	4742.86 ± 103.02[d]
3 mg L^{-1} BAP	4447.62 ± 98.80[c,d]
4 mg L^{-1} BAP	7200.00 ± 103.02[e]

One-ANOVA sample test; post-Hoc test (Bonferroni) in comparison of FRAP against samples of field-grown of purple variety of *O. stamineus* and *in vitro O. stamineus* (MS0, 1 – 4 mg L^{-1} BAP). Data were expressed as mean ± standard error mean (SEM) of analysis (N=15). Different letters (a,b,c,d) shown significant level between means at *p<0.05. The same letter indicates no significant difference between the means (p>0.05).

4.0 CONCLUSION

Of 1–4 mg L^{-1} BAP, 4 mg L^{-1} showed the highest number of shoots and the lowest value of fresh weight (g). All BAP concentrations attained 100% shoot induction. As for antioxidant assays, 4 mg L^{-1} BAP-treated shoot extracts obtained the strongest DPPH value (the lowest EC_{50} value) and the highest FRAP activity. As overall, BAP treated shoot extracts also showed better antioxidant activity than the field-cultivated samples. In conclusion, BAP did improve shoot biomass and the antioxidant activities of *O. stamineus*.

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Progressive Algal Biotechnology: A Sustainable and Viable Approach towards Bioeconomy

Imran Ahmad*, Norhayati Abdullah, Sasmitha Aulia Zahra, Laila Dina Amalia Purba, Thilagavathi Arumugham, Iwamoto Koji, Ali Yuzir, Shaza Eva Mohamad

Malaysia Japan International Institute of Technology, Universiti Teknologi Malaysia, Jalan Sultan Yahya Petra, 54100, Kuala Lumpur, Malaysia.

*mustafwibinqamar@gmail.com

ABSTRACT

Microalgae species are the potential source to produce protein, carbohydrates, lipids, pigments as well as other metabolites that are currently obtained from conventional agriculture which is having many limitations in terms of sustainability. Algae are the emerging area of study and due to continuous and progressive research they are expected to become bio-factories for the sustainable production of myriad products, especially biofuels as future energy sources. This paper will summarize different technologies involved in the biorefinery concept of microalgae from cultivation to valorisation to obtain various biofuels and other myriad bioproducts. The overall objective of the technological advancement in algal research is to tap its hidden potential in an economically viable and environmentally sustainable manner. To overcome the bottlenecks in algal cultivation and harvesting aspects researchers are going for the bioeconomy approach, so that the upstream cost issues can be compensated by downstream production. The paper encompasses the biorefinery concept of microalgae, summarizing the production of biofuels and bioproducts. Furthermore, it provides an insight to the contribution to bioeconomy. Algal biotechnology has an enormous potential for clean and green future. While algae biotechnology can be further improved to make it commercial. We expect a never-ending road for algal research ahead.

Keywords: microalgae; cultivation; biofuels; bioproducts; CO_2 sequestration

1.0 INTRODUCTION

Rapid urbanization, industrialization together with excessive energy demand and reliance on the fossil fuels have caused detrimental impacts on the environmental sustainability and wellbeing of human society. This led to the advent of algae that has the advantage to utilize light, fix atmospheric CO2 and produce adenosine triphosphate (ATP), termed as energy currency of life during their growth [1]. Algae can grow in varying environments (pH, salinity, temperature, light etc.) and have better photosynthetic efficiency to generate higher quantity and quality of biomass. They can be grown in non-arable land thus solving the food-fuel duel and their water footprint is also less as compared to other crops. The efficacy of microalgae in food and fuel is well accepted by the researchers [2]. The features and advantages of microalgae are summarized in Table 1. The compositional analysis and applications of some of the prominent microalgae species are listed in Table 2.

Table 1: Salient features and advantages of microalgae

Salient Features	Advantages
Microalgae or microphyte (3-10μm)	Eukaryotic, unicellular, & photosynthetic organisms[3]
Versatile metabolism	Autotrophic, heterotrophic & mixotrophic growth
Growth in varying environment and higher tolerance & adaptability	Can be grown in different environments (pH, temperature, salinity, etc)
Higher growth rate	Fast growth as compared to other plant species & less area is required
Can be grown throughout the year	Can be grown in open systems and closed photobioreactors (PBRs) [4]
Higher CO2 resilience & absorption	Increased potential for CO_2 sequestration
Potential for nutrient uptake & pollutant degradation	Assimilates N & P as nutrients, can be grown in wastewater and flue gases having high SO_X, CO_2, and NO_X [5]
larger cell size and filamentous morphology	Lowering the cost of harvesting and processing
High content product	Enriched Biomass that can be biotechnologically processed to obtain various biofuels & different myriad products [6, 7]

Table 2: Composition & Applications of some significant Microalgae species [8]

Microalgae Species	Carbohydrate (% dry matter)	Protein (% dry matter)	Lipids (% dry matter)	Products obtained	Applications
Spirulina platensis	8-14	46-63	4-9	Phycocyanin, biomass	Cosmetics & health supplement
Chlorella vulgaris	12-17	51-58	14-22	Biomass, Carbohydrate extract	Health food, animal feed food supplement,
Dunaliella salina	32	57	6	Carotenoids, β - carotene	Health food, food supplement, feeds
Haematococcus pluvialis	27	48	15	Carotenoids, astaxanthin	Pharmaceuticals, Health food, feeds
Nannochloropsis oculata	15	63	11	Biomass	Food for juvenile marine fish and larval
Scennedesmas spp.	21-52	47	1-9	Protein	Human nutrition, Aquaculture

2.0 ALGAL BIOREFINERY CONTRIBUTING TO BIOECONOMY

The biorefinery concept includes the objective of attaining biofuels and other biocompounds from the cultivated biomass. The generation of biofuels and value-added compounds in bulk can contribute the overall process economically viable [9]. The production of various biofuels and bioproducts after the harvesting of cultivated algal biomass is shown as a flow diagram in Figure 1.

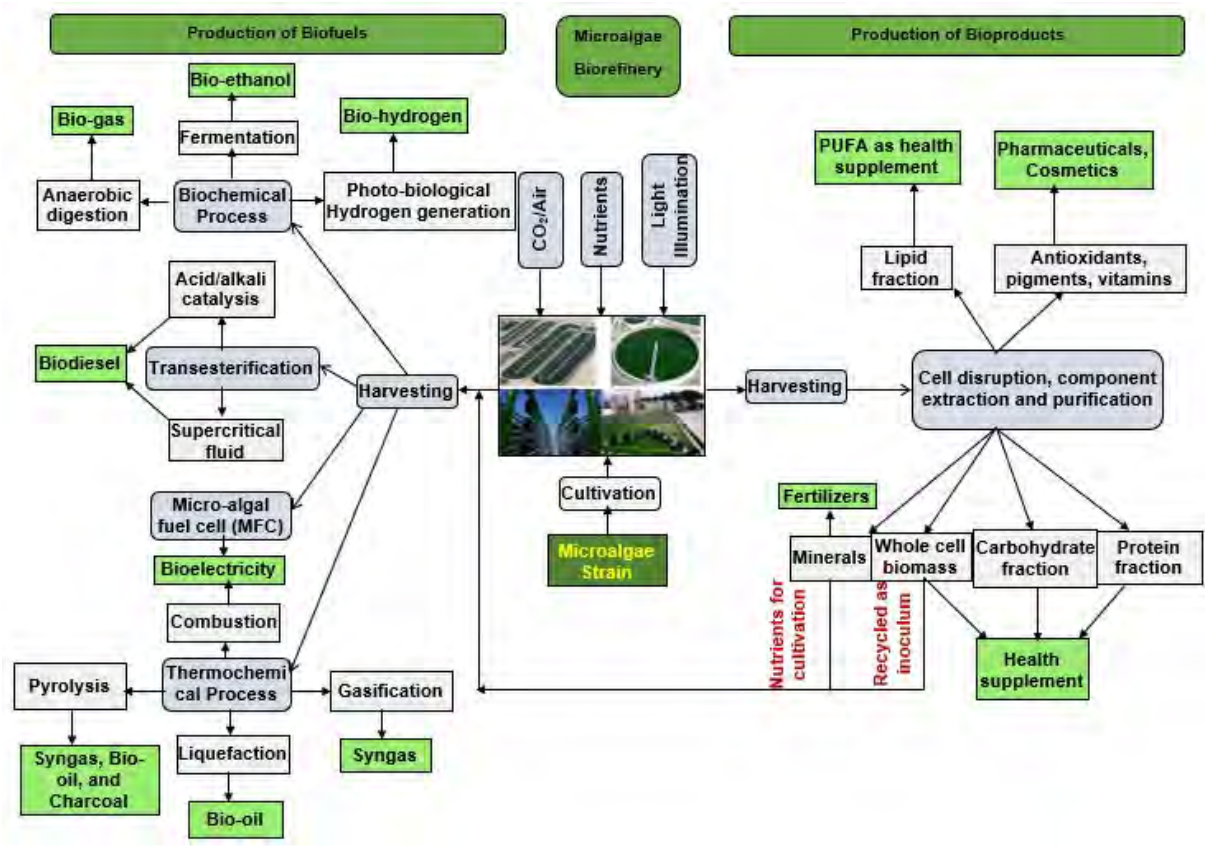

Figure 1: Biorefinery concept in microalgae cultivation to valorisation

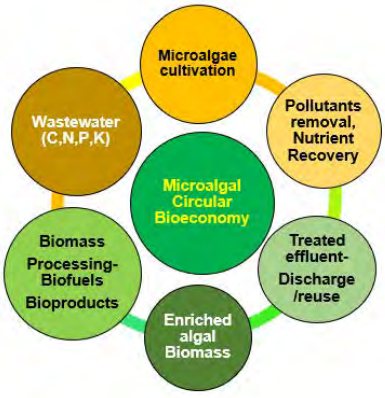

Figure 2: Concept of Circular Bioeconomy

Circular economy is the optimized utilization of resources in a close loop, thus opposing the linear way of utilization in petroleum-based economy. Microalgae based circular bioeconomy instigates the complete utilization of cultivated biomass by establishing a balance between sustainability and ecology [10]. The most dominant example is the bioremediation of wastewater using microalgae as it serves two purposes, i.e., (i) nutrient uptake & pollutant removal, and (ii) production of enriched biomass. The closed loop concept of microalgae-based bioremediation of wastewater is shown in Figure 2.

3.0 CONCLUSION

Microalgal biomass production and its capability to accumulate biomolecules make it a sustainable and environmentally friendly alternative to the depleting and polluting petroleum-based processes/products. The carbohydrates and lipids derived from microalgae are subsequently utilized to produce various biofuels (i.e., biodiesel, bioethanol, biogas, and biohydrogen, etc), as well as minerals, proteins, and pigments can be processed to attain pharmaceuticals, health supplements, fertilizers, and cosmetics etc. The microalgae-based bioremediation of wastewaters contributes to dual mode, firstly in the uptake of nutrients and degradation of pollutants and secondly in the production of enriched biomass. Progressive microalgal biotechnology is believed to endure the future issues of food-fuel and energy-water nexus, but the bottleneck lies in the high upstream and downstream cost. The economic viability can be achieved by effectively implementing the biorefinery concept (Figure 1).

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Microbial Community of Two Faecal Contaminant Sources: Sewage Treatment Plant Versus Goat Farm

Kayverne a/p Santhanasamy*, Saleha Shahar, Tan Zen Dong

Department of Biosciences, Faculty Science, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia.

*ksanthanasamy@gmail.com

ABSTRACT

Faecal contamination is a threat to water sources. Although faecal indicator bacteria are useful for monitoring faecal contamination, it could not differentiate the source of contaminants as human or animal due to its ubiquitous presence in the gut of all mammals. Microbes unique to each contaminant source would be better for tracking. This study aimed to identify these unique microbes. Dischargers from the sewage treatment plant and goat farm were subjected to 16S rRNA gene amplification of its V3-V4 hypervariable region, sequenced, and taxonomic assignment against the 16S rRNA database (SILVA release 132). Findings from the 16S rRNA sequencing showed that the microbial communities from both discharges featured the classic gut core phyla Firmicutes/ Bacteroidetes/ Proteobacteria, although at different portions. Goat farm discharge, but not sewage treatment plant, was best represented by gut bacteria, the sample's most abundant genera. On the other hand, human pathogens were the concern of effluent from the sewage treatment plant. In conclusion, the two faecal contaminant sources have different makeup to their microbial communities and with the possibility of differentiating both sources by their most abundant member.

Keywords: Sewage; Humans; Goat farm; Metagenome; effluent

1.0 INTRODUCTION

Maintaining clean and safe water sources is pertinent for sustainable livelihood and wellbeing. A challenge to this effort is to prevent contaminants from entering the water systems from one of the two entries, point sources or non-point sources. Point sources release contaminants from discrete carriage lines or drainage, whereas contaminants from different sources such as surface run-offs from land-use may accumulate and diffuse into the water system, thus referred to as non-point sources. Locally, point sources are diverse, with sewage treatment plant discharges contributing to a significant portion of all point source contaminations [1,2].

Whether for drinking or recreation, existing water quality standards and regulations were designed to respond to faecal-oral transmitted health issues. Faecal contamination is primarily measured by detecting and quantifying bacterial indicators known as faecal indicator bacteria (FIB). The FIB, represented mainly by enteric bacteria (Family *Enterobacteriaceae*), fit the purpose to indicate the presence of faecal materials [3]. However, FIB is widespread in the gut of every mammal; thus, it was unable to discern the origins of faecal contamination, either humans or otherwise, such as agricultural animals [4].

Metagenomic sequencing enabled a deeper and better resolution of microbial taxonomic characterisation, which traditional microbiological techniques could not provide. It was also considered an emerging tool, and along with the conventional methods, could make for a robust water quality monitoring package [5]. However, metagenomic studies of microbial communities in sewage treatment plants have focused on the system's efficiency in removing pathogenic bacteria, the source of antibiotic

resistance persistence, and the microbial environment responsible for the system's operation [6]. Meanwhile, studies of faecal or gut metagenome from agricultural animals focus on community changes throughout animal growth to understand the makeup of the gut microbiome and its contribution to animal health for better agricultural production [7]. None so far had compared STP and animal gut microbial communities as faecal contaminants representing humans versus animals' origin. Comparing faecal microbial communities from the perspective of water contaminants would give insight for tracking faecal contaminants to their sources.

