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Molecular Docking and Simulation of Transketolase from *Orthosiphon Stamineus*

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ABSTRACT

Orthosiphon stamineus, locally known as Misai kucing is a popular traditional medicinal plant and employed as folk medicine to treat various ailments in Southeast Asia. The pharmacological properties of the leaves have been reported including diuretic, antidiabetic, antihypertensive and so forth. Hence, the plant is commercialized as tea product and launched in the market aiming to benefit human health. Earlier proteome profiling of *O. staminues* leaves identified a multifunctional protein, transketolase. From the literature, transketolase require thiamine diphosphate for catalytic activity and believed that transketolase plays crucial role in prevention of cardiovascular, neurological and diabetes diseases. We generated 3D model of transketolase using I-TASSER and validated followed by docking with thiamine diphosphate using Autodock Vina to identify the key residues in the binding sites and binding mode and further proceeded to simulation at 37°C, 95°C for 50 ns using GROMACS. Docking result elucidated that thiamine diphosphate bound to transketolase through several interactions with residue Gln237, Ser242, Cys246, Arg283 and Phe284. MD simulation result revealed that the protein-ligand complex is stable with reasonable flexibility and compactness based on RMSD, RMSF and radius of gyration at both temperatures.

Keywords: *Orthosiphon stamineus*, transketolase, homology modelling, molecular docking, molecular dynamics simulation

1. INTRODUCTION

Cardiovascular diseases, heart failure and diabetes are the major causes of mortality in people nowadays. There are many drugs produced and available in the market in order to cure these diseases. Nevertheless, the drugs cannot reach the adequate level to control those diseases in patients and may cause side effects on patients in long term [1]. In recent years, computer-aided drug design became an alternative approach to produce drugs with higher effectiveness as it can accelerate new drug discovery and cheaper production. Until now, there are a few computer-aided drug design (CADD) molecules gaining approval from U.S Food and Drug Administration (FDA) and reached clinical stage of drug development [2] using computational methods such as molecular docking and molecular dynamics (MD) simulation. 11β-HSD1 has been identified as an inhibitor to treat diabetes as it can inhibit the production of active cortisol [3] since its presence can prevent insulin secretion from pancreatic-beta cells [4].

Orthosiphon stamineus from the Lamiaceae family is a well-known herb in South East Asia countries and have been extensively used as folk medicine to cure various ailments such as diabetes and arthritis. Moreover, its pharmacological activities have also been proven including antidiabetic, antioxidant, antihypertensive, anti-inflammatory, diuretic and hepatoprotective activities [5]. Meanwhile, transketolase (TKT) is one of the proteins identified from the plant and believed to be a new drug source to prevent certain diseases such as hyperglycemia due to its role in the biochemical reaction. TKT is a thiamine-diphosphate (TPP) dependent enzyme involved in pentose phosphate pathway. Malfunction of TKT highly affect the cardiovascular and nervous system since the cells in both system is very sensitive to deficiency of TKT [6]. Proper-functioning TKT is possible to ensure antidiabetic activities by

inhibiting three pathways associated with the pathogenesis of hyperglycemia and able to prevent diabetic retinopathy in diabetic rats [7]. Deficiency of TKT will also cause short stature, developmental delay and congenital heart defects [8]. Moreover, recent studies highlighted that engineered *E. coli* TKT has the ability to convert L-arabinose in sugar beet pulp into L-*gluco*-heptulose which posseses potential therapeutic value in cancer and hypoglycaemia [9]. Therefore, TKT is a potential therapeutic molecule that can be used to prevent certain ailments such as diabetes and heart diseases. To date, even though TKT from maize, human, bacteria and fungi have been reported, there are no work that provides insight about the structure and the behaviour of TKT from *O. stamineus* at different temperature that could serve as a new therapeutic agent to prevent disease such as diabetes. Hence, three-dimensional (3D) model of TKT from *O. stamineus* was constructed followed by docking with thiamine diphosphate which is crucial for TKT to perform catalytic activity. Simulation of the docked structure provides the key residues in the binding sites and reveals the structural properties to further understand the function and biological activities of TKT. We hope that the insights obtained will be useful to design new drug for diabetes patients and accelerate the drug discovery process.

2. MATERIALS AND METHODS

2.1 Homology Modeling and Evaluation

The peptide sequences of TKT identified from LC-MS/MS matched and replaced in the sequence. The 3D model of TKT was constructed via I-TASSER server by homology modelling and validated using several evaluation methods such as ERRAT, PROCHECK and VERIFY3D.

2.2 Molecular docking

AutoDockTools (ADT) 1.5.6 were used to prepare the input file for the protein and ligand, thiamine diphosphate (PubChem ID: 1132) and followed by molecular docking using Autodock Vina. The binding model with the lowest binding energy was selected and analyzed based on different interactions and the orientation of the ligand in the binding pocket.

2.3 Molecular Dynamics Simulation

The docked protein-ligand complex was used to perform MD simulation using GROMACS 5.0.4 packages with GROMOS96 54a7 force field [10] at 310 K and 368 K for 50 ns. MD trajectories were analyzed to determine root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg) and hydrogen bond distribution for the system. Salt bridge analysis was also performed using ESBRI program.

3. RESULTS AND DISCUSSION

3.1 Homology modeling and evaluation

TKT from *O. stamineus* was successfully generated using I-TASSER server through homology modeling as shown in Figure 1. The generated 3D model was proceeded to the secondary structure analysis followed by model validation. The secondary structure analysis of TKT was performed using YASARA program and revealed that TKT consists of 40.0% helix, 11.2% sheet, 16.1% turn, 31.3% coil and 1.4% 3_{10} helix. Whilst, the result obtained from several model validation methods such as PROCHECK-97.5% in allowed regions, 2.5% in disallowed regions, ERRAT-86.44%, VERIFY3D-94.24% indicated that the constructed TKT model is in good quality.

Figure 1. Predicted TKT structure of *O. stamineus* by I-TASSER.

3.2 Molecular docking

In order to find the binding affinities and key interaction of TKT with the ligand, Autodock Vina was used to dock the TPP into the TKT protein model as many diseases such as brain disease, heart disease[6] and diabetes [11] are closely related to lack of functional TKT with the loss of its catalytic properties. Figure 2 showed the docking mode between TKT and its ligand. Gln237, Ser242, Cys246, Arg283 and Phe284 made up the ligand binding pocket from TKT through several interactions including two hydrogen bonds (2.04Å; 3.02Å), a π-sulfur contact (4.74Å); a π-π stacking contact (4.60Å) and a π-alkyl contact (5.05Å) with thiazolium ring of the TPP.

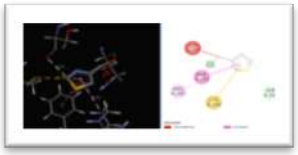

Figure 2. Docking mode of transketolase with its ligand, thiamine diphosphate.

3.3 MD simulation

TKT-TPP complex has RMSD value of about 0.37 nm and 0.7 nm at 310 K and 368 K respectively (Figure 3a). Higher RMSD value indicates the protein undergoes more structural changes and has lower stability. Even though the complex has higher RMSD value at 368 K, the complex is still considered as stable as the RMSD value is lower than 1 and the value was within similar range until end of simulation. From RMSF graph as shown in Figure 3b, higher peak and more fluctuations at 368 K with total of eight loops combined were detected; four loops at 310 K and another four loops at 368 K. Only one loop labelled as L3 and L6 are located at the same protein region at both temperatures. This may indicate that the arrangement of amino acid located at the loop does not change much even when exposed to high temperature and suggests that the function may not be affected. Besides, the curve showed no significant fluctuation at the ligand-binding site of the protein at 368 K which may elucidated that the protein-ligand interaction is static and probably not disrupted at elevated temperature. The compactness of the TKT-TPP complex was considered as low with high graph reading which is about 2.9 nm (Figure 3c) and do not change much at elevated temperature. The average numbers of hydrogen bonds are 562 and 550 for the 310 K and 368 K simulations respectively and shown in Figure 3d. There is only a slight difference in hydrogen bond number between 310 K and 368 K indicating the protein conformation has not change much and the protein function may remain intact. Salt bridge is one of the crucial factors that contributed to protein stability [12] which can be affected even if one salt bridge is disturbed. There are a total of 258 and 295 salt bridges detected at 310 K and 368 K respectively. Additional 37 salt bridges formed at 368 K may play a critical role to maintain protein stability. This finding is supported by Elcock where he mentioned that there are more salt bridges detected in hyperthermophilic proteins compared to mesophilic proteins [13].

Figure 3. a)RMSD graph of TKT versus time at both 310 K and 368 K. b)Graph of RMSF versus residue for ligand-bound transketolase at 310 K and 368 K. c)The radius of gyration graph versus time (50 ns) for ligand-bounded transketolase at 310 K and 368 K. d)The graph of number of hydrogen bond versus time for thiamine diphosphate-bounded transketolase at 310 K and 368 K. Black line=310 K, Red line=368 K.

4. CONCLUSION

The TKT model from *O. stamineus* was successfully constructed and docked with TPP. Gln237, Ser242, Cys246, Arg283 and Phe284 residues are determined as the possible ligand binding sites and the TKT-TPP complex is stable with reasonable flexibility and compactness at both 310 K and 368 K which means that this complex can withstand high thermal stress condition. These results are expected to provide some useful insights to design new therapeutic molecule for diabetes and accelerate the drug discovery process. For future works, it is interesting to make comparison between *O. stamineus* TKT and TKT from other species to find out the speciality of *O. stamineus* TKT.

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Full Factorial Design Approach for Formulation of Flexible Liposomes Loaded *Labisia Pumila*: A Storage Stability Study of the Optimized Formulation

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ABSTRACT

Labisia pumila (*Lp*), is a medicinal herb contained high antioxidant and phenol compound. Due to its high antioxidant properties, *Lp* extract is beneficial for anti-aging and could be applied topically. In this study, *Lp* was encapsulated into Flexible liposomes (FlexLipo) to protect its stability which comprises lecithin, β-sitosterol and surfactants as an edge activator. The present work focus on the design of FlexLipo-*Lp* nanocarrier and its storage stability. A 2^3 full factorial design was used to investigate the interaction of FlexLipo formulation and five parameters on the particle size, zeta potential, polydispersity index, elasticity and encapsulation efficiency. Results indicated that the particle size, zeta potential and polydispersity index affect the interaction among the factors. The final optimized nanocarrier formulation was obtained and used to study its stability during storage. The important outcomes of this research revealed that the formulation prepared from the design demonstrated a stable structure, high encapsulation efficiency and desired particle size. It could be concluded that Flexible liposomes loaded *Labisia pumila* are a new strategy in herbal transdermal delivery.

***Keywords*:** Full-factorial design, *Labisia pumila*, flexible liposomes, nanoparticles, stability.

1. INTRODUCTION

Flexible liposomes are known as deformable liposomes, transfersomes or elastic liposomes consist of phospholipid along with surfactant as an edge activator. Edge activators can increase the fluidity of bilayers in the stratum corneum by lowering the interfacial tension of the bilayer, thus enhancing the permeability of encapsulated active ingredients into the skin [1]. Mostly, a single chain surfactant was used such as SPAN 80, TWEEN 80 and sodium deoxycholate.

Labisia pumila (*Lp*) is also known as Kacip Fatimah (KF) was traditionally used in post –partum treatment to enhance energy and get stronger after giving birth [2, 3]. The extract contained estrogen – liked substituent which is believed will assist collagen synthesis and has strong antioxidant activity comparing ascorbic acid [4].They also indicated that *Lp* extract may have great potential to be developed as novel anti – inflammatory drug. Materials having anti-inflammatory efficacy could be used not only for inflammatory skin conditions but also as soothing ingredients for sensitive skins. In consequence, *Lp* extract shows a great potential as an active ingredient for an anti-aging cosmetic that is suitable for any types of skin.

In order to enhance the stability of the nanoparticles and its permeability in transdermal delivery, a stable formulation is crucial. A 2^3 full factorial design was applied to develop a stable formulation for FlexLipo loaded *Labisia pumila*. The formulation was optimized by the application of the design to evaluate the major interaction among the factors on the characteristics of FlexLipo-*Lp* such as encapsulation efficiency, zeta potential, particle size, deformability and polydispersity index. Furthermore, the

optimized formulation was evaluated in terms of its storage stability. This approach is to be known much more reliable as it is based on the mathematical model of the combined effects of the processing factors.

2. MATERIALS AND METHODS

2.1 Formulation of Flexible Liposomes using 2^3 Full Factorial Design

Formulation of flexible liposomes was developed by employing two level full factorial design using Design Expert (version 6.1). A total of 32 experiments were generated by Design Expert software to evaluate the interaction effect of L-α-phosphatidylcholine (PC), β-sitosterol, surfactants and time of sonication in the formulation. Table 1 defines the five adjustable factors and responses for the formulation of flexible liposomes.

Table 1: Experimental parameters for 2^3 Full Factorial Design for Flexible liposomes formulations.