In this study, we aimed to address two issues, (1) whether the microbial communities (microbiota) in sewage treatment plant (STP) and goat farm (GF) discharges vary; and 2) whether their abundant species could differentially identify the two communities. Therefore, we apply 16S rRNA gene sequencing on discharges from the two sources and carry out taxonomic profiling of their microbial communities.

2.0 MATERIALS AND METHODS

2.1 Materials

Sample representing faecal contaminants of human origin were taken from a mechanical sewage treatment plant (STP) which receive influents from three residential colleges inside Universiti Teknologi Malaysia (UTM). Sample representing faecal contaminants of small ruminant origin was collected from a commercial goat farm (GF) discharge. The 1-acre farm housed multiple breeds of small ruminants, 'British Alpine', 'Anglo Nubian, 'Saneen', 'Jamnapari' and 'Farel' goats, and sheep in freestall barn. 250 ml samples were collected from sampling sites into screw cap containers. The sampling sites were the final exit point of discharge before it reaches water surfaces - the clarifier chamber for STP and open drainage for GF. The STP sample was clear with brown-green sedimentation, whereas the GF sample was brown and contained sedimentation, including faecal matter. All samples were transferred on ice to the Virus Research Lab (T02) UTM, Skudai. Samples were filtered and extracted immediately on the day of sampling.

2.2 Methods

2.2.1 Genomic DNA extraction

Twenty-five ml sample was diluted with sterile water (1:1 ratio) and vortexed to release microbes attached to the particles into the water. The sample was then filtered through 125 mm Whatmann filter paper (Gred 541, 22 µm). The filtrate was centrifuged at 8,200 xg for 15 mins (Eppendorf, Model 5424). Pellet yielded from the centrifugation was subjected to genomic DNA extraction using the phenol/ chloroform DNA extraction protocol previously described by Tabatabaei (2010) [8]. Extracted DNA was resuspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and subjected to 1% TAE agarose-gel electrophoresis for visual inspection for any signs of degradation. DNA concentration was determined by spectrophotometer at A260/A280 (Implen NanoPhotometer N60/N50) with a reading between 1.8 to 2.0 for gDNA samples as acceptably free of contamination.

2.2.2 16S RNA gene sequencing and taxonomic assignments

The 16S rRNA V3-V4 hypervariable region of both samples were amplified by PCR using the V3 forward primer (5'- CCT ACG GGN GGC WGC AG) and V4 reverse primer (5'- GAC TAC HVG GGT ATC TAA TCC). Successfully amplified samples were processed for library preparation, then normalised to a final concentration of 4 nM using 10 mM Tris (pH 8.5) for even reading between samples. Libraries were then subjected to Illumina MiSeq sequencing with 75,000 read depth. Subsequently, sequence reads were cleaned from adaptors and low-quality reads, and merged using USEARCH v11.0.667. Reads were then aligned with 16S rRNA (SILVA Release 132) and inspected for chimeric errors using VSEARCH v2.6.2. Reads that passed these quality assessment steps were clustered *de novo* into

Operational Taxonomic Units (OTUs) at 97% similarity using UPARSE v11.0.667. A single representative sequence from each OTU was then randomly chosen and aligned using PyNAST to construct a phylogenetic tree against SILVA 132 16S rRNA database. The QIIME V1.9.1 platform was used to taxonomically assign each OTU against the Silva 132 16S rRNA database

3.0 RESULTS AND DISCUSSION

3.1 Quality of reads

Genomic DNA from sources representing faecal contaminants of human and goat farm origin were successfully extracted from the discharges of STP and GF, respectively. Sequencing generated 160,133 reads and Phred Mean Quality Score above 20%. After removing single reads (singletons) and sequences less than 150 bp and more than 300 bp, de novo clustering yielded 873 OTUs of which 34 OTUs were found in both GF and STP (Figure 1). Finally, a total of 426 genera affiliated with 30 phyla were identified from both sources.

Figure 1: OTUs identified in the samples were delegated either as exclusive to each sample or shared in both

3.2 Microbial diversity of sources

Alpha diversity analysis (Table 1) on rarefied sequence library by Shannon and Fisher indexes indicate both sources were different in richness (number of individuals) and diversity (identity of individuals). STP with indexes higher than GF indicates a richer and diverse community than the latter. Although GF was estimated to have more rare taxa in its community based on a higher Chao1 index than the observed reads. STP was shown to have a slightly different Simpsons index (0.976) compared to GF (0.987), whereby an index number closer to 1.0 indicates an even community. The higher index reflects uneven species distribution in STP, suggesting at least one OTU that dominated the rest of the community.

Table 1: Alpha diversity of microbial community in STP versus GF

	Observed	Chao1	Simpsons	Shannon	Fisher
STP	553	553	0.976	5.26	81.44
GF	345	352	0.987	4.96	47.01

3.3 Genus unique to sources

Focus was given to identify abundant members making up more than 1% of the total microbial community in each source (Figure 2). Seventeen abundant genera identified in GF have an abundance

range from 1.2% to 7.9%. All identified genera in this category are common mammalian gut bacteria - four of which, Rikenellaceae RC9 gut group, Prevotellaceae UCG-003, Ruminococcus 1, and Fibrobacter are associated with ruminants [9]. Nine identified genera in STP have an abundance range between 1.1% to 1.6%. Unlike GF, most of the abundant genera in STP were linked with the environment; however, two – Mycobacterium spp. and Haematospirillum spp. are human health concerns [10].

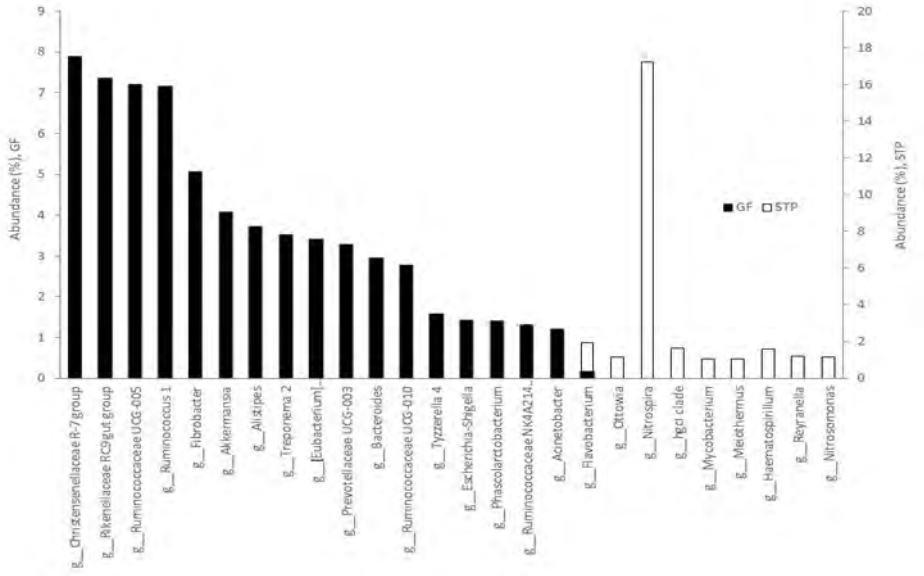

Figure 2: Abundant genera identified in each source. Take note of the different y-axis scale.

4.0 CONCLUSION

This study showed that STP and GF discharges can be differentiated by their microbial communities and that identifying abundant species unique to each source was possible. While this is encouraging to finding source-specific bacterial indicators, it is not known for now whether the unique abundant species identified here would withstand the pressure from changing rainy and dry seasons. Ultimately, the influence of local seasonal change will be the focus of our future work.

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Synergistic Effect of Antibiotic Agents Against *Cupriavidus* Species Bacteria

Yung Yung Fua, Kar Yern Kam, Nor Azimah Mohd Zain*

Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.

*norazimah@utm.my

ABSTRACT

Antibiotics are commonly prescribed in the medical setting to fight against bacterial infection. However, the rising of antibiotic resistant bacteria has caused a severe consequence includes a high mortality rate in the population. *Cupriavidus metallidurans* (*C. metallidurans*), a pathogen that was known to cause a wide range of diseases was isolated from the environment setting in Johor, Malaysia is the main focus of this study. Antibiotic combination therapy is proposed to tackle the issue of multiple antibiotic-resistant (MAR) bacteria and prevent virulence of MAR bacteria in the future. The bacteria were isolated from the river (P3W1), and fish species, *Lates calcarifer* (G2) respectively. They were subjected to antibiotic susceptibility test by broth microdilution method for determining minimum inhibitory concentration (MIC) towards seven antibiotics namely, ampicillin, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, sulfafurazole and tetracycline. The antibiotic activity was assessed by minimal bactericidal concentration (MBC) later. Double antibiotic combinations were carried out to determine synergistic effects and further validated with time-kill assay. P3W1 was susceptible to all the antibiotics tested while G2 was resistant to tetracycline, intermediate to ciprofloxacin and susceptible to other antibiotics. All the antibiotics exhibited bacteriostatic towards P3W1. Chloramphenicol, gentamicin and sulfafurazole exhibited bacteriostatic towards G2 while the other antibiotics act bactericidal. The double antibiotic combination between ciprofloxacin and tetracycline exhibited a synergistic effect on G2 with fractional inhibitory concentration index of 0.250. The synergistic effect of ciprofloxacin and tetracycline was validated in time-kill assay. In-vivo testing could be carried out in order to provide stronger evidence on the antibiotic combination effect. As the MAR issue is growing and creates health concerns towards the public, this study provided insight into the synergy effect of combination drugs, to tackle multidrug-resistant bacteria for shorter treatment and hospitality duration and create awareness of MAR and provide a better understanding to the public.

Keywords: Antibiotic resistance bacteria; *Cupriavidus metallidurans*; Antibiotic combinations; Synergistic effect; Time kill assay

1.0 INTRODUCTION

Antibiotics are commonly prescribed in the medical setting to fight against bacterial infection, including pneumonia, foodborne diseases and urinary tract infections [1]. However, the emergence of antibiotic-resistant bacteria has become a problem worldwide as no less than 700,000 deaths are reported globally due to drug resistance illnesses. The death toll is expected to reach 10 million cases by the year 2050 if there is still no action taken as a more intensive treatment will be needed to treat the infections with longer hospital stays, higher medical costs and an increase in mortality rate [2].

Cupriavidus spp., the bacteria that are commonly found in environmental and human clinical sources, has gained its attention in the medical field where a number of different species that caused fatality cases has been isolated from immunocompromised or even non-immunocompromised patients [3, 4].

Since the common treatment of antibiotics course does not show impactful outcome on the bacteria, an empirical combination antibiotics therapy is proposed. Combined antibiotics therapy is favoured over the single use of antibiotics counting on its broader antibacterial spectrum, lower risk of developing antibiotics resistance bacteria and most importantly, combined antibiotics can achieve synergism effects [5]. Synergy is a well-defined concept in microbiology field where the use of paired or triple combination of inhibitory agents shows positive interaction and greater activity than each agent alone that inhibits the growth of targeted microorganisms [6].

In this study, we aim to provide insight of the unrevealed synergy effect of antibiotics on *C. metallidurans* isolated from river and aquaculture sources, to provide the best selection of antibiotics to treat the bacteria infection as to shorten the duration of treatments.

2.0 MATERIALS AND METHODS

2.1 Materials

Two isolates of *C. metallidurans* that have been previously characterized to be resistant to several antibiotics through disk diffusion method from fish (G2 isolate) and river (P3W1 isolate) source were cultured from the glycerol stock. The glycerol stock of the isolates that were stored at -80°C was scraped with a sterile loop to obtain frozen bacteria off the top. Later, it was streaked on MHA and incubated overnight at 37°C.

2.2 Methods

2.2.1 Determination of MIC and MBC

The *C. metallidurans* isolates were subjected to antibiotic susceptibility test by broth microdilution method for determining minimum inhibitory concentration (MIC) towards seven antibiotics namely, ampicillin, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, sulfafurazole and tetracycline, according to the Clinical and Laboratory Standards Institute (CLSI) standard [7]. Resazurin assay was used for indication of viable bacterial cells. The antibiotic activity was assessed by minimal bactericidal concentration (MBC) as described by [8].

2.2.2 Synergistic Double Antibiotic Combinations via Checkerboard Assay

Checkerboard assay as referred to the MICs of antibiotics was carried out to determine the synergistic effect of double antibiotic combination by accessing FICI. The assay was performed on 96-well plates by the two plates method as described by [9] with modification. The double antibiotic combinations for G2 were Tetracycline + Ciprofloxacin; Tetracycline + Rifampicin, while for the P3W1 were Tetracycline +Ciprofloxacin; Sulfafurazole + Rifampicin. To evaluate the combined effect of the antibiotics, FICIs values will be calculated based on Equation (1). The interpretation of FICI is as followed where synergy, FICI ≤ 0.5; indifference, 0.5 < FICI ≤ 4; and antagonism, FICI ≥ 4.0.

$$FICI = \frac{MIC\ A\ in\ combination}{MIC\ A\ Alone} + \frac{MIC\ B\ in\ combination}{MIC\ B\ Alone} \quad (1)$$

2.2.3 Time-kill Assay

Time-kill assays were performed only for the combination of double antibiotics that was found to be synergistic from the checkerboard assay. Synergism is said to be achieved when a decrease of at least 2 log10 in CFU/ml is observed in the colony treated by antibiotic combination compared to the most active single agent and the final bacterial count at 24h was 2 log10 lesser than the starting inoculum

3.0 RESULTS AND DISCUSSION

3.1 Antibiotic Susceptibility Profile

The susceptibility of the isolates towards each antibiotic were then determined by comparing MIC with breakpoints in CLSI M100. *C. metallidurans* of G2 isolate is sensitive to chloramphenicol, gentamicin and sulfafurazole while P3W1 isolate is sensitive towards all of the antibiotics tested includes chloramphenicol, ciprofloxacin, gentamicin, sulfafurazole and tetracycline. Through the determination of MBC, the activity of antibiotics can be accessed where MBC less than 4X MIC denotes a bactericidal antibiotic while MBC more than 4X MIC represents a bacteriostatic antibiotic. Table 1 showed the summary of the MIC, MBC and antibiotic activity of G2 and P3W1. Ampicillin, ciprofloxacin, rifampicin and tetracycline are bactericidal antibiotics while chloramphenicol, gentamicin and sulfafurazole are bacteriostatic antibiotics toward G2 isolate with growth observed at 4X MIC. On the other hand, all of the antibiotics tested were bacteriostatic towards P3W1 isolate with positive growth observed at all dilution plated up to 4X MIC.