Independent variables	Coded unit	Low (-1)	High (+1)
Phosphatidylcholine	X_1	250mg	500mg
B-sitosterol	X_2	50mg	100mg
Tween 80	X_3	12.97µl	39.95µl
Span 80	X_4	17.52µl	49.07µl
Time of sonication	X_5	3 minutes	7 minutes
Dependent variables		Unit	
Encapsulation efficiency	Y_1	%	
Particle size	Y_2	d.nm	
Zeta potential	Y_3	mV	
Polydispersity index	Y_4		
Deformable index	Y_5		

The assumption of linearity in the factor effect was expressed using regression equation with five parameters and the interaction can be given by the following equation;

$$Y_i = b_0 + b_1X_{1i} + b_2X_{2i} + b_3X_{3i} + b_4X_{4i} + b_5X_{5i} \ldots + b_{12345}X_{1i}X_{2i}X_{3i}X_{4i}X_{5i} \quad \text{(Eq. 1)}$$

Where Y_i is the response, X_{ji} values j = 1, 2, 3, 4, 5 and i = 1, 2, 3, 4, 5 indicated the corresponding parameters; b_0 is the average value of the result, b_1, b_2, b_3, b_4 and b_5 are the linear coefficient; and b_{12}, b_{123}, b_{1234}, b_{12345} represent the interaction coefficient [5]. Optimised formulation was validated and analysis of variance (ANOVA) was used to analyse the results.

2.2 Preparation of Flexible liposomes

Flexible liposomes were prepared by a standard thin –film hydration method. Chloroform and ethanol (1:1) were used as a solvent to produce lipid thin film. β -sitosterol was dissolved in ethanol [6] prior mixed with chloroform. L – α – phosphatidylcholine was dissolved in chloroform and followed by TWEEN 80 and SPAN 80 as an edge activator to enhance the elasticity of the vesicles. *Labisia pumila* extract was dissolved in a hydration medium using phosphate buffer solution (PBS), 0.1mM at 6.8 pH before added to the dried thin film. After complete hydration, the suspension then sonicated using probe

sonicator at 30% amplitude to obtain smaller vesicles size. The sample was respectively stored at 4°C for analysis.

2.3 Characterization of the nanoparticles and Stability Study

Average particle size, zeta potential (ζ) and polydispersity index (PDI) were measured using Zetasizer Nano ZS (Malvern, United Kingdom). A comparative measurement of elasticity for flexible liposomes and liposomes were carried out using extrusion method [7]. To analyse the stability of the nanoparticles, the samples were stored at different temperature (4°C, 25°C) and the physical stability was examined.

2.4 Encapsulation Efficiency and Leakage Rate

Freshly prepared flexible liposomes encapsulated *Labisia pumila* was separated from unentrapped extracts using mini-column centrifuge method [8,9]. The released *Labisia pumila* extracts were assayed using Total Phenolic Content (TPC). The TPC was expressed as Gallic acid equivalent (GAE) in mg of dry weight *Labisia pumila* extract. All assays were done in triplicate [10]. The amount of entrapment extract and leakage ratio were expressed in % and calculated from the following equation:

$$\text{Encapsulation Efficiency (EE) \%} = (\text{Encapsulated extract} / \text{Total extract}) \times 100 \quad \text{(Eq.2)}$$

$$\text{Leakage ratio} = (1 - LE_{\text{during storage}} / LE_{\text{before storage}}) \times 100\% \quad \text{(Eq.3)}$$

**LE = Loading efficiency*

3. RESULTS AND DISCUSSION

The accuracy and the convenient of the model was expressed by the determination of coefficient value (R^2). The R^2 value of the model was calculated, indicating that the developed mathematical equation defined the model as shown in Table 2. The formulation of FlexLipo-*Lp* affected Y_1, Y_2 and Y_3 statistically.

Table 2: Results of regression analysis of variance for all dependent variables.

	Y_1	Y_2	Y_3	Y_4	Y_5
F	4.03	26.95	379.35	2.15	3.92
P value	0.0446	0.0028	0.0406	0.0728*	0.1429*
R^2	0.9438	0.9945	0.9999	0.6966	0.9734
Adjusted R^2	0.7097	0.9576	0.9973	0.373	0.7252
Predicted R^2	0.5978	0.6501	0.91	0.3808	2.0262
Adeq. Precision	7.2940	23.2780	77.108	6.078	8.7592

*Y_4 and Y_5 models were considered statistically not significant where $p>0.05$.

The formulation was assessed for 120 days of storage stability and the formulation was observed not stable at 25°C after 120 days. Table 3 shows the storage stability outcomes of the storage stability of FlexLipo-*Lp*.

Table 3: Stability of Flexible liposomes loaded *Labisia pumila*

Parameters	At first day	After 120 days	
		4°C	25°C
Particles size	182.4 d.nm	111.2 d.nm	
Polydispersity index	0.216	0.217	
Zeta potential	-31.3	-31	Not Available
Encapsulation efficiency	87.12%	-	
Loading efficiency	-	68.36%	
Leakage ratio		21.5%	

It was observed that a leakage ratio during storage was 21.5% from the first day prepared, due to saturating of lipid compartment and aqueous compartment with the active ingredients. The low polydispersity index (0.216) confirmed the homogeneity of developed nanocarrier and it was stable throughout the storage.

4. CONCLUSION

According to ANOVA results, the formulation of FlexLipo-*Lp* was designated as the optimum formulation due to its physiochemical properties. This research demonstrated that the optimum FlexLipo-*Lp* nanoparticles designed in this study may be considered for transdermal delivery application. To evaluate the effectiveness of this nanocarrier, further studies are strongly required to identify its pharmacological properties.

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Assesment on the Demographic Data and Risk Factors of Oral Squamous Cell Carcinoma Patients in Tertiary Care Setting

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ABSTRACT

The epidemiological study on oral squamous cell carcinoma (OSCC) differs significantly among continents. Smoking, betel quid chewing and alcohol consumption are the most important risk factors. This is a hospital based retrospective study. Medical record of 55 confirmed OSCC patients was reviewed. The mean age of OSCC patients was 58 years old (SD 14.87) with female predominates. Patients of Indian ethnicity were the highest associated with OSCC. Betel quid chewing was the major risk factor for the development of OSCC followed by alcohol consumption and smoking. The most common site of tumor was in buccal mucosa region, followed by tongue, gingiva, floor of the mouth, palate region and lip. Majority of the patients (45.5%) was diagnosed at stage IV. There was correlation between age and stage of cancer, $p<0.005$. No significant correlation ($p> 0.05$) was noted between two variables among age, gender, ethnic, habits, staging, site of tumor, history of head and neck cancer and family history of cancer. OSCC was predominantly found in elderly female with habits of betel quid chewing. It was found within buccal region and the majority of the cases presented were at advanced stage.

Keywords: Demographic data, risk factor, oral cancer, oral squamous cell carcinoma.

1. INTRODUCTION

Etiologically, oral carcinoma may arise afresh or from different pre-malignant lesions with different malignancy potentials likes leukoplakia, erythroplakia or a mix of the two (erythroleukoplakia) with an ulcer (Yardimci *et al.*, 2014). Despite the vital role played by genetic factors, oral carcinoma is tremendously influenced by environmental and behavioural risk factors (Antunes *et al.*, 2013). Chronic exposure by carcinogens like tobacco or alcohol will inactivating tumor suppressor genes and causing over expression of oncogenes (Kerawala *et al.*, 2016, Sousa *et al.*, 2014). In addition, betel quid chewing, chronic exposure to sunlight and human papillomavirus are among well-known risk factor for oral squamous cell carcinoma (OSCC) (Kaminagakura *et al.*, 2012, Lee *et al.*, 2012, Shenoi *et al.*, 2012, Vargas-Ferreira *et al.*, 2012). OSCC is an invasive lesion with the manifestation of perineural growth. Approximately, 90% of epithelial malignancy found within oral mucosa is squamous cell carcinoma (SCC) (Paul, 2012). It is one of the most difficult malignancies to control and has been associated with poor prognosis that may be explained by frequent lymph node metastases and local invasion characteristic (Noguti *et al.*, 2012).

According to the latest World Health Organization (WHO) data published in May 2014, oral cancer deaths in Malaysia reached 1,060 or 0.83% of total deaths (WHO, 2014). Malaysia population comprises many ethnic groups, the largest being the Malays followed by the Chinese, the indigenous peoples of Borneo and the Indians (Department of Statistics Malaysia, 2014). Of these ethnic, the highest prevalence

of oral cancer are Indians and indigenous groups. A traditional stimulant mixture of areca nut and/or tobacco with the betel leaf (Piper betel) is common in Malaysia found associated in these ethnic groups (Razak *et al.,* 2010). Among the Chinese and Malays, where mouth cancer is less prevalent, the major risk factors are tobacco smoking for Malays and alcohol consumption for Chinese (*e*cancer, 2010).
Above all, oral cancer is a preventable disease. The malignant lesions that occur in an anatomic region is easily accessed and reachable during physical examination. The lesion can be easily explored visually and tactilely (Sousa *et al.,* 2014). Hence, knowledge on the risk factors and lesion malignancy will aid health care professionals in determining the extent of the disease together with decision making in health policy.

2. MATERIALS AND METHOD

This is a hospital based retrospective study, conducted at Oral Cancer Research & Coordinating Centre (OCRCC) Universiti Malaya, from January 2006 to December 2014. Written informed consent was obtained from all participants prior to data collection. This study was approved by the Ethics Committee (Human) of Universiti Malaya (UM) [OI DF1601/0072 (P)]. All 55 patients were confirmed cases of OSCC by histopathological staining. Patients medical record were reviewed to obtain the information regarding the oral cancer status. This included medical history, age, gender, habits of tobacco ingestion, and alcohol intake, site of the primary tumor and size of tumor (TNM staging).

All data were analysed using SPSS version 22.0 (SPSS Inc, Chicago, IL, USA). Descriptive analysis was calculated and was expressed as mean and median. The correlation between categorical variables were analysed using chi-square test between the two variables, i.e., site to habits, staging to site involved, staging to history of the disease, staging to habits, and staging to age of the patient were done. The significant level was set at $p<0.05$ at 95% Confidence Interval (CI).

3. RESULTS

There were 55 cases of OSCC confirmed by biopsy from 2006 to 2015. Table 1 showed the demographic data of the OSCC patients. Thirty-four patients (61.8%) were females and 21 (38.2%) were males. The largest number of patients in the study were seen in the age group 51-60 (25.5%). The youngest of all patients affected was 20 years old and the oldest was 90 years old. The least number of patients were in the age group 20-30 (5.5%) and the mean age of OSCC patients was 58 years old. Indian ethnic dominates with 33 cases (60.0%) and the least were from Iban and Bidayuh ethnic.Table 2 showed the habits of OSCC patients. By looking at personal habits, most patients were betel quid chewer (47.3%). Both smoking and alcohol consumption represented 14 cases each (25.5%).
Table 3 showed the relationship and history of cancer. Among 55 OSCC cases, 11 (20.0%) of the patients have family history of cancer while four patients (7.3%) were previously diagnosed with head and neck cancer.Table 4 showed the site distribution of the lesions in oral cavity. The buccal mucosa was the most frequently involved site, accounting for 22 cases (40.0%), followed by tongue for 14 cases (25.5%). Gingiva was involved in 11 cases (20.0%) and floor of the mouth in 4 cases (7.3%) patients. The least site affected was in palate region represented only 2 cases (3.6%).Table 5 showed the staging of the tumor during diagnosis. Majority of patients, 25 (45.5%) were in stage IV. Fourteen patients (25.5%) presented in stage III. Nine patients (16.4%) were in stage I and seven (12.7%) in stage II.
Correlation using chi-square test between the two variables, i.e., site to habits, staging to site involved, staging to ethnic, staging to habits, and staging to age of the patient were done. All the above-said correlation was found to be statistically nonsignificant ($p>0.05$). Although, it was seen that patients with older age (>40 years old) were at diagnosed at stage IV [Table 6] and younger age (<40 years old) were diagnosed at stage I.

Table 1: Demographic data

Demographic data	No of patients	Percentage
Gender		
Male	21	38.2%
Female	34	61.8%
Age group (years)		
20-30	3	5.5%
31-40	5	9.1%
41-50	7	12.7%
51-60	14	25.5%
61-70	13	23.6%
> 70	13	23.6%
Ethnic		
Malay	10	18.2%
Chinese	10	18.2%
Indian	33	60.0%
Other	2	3.6%

Table 2: Personal habits

Habits	No of patients	Percentage
Smoking	14	25.5%
Alcohol consumption	14	25.5%
Betel quid chewing	26	47.3%
No habits	3	5.5%

Table 3: History of cancer

History	No of patients	Percentage
History of Head and neck cancer	4	7.3%
Family history of cancer	11	20.0%

Table 4: Primary site

Site	No of patients	Percentage
Gingiva	11	20.0%
Lip	1	1.8%
Buccal mucosa	22	40.0%
Floor of the mouth	4	7.3%
Tongue	14	25.5%
Palate	2	3.6%

Table 5: Staging

Staging	No of patients	Percentage
Stage I	9	16.4%
Stage II	7	12.7%
Stage III	14	25.5%

Stage IV	25	45.5%

Table 6: Correlation between the variable

		< 40		>40		p-value
		n	%	n	%	
Site	Buccal mucosa	2	3.6	20	36.4	0.349
	Gingiva	1	1.8	10	18.2	0.566
	Tongue	5	9.1	9	16.4	0.321
	Floor of the mouth	0	0	4	7.3	0.349
	Palate	0	0	2	3.6	0.552
	Lip	0	0	1	1.8	0.677
Habits	Smoking	4	7.3	12	22.2	0.159
	Alcohol	4	7.3	12	22.2	0.159
	Betel quid chewing	2	3.8	25	45.5	0.140
Stage	Stage I	5	9.1	4	7.3	0.000*
	Stage II	0	0	7	12.7	0.243
	Stage III	2	3.6	12	21.8	0.975
	Stage IV	1	1.8	24	43.6	0.043*
Cancer history	Previous head & neck cancer	1	1.8	3	5.5	0.538
	Family history of cancer	1	1.8	9	16.4	0.65

4. DISCUSSION

Of Asia continent, Bangladesh being the highest in prevalence of oral cancer (WHO, 2016). Early identification and prompt treatment are important as it pilot to early institution of therapy that translates in a better prognosis (Shenoi *et al.*, 2012). The OSCC cases in this study were found higher in female than male. Sex distribution or the male-to-female (M:F) ratio in the present study is 1:1.6. This is disagreement with the result of previous studies where males were more often affected than females because males were more likely indulge in major risk habits (Johnson *et al.*, 2011, Udeabor *et al.*, 2012). However, it should be noted that in Malaysia, with different ethnicity, the betel quid chewing habit is common among women. This present results showed that majority of the female patients are from Indian ethnic. Study by Razak et al, (2010) showed that betel quid, a traditional stimulant was common in Malaysia associated in these ethnic groups (Razak *et al.*, 2010). Betel-quid is a masticatory mixture combining the areca nut, betel leaf, slaked lime and locally varied flavorings. It is a the fourth most frequently consumed psychoactive substance worldwide. Moreover, our results were also supported as in study by Lee et al, (2012) which reported that in Malaysia, habits of chewing betel leaf are higher among female rather than male (Lee *et al.*, 2012).