Table 6: Summary Table on the antibiotic susceptibility profile of G2 and P3W1 Isolates

Antibiotic	G2			P3W1		
	MIC (µg/ml)	MBC (µg/ml)	Activity	MIC (µg/ml)	MBC (µg/ml)	Activity
Ampicillin	0.500	2.000	BC	64.000	> 256.000	BS
Chloramphenicol	2.000	> 8.000	BS	8.000	> 32.000	BS
Ciprofloxacin	2.000	2.000	BC	0.031	> 0.125	BS
Gentamicin	0.063	> 0.250	BS	0.500	> 2.000	BS
Rifampicin	2.000	8.000	BC	4.000	> 16.000	BS
Sulfafurazole	128.000	>512.000	BS	128.000	> 512.000	BS
Tetracycline	32.000	128.000	BC	2.000	> 8.000	BS

†BC: Bactericidal; BS: Bacteriostatic

3.2 Checkerboard Assay

Four double antibiotic combinations each was carried out to determine the synergism of antibiotic towards *C. metallidurans* of G2 and P3W1 isolate. The combinations of antibiotics for *C. metallidurans* G2 isolate is based on the antibiotic susceptibility profile where the combinations were done between resistant and intermediate response antibiotics for G2 isolate, while the antibiotic combination for P3W1 isolate was done with combinations that are commonly shown to be synergism in other gram-negative bacteria. From the result of the checkerboard assay, tetracycline and ciprofloxacin exhibited a significant synergistic effect on *C. metallidurans* of G2 strain with FICI of 0.250 at 2.000 µg/ml and 0.250 µg/ml, respectively (Figure 1). The MIC of G2 isolate towards tetracycline and ciprofloxacin reduced 8-fold from the MIC towards these antibiotics alone. This result corresponds to the finding of Sulieman (2008), where synergism was achieved between combination of tetracycline and ciprofloxacin on gram-negative bacteria particularly on *Klebsiella pneumoniae* and *Enterobacter* [10].

Blank	1	2	4	8	16	32	64			Growth Control	Sterility Control
1/16	0.094	0.156	0.281	0.531	1.031	2.031	4.031				
1/8	0.125	0.188	0.313	0.563	1.063	2.063	4.063				
1/4	0.188	0.250	0.375	0.625	1.125	2.125	4.125				
1/2	0.313	0.375	0.500	0.750	1.250	2.250	4.250				
1	0.563	0.625	0.750	1.000	1.500	2.500	4.500				
2	1.063	1.125	1.250	1.500	2.000	3.000	5.000				
4	2.063	2.125	2.250	2.500	3.000	4.000	6.000				

Figure 1: FICI value of G2 isolates on tetracycline and ciprofloxacin combination

3.3 Time-kill Assay

The combination effect of antibiotic combination 1, tetracycline and ciprofloxacin on *C. metallidurans* of G2 isolate was validated with time-kill assay method. The effect of synergism started to take place at 4 hours onwards with the viable cell number decreasing over time. About 4 log reductions in cell number can be observed when the colony treated by antibiotic combination is compared to the most active single agent alone of tetracycline and ciprofloxacin. Besides, a 2-log reduction was observed at 24h compared with the starting inoculum of antibiotic combination. The result obtained agreed with checkerboard assay result which suggested that combination of tetracycline and ciprofloxacin may be used to enhance antibacterial activities against *C. metallidurans*.

4.0 CONCLUSION

The *C. metallidurans* of G2 isolate exhibited a synergistic effect combination of tetracycline and ciprofloxacin through the checkerboard assay and time-kill assay. Investigation of such combination in-vivo could be done to further analyse the biological effects of the antibiotic This study would contribute to the biological data of synergy effect of antibiotic agents in the treatment of *C. metallidurans* infection to tackle multidrug-resistant bacteria for shorter treatment and hospitality duration

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Genetic Variation Analysis Between Fish Eggs and Fish Muscles of *Pimelodus maculatus* Species in Parana River Through Co1 Gene

Ain Najihah S., Ahmad L.W.Z, Kambol R., Syed Mohamad S.A., Farizan A., Nurul Aili Z. & Norfatimah M.Y.*

Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia

*norfatimah9778@uitm.edu.my

ABSTRACT

Pimelodus maculatus known as yellow catfish or mandi is a migratory fish and important for commercial fisheries. The construction of hydroelectric dams may disrupt the migratory behaviour of the fish in order to spawn which leading to reduction of population of the fish. Fish eggs have a difficulty on morphology observation since they contain similarity in terms of shape, size and colour in order to study the distribution/population of an organism. In order to identify the species, CO1 genetic marker were used to identify the genetic variation between fish egg species with muscles from different geographical areas, Brazil and Argentina, as well as, to study the population of the fish species. By using MEGA software, all sequences were aligned by using ClustalW to construct a phylogenetic tree. DAMBE software and ABGD tool were used to test substitution saturation and species delimitation. The theory of Yoshimi Sato stated that the *P. maculatus* undergoes short-distance migrants that contradict with the studies of da Silva which stated otherwise. Genetic distance range obtained shows less than 2% between eggs from Brazil with the muscles from Argentina, where there is probability, the fish undergo migration in order to spawn. However, the ABGD and phylogenetic tree shows a conflict for a few individuals from Brazil where there is probability that a few muscles from Brazil are not *P. maculatus* species. The findings could reveal the effectiveness of CO1 gene as a good genetic marker in order to identify the species of fish and discover the population of the fish.

Keywords*: Pimelodus maculatus*; Fish egg; Phylogenetic tree; Species delimitation

1.0 INTRODUCTION

Pimelodus maculatus belongs to the Pimelodidae family that is known as yellow catfish or mandi is one of the migratory species that is widely distributed in South-eastern Brazil especially Parana River and Sao Francisco River. It is also very important for commercial fisheries in Brazil [1] that are used for nutritional and recreational purposes [2]. However*, P. maculatus* is among the threatened habitat including their eggs due to the construction of hydroelectric dams leading to extinction of habitat [3]. Brazil has a hydroelectric potential that plays an important role in producing energy/electricity by using flowing water from the river [4]. Hydroelectric dams that are prone to predators and may interrupt the migratory behaviour can cause the reduction in population size [5]. Other than that, the fish eggs contain similar phenotypic characteristics such as colour, size and shape [6]. These characteristics bring difficulty to the scientist in order to study the distribution/population of an organism in particular areas. Therefore, CO1 barcoding gene approach is a very useful tool to reveal the identification of the species of the fish eggs species. CO1 encodes part of the terminal enzyme of the mitochondrial respiratory chain that is a particularly promising target for species identification [7]. Sequence divergences within the 5' region of CO1 gene for animal taxa are much greater between species than within species which in turn suggest that this approach is applicable among phylogenetically distant animal groups [8]. Therefore, in order to identify an organism, the verification used is the sequencing method of

mitochondrial cytochrome oxidase. Cytochrome c oxidase subunit 1 (CO1) gene is a universal genetic marker that is used to identify animal species especially fish [9]. There are studies showing the effectiveness of CO1 genes with 99% identity in DNA barcoding in order to identify and distinguish the fish species which have been conducted by Sasaki, et al. [10] and Kuguru, et al. [11].

Molecular technique is the technique that has been used by other researchers in order to determine taxonomy and systematic relationships by constructing a DNA barcode library of all the world's fish [12]. Taxonomy is the branch of science that is concerned in identifying, classifying into categories and naming the organism. DNA barcoding is one of the taxonomists methods used to identify organisms based on short, standardized fragments of genomic DNA to obtain taxonomic information about unidentified organisms and evaluate the variation of species differences [13]. These techniques are quite reliable; however, its process is slow and expensive [14]. There are studies that shows the DNA barcoding plays an important role in fisheries management regarding the identification of the species, the distribution of spawning activity and the response of the whole ecosystem to environment variability [15]. The sequences of CO1 gene will be assembled and aligned by using Molecular Evolutionary Genetics Analysis (MEGA) software. MEGA is a tool to compute the sequence alignment, reconstruction of phylogenetic tree, and estimation of sequence divergences for evolutionary relationships purposes. Hence, MEGA software provides some collection of programs to statistically analyze DNA and protein sequence data [16]. Along the time, MEGA has been advanced into MEGA-X, which provides a modernized and organized look, but maintaining the familiar structure of the previous versions of MEGA [17] which make it easier to use for analysis. There are also several enhancements over the previous versions of MEGA such as addition of the utility for calculate the Evolutionary Probabilities (EP) and a method (CorrTest) for detecting auto-correlation of evolutionary rates in large phylogenies, as well as runs natively on macOS systems without using WINE compatibility layer.

The previous study of Lima, et al. [18] showed the samples of eggs and muscles of the fish were obtained only in a particular area which is Brazil. There is absence of the compilation of muscle samples of *P. maculatus* species from Argentina which lead to the variation between species from both countries may not be well-studied. Therefore, this study was performed with two aims which are: 1) to identify the genetic variation between fish egg species with muscles from two different countries, Brazil and Argentina, and 2) to study the population of the fish species.

2.0 MATERIALS AND METHODS

2.1 Data Retrieval

The data was retrieved from a certain journal by its accession number to be assembled and aligned in the MEGA-X software. Table 1 shows the summary of the fish egg species and muscle fish species to be studies with its journal. All the sequence was obtained from the BOLD databases before the sequence was aligned in MEGA software.

2.2 Map Distribution

In this study, the samples were obtained from two countries, Brazil and Argentina. The samples of the fish egg species were obtained from the major tributary of the Parana River, which is Paranapanema River. While, the samples of the fish muscle species were obtained from two countries, which are Brazil including Sao Francisco River, upper Parana River and Paraiba do Sul River and lower Parana River, Argentina (Figure 1).

Table 1: The summary of fish egg and fish muscle species information

No.	Accession No.	Species	Type	Size (bp)	Geographical Area	Journal
1.	CAPV082-17	*P. maculatus*	Egg	601	Brazil	Lima, et al. [18]
2.	CAPV104-17					
3.	CAPV095-17					
4.	CAPV076-17					
5.	CAPV073-17					
6.	CAPV063-17					
7.	CAPV078-17					
8.	CAPV111-17					
9.	CAPV088-17					
10.	CAPV066-17					
11.	CAPV085-17					
12.	CAPV114-17					
13.	CAPV120-17					
14.	BSB378-10		Muscle	648		de Carvalho, et al. [19]
15.	BSB379-10			652		
16.	BSB380-10					
17.	BSB459-10			596		
18.	BSB165-10			633		
19.	FUPR674-09			606		Pereira, et al. [20]
20.	FUPR673-09			651		
21.	FPSR177-09			655		
22.	FPSR173-09					
23.	FPSR175-09					
24.	FPSR176-09					
25.	FPSR174-09					
26.	LARI148-12			562	Argentina	Díaz, et al. [22]
27.	LARI147-12			639		
28.	LARI150-12			592		
29.	LARI151-12			647		
30.	LARI149-12					
31.	CAPV101-17	*L. friderici*	Egg	592	Brazil	Lima, et al. [18]

Figure 1: Distribution of fish eggs and fish muscles species from two countries

2.3 Data Sequence Analysis

The sequences were assembled and aligned by using MEGA-X software. The multiple sequence alignments were built by automated aligner ClustalW algorithm (command line interface). ClustalW is a system that commonly used to align any number of homologous nucleotide or protein sequences which can be access easily in any operating system [23]. The information for the align selected by codons were followed as in appendix A. The multiple sequence alignment data were analysed and recorded such as Variable region, Parsimony Informative region and Conserve region. Bootstrapping method was used in order to estimate maximum likelihood and phylogenetic tree under the best fit substitution models, which is Kimura 2-parameter (K2) model. The pairwise distance also was calculated automatically by using the same model [24]. Both the phylogenetic tree and pairwise distance were recorded and analysed to identify the genetic variation between fish eggs and fish muscles of *P. maculatus* species.

The aligned sequences also were analysed to observe the level of substitution saturation by using DAMBE software which determines an “index of substitution saturation”, based on the notion of entropy in information theory [24]. The data for plotting the number of transition and transversion versus Kimura

2-parameter distance (models) were analysed to evaluate the usefulness of the sequences for phylogenetic analyses. In order to validate the data, species delimitation was performed in ABGD tool, by using Kimura (K80) relative gap width, X of 1.5 and Nb bins (for distance distribution) of 20 [25].

3.0 RESULTS AND DISCUSSION

3.1 Multiple Sequence Alignment

Multiple sequence alignment shows that all data analysis such as conserved region, Parsimony informative and variable region were obtained from MEGA-X software by using ClustalW algorithm (Appendix B). The alignment of all 517 nucleotide sequences shows 407 (78.72%) Conserved, 110 (21.28%) Variable and 12 (2.32%) Parsimony Informative (Table 2).

Table 2: The percentages of conserved region, variable region, and Parsimony informative region during multiple sequence alignment

Region	Percentages (%)
Conserved (C)	78.72
Variable (V)	21.28
Parsimony Informative (Pi)	2.32

3.2 Phylogenetic Analysis

The maximum likelihood method shows Kimura 2-parameter incorporating discrete gamma rate categories (K2+G) was used as a best fit substitution model to construct the Neighbor-Joining tree (NJ) (Appendix C).

The NJ tree and Parsimony tree (Appendix D) shows two different clades which are: Clade 1 shows high similarity between eggs from Brazil with the muscles from Argentina and Brazil, where there is a probability the fish eggs are from Argentina (Figure 2). The genetic variation shows 99% similarities between the eggs of *P. maculatus* from Brazil with muscles from both Argentina and Brazil (Table 3). Clade 2 shows there is a slight difference between eggs and a few muscles from Brazil, which there is probability the fish is not *P. maculatus* species [26], since the variance shown in genetic distance table reaches to 2%. *L. friderici* acted as an outgroup. The similarity between fish eggs and fish muscles from Brazil and Argentina proves that the *P. maculatus* may be undergoing migration in order to spawn [27].

The theory of Yoshimi Sato from Zaniboni-Filho and Schulz [28] stated that the *P. maculatus* is a short distance migrants which contradict with the study of da Silva, et al. [29] stated that the *P. maculatus* is a long distance migrants with the features that having a swimming strategy near the bottom in order to migrate to other places [30]. Short distance migrants are the species that need short-free flowing rivers to spawn, and long-distance migrants are the species that migrate upriver to spawn in specific localities such as lakes for their initial development [31]. Parana River has a distance of 4,695 km that covers South-Central Brazil until northern Argentina [30]. It proves that the *P. maculatus* which is the most abundant species in southeastern Brazil probably originated from Argentina which migrated to Brazil to spawn especially in the rainy season because it may be the best time for *P. maculatus* to migrate [32].

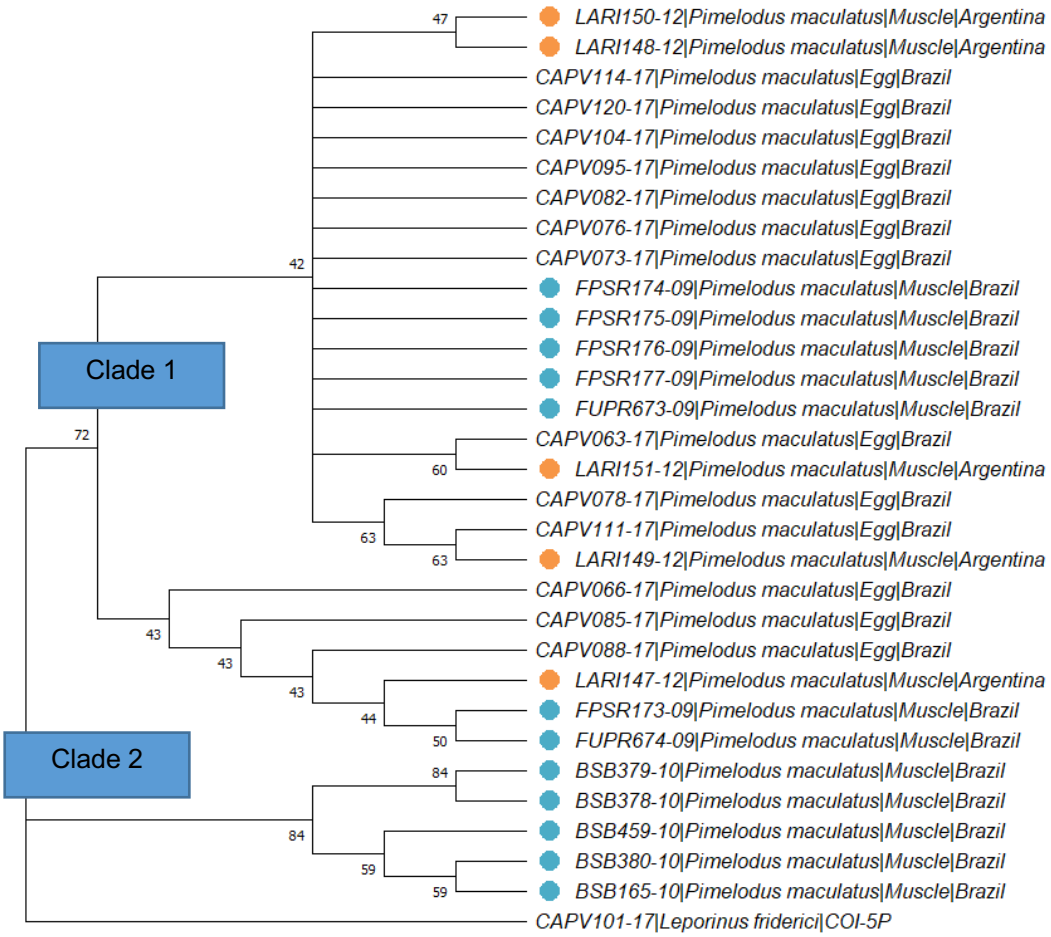

Figure 2: The phylogenetic tree represents the similarity between fish eggs and fish muscles from Brazil (blue) and Argentina (orange) which are divided by two clades. The Kimura 2-parameter model was chosen from the maximum likelihood method to construct a NJ tree with a bootstrap method by using MEGA-X software.

Table 3: Range of the genetic pairwise distance analysis that was computed by using Kimura 2-parameter as a fit substitution model to observe the genetic variances between eggs and muscles of *P. maculatus* from both countries.

	1	2	3	4
P. maculatus Muscle Brazil	0.0000-0.0221			
P. maculatus Egg Brazil	0.0000-0.0200	0.0000-0.0039		
P. maculatus Muscle Argentina	0.0000-0.0202	0.0000-0.0020	0.0000-0.0039	
L. friderici Outgroup	0.2840-0.2922	0.2840-0.2881	0.2840-0.2905	0.0000-0.0000

3.3 Substitution Saturation Test Analysis

Substitution saturation tests for the whole 517 bp sequences were tested by using DAMBE before reconstructing the phylogenetic tree. DAMBE software functions to plot the transition and transversion against a corrected genetic distance [33]. Estimation of saturation shows that the base saturation by transition was higher than the base saturation by transversion (Figure 3). Based on the data sets, the graph was directly proportional which indicated the CO1 sequence is not saturated [34]. It proves that the nucleotide sequences were aligned appropriately and can be used to construct the phylogenetic tree.

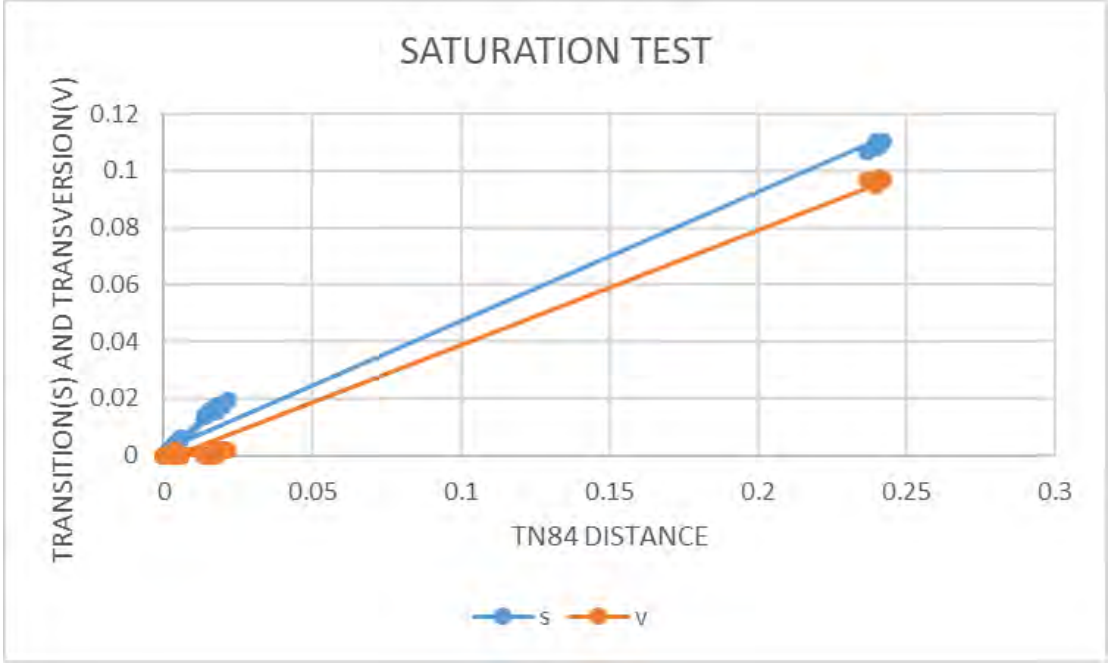

Figure 3: Graph of DAMBE substitution saturation test that was plotting transitions(s) and transversions(v) against TN84 genetic distance.

3.4 Automatic Barcode Gap Discovery (ABGD) analysis

In order to delimit the species, the data set of sequences were analysed by using ABGD tool, using K80 Kimura distance. Basically, the ABGD tool acted as a secondary tool in order to validate the data in this study. ABGD was introduced with a barcode gap to observe the divergence among an organism [25]. The prior of maximal intraspecific divergence lies between 1% and 3% of divergence [25]. The ABGD analysis shows there is a divergence between *P. maculatus* (Figure 4). The ABGD analysis and phylogenetic tree shows a conflict for a few individuals from Brazil which have a slight difference with the eggs. This suggests that a few muscles from Brazil are not a species of *P. maculatus,* instead, it probably includes other species, such as *P. albicans*, *P. blochii* or *P. fur* (Appendix E).

From the results obtained, it shows that the genetic identification by using CO1 gene helps to reveal the exact identity species of the fish eggs to prevent mislabeling that can lead to erroneous identification of the species. In the study of Pappalardo, et al. [35] shows the mislabeling of the fish eggs may cause health problems for individuals with allergies. Other than that, it shows that the CO1 gene is an effective marker to discriminate between the egg samples and muscle samples of the fish which will help the scientist to discover and protect the population of the fish species, as well as, to prevent the extinction of the fish species. For future studies, the CO1 gene should be added with another genetic marker, such as *cytb*, in order to get a better result that can resolve the low resolutions of the CO1 gene alone [8]. In addition, the method for this study can also be used for future studies on the identification of genetic variation of fish eggs and fish muscles in Malaysia.

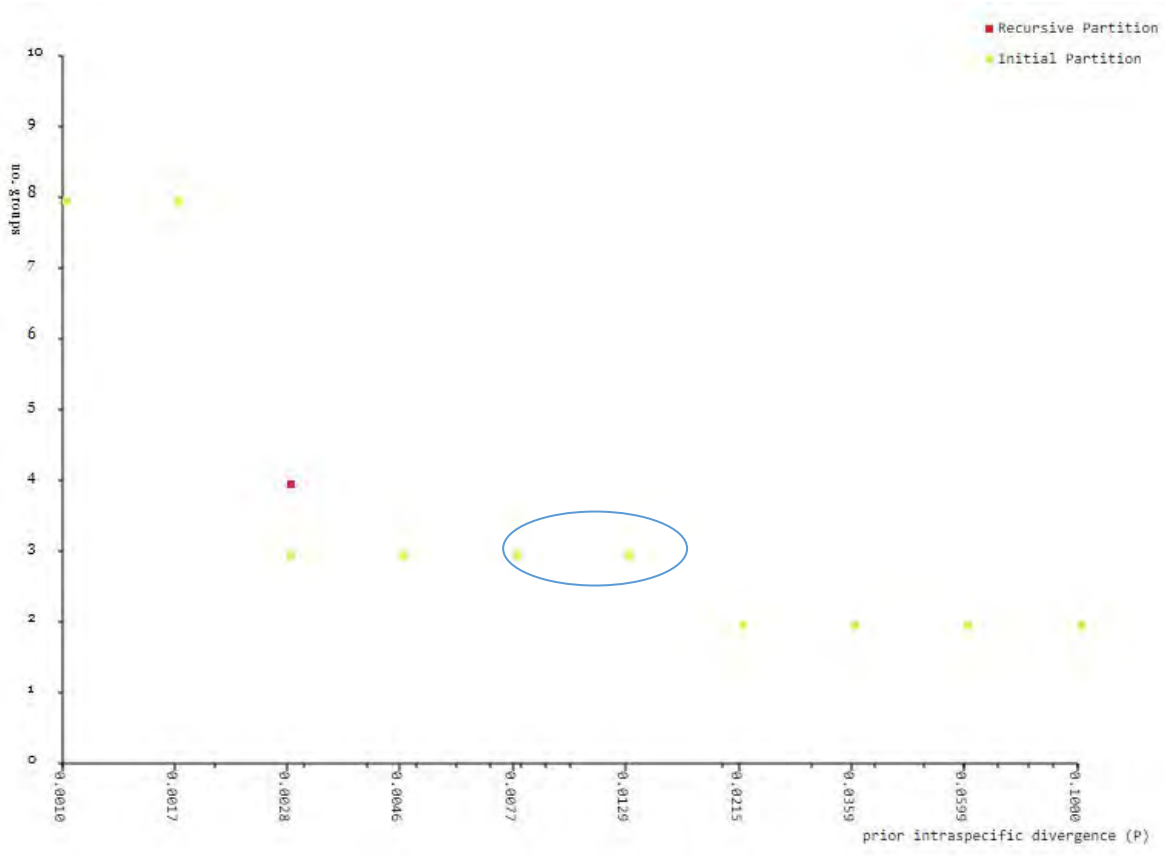

Figure 4: ABGD represents the number of groups against prior intraspecific divergence (P).

4.0 CONCLUSION

In conclusion, this study verifies that the CO1 gene acts as an effective marker to identify the species of *P. maculatus* and discover the population of *P. maculatus* that undergo long distance migration from Argentina to Brazil to reproduce. In the recommendation, a few samples of muscles from Brazil should be taken into serious consideration for revision to avoid misinterpretation on the species.

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Synthesis and Characterization of Bacterial Cellulose produced from Local papaya isolate

Nur Adila Binti Jamaludinr[1], Farizan Aris[1], Mohd Taufiq Mohd Jalil[1], Khairunnisa Ahmad Kamil[2], and Nurul Aili Zakaria[1*]

[1]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.
[2]Faculty of Applied Sciences, Universiti Teknologi MARA, Perlis Branch, Arau Campus, 02600 Arau, Perlis, Malaysia.