According to Surveillance, Epidemiology, and End Results (SEER) database, the mean diagnosis age of oral cancer is 62 years while it is 52.07 years old in another study from Eastern India (Shenoi *et al.*, 2012, Chi *et al.*, 2015). Predictably in our study, the most affected age group was 51-60 years, youngest of all patients affected was 20-years old and the oldest was 90 years old. The mean age of patients of oral

cancer was found to be 58 years old. Like most cancer, oral cancer is disease of older age. It is diagnosed mostly in people above 40 years old (Silverman and Society, 2003). On the other hand, there are also trends nowadays where oral cancer was diagnosed in younger generation especially in developing countries (Llewellyn *et al.*, 2004). In this study 14% of OSCC patients were at age ≤ 40 years old. Our results are quite high showing significant value ($p<0.005$) compare to other reports (Komolmalai *et al.*, 2015, Udeabor *et al.*, 2012). The high cases in younger population in this country might due to the ease of getting alcohol, tobacco and its related products at very affordable prices at the grocery stores. Even though there are strict warning from the Health Ministry not to sell the tobacco product for teenagers <18 years old, yet many smuggled cigarettes are accessible to them (Hizlinda *et al.*, 2012). By looking at site of infection, our results are in agreement with de Camargo *et al.*, (2010) which reported that the common site affected is on buccal region (de Camargo Cancela *et al.*, 2010). Epidemiological studies have shown that the sites of occurrence for oral cancer differ widely (Kerawala *et al.*, 2016). It is stated that tumor site is related to specific risk factors (Warnakulasuriya, 2009, Johnson *et al.*, 2011). Tumors of the tongue and the floor of the mouth may be associated with excessive alcohol consumption and cigarette-smoking habits, whereas lesions on the buccal mucosa and gingiva may be related to tobacco/betel quid chewing habits (Johnson *et al.*, 2011, de Camargo Cancela *et al.*, 2010, Oo *et al.*, 2011, de Camargo Cancela *et al.*, 2012). Taken together, these factors could explain the high percentages of tumors on the buccal mucosa since OSCC population in this study have betel quid chewing habits. Second affected site were on tongue followed by gingiva. The least was on lip site. Majority of patients, 25 (45.5%) were in stage IV. Fourteen patients (25.5%) presented in stage III. Seven (12.7%) in stage II and nine patients (16.4%) were in stage I. There was delay in reporting and diagnosis of the cancer. The delay in diagnosis could be correlated to patient delay (in looking for professional care), professional delay (in reading a diagnosis), or both. Moreover, the time interval between the onset of symptoms and the start of treatment depends on various factors such as patient behavior, clinical course of the illness and the quality of the health services (Kerdpon and Sriplung, 2001, Esmaelbeigi *et al.*, 2014, Naseer *et al.*, 2016). In our study however, these factors were not analyzed yet no conclusion can be made.

Along with other factor, genetic also contribute to OSCC. In this study, 20% have family history of cancer and 7.3% previously diagnosed with other head and neck cancer. It is now established that up to 10% of all cancers have a strong hereditary component. There were study showing that, a clustering of oral cancer has been seen in certain ethnic groups, like Askenazi group in Israel which the incidence being double as compared to other Jewish population in that country. However, the basis of this genetic susceptibility is not well understood, as yet (Ram *et al.*, 2011).As conclusion, OSCC was predominantly found in elderly female with habits of betel quid chewing. It was commonly found within buccal region and the majority of the cases presented were at advanced stage IV.

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Characterization of Screen Printed Carbon Electrode Modified with Gold Nanoparticles and Cysteine

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ABSTRACT

The main purpose of this work was to characterize a screen printed carbon electrode (SPCE) modified with gold nanoparticles (AuNPs) and cysteine using cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), field emission scanning electron microscope (FESEM) and X-ray diffraction (XRD). The AuNPs-cysteine modified SPCE was characterized using electrochemical methods to study its reproducibility, stability, and estimated surface coverage of AuNPs-cysteine. FESEM and XRD were used to describe the topography and elemental information of the surface of SPCE after modification with AuNPs and cysteine. The cysteine/self-assembled AuNPs/thiourea/electrodeposited AuNPs/SPCE (ETSC) was selected as the optimum modified SPCE layer. The surface was smooth with uneven distribution of cysteine and AuNPs molecules deposited on the carbon surface of the SPCEs. The ETSC modified SPCE showed good reproducibility with an RSD of 4.31% for Ipc responses. The Rct was quite stable with the decrease to only about 73% of its original value after four weeks at room temperature. The SPCE has an estimated surface area of 0.1348 cm^2 after modifications with AuNPs and cysteine. The acceptable reproducibility and stability of the modified SPCEs resulted from the fabrication of SPCE using a combination of self-assembled monolayers (SAM) and electrodeposition of Au techniques. The results obtained during this research work suggest that the combination methods provide a good alternative to modify disposable SPCEs using AuNPs and cysteine for potential use as a biosensor for skin sensitizer detection.

***Keywords*:** cysteine; screen printed carbon electrode; cyclic voltammetry; impedance; gold nanoparticles.

1. INTRODUCTION

These days, the dependence on conventional three electrochemical cells and massive electrodes is unfavorable; instead, it is preferable to have quick, miniature, convenient, low-cost and disposable electrode systems. Screen-printed electrode (SPE) addresses the issue of cost viability, portability with simple and inexpensive analytical methods (Hayat & Marty, 2014). The great versatility presented by the SPEs may directly modify the composition with current or may just deposit the substances on the surface as demonstrated in recently published papers. Although the AuNPs-modified SPEs are already commercially available, it is not economical for the researcher to afford (Bernalte *et al.*, 2012). Besides, with the combination of SAM and electrodeposition method to modify the SPCE, it has a great stability and reproducibility effects.

Currently, the application of a SAM technique in the construction of SPCE has attracted considerable attention, as there are many advantages, such as ease of preparation, great stability, reproducibility, versatility and the possibility of incorporating different chemical functionalities to produce a high molecular order of monolayers. SAMs-modified with AuNPs is widely used in the fabrication of SPCE since excellent selectivity and sensitivity for studies of the electron transfer mechanism is exhibited (Pooi See *et al.*, 2011). Other than that, electrodeposition also is the most familiar that uses electric current to reduce cations of a desired material from a solution and coat that material as a thin film onto the conductive substrate surface. It is a simple technique and equivalent to the electroplating process (El-Deab *et al.*, 2009). In this work, the SAM and electrodeposition of AuNPs technique are combined to produce good reproducibility of modified SPCE.

AuNPs have been used increasingly in many electroanalytical systems to improve analytical selectivity and sensitivity due to their excellent biological compatibility, high conductivity, large surface area on the electrode and good catalytic properties to modify the SPCE. In the literature, many examples showed the electroanalysis of cysteine and related chemicals based on AuNPs (Pingarrón *et al*., 2008). The modification of electrode surfaces with AuNPs also has been proposed as an appealing approach for enhancing the electron transfer process (Pooi See *et al*. 2011).

A molecular level investigation of the various interactions of the SAM and electrodeposition forming the AuNPs molecule and cysteine structure is very important in this work. Therefore, to understand the packing density and distribution of SAMs and electrodeposition methods, various techniques including XRD and FESEM were investigated. Further, the electrochemical techniques like CV and EIS were used effectively to show that the dynamics of charge transfer at the electrochemical interface is strongly controlled by the electrode surface (Kulkarni *et al*., 2006).

In this work, SPCEs were modified with AuNPs and cysteine using SAM and electrodeposition methods. Cysteine is unable to immobilize directly to a working surface of SPCEs. Instead, AuNPs was used to immobilize on the working surface electrode and then cysteine was self-assembled on AuNPs (Pooi See *et al*., 2011). Cysteine is the amino acid that has a thiol group, which is a representative thiol linker that can bind to AuNPs (Abraham *et al.*, 2010). FESEM, XRD, CV and EIS analysis were conducted to characterize the AuNPs-cysteine modified SPCE. The combination of modified method of AuNPs and cysteine was determined to choose the best-modified layer that can be used for next experiment. The stability and reproducibility of the modified SPCE also was tested by using CV and EIS measurement. Besides, the estimation of surface area for AuNPs-cysteine modified SPCE has analysed using Randles-Sevcik equation for better results.

2. MATERIALS AND METHODS

2.1 Chemicals Reagents

Potassium ferricyanide ($K_3Fe(CN)_6$), gold chloride ($HAuCl_4$), cysteine ($C_3H_7NO_2S$), trisodium citrate dehydrate ($Na_3C_6H_5O_7$), and potassium chloride (KCl) was obtained from Sigma-Aldrich, Malaysia. Unless noted otherwise, all chemicals were used as received.

2.2 Modifying SPCE with AuNPs and Cysteine

Bare SPCEs was washed with 85% ethanol solution to remove excess dirt on the surface of the carbon-working electrode. SPCEs were modified with AuNPs using three methods. In the first method, 100 uL $HAuCl_4$ was added with $Na_3C_6H_5O_7$ solutions to reduce the size of the gold solution to become AuNPs. AuNPs solution was used to electrodeposit the SPCEs at +1.1V for the 60s. In the second method, 6µL 0.25mM thiourea was deposited onto the AuNPs modified SPCE. Next, AuNPs solution was deposited onto the layer consisting of AuNPs and thiourea. Thiourea functioned as a cross-linker between the two gold layers. In the third method, another layer of thiourea followed by AuNPs solution was added to the whole assembly. Lastly, 6uL of 50mM cysteine was dropped onto the AuNPs modified SPCEs and let to dry for several minutes in a petri dish under air flow. The summary of three methods is as shown in Figure 1. The CV was conducted and measured the Ipc respectively when the modified SPCEs immersed in 1mM of $K_3Fe(CN)_6$ in 0.1M KCl.

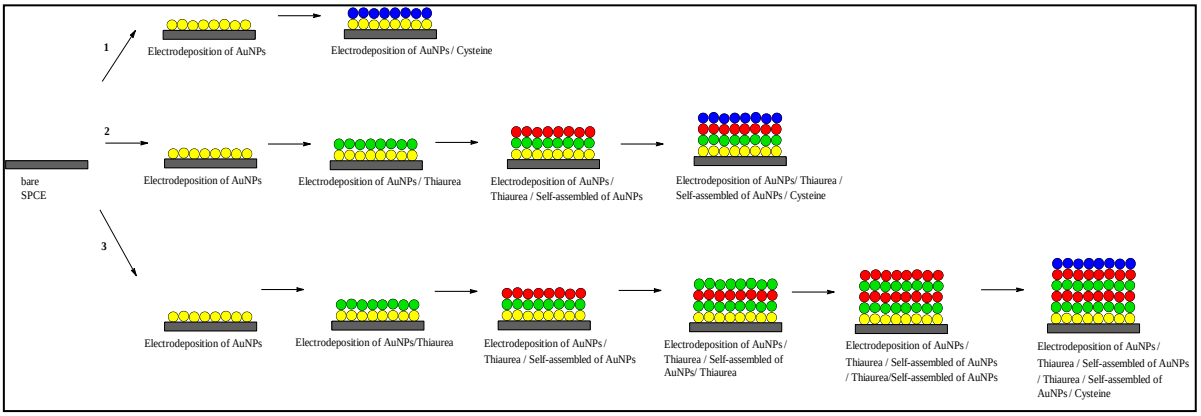

Figure 1 Summary of the modifications of SPCEs

2.3 Reproducibility and Stability of AuNPs-Cysteine Modified SPCE

The reproducibility of cysteine-AuNPs modified SPCEs was repeatedly prepared with ten pieces of SPCE to determine the stable measurement of Ipc (Dolati *et al*., 2011). The reproducibility was measured by CV, and calculated for RSD. For stability over storage condition and times, the AuNPs-cysteine modified SPCEs that was kept in the sealed aluminum foil bag was left in room temperature and cold room at 4^0C for four weeks. The cysteine-AuNPs modified SPCEs was prepared per pieces for every week measured by EIS, and calculated for changes of Rct. Both results were collected when the modified SPCEs has immersed in 1mM of $K_3Fe(CN)_6$ in 0.1M KCl.