*nurulaili@uitm.edu.my

ABSTRACT

Bacterial cellulose (BC) is widely used due to its distinct physicochemical properties when compared to plant cellulose. BC is primarily concerned with the biosynthetic process in order to achieve low-cost preparation and high cellulose production. BC has displayed significant advantages over cellulose obtained from plants due to the absence of lignin, hemicellulose, pectin, and other contaminating materials of animal origin. Hence, it possesses high purity, crystallinity index, and biocompatibility. This study aims to isolate cellulose-producing bacteria from *Carica papaya* and characterize the bacterial cellulose produced using SEM, FTIR, XRD. Initially, the fermentation of the isolates was carried out under static and agitated fermentation with an initial pH of 6, a temperature of 37°C, and a fermentation period of 14 days. Isolate PP4 produced approximately 1.22g/mL pellicles under static condition at 37ºC for 14 days. Some FTIR peaks of the pellicle analysed indicate the absence of a hydrogen bond, while sharp intensity peaks contain an oxygen-related group. There is also a narrow band indicating the presence of aliphatic compounds. A fingerprint peak shape representing the aromatic ring and Vinyl-related compound was also detected. XRD analysis revealed 15 distinct diffraction peaks, indicating that the pellicle belonged to the monoclinic type with 95.0% of crystallinity index. Lastly, the SEM images showed that the pellicle had clustered microfibrils, non-microfibrils cellulose interaction, which had gaps between the fibres and not intertwined closely.

Keywords: Bacteria cellulose; Carica papaya; Henstrin Schramm; FTIR; XRD; SEM

1.0 INTRODUCTION

Cellulose, an embedded extracellular mass of cellulose, called pellicles, was described by Anselme Payen as the primary component of the higher plant cell wall. It is primarily produced by plants but can also be produced by bacteria (*Acetobacter* spp., *Acanthamoeba* spp., and *Achromobacter* spp.) which was named *Bacterium xylinum* by A.J Brown (1886) after he observed a 'jelly like translucent' on his acetic acid fermentation, algae and fungi. Meanwhile, Hibert and Barsha (1931) found bacteria cellulose (BC) was identical in the molecular formula of plant-cellulose but differed in its properties. The BC has unique properties which are high in purity due to the absence of lignin and hemicellulose, porosity, crystallinity (generally above 60%), and surface area, which improves the water retention and the liquid-absorption capacity but still lacking in broad-spectrum applications in various field such as in nanomaterials, and polymers [1]. BC also has smaller microfibrils which are 100 smaller than plant cellulose [2]. Cellulose $(C_6H_{10}O_5)n$, linked at (1-4) β-glycosidic bond of glucose residues and forming hydrogen bond which make it as non-soluble compound and there are three classes of cellulose to identify cellulose ability to dissolve in optimum concentration sodium hydroxide (NaOH) solution which are α, β, and γ. Furthermore, BC has a high degree of polymerization (DP), ranging between 16,000 and 20,000, compared to plants, which average between 13,000 and 14,000 [3]. There are two distinct groups at each end of cellulose: a closed ring of the non-reducing group and a reducing group

composed of aliphatic and carbonyl structures. The crystallinity of cellulose can be determined using (FTIR), (XRD), and (SEM), and it consists of four allomorphs: type I (the most abundant), type II (the most stable), type III, and type IV. Type I has two allomorphs, I (triclinic) and I (monoclinic), determining the hydrogen bond between celluloses. When cellulose I and cellulose III are regenerated, monoclinic cellulose III is formed, used as a precursor for cellulose derivatives. In comparison, high temperature treatment of cellulose III with glycerol results in the formation of type IV [4]. Static culture promotes the formation of a gelatinous membrane, which results in the formation of pellicle forms. Still, it is unsuitable for large-scale production due to the low yield and high cost of production. Simultaneously, agitated conditions produced the highest yield of BC and created harsh conditions, which means Acetobacter xylinum cannot adapt to the culture for growth due to high oxygen exposures caused by the oxidation of many substrates [5]. However, BC production is preferable in agitated culture in this study since oxygen dissolves more efficiently to promote growth at certain rpm. Meanwhile, the agitated culture leads to abnormalities structure of BC because high pressure is induced in this culture.

2.0 MATERIALS AND METHODS

2.1 Materials

Hestrin-Schramm (HS) was used as a culture medium for bacteria producing cellulose. The preparation of standard HS medium consists of 0.5% (w/v) of yeast extract, 0.5% (w/v) peptone water, 2% (w/v) D-glucose, 0.115% (w/v) citric acid ($C_6H_8O_7$) and 0.27% (w/v) disodium hydrogen phosphate (Na_2HPO4). All these mixtures were mixed and transferred to a 250 mL Schott bottle, and its pH was adjusted around 5.5 to 6.0 using 1 M of acetic acid. After that, the mixture was autoclaved at 121 ºC for 15 minutes before use. For HS agar, the sterilized HS medium was left to cool, and 1.5% (w/v) agar was added. All chemicals used were of analytical grade.

2.2 Methods

2.2.1 Isolation and screening of cellulose-producing bacteria

The *C. papaya* fruit was peeled and cut into small pieces. Approximately 25 g of the fruit peel is homogenized in a stomacher bag containing 225 mL of 0.1% (w/v) sterile buffered peptone water. The solution was diluted (10^{-1} to 10^{-6}) using 0.85% (w/v) sterile saline solution. After that, 100 µL of dilution was spread onto HS agar plates with pH 6 and incubated for 24-48 hours at 30ºC-37ºC. Each distinct colony grown on agar plates was isolated and purified by repeatedly streaking onto new HS agar plates. The purified bacterial isolate was then inoculated individually into a universal bottle containing 10 mL of HS medium at 30ºC for 24-48 hours. Only the bacterial isolate that showed pellicle formation in the culture medium was chosen as the potential cellulose-producing bacterial. The potential cellulose-producing bacterial isolate was stored in 50% glycerol at -80 ºC for further studies.

2.2.2 Synthesis of bacterial cellulose (BC)

The potential cellulose-producing bacterial isolates were plated on HS medium. A 10% (v/v) culture medium was transferred into a conical flask containing 100 mL of HS medium, incubated statically at 30ºC for 10-14 days. The pellicle formed was harvested via centrifugation at 14000 g for 1 min at room temperature. The harvested pellicle was rinsed with sterile distilled water several times to remove leftover medium and other contaminants and boiled with 2.0% NaOH solution for 30 minutes at 80 ºC then washed thoroughly with distilled water (2-3 times) to remove the attached bacterial cells. Next, the pellicles were neutralized with 4% acetic acid, followed by repeated washing with distilled water (2-3 times). Finally, the pellicles were dried at 60 ºC until constant weight, and the dried yield was determined as equation (1), where *m* is the dry weight of cellulose (g), and *c* is the weight of carbon source (g).

$$\text{Yield (\%)} = \left(\frac{m}{c}\right) \times 100 \quad \text{.....(1)}$$

2.2.3 Characterization of Bacterial Cellulose

The production of BC pellicle produced was characterized by Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and X-ray Diffraction.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectrum of BC pellicles was recorded in the spectral range of 650 – 4000 cm^{-1} at a resolution of 4 cm^{-1}. The pellicle must be in KBr pellet form. Thus, 0.8g of KBr powder and 0.01g of sample pellicle were homogenized by pestle and mortar and pressed using hand press to form a KBr pellet.

X-ray Diffraction (XRD)

X-ray diffraction (XRD) is a technique used in the qualitative identification of crystalline phases by their diffraction pattern. In this study, XRD analysis was utilized to characterize the crystallinity index of the BC sample. The X-ray diffraction spectra were recorded using Cu Kα radiation as an X-ray source at 25°C. X-ray diffraction spectra were recorded using a diffractometer at a plate current intensity of 40 mA and an accelerating value of 40 kV.

Scanning Electron Microscopy (SEM)

The samples were prepared in 0.1% (w/v) water. Before SEM observation, several drops of dried pellicle were applied on the aluminium stage, covered by the carbon tape, and left to air dry with HEPA filters. Next, the samples were coated with an ultrathin layer of gold using an ion sputtering machine such as The ETD detector. Since BC does not conduct electricity, the dried pellicle was gold-coated for the 90s so the structure of the BC can be seen easily. The structure of the BC was then analysed by SEM operating at 15.0 kV. The micrographs were acquired at a magnification of 20,000

3.0 RESULTS AND DISCUSSION

3.1 Production of Bio cellulose from bacterial isolates

Initially, PP4 isolate was incubated overnight at 37ºC in the universal bottle, and only 0.05g/mL of pellicle formed. This is due to the small surface area to volume ratio of the universal bottle, which contains 10 mL of HS medium. In stationary conditions, the surface/volume (S/V) ratio is critical for BC synthesis. This was demonstrated in an experiment using six plastic trays with the same surface area of 425 cm^2 but varying medium volumes. In that study, the optimal S/V ratio was determined to be 2.2 cm. The authors discovered, however, that a S/V ratio of 0.71 cm results in the highest BC pellicle [6]. The HS medium was then transferred to a 250 mL conical flask and incubated for 14 days in two conditions: agitated (120 rpm) and stationary at 37ºC. Pellicles were successfully developed in the flask starting on day 11 and produced approximately 1.22g/mL pellicle (Figure 1).

Figure 1: Pellicle of bacteria producing cellulose

BC membranes formed a flat pellicle and accumulated on the surface of the HS medium as a thin gelatinous membrane [7]. Pellicles are formed primarily from transparent soft cellulose in all samples. According to a recent study, BC derived from ripe fruit or vegetables would have a pellicle shaped like the interconnections of a three-dimensional net-like structure [8]. In this experiment, the pellicle grew

better in stationary culture than in agitated culture, as the pellicle began to degrade, clouding the broth. This is because stationary conditions inhibit uniform medium aeration, and Cel^{+} colonies dominate in the medium meanwhile in agitated conditions. BC is affected by environmental factors such as acidity and ionic strength to a minimum and also resulting in the production of cel^{-} mutants, a non-cellulose producer that decline the BC thus do not allow the binding of cellulose to the edge of the flask (Zhong, 2020). Previous study stated that they discovered in static conditions, the BC pellicle formed as a membrane sheet at the medium surface exposed to air. In contrast, the BC particles were spherical or apostrophe in shape in agitated conditions. The shaking effect on BC results in a different structure than those without agitation(Raghavendran et al., 2020). Under higher oxygen conditions, glucose can be used as a carbon source for conversion to gluconic acid, resulting in low BC production due to competition where glucose is converted to gluconic acid (Krusong et al., 2016).

3.2 Characterization of bacterial isolates and bacterial cellulose

The Fourier Transform Infrared Spectroscopy (FTIR) determines the interpreted bonds, functional groups, and possible nutrient types in the positive pellicles. The pellicle was then prepared into a KBr pellet and analysed at a resolution of 4 cm^{-1}. Table 1 and Figure 2 summarise the corresponding wavenumbers from the FTIR analysis of the PP4 isolate. he FTIR spectrum contains more than five peaks, indicating that the chemical being examined is a complex molecule [11]. There is no broad absorption band between 2500 and 4000 cm^{-1}, indicating the absence of a hydrogen bond. Additionally, several strong intensity absorptions at 3670 and 3550 cm-1 were observed, indicating the presence of an oxygen-related group such as alcohol or phenol [12]. A narrow band below 3000 cm^{-1}, at 880.60 cm^{-1}, indicates the presence of aliphatic compounds, while the peak 2497 cm^{-1} and 930.43 cm^{-1} exhibit fingerprint peak shapes, indicating the presence of an aromatic ring [13]. Additionally, a vinyl-related compound was detected at these fingerprint peaks. The peaks at 1050.8 cm^{-1} and 702.92 cm^{-1} indicate that the BC sample was properly washed in the range 1050 cm^{-1} to 517.08 cm^{-1} during purification [12].

Table 1: FTIR peaks analysis associated with interpreted bonds, functional groups, and possible nutrient type

Peaks cm^{-1}	Type	Vibration Type	Functional group	Possible nutrient type
517.08	Narrow	Stretching	C-I	-
702.92	Broad	Bending	C=C (alkene)	-
803.72	Narrow	Bending	C-C (Methyne)	-
880.60	Narrow	Stretching	C-Cl	-
930.43	Narrow (Fingerprint)	Bending	C≡C	Carbon
1050.8	Broad	Vibration	C-C (Methylene)	Carbohydrate
1440.98	Narrow	Stretching	C-C (Methyl)	
2364.15	Narrow	Bending	C-O	-
2497.65	Narrow (Fingerprint)	Stretching	C≡C	Amino related
3445.90	Narrow	Vibration	OH stretch	-
3525.96	Narrow	Vibration	OH stretch	-
3547.41	Narrow	Vibration	OH stretch	-
3589.06	Narrow	Vibration	OH stretch	-
3650.22	Narrow	Stretching	OH stretch	-

3672.99	Narrow	Stretching	Oxygen related	-
3712.84	Narrow	Stretching	Oxygen related	-
3736.65	Narrow	Stretching	Oxygen related	-
3747.89	Narrow	Stretching	Oxygen related	-
3839.25	Narrow	Bending	Oxygen related	-