2.4 Surface Characterization and Estimation of Surface Areas of AuNPs-Cysteine Modified SPCE

The FESEM and XRD have analysed the ETSC of modified SPCE for surface characterization and element compounds that was successfully embedded on the carbon surface of SPCEs. The estimation of the surface area of AuNPs-cysteine modified SPCE was tested by comparing the EC, ETSC, and ETSTSC layers. CV was conducted by difference of scan rate from 10mV/s to 200mV/s for each type of modified layer.The Ipc was calculated using Randles-Sevcik equation after the modified SPCEs was immersed in 1mM of $K_3Fe(CN)_6$ in 0.1M KCl (Ferreira, A. AP *et al*., 2009).

3. RESULTS AND DISCUSSION

3.1 Modifying SPCE with AuNPs and Cysteine

Figure 2 shows the CV in 1mM of $Fe(CN)_6^{3-/4-}$ containing 0.1M KCl at 10mV/s: (a) cysteine/electrodeposition of AuNPs on modified SPCE (EC) (b) ETSC and (c) cysteine/self-assembled of AuNPs/thiourea/self-assembled of AuNPs/thiourea/electrodeposition AuNPs/modified SPCE (ETSCSC). As can be seen, the CV of ETSC has increased and is higher compared to ETSTSC and EC. The cathode peak current (Ipc) from CV has detected binding of cysteine, AuNPs, and thiourea on the surface of AuNPs electrodeposition on modified SPCEs. It has impeded the access of an electroactive redox couple $Fe(CN)_6^{3-/4-}$ to the modified SPCEs, therefore increasing the redox current occurred in the measurement (Jun Wu *et al*., 2011). Thus, ETSC modified SPCE was reported as the optimum combination methods for modification SPCE layer.

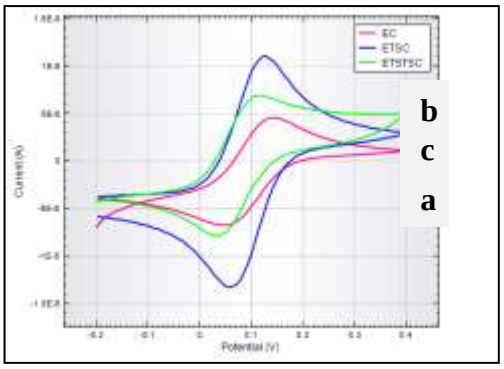

Figure 2 CV for (a) EC (b) ETSC and (c) ETSTSC in 1mM of $Fe(CN)_6^{3-/4-}$ containing 0.1M KCl at 10mV/s.

3.2 Reproducibility and Stability of AuNPs-Cysteine Modified SPCE

The increase of RSD in reproducibility data of EC, ETSC, and ETSTSC on AuNPs-cysteine modified SPCEs was 14.64%, 4.31%, 10.48%, respectively. The ETSC on modified SPCEs shows the lowest RSD at 4.31% less than 10% which is deemed as great values for RSD that was selected as the optimum type modified layer for SPCEs (Thevenot, 1999). The Ipc data of 10 modified SPCEs data was calculated to achieve RSD. In addition, it is clear that the ETSC on modified SPCEs was determined as the best choice for modified layer in the next experiment.

The stability of AuNPs-cysteine modified SPCEs was conducted at room temperature and cool storage within four weeks when not in use. The changes of Rct from its original value after 4 weeks for EC, ETSC, and ETSTSC of modified SPCEs in room temperature was maintained at 10.2%, 73%, 62.5%, respectively. While in cool storage, the changes of Rct from its original value after 4 weeks for EC, ETSC, and ETSTSC of modified SPCEs was reported at 30.97%, 55.28%, and 37.74% respectively. This percentage shows that good stability for ETSC modified SPCEs kept in the room temperature can be selected for the best modification layer among the others SPCEs. Thus, the combination of electrodeposition and SAM of AuNPs with thiourea is quite efficient for retaining the stability of modification layer of SPCEs. It has attributed to the strong interaction between cysteine and AuNPs.

3.3 Surface Characterization and Estimation of Surface Areas of AuNPs-Cysteine Modified SPCE

It can be seen that the surface characterization for ETSC immobilization layer by FESEM in Figure 3(i) shows that the surface is smooth with uneven distribution of cysteine molecules that was deposited on the surface of carbon on SPCEs. There is a lot of remaining cysteine molecule attachment due to the covalent bond attached with AuNPs molecules that can increase the chances on the attachment of allergen in the next experiment. It is also suggesting that the strong interaction between the Au-S that was obtained on the surface of carbon and FESEM images supports the evidence (Dolati *et al*., 2011).

Figure 3(ii) shows the XRD graph for ETSC on modified SPCEs with element compound of O, C, Au, S, and N bond. As can be seen, the XRD patterns of the ETSC on modified SPCE with different diffraction peak as for O (4.5), C (4.1), Au (2.7) and S (0.4) indicate that the ETSC on modified layered Au-S grows well during immobilization process. There was a little difference with diffraction of element compound of N that the XRD shows much less influence with ETSC on modified SPCEs that cannot be detected, indicating that the N element compound was not dismissed during the immobilization between layers. Additionally, it is worth noticing that the intensity of all the diffraction peaks of O, C, Au, and N decrease with proportion in the element compound, which

indicates that the incorporation of the C-Au-S considerably inhibits the ETSC on modified SPCEs (Ali, Mohamed, Azzam, & Abd-Elaal, 2014).

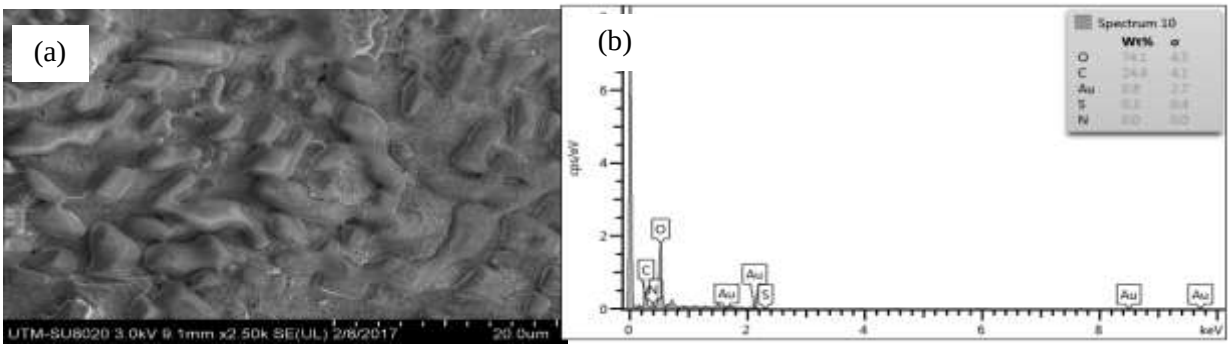

Figure 3 (a) FESEM images for ETSC for 20µm size and (b) XRD graph for ETSC with a compound of O, C, Au, S, and N bond.

The estimation of the surface area of AuNPs-cysteine for all type modified layer for SPCEs was conducted by CV and measured using Randles-Sevcik (equation 1). The CV was analysed by different scan rate as in Figure 5 and calculated in calibration plot as in Figure 4. The estimation surface area for EC, ETSC and ETSTSC was 0.0944 cm^2, 0.1348 cm^2 and 0.1214 cm^2, respectively. The estimation surface areas have shown that ETSC has higher values of the area, which is 0.1348 cm^2 with full coverage of cysteine and AuNPs. Hence, it is reported that the best-modified layers were used for next experiments (Siswana, Ozoemena, & Nyokong, 2006).

$$I_{pc} = (2.69 \times 10^5)\, n^{2/3}\, A\, D^{1/2}\, v^{1/2}\, C_o \qquad \text{Equation 1}$$

with the D= Diffusion coefficient, C_o = concentration of redox probe, v = scan rate and A = surface area.

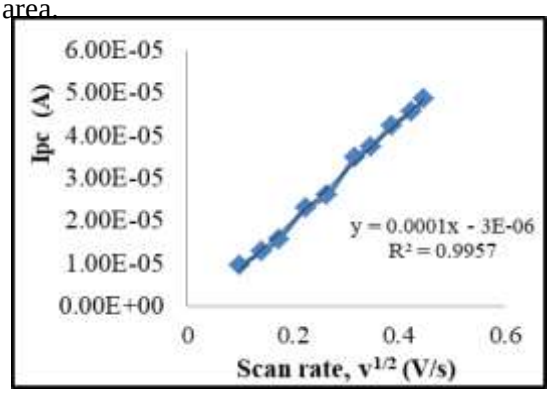

Figure 4 Calibration plot for ETSC based on Ipc with different scan rate ($v^{1/2}$)

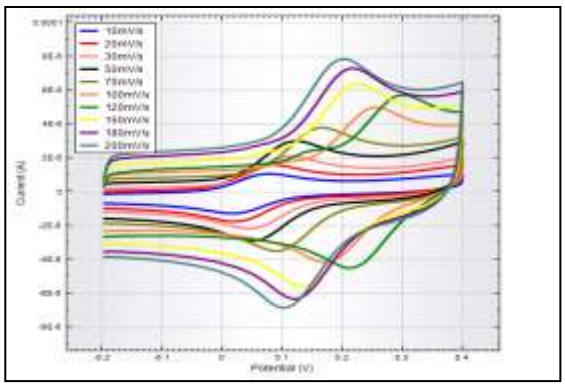

Figure 5 CV in 1mM of $Fe(CN)_6^{3-/4-}$ containing 0.1M KCl at 10mV/s of ETSC with various scan rate.

4. CONCLUSION

ETSC was selected as the optimum modified SPCE layer with excellent characterization. The ETSC modified SPCE has great reproducibility with 4.31% RSD. It was also found that the response of the modified SPCE was quite stable where the value of Rct only decreased to about 73% of its original value after four weeks storage at room temperature. The SPCE has a surface area of 0.1348 cm2 after modification with AuNPs and cysteine. The results obtained during this research work suggest that the combination methods provide a good alternative to modify disposable SPCEs using AuNPs and cysteine for potential use as a biosensor for skin sensitizer detection.

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Comparison of Human Papillomavirus Detection in Saliva, Blood and Tissue by PCR

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ABSTRACT

Infection of the oral cavity with human papillomavirus (HPV) has been implicated as one of the risk factors for the development of oral squamous cell carcinoma (OSCC). The presence of HPV found in different studies varies due to many factors such as sampling methods, HPV detection assay and the anatomical location of the tumor. The aim of this study was to compare the presence of high risk HPV in genetic materials obtained from saliva, blood and tissues of OSCC patients. The genomic DNA was extracted from saliva (n=13), blood (n=62) and tissues (n=57) specimens and was screened for high-risk HPV by polymerase chain reaction (PCR). Extracted DNA from all samples were tested for amplification of human beta globin gene with GH2O/PCO4 primers to confirm the DNA presence and integrity. Positive samples for this gene were then tested for HPV DNA by a combined nested PCR using MY11/09 and GP5/6 consensus primers, followed by DNA sequencing for confirmation. Two saliva samples (13.3%) were found to harbor HPV 16. One tissue sample (1.75%) was shown to be positive for HPV 18. None of the blood samples were detected positive for high risk HPV. From the results, HPV are more likely to be found in saliva of OSCC patients compared to blood and tissue samples. Analysis of HPV DNA using saliva that is a noninvasive approaches may allow early detection of HPV in OSCC patients.

Key words**:** HPV**,** oral squamous cell carcinoma, nested PCR.

1.0 INTRODUCTION

Oral cancer is nominated as the sixth most common malignancy around the globe and approximately 90% of oral cancer comprised of oral squamous cell carcinoma (OSCC) (Bagan *et al.*, 2010). The etiological factors of OSCC are multifactorial ranging from epigenetic, genetic, environmental to immunosuppression factors (Kumar *et al.*, 2016). Initially, OSCC development was etiologically related to common risk factors such as betel quid chewing, heavy tobacco and alcohol consumption (Subapriya *et al.*, 2007). In recent years, epidemiological and molecular data have found a new causative factor in OSCC cases where patients have denied any traditional risk factors. It is the involvement of high risk human papillomavirus (HPV) mainly HPV genotypes 16 and 18. Several investigators has reported the presence of HPV DNA in OSCC (Krüger *et al.*, 2014) and even in healthy oral mucosa (Wimardhani *et al.*, 2015).

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HPV is a non-enveloped DNA virus that has the capability to infect squamous epithelial layer. More than 200 different genotypes of human papillomavirus have been identified and they were classified based on their epidemiological association which are low risk and high risk subtypes, where it can cause benign and malignant lesions, respectively (Conway and Meyers, 2009). High risk subtypes have an oncogenic potential and among the high risk subtypes, HPV 16 and 18 are the most common infective agent in oral cancers (Kreimer, 2014). Each HPV DNA contains two early genes, E6 and E7 that have an ability to interrupt the function of two tumor suppressor genes, p53 and pRb that will eventually cause malfunction of cell apoptosis and lead to uncontrolled cell growth (Duensing and Münger, 2004).

HPV-positive OSCC that are associated to oral HPV infection and sexual habits shows an inclined in its incidence worldwide. HPV have been detected in a variable proportion of oral cancer in many countries ranging from 0% to 100%. In Malaysia, the prevalence of oral cavity cancer caused by HPV was 3.33% in 2012 (Goot-Heah *et al.*, 2012). The percentage varies in different studies due to many factors such as sampling methods, HPV detection assay, variation of genetic materials used and anatomical location of the tumor. Polymerase chain reaction (PCR) based assays is a widely used assay for detection and typing of HPV since it is one of the most sensitive and flexible among all DNA analysis techniques. To monitor HPV, saliva has been extensively used to estimate the disease risk either in healthy oral mucosa (Wimardhani *et al.*, 2015) or OSCC patients (Dang *et al.*, 2015). Metastasized cancer cells or cell debris that being shed from HPV virus in blood could also be used to investigate HPV infection (Bodaghi *et al.*, 2005). Not only that, *Papillomas* also can be found throughout the oral mucosa of biopsies tissue (Mravak-Stipetić *et al.*, 2013).

This study was aimed to compare the presence of high risk HPV in genetic materials obtained from saliva, blood and tissues of OSCC patients using the gold standard HPV PCR.

2. MATERIALS AND METHODS

Subjects and specimens

A total of 118 specimens comprising of 13 saliva, 62 blood and 57 tissue samples was collected from OSCC patients. These specimens were obtained from a retrospective compilation of Oral Cancer Research & Coordinating Centre (OCRCC), Faculty of Dentistry, University Malaya (Kuala Lumpur, Malaysia) and also from patients admitted to Hospital Universiti Sains Malaysia (Kubang Kerian, Kelantan). The study was approved both by the Medical Ethics Committee, Faculty of Dentistry, University Malaya (UM) OI DF1601/0072(P) and Research Ethics Committee (Human), Universiti Sains Malaysia (USM) USM/JEpeM/15020050.

Sample collection

Saliva sample

The patients were asked to fast for about 30 minutes before saliva collection . Prior to saliva collection, their mouths were thoroughly rinsed with water to reduce contamination. Then, fresh saliva sample was collected and transferred into a sterile collection cup. The collected saliva was stored at -80°C for later analysis.

Blood sample

About 10 ml of blood was collected in heparinized tube and stored at -80°C for further analysis.

Fresh Tissue sample

The tissue specimens were collected from each patient at the lesion area of the tumor before any cancer treatment. Tissue specimens were placed in formalin solution and kept at −80°C fur further analysis.

DNA extraction

All samples were subjected to DNA extraction using a DNA mini kit (Qiagen, USA) according to the manufacturer's protocol. The purity (A260/A280 ratio) and concentration of DNA samples was measured using a spectrophotometer (Eppendorf, USA) and the samples were stored in 20°C for further analysis.

Beta-globin PCR

Initially, all specimens were tested for the presence of.beta-globin DNA with GH2O/PCO4 primers (Table 1) in order to check the sample quality and adequacy. Eventually, only samples that tested positive in beta-globin PCR were subjected to HPV detection by nested PCR. DNA from samples that are negative in beta-globin amplification were extracted again and amplification was also repeated. The amplification of beta-globin was as followed: 94°C for 4 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. This was followed by a final extension of 72°C for 10 min.

Detection of HPV by nested PCR assay

The samples were detected for the presence of HPV using standard nested PCR with MY11/09 and GP5+/6+ primer sets (Table 1). The beta-globin gene as a housekeeping gene, was used as an internal control and HPV 16 ATCC as positive control. Briefly, in first round of DNA amplification was done in a 20µl reaction mixture consisting 2 µl of sample, MY11/09 (20 pmol each), 2µl 10x PCR Buffer, 2.5 U *Taq* DNA polymerase, 25 mM $MgCl_2$ and 0.2 mM dNTPs. First round amplification was done with MY11/09 primer with an expected size of about 450 bp using Applied Biosystems thermal cycler machine according to the following conditions: 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. This was followed by a final extension of 72°C for 5 min.

In second round amplification, the reaction mixture were similar as the first round PCR which consisted of 2 µl of sample, GP5+/6+ (20 pmol each), 2µl 10x PCR Buffer, 2.5 U *Taq* DNA polymerase, 25 mM $MgCl_2$ and 0.2 mM dNTPs. Two microliters of thefirst round PCR products was used as the template for the second PCR using GP5+/6+ primer set which resulted a ~140 bp product. The amplification was performed using the following conditions: 95°C for 5 min, 40 cycles of 94°C for 1 min, 40°C for 2 min and72°C for 1 min and finally a final extension of 72°C for 5 min. The PCR products were visualized by ultraviolet illumination on 1.5% agarose gel in 1X TBE buffer stained with fluorosafe for 50 min. The products from the HPV-positive OSCC were submitted for direct DNA sequencing. The sequences were compared with HPV genomes database that are available in the NCBI-GenBank using the basic local alignment search tool (BLAST, NCBI).

Table 1. Sequences of primer sets used in PCR

Primers	5' → 3' sequence	Size (bp)	Reference
First step outer primer			
MY11	GCA CAG GGA CAT AAC AAT GG	~ 450 bp	(Şahiner *et al.*, 2014)
MY09	CGT CCA AAA GGA AAC TGA TC		
Second step inner primer			
GP5+	TTT GTT ACT GTG GTA GAT ACT AC	~ 140 bp	(Asadi-Amoli *et al.*, 2011)
GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C		
Human β globin gene			
GH2O	GAA GAG CCA AGG ACA GGT AC	~ 248 bp	(Simonato *et al.*, 2016)
PCO4	CAA CTT CAT CCA CGT TCA CC		

3. RESULTS

Betaglobin PCR

A total of 118 DNA extracted from saliva, blood and tissue were tested in a PCR with β globin primer to access for the presence of cellular DNA prior to HPV detection (Figure 1). Beta-globin DNA was not detected in two of thirteen saliva samples and therefore were excluded from the study, leaving a total of 116 samples for further analysis.

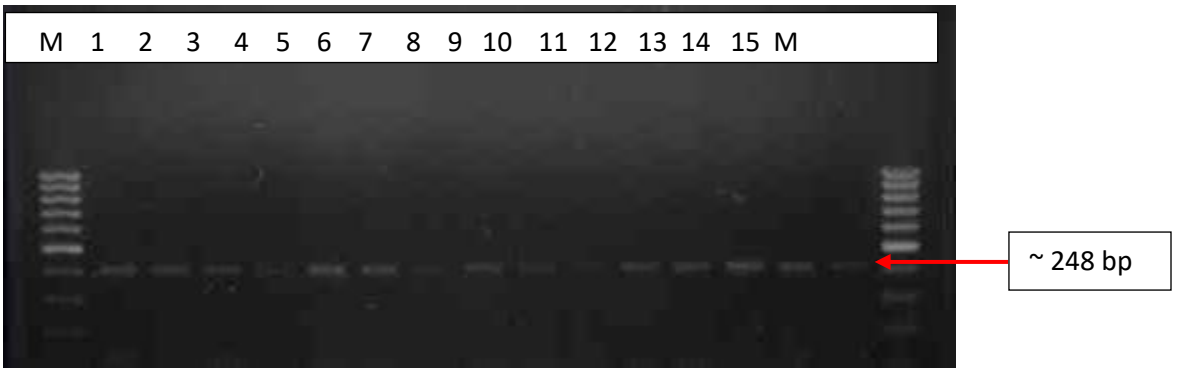

Figure 1: Electrophoretic gel analysis of beta-globin PCR products of extracted DNA samples using GH2O/PCO4 primers; M; 100 bp ladder (Axon Scientific), 1 to 15; DNA samples.

Detection of HPV in saliva, blood and tissue specimens by nested PCR

A total of 11 saliva, 62 blood and 57 tissue samples were analyzed for detection of HPV-DNA by two different PCR methods. They were first analyzed by using the MY11/09 primers (450 bp), followed by nested PCR using GP5+/6+ primers (140 bp) (Figure 1). All 116 samples were amplified for HPV DNA and genotypes were further confirmed by DNA sequencing. Three out of 116 patients that were evaluated using two different consensus primer MY11/09 and GP5+/6+ tested positive for HPV. Two saliva samples (13.3%) were found to harbor HPV 16 (Figure 2) and one tissue sample (1.75%) was shown to be positive for HPV 18 (Figure 3). Meanwhile, none of the blood samples was detected positive for high risk HPV (Figure 4).

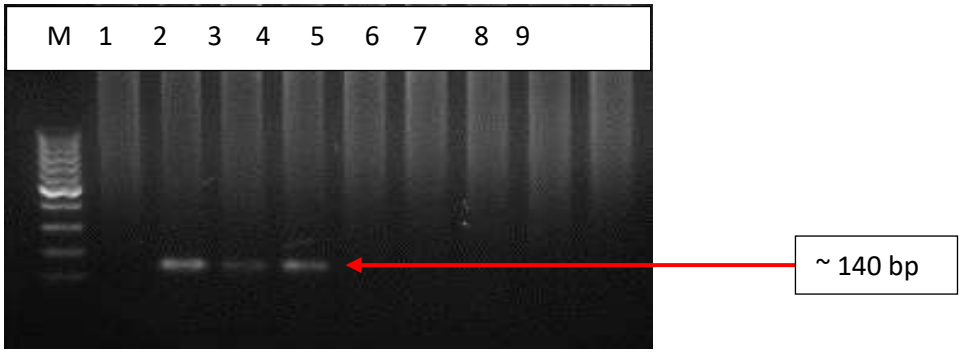

Figure 2: Electrophoretic gel analysis of PCR products of extracted DNA from saliva samples (a) M; 100 bp ladder, 1; negative control, 2; positive control (HPV 16), 3 to 9; DNA samples.

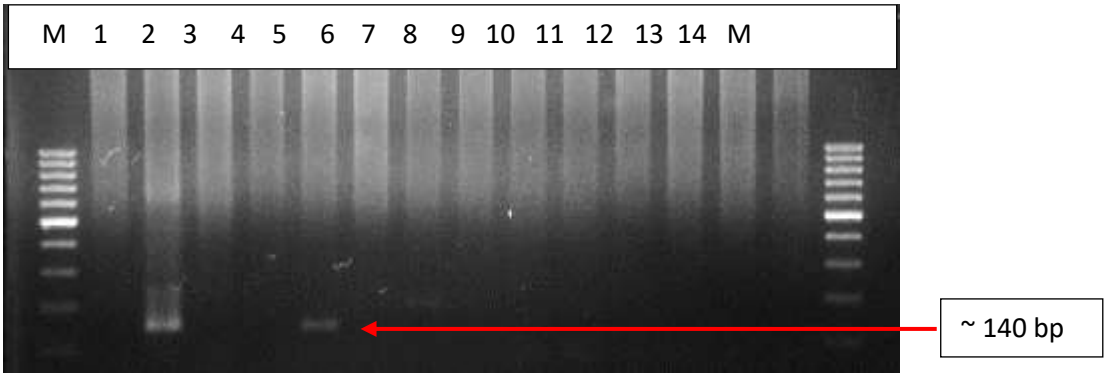

Figure 3: Electrophoretic gel analysis of PCR products of extracted DNA from tissue samples M; 100 bp ladder, 1; negative control, 2; positive control (HPV 16), 3 to 14; DNA samples.

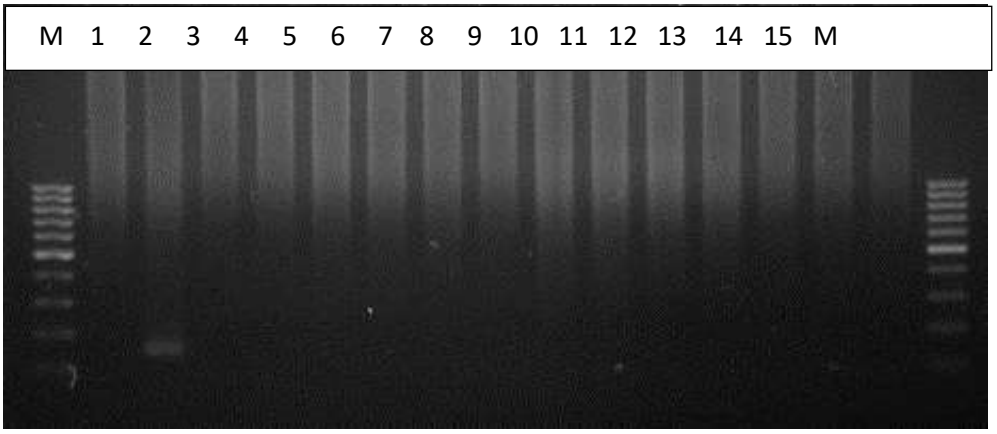

Figure 4: Electrophoretic gel analysis of PCR products of extracted DNA from tissue samples M; 100 bp ladder, 1; negative control, 2; positive control (HPV 16), 3 to 14; DNA samples

4. DISCUSSION

HPV detection and typing by nested PCR with consensus primer is simple yet sensitive method. The consensus PCR primers used in nested PCR were developed specifically to target and amplify the highly conserve region in HPV genome and it is potentially capable to detect all mucosal HPV types. Thereby, they have been extensively used in epidemiological studies and contributed to an effective detection of HPV in clinical samples. Previous studies have demonstrated that the use of MY11/09 and GP5+/6+

primer pairs increase the sensitivity and rate of HPV detection hence promise a better results in nested PCR assay compared to a single round PCR assay (Coser *et al.*, 2011; Erhart *et al.*, 2016; Jalouli *et al.*, 2015). It was proven that nested PCR has also enabled detection of investigated samples which contains low level of HPV DNA (Winder *et al.*, 2009).