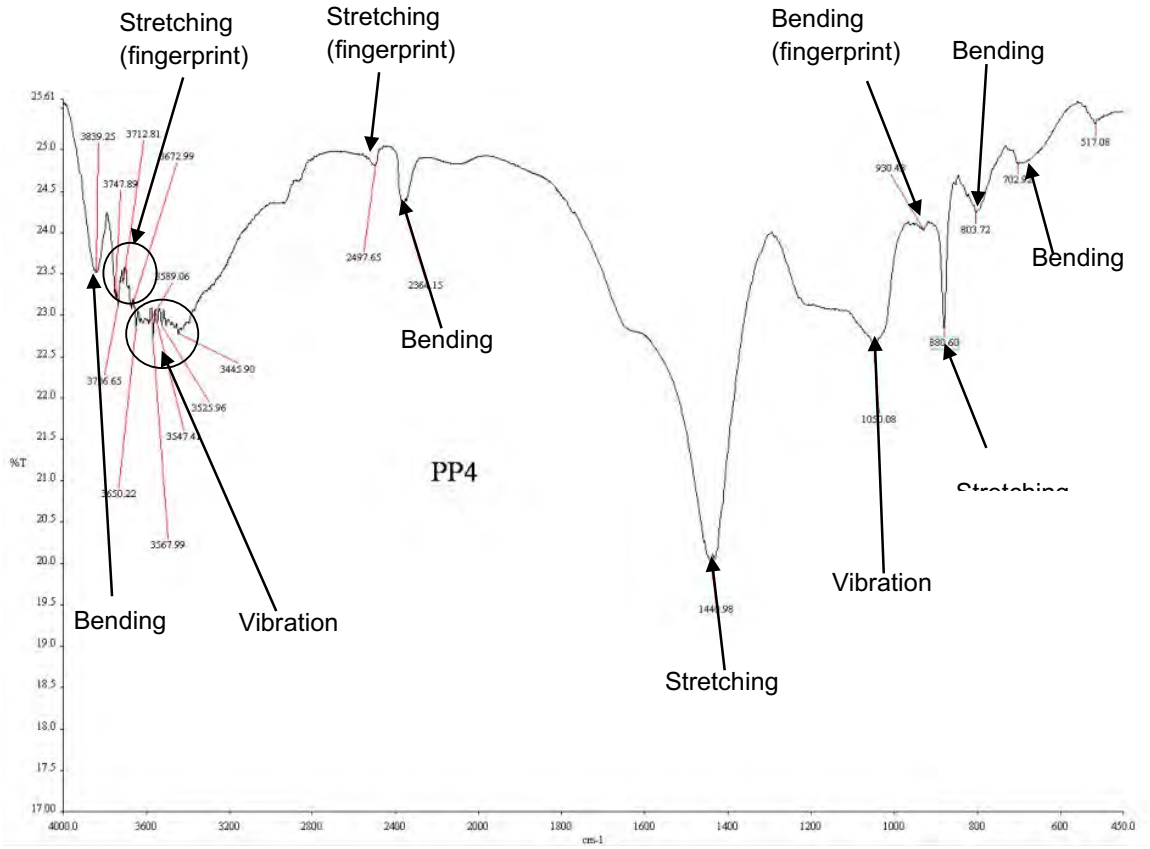

Figure 2: FTIR peak of bacterial cellulose from PP4 isolate

For crystallinity index examination, the pellicle also subjected to XRD analysis. Figure 2 shows the diffraction peaks of the pellicle. There are 15 distinct diffraction peaks, which are 17º, 25º, 30 º, 38 º, 40º, 43º, 45 º,47º, 49º, 53º, 57º, 62º, 65 º, 78 º, 80º, and 83º. XRD analysis showed that the pellicle was monoclinic (Iβ), supposedly dominant in plants (Peter, 2021). Based on a recent study, BC also contains Iβ, which is from nanocrystalline cellulose (NCC) that can be obtained from cellulose fibres by tuning out the amorphous part, and it is a new and renewable nanomaterial that can be isolated in acidic solution and contain high crystallinity index shown in Table 2 (Rongpipi et al., 2019). However, there is no exact study of crystallinity BC from *C.papaya* since most studies use papaya waste in isolated BC.

Table 2: The crystal size and crystal index of BC

Sample	Crystal size (X 10^{-4} nm)	Crystallinity index (%)
PP4	18.4	95.0

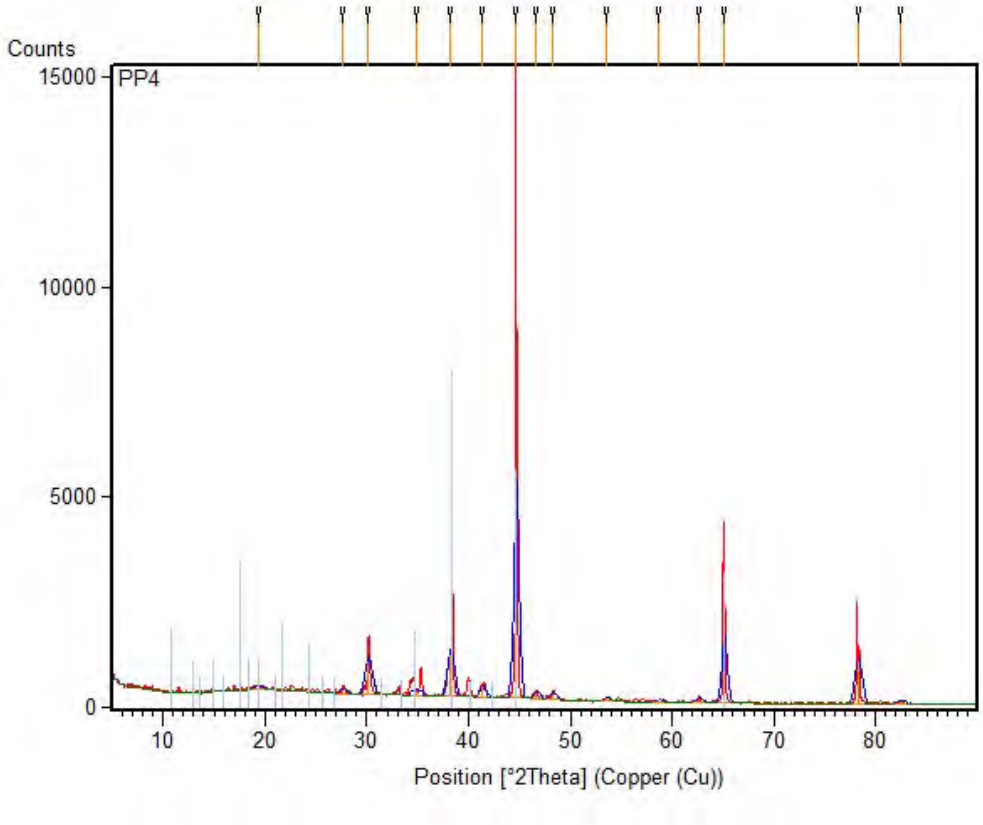

Figure 3: XRD diffraction of pellicle

Lastly, for morphology, SEM analysis was carried out. SEM image of threadlike cellulosic microfibrils without visible bacterial cells in Figure 4 demonstrate that the fibrils are tightly packed, indicating that they are composed of amorphous cellulose and may exhibit morphological characteristics similar to pure microcrystalline cellulose(Jia et al., 2017). There is also cellulose interaction between non-microbial cellulose samples because of discontinuous and fragmented formation present. The micrograph also shows that some microfibrils are cluster together (Auta et al., 2017). The non-microbial cellulose shows cellulose sheets having gaps between the fibres and not intertwined closely (Raghavendran et al., 2020).

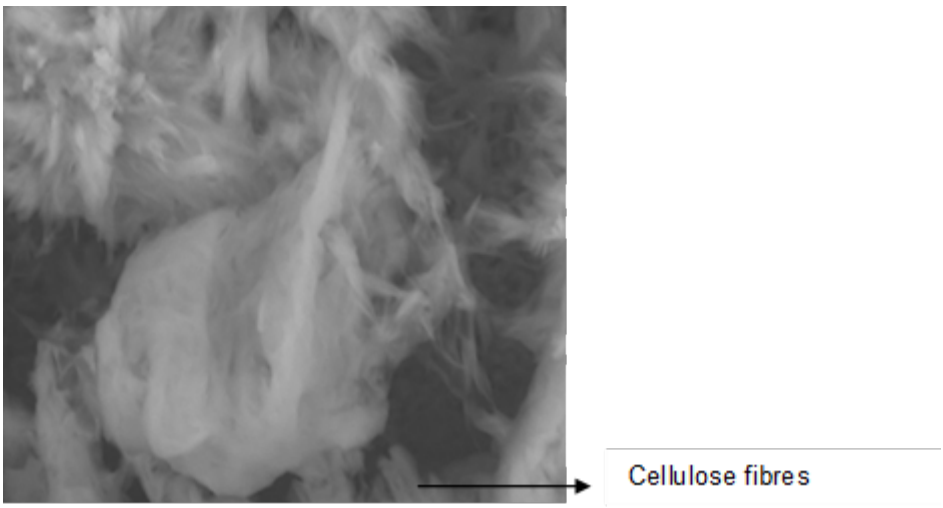

Figure 4: Morphological of cellulose pellicle under SEM

4.0 CONCLUSION

The findings suggest that isolate PP4 from *C. papaya* peel harbour a potential as cellulose producing bacteria. The structural and morphological characterization of the bacterial cellulose from PP4 also revealed that it possesses an excellent crystallinity index and morphology. However, further investigation of the fermentation conditions and bacterial cellulose synthesis is necessary to optimise the yield of bacterial cellulose obtained.

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Screening of Schiff Base Ligands Derived from Phenylenediamine and Its Metal Complexes as A Potential Efflux Pump Inhibitor Against *K. Pneumoniae*

Norshahida Mat Jaya[1], Anis Low Muhammad Low[2,3], Suhaidi Ariffin[2], Siti Solihah Khaidir[1], Hadariah Bahron[1], Nor Hadiani Ismail[1,3], Sharifah Aminah Syed Mohamad[1,3*]

[1]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
[2]Faculty of Applied Sciences, Universiti Teknologi MARA, Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000 Kuala Pilah, Negeri Sembilan, Malaysia
[3]Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Level 9, Building FF3, Universiti Teknologi MARA, Cawangan Selangor, 42300 Bandar Puncak Alam, Selangor, Malaysia

*sharifah459@uitm.edu.my

ABSTRACT

Treatment of infectious diseases is becoming more complex and ineffective as the rate of multidrug resistance (MDR) bacteria rises. Schiff base is synthesized from condensation reaction of primary amines with active carbonyl compounds and considered as “privileged ligands” because of their coordination with many different metals and able to stabilize metals in various oxidation states. These compounds have showed promising antibacterial agents but their potential as efflux pump inhibitor in bacterial resistance mechanism is still limited. This study focusses on the screening of 40 Schiff base compounds that consist of ligands and metal complexes as efflux pump inhibitor against *K. pneumoniae* by resazurin microtiter-based assay using nalidixic acid and chloramphenicol as antibiotics at their sub-MIC concentrations. It was found that, Co4[OVan(Me)MPD] displayed the highest inhibition at 86.5% when Schiff base compounds and *K. pneumoniae* ATCC 700603 were treated with sub-MIC of chloramphenicol (20 µg/mL). Metal of copper exhibits the highest activity than other metal complexes and this agree with Overton’s and Tweedy’s chelation theory, where metal complexes have better potential as antibacterial agents than their parent ligands. As a conclusion, unique tetranuclear complexes possess potential for future development as efflux pump inhibitors.

Keywords: Schiff base; efflux pump inhibitor; *K. penumoniae*; bacterial resistance

1.0 INTRODUCTION

The emerging of MDR bacterial strains is of great concern and considered by the World Health Organization (WHO) as one of the three most important public health threats of the 21st century. This situation is worsened by a dramatic reduction in antibiotic’s development, due to the high development costs resulting in the emergence of infections that are almost untreatable with no reliable alternatives to treat infectious diseases [13]. Over the last 20 years, multidrug-resistant *K. pneumoniae* becomes an important pathogen in both the community and the hospital setting associated with healthcare infections [8]. The primary mechanism of multidrug resistance (MDR) bacteria to resist the action of antibacterial agents is via efflux pump extrusion. The development of broadly active efflux pump inhibitors is considered desirable, especially for the reclamation or reactivation of existing antibiotics [5]. Combination of synthesized compounds such as Schiff base with conventional antibiotics may hold greater promise as antibacterial agents [11]. The term of “Schiff bases” was reported in 1864 by German Chemist, Hugo Schiff, the first researcher that synthesized the Schiff base compound from a condensation process of primary amines with active carbonyl compounds [3]. Schiff base compounds are considered as “privileged ligands” due to their facile preparation, good chelating agents, ability to

coordinate with many different metals and can stabilize metals in various oxidation states [1]. It has been reported when Schiff base was coordinated to metals ions such as copper, nickel, zinc and cobalt; the biological activities was much greater compared to their parent ligand [7]. Metal complexes have been reported to display good bioactivity acting as lipophilic moieties that assist cell penetration and the results have shown that polynuclear complexes generally display higher bioactivity than their mononuclear counterparts [2]. Even though many complexes derived from phenylenediamine and its metal complexes have been successfully synthesized and characterized, there is still not much work reported on the potential of Schiff base compound act as efflux pump inhibitors (EPI). This study focusses on the screening of 40 Schiff base compounds that consist of ligands SalOPD, OVanOPD, OVanMPD, OVan(Me)MPD and OVanMPD(Cl) and metal of copper(II), nickel(II), cobalt(II) and zinc(II) metal salts for their activity as efflux pump inhibitor against *K. pneumoniae* by resazurin microtiter-based assay. Screening these metal complexes continues to be of interest in order to evaluate their bioactivity potential as antibacterial agents as well as EPI for existing antibiotics to work more efficient against multidrug resistance bacteria. The synthesized Schiff base compounds have a potential as EPI agents due to their privileged ligands that have conformational flexibility and ability to adopt a variety of geometries. Thus, this research is important for a wider scope of future studies that enables the development of alternative target against MDR bacteria.

2.0 MATERIALS AND METHODS

2.1 Preparation of Schiff Base Compound Stock Concentration

A total of 40 samples of Schiff base ligands and its metal complexes derived from phenylenediamine, consisted of 5 Schiff base ligands, 8 mononuclear complexes, 15 dinuclear complexes and 12 tetranuclear complexes were provided by Siti Solihah Khaidir, Coordination Chemistry Laboratory, Faculty of Applied Sciences, UiTM (Table 1). The stock concentration was prepared at 1 mg/mL in DMSO.

Table 1 : Schiff base ligands and its metal complexes with its designation (L1 – L40)

Ligands	**SalOPD(L1)**	**OVanOPD(L6)**	**OVanMPD(L14)**	**OVan(Me)MPD(L23)**	**OVan(Cl)MPD(L32)**
Mononuclear	Co(SalOPD)(L2)	Zn(OVanOPD)(L7)			
	Zn(SalOPD)(L3)	Cu(OVanOPD)(L8)			
	Cu(SalOPD)(L4)	Ni(OVanOPD)(L9)			
	Ni(SalOPD)(L5)	Co(OVanOPD)(L10)			
Dinuclear		Zn_2(OVanOPD)(L11)	Cu_2(OVanMPD)(L15)	Cu_2[OVan(Me)MPD](L24)	Cu_2[OVan(Cl)MPD](L33)
		Cu_2(OVanOPD)(L12)	Ni_2(OVanMPD)(L16)	Ni_2[OVan(Me)MPD](L25)	Ni_2[OVan(Cl)MPD](L34)
		Co_2(OVanOPD)(L13)	Co_2(OVanMPD)(L17)	Co_2[OVan(Me)MPD](L26)	Co_2[OVan(Cl)MPD](L35)
			Zn_2(OVanMPD)(L18)	Zn_2[OVan(Me)MPD](L27)	Zn_2[OVan(Cl)MPD](L36)
Tetranuclear			Cu_4(OVanMPD)(L19)	Cu_4[OVan(Me)MPD](L28)	Cu_4[OVan(Cl)MPD](L37)
			Ni_4(OVanMPD)(L20)	Ni_4[OVan(Me)MPD](L29)	Ni_4[OVan(Cl)MPD](L38)
			Co_4(OVanMPD)(L21)	Co_4[OVan(Me)MPD](L30)	Co_4[OVan(Cl)MPD](L39)
			Zn_4(OVanMPD)(L22)	Zn_4[OVan(Me)MPD](L31)	Zn_4[OVan(Cl)MPD](L40)

2.2 Determination of Minimum Inhibition Concentration (MIC) and Sub-MIC by Resazurin Microtiter Plate Based Assay

A 96-well microtiter was labelled as in Figure 1. Column (1-10) row (A-G) was loaded with 50 µL of antibiotics with respective concentration. Bacterial suspensions at a density 0.5 McFarland (Densimat; bioMérieux) were diluted 1:10 (to give a final concentration of 1×10^6 cfu/mL) and 50 µL was loaded to each row (A-F) for columns (1-11). For growth control, 50 µL of sterile MHB was loaded into the wells (column 11A, 11B and 11C) and 100 µL of sterile MHB was loaded into wells (column 12D, 12E and 12F) as sterility control. For negative control, 50 µL of 2% DMSO was loaded into wells (column 11D, 11E and 11) and for positive control, 50 µL of 20 µM gentamicin was loaded into wells (column 12A, 12B and 12C). The final volume in each well was 100 µL. The microtiter plates were covered and incubated at 37 °C for 18 - 24 hours. After incubation, 10 µL of 0.1 mM resazurin was added to the well and the plate was re-incubated for another 2 - 4 hours at 37 °C. The result was observed and recorded as positive for blue colour or negative for pink colour and was compared with Performance Standards for Antimicrobial Susceptibility Testing, Clinical and Laboratory Standards Institute (2018).

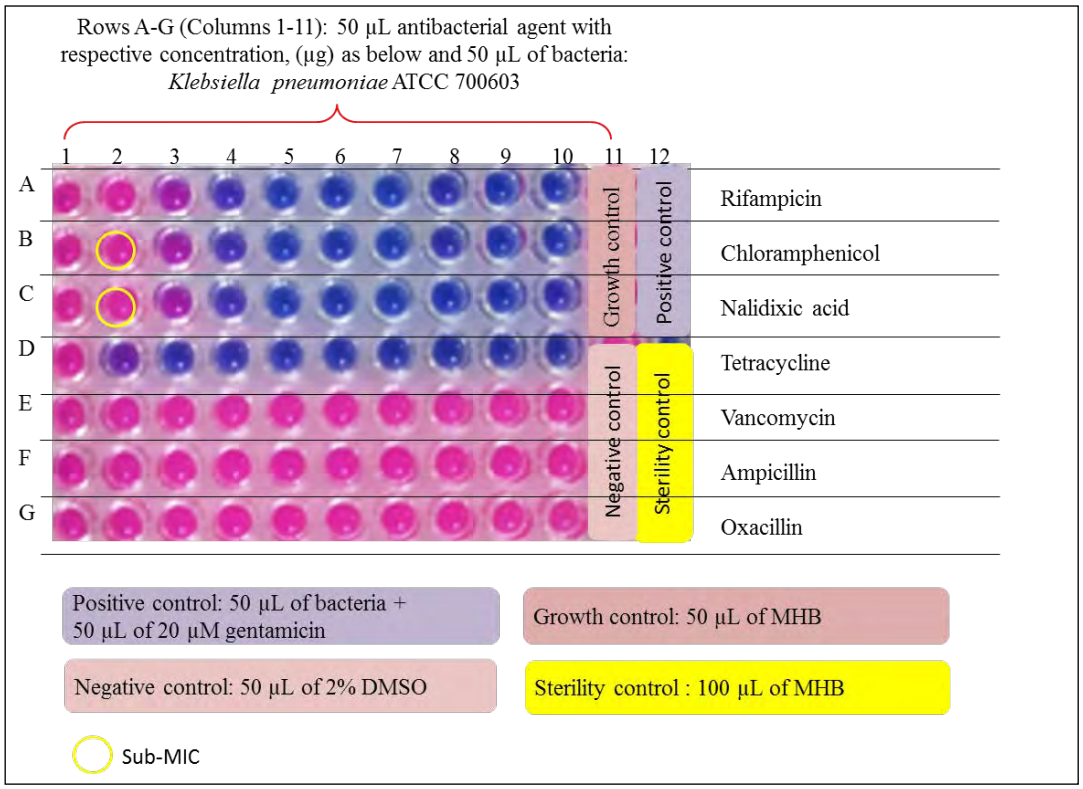

Figure 1: Determination Minimum Inhibition Concentration (MIC) and Sub-MIC by Resazurin Microtiter Plate Based Assay

2.3 Screening for Potential Efflux Pump Inhibitors

The screening for potential efflux pump inhibitors among 40 Schiff base compound was done as described in Figure 2. The antibiotics selected to tested with test sample compounds are chloramphenicol (20 µg/ml) and nalidixic acid (25 µg/ml). The screening comprising of 20 µM Schiff base compounds (50 µL), 50 µL of antibiotics and 50 µL of bacterial suspension were loaded into the wells of row A, B and C, accordingly. In addition, blank sample comprised of 50 µL test sample compounds and 50 µL of bacterial suspension were loaded in wells D, E and F. A 50 µL volume of each bacterial suspension, 200 µg/mL Phe-Arg-β-naphthylamide (PaβN) and antibiotics were pipetted into wells A10, B10 and C10 which served as a positive control. Wells A11, B11 and C11 served as negative

control were loaded with 50 µL each of bacterial suspension and 2.0 % DMSO. For growth control, wells A12, B12 and C12 were loaded with 50 µL each of bacterial suspension and sterile MHB whereas 100 µL sterile MHB was loaded into wells D12, E12 to F12 that served as sterility control. The microtiter plate was later incubated at 37 °C for 18 to 24 hours followed by the addition of 10 µL resazurin (0.1mM) to all wells and incubated further at 37 °C for 2 to 4 hours. Changes of colour was observed and recorded, and the absorbance of each microtiter plate was read at an OD570nm and OD600nm. The percentage of inhibition for each sample was calculated as in Formula 1. The assay was done in triplicates.

$$\frac{\text{Growth Control Reading} - \text{Test Sample Reading}}{\text{Growth Control Reading}} \times 100 \qquad \text{(Formula 1)}$$

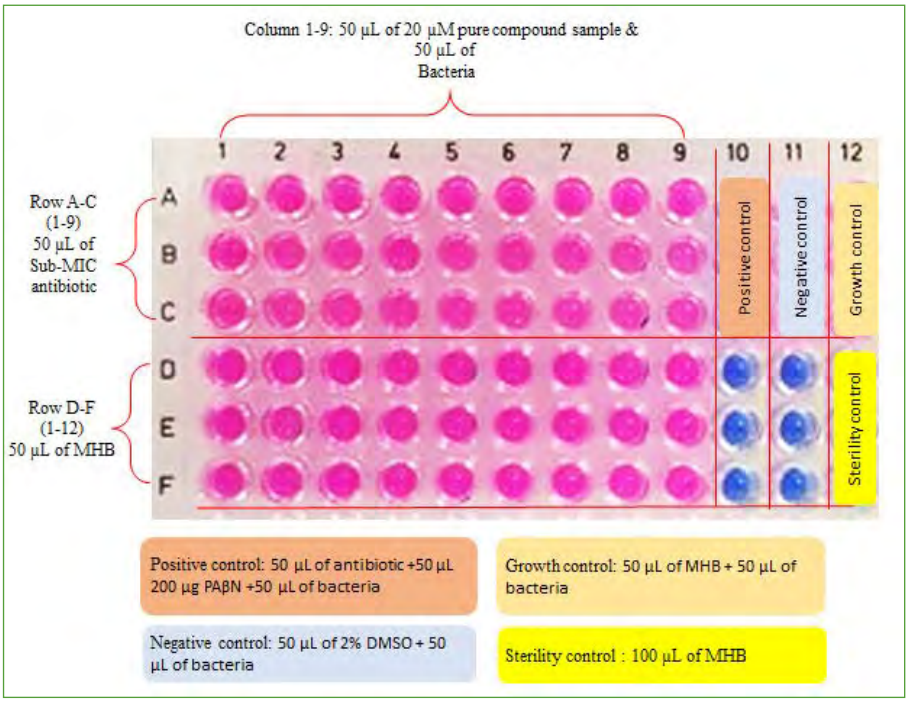

Figure 2: Efflux Pump Inhibitor Screening of Schiff Base Compounds against K. pneumoniae ATCC 700603

3.2 RESULTS AND DISCUSSION

Many studies have shown the efflux pump resistance is the main contributor to resistance mechanism as they evade most substrates as well as broad types of antibiotics [10]. The ability of *K. pneumoniae* ATCC 700603 that was used in this study to remove antibiotics with and without the efflux pump inhibitors, of phenylalanyl arginyl β-naphthylamide (PaβN) were evaluated and compared with Laudy et al. [9]. A basic *in vitro* screening for the potential of Schiff base compounds as efflux pump inhibitor against *K. pneumoniae* ATCC 700603 was also done using resazurin microtiter-based assay as described by Laudy et al. [9] with some modification. The selection of antibiotics was based on the determination of Minimum Inhibition Concentration (MIC) and Sub-MIC as described in the method section. The results (Figure 1) showed rifampicin recorded MIC values ≥ 4 µg/mL, blue color in the well A4 (4 µg/mL), chloramphenicol and nalidixic acid showed MIC values ≥ 30 µg/mL, blue color in the well B4 and C4 (40 µg/mL) respectively, while tetracycline recorded MIC values ≥ 16 µg/mL, blue color in

the well D2 (20 µg/mL). Thus, sub-MIC of chloramphenicol (20 µg/mL) and nalidixic acid (20 µg/mL) were selected as positive control in an assay for screening the potential of Schiff base ligands as EPI against *K. pneumoniae* ATCC 700603, where many studies have shown that sub-MICs of antibiotics can act as signal molecules and may alter their physicochemical characteristics as well as the expression of bacterial virulence [6].

The efflux pump inhibitor activity of 40 Schiff base compounds against *K. pneumoniae* ATCC 700603 in the presence of sub-MIC chloramphenicol (20 µg/ml) in Figure 3 showed all compounds exhibited potential inhibitor activity. The highest activity was shown by L30 compound known as Co4[OVan(Me)MPD] (L30) at 86.5% inhibition. In general, metal complexed compounds displayed better inhibition activity in the presence of chloramphenicol compared to the parent ligand.

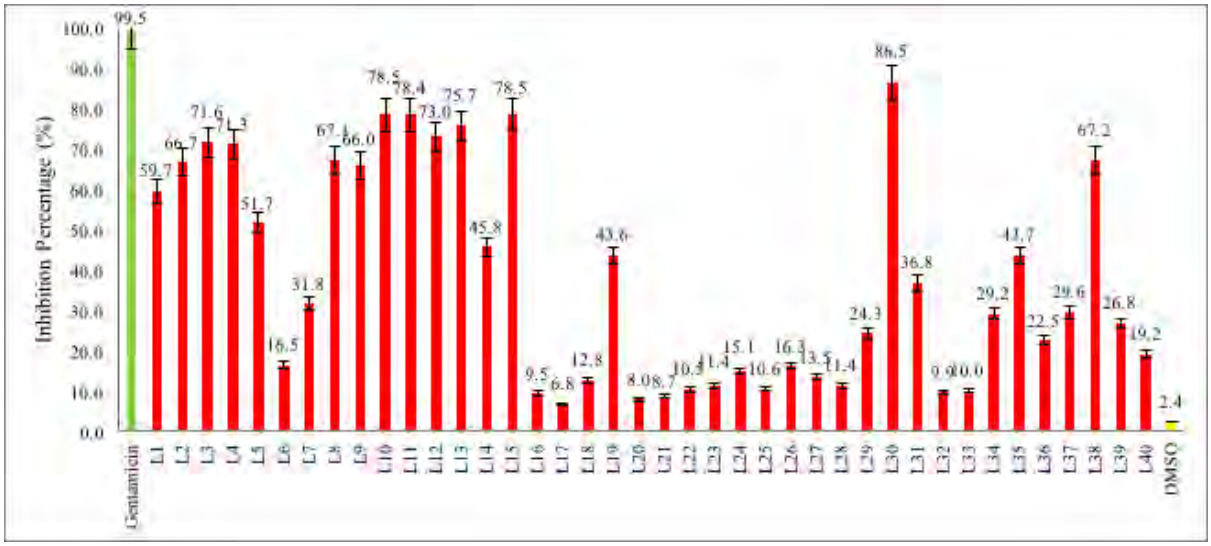

Figure 3: The Efflux Pump Inhibitor Activity of Schiff Base Compounds against K. pneumoniae ATCC 700603 Treated with Sub MIC of Chloramphenicol (20 µg/ml)