Here we describe the detection of high-risk HPV in the several genetic materials. Prevalence of high risk HPV related OSCC in our study varied considerably by the different sources of materials whereas HPV were more likely to be found in saliva of OSCC compared to blood and tissue samples. As speculated by Montaldo *et al*, one possible reason is saliva could serve as a main mode of virus transmission from individuals with high infectious dose (Montaldo *et al.*, 2007). Other studies also found that, salivary rinses has a high throughput of HPV detection and has potential to be developed as a molecular screening for HPV-related head and neck squamous cell carcinoma (HNSCC) (Wasserman *et al.*, 2017; Zhao *et al.*, 2004). The prevalence of HPV in saliva in our study was 13.3% and it is approximately similar to the reported studies where HPV 16 was detected in 7 of 35 (20%) saliva of OSCC patients (Khyani *et al.*, 2015). Saliva is a body fluid that appear to be adequate for the detection of HPV (Adamopoulou *et al.*, 2013; Kulkarni *et al.*, 2011), and because it is a easy to obtain and non-invasive method for collecting material. In addition to that, it presents cells of different regions of the oral mucosa, thus facilitating the analysis when seeking HPV.

Comparing the HPV presence in this study, tissue being the second most common location for HPV to be found. In many studies, tissue samples from OSCC biopsies may lead to poor tissue stability and quality for further HPV testing. A low prevalence of HPV in tissue could also be affected by overall DNA concentration, pH of the solution, temperature and type of tissue storage (Noori *et al.*, 2012). Though blood circulation is one of the possible route of HPV transmission to the site of cancer however, in the present study no HPV was detected in blood of OSCC patients. As reported by Wang and colleagues, saliva is preferentially enriched for tumor DNA from the oral cavity, meanwhile plasma is preferentially enriched for tumor DNA from the other sites (Wang *et al.*, 2015). This is probably the reason why HPV DNA is hardly detected in blood since our study subjects comprised of all OSCC patients.

Overall, in our study HPV were highly detected in saliva compared to blood and tissue of OSCC. The detection rate of HPV shows that HPV 16 is the most detectable subtypes and this finding is supported by a study which indicated that HPV 16 was the predominant type found in OSCC patients in Malaysia (Saini *et al.*, 2011) and also worldwide (Gillison *et al.*, 2012; Kreimer *et al.*, 2005). Meanwhile, the second most common subtypes found is HPV 18, which is also the second most common oncogenic types. It is corresponding to previous study which identified only HPV 18 and no other subtypes detected in squamous cell carcinoma of head and neck (Quintero *et al.*, 2013).

5. CONCLUSION

Analysis of HPV DNA using saliva displayed good approach to be an alternative ways for screening and detection of HPV in OSCC . It is noninvasive, quick and convenient sampling method for the patients. However, further studies with larger sample size need to be carried out before a routine HPV saliva screening for OSCC patient can be implemented in clinical settings.

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Fouling Properties of PES/LICL/MWCNT Membranes Fabricated Via Tips and Nips

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ABSTRACT

Membrane filtration has been long used for solid-liquid separation and for algae harvesting. However, the issue of using membrane is it prone to fouling of solute. Microalga usually releases transparent exopolymer particles (TEP) in an aquatic condition which are the source of fouling. This paper represented the study of microalgae fouling mechanism on blend polyethersulfone (PES) polymer with lithium chloride (LiCl) and functionalized multiwall carbon nanaotubes (F-MWCNT) as additives membranes fabricated via temperature induced separatinon (TIPS) and non-solvent induced separation (NIPS). The membrane was prepared by blending 18wt% of PES, 1-4wt% of LiCl and 1wt% of F-MWCNT into a homogenous solution. All membranes were having molecular weight cut off at 145 kDA which indicated ultrafiltration/microfiltration. Increased of LiCl content in membrane does not help to increased membrane hydrophilicity further. Water permeation results shows that membrane fabricated via TIPS demonstrated higher flux compare to membrane fabricated via NIPS. Although no significant effect observed in membrane contact angle, the water permeability result from membranes with LiCl additives were improved. In the other hand, F-MWCNT has help to maintained LiCl additives in the membrane matrices. In this study NIPS membrane of PES 18/MWCNT/LiCl 4wt% is preferable since it demonstrated acceptable flux and good antifouling.

Keywords: ultrafiltration; lithium chloride; functionalized multiwall carbon nanotubes; microalgae harvesting; algae fouling.

1. INTRODUCTION

Membrane filtration has long been used for solid-liquid separation. The advantage of membrane filtration is it is simple and flexible in operation. Microalgae are a unique unicellular organism that contains many valuable components for green product such as biofuel Ultrafiltration and microfiltration are both membrane filtration that their separation is based on sieving mechanism and are suitable for algae harvesting. However, the issue of using membrane is it prone to fouling. Microalga usually releases transparent exopolymer particles (TEP) in an aquatic condition. TEP are in gel form and consist of largely acidic polysaccharides. TEP are highly sticky and tend to agglomerate with other particles. The stickiness of TEP is due to the presence of half-ester groups which can form metal ion bridges and hydrogen bonds with other substances. Therefore TEP is easy to deposit on membrane surface and created fouling.

There are only a few works reported related to membrane modification with antifouling property for microalgae harvesting application. Generally, membranes can be modified through the addition of additives via blending and surface coating. However, blending has more advantages than surface coating Hwang et al. 2015. Thus, this paper represented the study of microalgae fouling mechanism on blend polyethersulfone (PES) polymer with lithium chloride (LiCl) and functionalized multiwall carbon nanaotubes (F-MWCNT) as additives membranes.

2. MATERIALS AND METHODS

2.1 Membrane Fabrication

The membrane was prepared by blending 18wt% of PES, 1-4wt% of LiCl and 1wt% of F-MWCNT into a homogenous solution. The solution was casted on a glass plate and immediately immersed into the coagulation bath. For temperature induced phase separation (TIPS), casting solution was at ≈ 90^0C while for non-solvent induced phase separation (NIPS) casting solution was at room temperature. The fabricated membranes were store in distilled water before used.

2.2 Membrane Characterization

The membrane was characterized on their pore size, hydrophilicity and chemical content properties through molecular weight cut-off (MWCO) experiment, contact angle and Fourier transform infrared spectroscopy (FTIR) and energy dispersive x-ray spectroscopy (EDX) analysis respectively.

2.3 Membrane flux

A flat sheet ultrafiltration module was to determined membrane fluxes. The membrane flux during filtration can be described by Darcy's law equation.

2.4 Fouling Determination

Algae fouling behavior on membranes were determined in term of irreversible fouling (IF) and reversible fouling (RF) using equation stated elsewhere.

3. RESULTS AND DISCUSSIONS

3.1 Membrane Characteristics

PES/MWCNT/LiCL membranes have been successfully fabricated via TIPS and NIPS process. The additives used have their own function on the membrane characteristic. F-MWCNT act as an anchoring agent for LiCL when EDX results (Table 1) shows that without F-MWCNT, chlorine compound in membrane was less. Meanwhile, the role of LiCl is to form membrane with pores of UF/MF as shows in Figure 1. The Figure 1 depicted that all membranes were 100% rejected PVA with molecular weight of 145 kDA which indirectly represent the pore size of membrane. Increased of LiCl content in membrane does not help to increased membrane hydrophilicity further. However all membranes were hydrophilic since contact angle results were below 80^0.

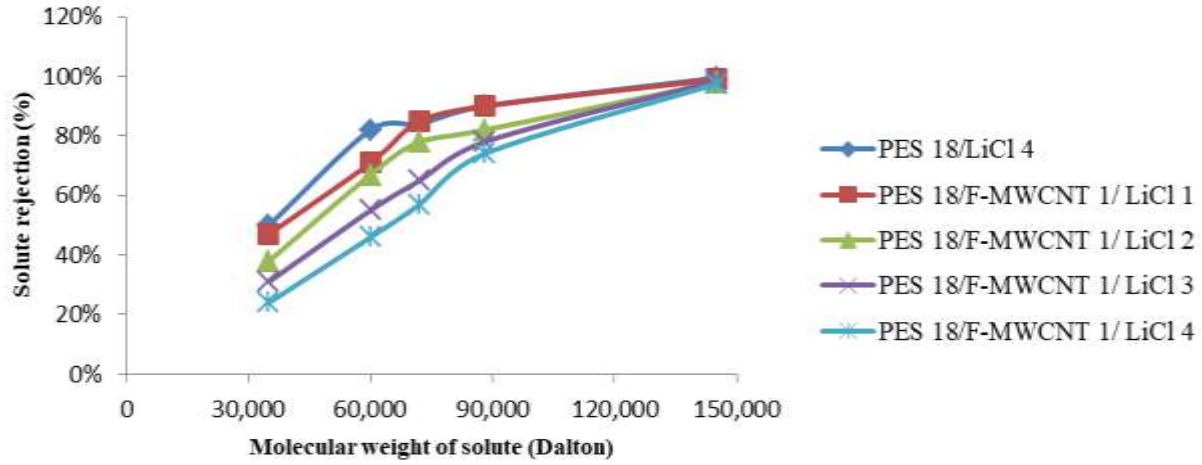

Figure 1. PVA rejection of PES membranes for MWCO determination

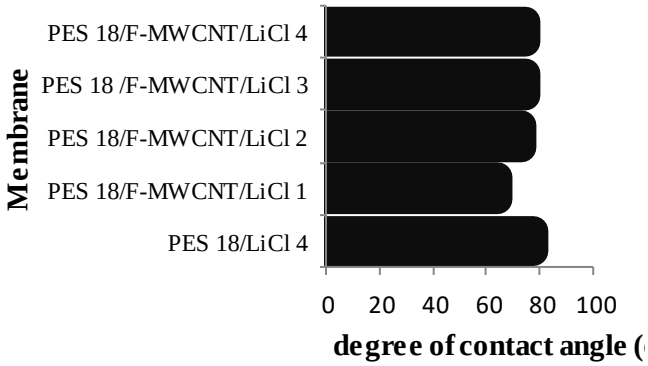

Figure 2. Contact angle analysis of varies membranes

Table 1. Chlorine content in various membranes from EDX

PES Membrane	Chlorine weight%
PES 18 /LiCl 4	0.02
PES 18 /LiCl 1 /F-MWCNT 1	0.42
PES 18 /LiCl 4/F-MWCNT 1	1.30
PES 18/ Licl 4/ F-MWCNT 0.2	0.35

3.2 Permeation

Water permeation results shows that membrane fabricated via TIPS demonstrated higher flux compare to membrane fabricated via NIPS. This proves that membranes from TIPS were highly porous.

Membrane thickness = 0.1mm Pressure = 3 bar		
	NIPS	
Membrane	IF	RF
PES 18/MWCNT/LiCl 1wt%	0.13	0
PES 18/MWCNT/LiCl 4wt%	0.00	16.61
	TIPS	
PES 18/MWCNT/LiCl 1wt%	0.38	0.00
PES 18/MWCNT/LiCl 4wt%	17.24	52.54

Table 2. Water permeation of membranes of NIPS and TIPS

3.3 Fouling properties

Fouling occurred in all types of the membranes (Table 3). Reversible fouling is better in any process since it can be easily removed by simple washing that would not damage the membrane. In this study, membrane with less licl performed no reversible fouling and fouling that occurred was the irreversible type which is undesired. Overall, the NIPS membranes have low fouling and compared to TIPS which can be related to the terminology of highest flux, highest fouling. Since NIPS membranes does not permeated higher flux so it had low fouling. No occurance of irreversible fouling was recorded for NIPS membrane with highest Licl.

Membrane thickness = 0.1mm Pressure = 3 bar	Water Flux rate (L/m^2h)	
Membrane	TIPS	NIPS
PES 18/LiCl 4	31.3	10.5
PES 18/MWCNT/LiCl 1wt%	52.0	29.2
PES 18/MWCNT/LiCl 2wt%	244.4	38.9
PES 18 /MWCNT/LiCl 3wt%	313.3	83.3
PES 18/MWCNT/LiCl 4wt%	548.2	161.2

Table 3. Irreversible and reversible fouling properties of the membranes

4. CONCLUSION

LiCl additives has improve the membrane permeation and helped to reduced fouling. Although no significant effect observed in membrane contact angle, the water permeabilities result from membranes with LiCl additives were improved. In the other hand, F-MWCNT has help to maintained LiCl additives in the membrane matrices. In this study NIPS membrane of PES 18/MWCNT/LiCl 4wt% is preferable since it demonstrated acceptable flux and good antifouling property.

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Preliminary Study on the Effects of Processing Parameters on Tannin Extracted from *Quercus infectoria* Galls

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ABSTRACT

Preliminary phase is greatly essential to assess the possibilities, outcomes and patterns of the experimental data before proceeding to further stage. Processing parameters are the main crucial factors affecting the whole extraction system and they are extraction time, temperature, solvent to raw material ratio and many others. These factors play a significant role especially in the extraction of desired active compounds from its sources. Hence, the effects of processing parameters (solvent to raw material ration, extraction temperature and duration) on tannin extraction from *Quercus infectoria* galls are studied. Series of experiments were designed using One Factor at a Time (OFAT) involving three different parameters to be analysed. Extraction temperature was set at the range between 50°C to 100°C, extraction duration (60 min to 210 min) and solvent to raw material ratio (15:1 to 30:1) was analysed accordingly using aqueous extraction method. Active compound found abundantly in *Q. infectoria* galls; tannin was quantified using High Performance Liquid Chromatography (HPLC) while yield of extract and antioxidant activity present in the extract was examined thoroughly. Overall, this study mainly present the preliminary work involved in the extraction of tannin from *Q. infectoria* galls. Conclusively, processing parameters functions at its best condition at the temperature of 70°C, 120 min of extraction duration and solvent ratio of 1:21.

Keywords: *Quercus infectoria*, Tannin, Aqueous extraction, Antioxidant, Preliminary study

1. INTRODUCTION

Quercus infectoria Olivier (Family: Fagaceae) is a small tree being widely distributed in Greece, Asia and the Middle East. This tree comprises its own galls that emerge on its shoot as a result from the attack of gall wasp, *Cypnis gallae-tincotoriae* (Samuelson, 1992). The galls are commonly known as Manjakani ni Malaysia and is common in traditional medicines practices since decades ago. Locally, the galls are used with combination of other herbs as a drinking remedy by women after childbirth. This to ensure the uterine wall elasticity is restored. On the other hand, in India this medicinal plant is widely known as Majuphal which has been used extensively as dental powder and also in the treatment of toothache and gingivitis. Besides that, scientific literature has stated that gargling Q. infectoria galls extract with hot water can subsequently reduce inflammation of tonsils while direct usage of boiled galls on skin can effectively cures any swelling or inflammation (Chopra *et al.,* 1956). The constituents of *Q.infectoria* galls comprise a large amount of tannin (50-70%), traces amount of gallic, syringic and ellagic acid (Ikram and Nowshad, 1977; Dar and Ikram, 1979; Hwang *et al.,* 2000). The main constituent of this galls are tannins which posses various beneficiary in terms of biological and chemical functions can actually being studied thoroughly for further scientific applications. *Q.infectoria* galls able to exhibit high potency in antioxidant (especially in scavenging free racidals) and anti-inflammatory properties as well as pharmacologically proven to be astringent, antiparkinsonian, antidiabetic and antitremorine (Dar *et al.,* 1974; Dar and Ikram, 1979; Hwang *et al.,* 2000; Kaur *et al.,* 2004; 2008). Hence, this particular medicinal gall has been studied extensively in terms of its active component identification, extraction processes and its relative affecting factors, biological and chemical functions for pharmaceuticals, medicinal and herbal technology industry.

Extraction can be defined as the separation of active components of plant or animal tissues from other inactive or inert components by using several solvents selectively chosen in accordance to standard extraction procedures. Standardization of extraction procedures contributes to a defined and final quality of the herbal product (Handa, 2008). The extraction of these standardized herbal extracts with its specific amount of bioactive compounds is highly important. Hence, varying processing conditions will give a great impact on every extraction processes. In this preliminary study, the effects of *Q. infectoria* galls active constituent; tannin, antioxidant ability and its overall yield will be evaluated based on the processing conditions selected. This is to ensure that despite from different processing parameters applied on the extraction process, the quality and quantity of active constituents extracted can still be well preserved and is able to exhibit its biological and chemical functions greatly. Previous literatures have only focused on the extraction process and the identification of active components found in *Q. infectoria* galls. There is no scientific studies have been performed in assessing the best conditions for processing parameters to extract out the active components at its best form and quantity. Hence, a preliminary study on these processing parameters is crucial to determine the relative effects of different range used in the selected processing parameters against the extraction of tannin from *Q. infectoria* galls. Different range of processing parameters can possibly display different extraction conditions and how this scenario can actually affects the quality of extracted compounds. Overall, this study aims to determine the significance of processing parameters range selected towards its response variables during the extraction of tannin from *Q. infectoria* galls.

2. MATERIALS AND METHODS

Materials

Quercus infectoria galls were purchased from a local herbal shop at Pasir Larkin, Johor Bahru. They materials were sent to Institute of Bioproduct Development, Universiti Tekonologi Malaysia, Skudai, Johor for further treatment. The galls were sent for a pre-treatment process where they were cleansed thoroughly with tap water and dried at 40^{o}C using drying oven (Memmert, Germany) until excess water has completely drained out. The powdered galls were stored in a sealed plastic container and stored in a dry cool place until further usage.

Chemicals and reagents

All the chemicals used in this experiment were in analytical and also HPLC (High Performance Liquid Chromatography) grade. Tannic acid, ortho-phosphoric acid (85%), acetonitrile (41.05 g/mol), L-ascorbic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (394.32 g/mol) were purchased from Sigma- Aldrich Malaysia Sdn Bhd.

Extraction of tannin from *Q. infectoria* galls

An amount of *Q.infectoria* galls were weighed accordingly and subjected to decoction extraction method using water as solvent. Using hot plate stirrer, the process of extraction was carried out using several selected temperatures, solvent ratio and duration of extraction as illustrated in Table 1 below. Once extraction process has completed, the filtrate was sent to rotary evaporator to remove excess water and therefore sent to drying process in a drying oven for 48 hours at 40^{o}C.

Table 1: Processing parameters in OFAT preliminary study

Parameter	Conditions
Group A	
Extraction Temperature	50°C, 60°C, 70°C, 80°C, 90°C, 100°C
Extraction Time	180 min
Solvent to Raw Material Ratio	30:1
Group B	
Extraction Time	60 min, 90 min, 120 min, 150 min, 180 min, 210 min
Solvent to Raw Material Ratio	30:1
Extraction Temperature	Selected from Group A
Group C	
Solvent to Raw Material Ratio	15:1, 18:1, 21:1, 24:1, 27:1, 30:1
Extraction Temperature	Selected from best results in Group A
Extraction Time	Selected from Group B

Preliminary study of processing parameters range

The preliminary study was mainly assessed on the effect of processing parameters towards the extraction of tannin from *Q. infectoria* galls. The range was set accordingly using OFAT preliminary study. As such, the experiment was carried out one at a time to thoroughly assed the significance of using such range in the extraction process or any further experiments. In common cases, OFAT is usually being used in order to analyze abundance of data and to predict the significance of most data before proceeding to other experimental designs. The parameters with its own conditions involved in this OFAT study were classified into three different groups (A, B, C) with their respective response variables; were further illustrated in Table 1. All experiments were carried out in triplicate and all data represented in this study are assessed statistically using Analysis of Variance (ANOVA) test.

Yield of extract

Yield of *Q. infectoria* aqueous extract was calculated based on the following formula:

$$\text{Extraction yield (\%)} = \frac{\text{Dry mass of crude extract (g)}}{\text{Mass of sample used for extraction (g)}} \times 100$$

Quantification of Tannic Acid

Tannin from *Q. infectoria* extract was determined through High Performance Liquid Chromatography (HPLC) using the method described by Asghari *et al.* (2011) with slight modifications. 1mg/ml of *Q. infectoria* extract was mixed thoroughly with distilled water as solvent and was filtered using 0.45 µm nylon filter membrane. The mixture was then injected to the HPLC system. The chemical marker used for the quantification of tannin was tannic acid. The mixture was injected with column C18 with particle size of 5 micron. 1 % of ortho-phosphoric acid was used as solvent A while 100% acetonitrile as solvent B. With isocratic pump mode, the separation of tannic acid was conducted on the basis of 95% solvent A and 5% solvent B with flow rate of 1ml/min at 280 nm. Every injection was set until they achieve 20 µl. Data was collected and sample peaks were identified by comparing with standard peaks of tannic acid solution obtained from the assay. The amount of tannic acid was calculated using appropriate calibration curves.

Scavenging of free radicals

This assay was conducted according to the method of Miliauskas *et al.* (2004) with slight modifications. In order to conduct scavenging assay, 1mL of methanolic solution of gall extract was incubated with

0.5mL of 0.05mM DPPH solution for 30 min. Absorbance was read there-after at 517nm measured using UV-vis spectrophotomer. A decrease in absorbance reading indicated that antioxidant activity is high and DPPH radicals are greatly scavenged. The capability to scavenge DPPH radicals was calculated using the formula below:

$$\text{DPPH quenched (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

Statistical Analysis

All values were expressed as mean ±S.E. Statistical analyses were performed using Analysis of Variance (ANOVA) test. Correlations were established using Pearsons correlation coefficient (r) in bivariate linear correlations ($p < 0.05$). These statistics were calculated using Microsoft office Excel 2007 and SPSS version 21.0 (IBM corporation, New York, U.S.A).The values of P lower than 0.05 were considered as significant (P is probability).

3. RESULTS AND DISCUSSIONS

Table 2: Effects of extraction temperature on yield of extract, tannic acid content and antioxidant activity of *Q. infectoria* aqueous extract

Factor / Response	Temperature (ºC)					
	50	60	70	80	90	100
Yield	52.477 ±0.000	65.850 ±0.001	82.543 ±0.000	80.643 ±0.001	78.940 ±0.000	73.840 ±0.004
Tannic Acid	1550.840± 0.039	1757.380 ±0.004	3155.243± 0.004	3010.823 ±0.012	2915.817 ±0.001	2011.440± 0.012
Antioxidant	71.560 ±0.000	74.993 ±0.001	91.150 ±0.000	90.84 ±0.000	88.673 ±0.004	81.820 ±0.001

Constant variable: Extraction time (180 min); Solvent ratio (1:30)

Table 3: Effects of extraction time on yield of extract, tannic acid content and antioxidant activity of *Q. infectoria* aqueous extract

Factor / Response	Time (min)					
	60	90	120	150	180	210
Yield	58.467 ±0.000	69.057 ±0.000	85.003 ±0.001	81.483 ±0.001	76.753 ±0.001	63.840 ±0.002
Tannic Acid	1350.373± 0.028	1658.62 ±0.014	3344.773 ±0.004	2996.823 ±0.018	2533.483 ±0.038	2196.167 ±0.014
Antioxidant	70.080 ±0.001	75.493 ±0.001	91.163 ±0.000	89.940 ±0.001	88.820 ±0.001	72.127 ±0.000

Constant variable: Extraction temperature 70ºC (selected from Table 4.1 results); Solvent ratio (1:30)

Table 4: Effects of solvent to raw material ratio on yield of extract, tannic acid content and antioxidant activity of *Q. infectoria* aqueous extract

Factor / Response	Solvent Ratio (ml)					
	15	18	21	24	27	30
Yield	68.240 ±0.001	75.733 ±0.028	84.677 ±0.000	82.303 ±0.001	78.683 ±0.001	62.513 ±0.001
Tannic Acid	1358.997	1678.030	3175.810	2897.203±	2734.637	2193.210

	±0.000	±0.012	±0.002	0.004	±0.001	±0.001
Antioxidant	70.290 ±0.002	76.073 ±0.002	91.860 ±0.000	89.020 ±0.0012	78.217 ±0.004	72.123 ±0.001

Constant variable: Extraction temperature 70°C (selected from Table 4.1 results); Extraction time at 120 min (selected from Table 4.2 results)

Table 5: Best parameters condition based on OFAT preliminary study

Extraction Temperature (°C)	Duration of Extraction (min)	Solvent to Solid Ratio
70	120	21

Based on Table 2, 3, and 4 it can be seen that yield of extract, tannin content as well as antioxidant activity is greatly affected from the processing parameters which are extraction temperature, extraction time and solvent to raw material ratio. From the results itself, the trend of respective response variables are parallel with its processing parameters. When there is gradual increase of temperature, time and solvent ratio, there will be an increment of relative responses (yield, tannin content and scavenging activity). However, prolong heating; temperatures higher than 70°C as well as longer extraction time; more than 120 min will lead to a subsequent reduction of its yield of extract, tannin content and its scavenging activity.

The ability of *Q. infectoria* extract to scavenge free radicals is correlated with the high amount of tannin; active compound present in the extract itself. Since, free radicals attacks diverse classes of biomolecules including phenolic acids, esters and glycosides (Nimse and Pal, 2015), hence the basic principle lies in this concept is that tannin; phenolic compound present from *Q. infectoria* extract is strong enough to exhibit great antioxidant activity on its own compared to other phenolic compounds (Amarowicz *et al.*, 2004). Tannin has the ability to chelate metal ions and interfere in one of the reaction steps in Fenton reaction thereby retards oxidation process (Zhang *et al.*, 2004; Karamac *et al.*, 2006). Antioxidants are also crucial especially in halting oxidative damage which can implicate humans with several range of diseases such as cancers, cardiovascular diseases and also aging (Kehrer, 1993). However, they are many medicinal plants have found to be effective in combating these free radicals but in many scientific literatures, tannin has found to be the new natural antioxidants due to its effectiveness in oxidative damage defense mechanism (Amarowicz *et al.*, 2001; Pegg *et al.*, 2005; Amarowicz *et al.*, 2005). Rice-Evans and his colleagues also claimed that many naturally occurring phenolics with low molecular weight able to scavenge free radicals as effective as the antioxidant vitamins E and A. In fact, tannins have been tested clinically and were proven that both condensed and hydrolysable tannins are more effective natural antioxidants compared to small phenolics (Hagerman, 1988).