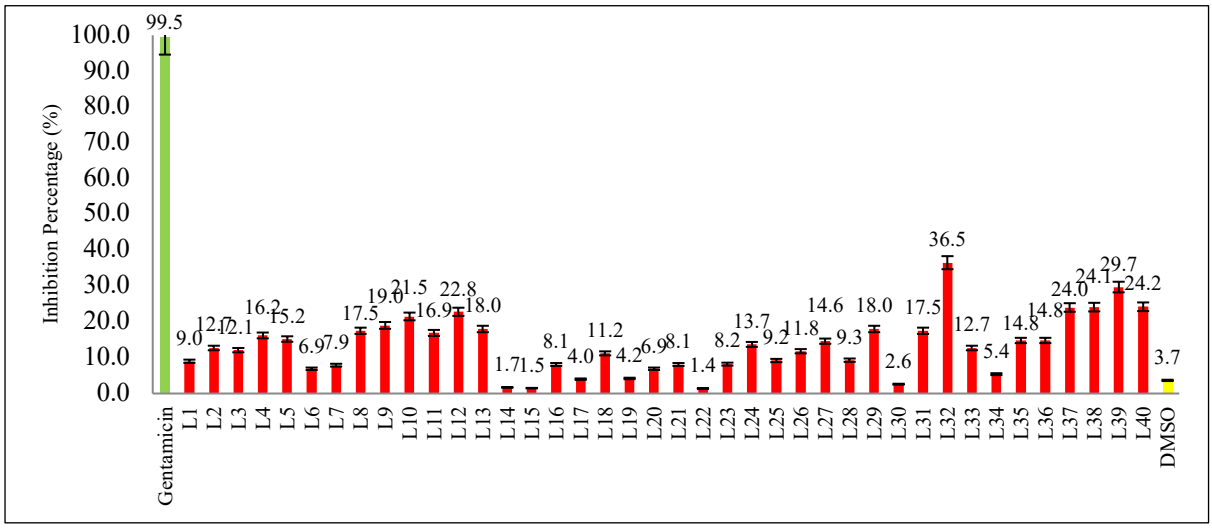

Figure 4: The Efflux Pump Inhibitor Activity of Schiff Base Compounds against K. pneumoniae ATCC 700603 Treated with Sub MIC of Nalidixic Acid (20 µg/ml)

The screening for efflux pump inhibition activity using sub-MIC of nalidixic acid (20 µg/ml) showed 36 complexes exhibited low inhibition with the highest activity was at 36.5% by OVan(Cl)MPD complexes (L32) and the lowest inhibition was at 5.4% by Ni2[OVan(Cl)MPD] (L34). These metal complexes disturb the respiration process of the cell and thus block the synthesis of proteins, which restricts further growth

of the organism. The variation in the activity of different complexes against different organisms depend on the impermeability of microbial cell wall against the compound [10].

4.0 CONCLUSION

This study demonstrated that tetranuclear complexes, Co_4(OVanMPD) showed the highest efflux pump inhibition (86.5%) when treated with sub-MIC of chloramphenicol (20 µg/mL) treatment. The highest inhibition in the presence of sub-MIC of nalidixic acid (20 µg/mL) was demonstrated by OVan(Cl)MPD at 36.5% against *K. pneumoniae* ATCC 700603. In general, metal complexes displayed to be more effective than their parent ligands due to the effect of the metal ions towards the cell's membrane. As such, a unique Co_4(OVanMPD); a tetranuclear complexes of Schiff base ligands derived from phenylenediamine and its metal complexes, possess strong potential for the future development of novel and broadly active efflux pump inhibitors targeting multidrug resistant bacterial infections.

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Bioactive Microbial Metabolites from Malaysian Rainforest Soil Fungi

Siti Hajar Sadiran[1*], Sadia Sultan[2], Kalavathy Ramasamy[2] Jean-Frédéric Faizal Weber[1,2,3]

[1]Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns)
[2]Faculty of Pharmacy, Universiti Teknologi MARA Selangor, Puncak Alam Campus, 42300 Kuala Selangor, Selangor, Malaysia.
[3]Laboratoire de Pharmacognosie, UFR Sciences Pharmaceutiques, Université de Bordeaux, 146 rue Léo Saignat, 33076 BORDEAUX cedex, France.

*hajarsadiran@uitm.edu.my

ABSTRACT

Microorganisms, especially soil fungi, are good sources of biologically active compounds. Twelve soil samples were collected from Sungkai (Perak), Pangkor Island (Perak), and Taman Negara (Pahang) and treated with phenol in order to select those fungal strains that would resist to that toxic compound. As a result, 91 strains were isolated and grown on a potato dextrose agar media at 28ºC. Their cultures were extracted with ethyl acetate and assayed for antimicrobial activity by disk diffusion assay against human pathogenic bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*), and fungi (*Aspergillus niger* and *Candida albicans*). Extract SHSF (1 mg/mL) showed remarkable activity with inhibition to almost all target organisms except *E. coli.* For example, its activity was similar to that of gentamicin (19 mm) when tested against *Bacillus subtilis.* The extract was then fractionated by HPLC. Twenty-five 0.5 ml-fractions were collected, dried under reduced pressure and subjected to the antimicrobial test. Three of these fractions showed significant activity in three out of five test microorganisms namely *S. aureus*, *A. niger* and *C. albicans*. A morphological study of the SHSF was carried out by scanning electron microscope (SEM). It is clearly appear as belonging to the genus *Aspergillus.* The result from internal transcribed spacer (ITS) showed that SHSF have 100% similarity with *Aspergillus longivesica*.

Keywords: Soil fungi; *Aspergillus longivesica*; antimicrobial activity

1.0 INTRODUCTION

Fungi are proven to be a rich source of antibiotics. Beyond antibiotics, fungi provided a number of drugs that are part of our current therapeutical tools, such as antiparasitic, antitumor, and hypocholesterolemic agents such as lovastatin. Recently, although there is strong competition with synthetic products, natural products, especially from microbes, remain the most promising source of novel bioactive compounds. Microbial metabolites derived from secondary metabolism of microorganism that evolved in nature in response to needs and challenges of the natural environment. An obvious explanation is that microbial products, primarily meant for the defense against competitors, may mimic metabolites of a variety of living systems, including mammal. They have drug-like properties, and as a result, it requires minimal modification to develop an effective, orally active and easily marketable product. Indeed, ascomycetes such as *Aspergillus, Penicillium,* and *Fusarium* are the most frequent producers of bioactive compounds among the fungal species [1].

In addition, a large number of compounds isolated from soil fungi show potent biological activity, which can be exploited as antibacterial, antifungal and antitumor in drug discovery programs. As an example, it was reported that rubratoxin B was isolated from fermentation of soil fungus *Penicillium purpurogenum* collected from Shanxi province, China [2], as well as from *Penicillium rubrum*. Rubratoxin B was found to show cytotoxic activity on human fibrosarcoma cell (HT 1080) [3].

Soil microorganisms from Malaysian forests could become a source of choice for the identification of new lead compounds in view of drug discovery. Furthermore, fungi isolated from ordinary surrounding such as soil, and plant (endophytic fungi) have been the source of wide range of bioactive natural products. Drugs derived from fungal origin mainly comes from fungi from temperate climate countries. However, tropical regions offer a much larger wealth of biotopes and biodiversity. Yet, very little work has been carried out on tropical microbes in general. When it comes to Malaysia, the microbial diversity either of terrestrial or marine origin is virtually unexplored.

2.0 MATERIALS AND METHODS

Twelve soil samples fungi were collected from Sungkai (Perak), Pangkor Island (Perak), and Taman Negara (Pahang). These samples were collected at the surface of the ground and at the riverbank using sterilized spatula. Soil fungi were isolated using phenol treatment method, a method modified from the acetic acid and ethanol treatment by Furuya and Naito [4]. The samples were treated by 5% of phenol solution before being rinsed twice with sterile distilled water. The suspension of treated soil was pipetted into 10 cm Petri dish. 15 mL of molten potato dextrose agar (PDA) with antibiotic was dispensed into the Petri dish over the soil suspension and agitated to disperse the soil particle evenly before being incubated at 28°C. The Petri dishes were observed every 24 hours and growing colonies were then sub-cultured into new PDA plates until purity was achieved.

Pure cultures were incubated for 14 days for the production of secondary metabolites before being extracted with ethyl acetate. The extracts were analyzed using a HPLC Agilent 1200 series system, comprising a quaternary pump, an online degasser, an automatic sample injector, a temperature column compartment, a diode array detector and a fraction collector fitted with up to 4 deep-well microtiter plates. The standard procedure that was developed and used throughout this work includes the following conditions: All analyses and separations were carried out in a reverse phase mode, using a Synergy 4µ Hydro-RP 80Å column (150 × 4.6 mm, 4 µm particle size, Phenomenex®, USA) with a guard column. The column temperature was maintained at 36°C. The DAD collected the full UV spectrum (190 nm to 600 nm) and was set to display the absorbance at the following wavelength: 220 nm, 254 nm, 280 nm, and 360 nm. The mobile phase consisted of purified water (solvent A) and acetonitrile (solvent B). The separations were carried out at flow rate of 1 mL/min with the following elution gradient: 0 min 10% B, 10 min 46% B, 14 min 70% B, and 20 min 100% B. Aliquot of 10 µL of each extract were injected in order to record their chromatographic profiles. 30 µl of aliquot were then injected two times consecutively for collection of fractions. Fractions were collected every 0.5 to 28 minutes into the 2-ml 96-deep well plate (polypropylene) and then dried using a Genevac EZ-2 centrifugal evaporation system.

All fungal extracts were subjected to antimicrobial assay screening by disk diffusion assay. The protocol used for this assay is adapted from Bauer *et al*. [5] with slight modifications. Those extracts showing significant activity had their fractions individually tested by as follows: Their fractions were transferred into the wells of a 96-well microtiter plate containing a bacterial suspension. The plate was incubated for 24 hours, and the turbidity was measured at 620 nm. This allowed to identify which fraction was responsible for the biological activity. The morphological study of the fungus was carried out by SEM and the molecular study by using internal transcribed spacer (ITS).

3.0 RESULTS AND DISCUSSION

Ninety-one fungi were isolated from 12 soil samples from three different places in Malaysia. Based on the screening results, 12 fungi were selected for disk diffusion assay and 4 of them were subjected for antimicrobial assay of HPLC fractions. Here, we wish to report more specifically on fungus SHSF which were obtained from the Sungkai soil sample. In disk diffusion assay, extract SHSF showed good

antimicrobial activity with inhibition to almost all target organisms except *E. coli*. The inhibition zone was similar to that of gentamicin (19 mm) when tested against *Bacillus subtilis.* The results of antimicrobial assay of HPLC fractions showed that only three fractions with growth inhibition of 92% and above being observed. The chromatographic peaks corresponding to these active fractions are shown in Figure 1. These fractions were active against *Staphylococcus aureus, Candida albicans* and *Aspergillus niger.*

Morphologically, this fungus forms fast growing colonies white to cream in color, with grayish blue-green conidial heads in 14 days culture. SEM examination (Figure 2) showed clearly the presence of conidial head, typical of the genus Aspergillus. The result from internal transcribed spacer (ITS) showed that SHSF have 100% similarity with *Aspergillus longivesica*. This species was first isolated from rainforest soil in Nigeria [6]. This fungus is white to cream in color and have grayish blue-green conidial in 14 days cultures. It was reported to produce patulin, tryptoquivalone, tryptoquivalines, antafumicins, and pyripyropens [7]. Identification of the active compounds is in process.

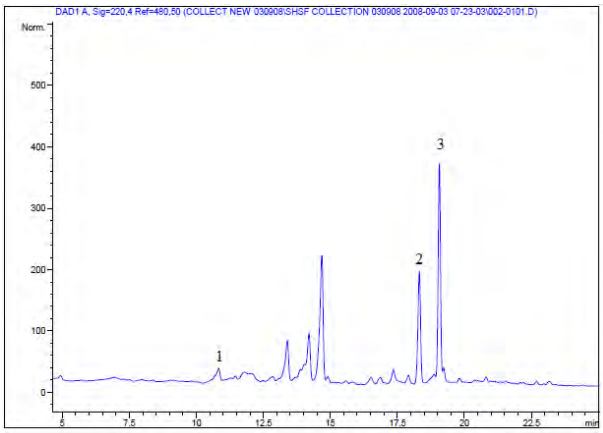

Figure 1: Chromatogram of extract SHSF. The active peaks were labelled as 1,2 and 3

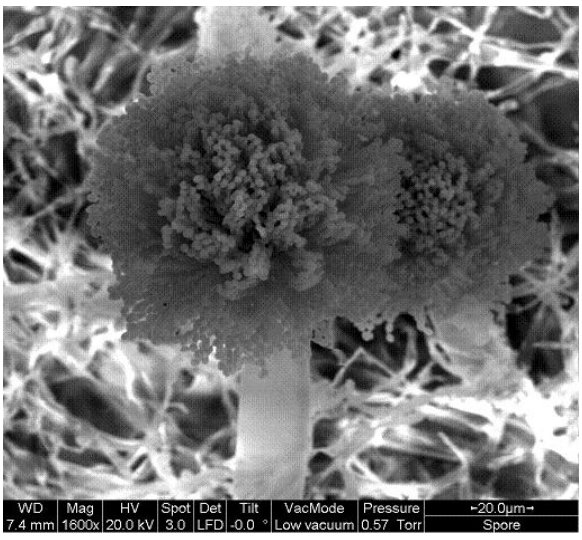

Figure 2: Fungus SHSF observed under SEM

4.0 CONCLUSION

Soil fungi were successfully isolated using phenol treatment and it was demonstrated that phenol treatment was an effective method to select manageable number of Ascomycetes from soils. From this work, it was revealed that Malaysian soil fungi especially *Aspergillus longivesica*, SHSF showed remarkable antimicrobial activity against *A. niger, C. albicans and S. aureus.*

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