Besides that, from the trend of data acquired it portrays a finding; prolong exposure to heat, longer extraction time and great difference in solvent to raw material ratio will greatly impact medicinal plants' yield of extract, active compound content as well as its scavenging ability. Therefore, the best range has been selected for each processing parameters examined and the results were tabulated in Table 5. Highest extract yield (85.003% ± 0.001), tannin content (3344.773 ppm ± 0.001) and most active scavenge activity (91.860% ± 0.002) was acquired at extraction temperature of 70°C, 120 min of extraction duration and solvent to raw material ratio of 1:21.

Figure 1 Effects of temperature, time and solvent ratio on *Q. infectoria* aqueous extract

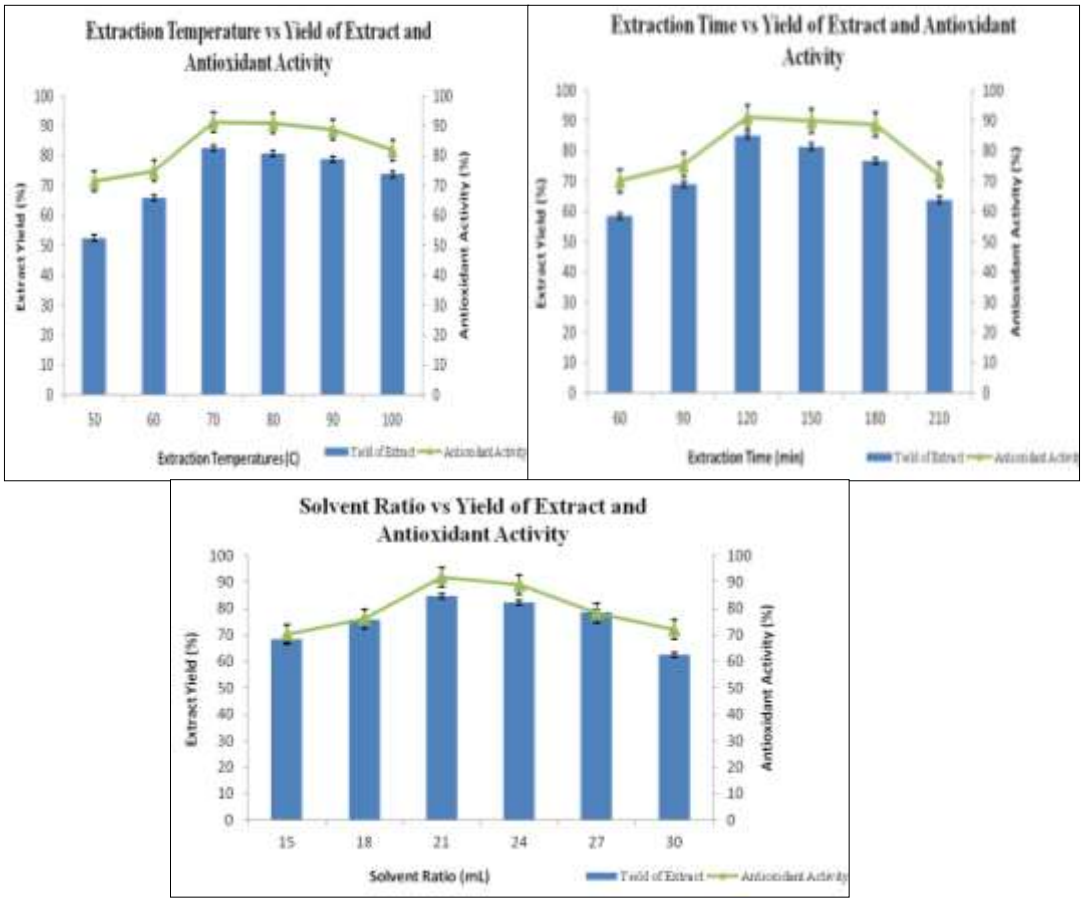
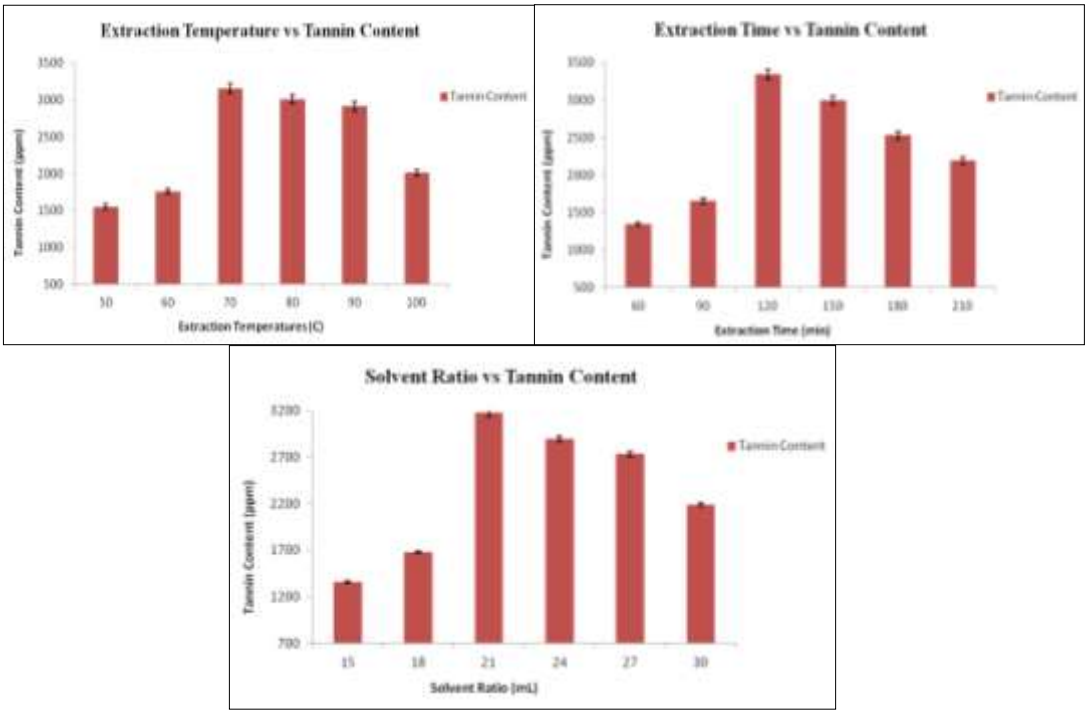


Figure 2 Effects of temperature, time and solvent ratio on tannin content of *Q.infectoria* extract



On the other hand, Figure 1 illustrates the effect of extraction temperature, time and solvent ratio towards extraction process of *Q. infectoria* galls. Higher temperature gives a significant rise of extract yield, tannin content as well as antioxidant activity. However, extraction temperatures should not exceed the right limit to avoid thermal degradation of active compounds from happening. High temperatures can actually destroy protein complexes and its structures therefore damaging the active compound desired. Different compounds have different molecular weight and thermal capacity. Hence, preliminary study is absolutely crucial to screen for the right extraction temperatures and also other processing parameters before proceeding to the other phases of extraction. By regulating these factors prior the extraction process, such thermal degradation can be avoided and the right active compound can be successfully extracted from the selected medicinal plants. Additionally, Figure 2 the graph illustrates the effects of temperature, time and solvent ratio towards tannin content; the main active compound constitutes in *Q. infectoria* extract. From the results, it clearly depicts that gradual increase of time, temperature and solvent ratio leads to a subsequent increment of tannin contained in the extract. However, after prolonged extraction process leads to relative thermal degradation. Tannin content also reduces after more than 120 mins of extraction duration and solvent ratio higher than 1:21. This explains that every parameter convey their own limits to optimally produce the active compound. Conclusively, the range of parameters that have been considered to give significant impact towards these response variables are extraction time at 120 min, extraction temperature of 70^{o}C and solvent to raw material ratio of 1:21.

4. CONCLUSION

The results concluded processing parameters range gave significant effects towards its response variables whether enhancing or diminishing the total extraction process. High temperatures could lead to partial or full thermal degradation of the active compounds by disrupting the protein structures. Imbalance of solid to raw material ratio and long duration of extraction will negatively affect the extraction of

phytochemicals and the quality and quantity of active compound extracted. Hence, the preliminary studies have clearly depicted the best appropriate range to be employed in further optimization study of extraction process or any other phytochemical related studies.

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Astaxanthin Production by Haematococcus Pluvialis under Different Light Emitting Diodes (LEDs) Illumination

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ABSTRACT

The freshwater phototrophic green microalga *Haematococcus pluvialis* is a well-known microorganism which is rich with powerful antioxidant astaxanthin and beneficial in pharmaceutical and cosmetic industries. The aim of this study is to investigate the effect of different LEDs illumination on the astaxanthin accumulation in the microalgae cell. Experiments were carried out by growing the microalgae using fluorescent light until it reached stationary phase. The growth was calculated by measuring the optical density (OD) spectrophotometrically at 750nm, cell counting and cell dry weight (CDW). The culture began its exponential phase on day-2 and reached stationary phase on day-13 with cell density, CDW, specific growth rate, μ and division time of 3.0 x 10^7 cells/mL, 6.58 g/L, 0.272 day^{-1} and 0.392 respectively. The *Haematococcus pluvialis* culture were then subjected to astaxanthin induction process by exposing the culture to (i) red, (ii) blue and (iii) red-blue LEDs at light intensity of 200 μmol $m^{-2}\ s^{-1}$. The astaxanthin accumulation were observed for 14 days and results found that mix red-blue LED exhibited the best result in terms of astaxanthin accumulation at 3.04mg/L compared to other red and blue LED, at 2.17mg/L and 2.49mg/L respectively. The usage of LED in astaxanthin production is proven effective and more study needed to determine the optimal induction setting for maximum astaxanthin production from *Haematococcus pluvialis*.

Keywords: *Haematococcus pluvialis*; astaxanthin; LEDs, cultivation; extraction.

1. INTRODUCTION

The freshwater green microalgae *Haematococcus pluvialis* is the best microbial source of astaxanthin [1]. Astaxanthin (3,3'-dihydroxy-diketo-β, β'-carotene-4,4'-dione) is one of the most powerful antioxidants among carotenoids with many applications in nutraceuticals and in the food and feed industries because of its strong anti-aging, anti-inflammatory, sun proofing, and immune system boosting effects on organisms. *Haematococcus pluvialis* is believed to demonstrate the highest capacity to accumulate natural astaxanthin under environmental stress conditions [2] which protects itself by secreting the astaxanthin when faced to the undesirable conditions as a response to adverse conditions that result in the cessation of growth while photosynthesis is still active [3] [4]. *Haematococcus pluvialis* species is considered as the most promising producer of astaxanthin, as it accumulates the (3*S*, 3'*S*)- isomer of astaxanthin, mostly in its mono- and di-ester forms in cytoplasmic lipid bodies as a secondary carotenoid [5]. There has been an emerging development in facilitating the commercial mass production of *Haematococcus pluvialis* due to its ability to secrete and produce powerful antioxidants for pharmaceutical and nutraceutical industry. This carotenoid also principally consumed in aquaculture industry specifically for salmon and lobster colorant. Hence, more commercial cultivation farm being constructed and research began to venture on alternative light source for astaxanthin production to replace sunlight. Conventional artificial light source was reported to produce higher biomass due to large illumination area, high stability of light source and low construction cost [6]. LEDs can serve as an ideal light source for algal growth due to its advantages: (i) narrow spectral output, which can overlap with the absorption spectra of microalgae; (ii) high electric-to-light conversion efficiency, which generates less heat; (iii) no emission outside of photosynthetic active radiation (PAR), such as ultraviolet and infrared regions, which makes the light delivery system simpler; (iv) small weight and volume characteristics,

which makes LEDs to be incorporated into virtually all types of PBRs for both internal and external light sources; (v) many other advantages, such as long life expectancy, solid state, safe (powered by low DC voltage), extremely short rise and fall time [7].
In the present study, the potential of LED light to induce astaxanthin accumulation in *Haematococcus pluvialis* is studied. The different wavelength of LEDs (i) red, (ii) blue and (iii) red-blue LED illumination bars were chosen as alternative artificial light source throughout the astaxanthin induction process to test their effect on astaxanthin accumulation in *Haematococcus pluvialis* culture.

2. MATERIALS AND METHODS

2.1. Collection of Microalgae Sample and Culture Maintenance

The strain of *Haematococcus pluvialis* was obtained from the Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah (UMS), Malaysia and maintained both in liquid culture and agar plates. Agar transfer was performed every month and single colony was picked from agar plates which then transferred into Erlenmeyer flasks containing 50mL culture media and grown at 21 ºC under continuous fluorescent light with intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 160 rpm as stock cultures.

2.2. Inoculation and Cultivation of *Haematococcus Pluvialis*

Modified Bold's Basal Medium (BBM) is the medium used for *Haematococcus pluvialis* cultivation. The freshwater microalgae species which was maintained in dormant condition (on agar plate) and liquid form (stock culture) was inoculated in modified BBM. The modified BBM formulation (per L) is as follows: 25 g $NaNO_3$, 2.5 g $CaCl_2$, 7.5 g$MgSO_4.7H_2O$, 7.5 gK_2HPO_4, 17.5 g KH_2PO_4, 2.5 g NaCl, EDTA Solution (50 g EDTA + 31 g KOH), trace element solution (8.82 g $ZnSO_4.7H_2O$, 1.14 g $MnCl_2.4H_2O$, 0.88 g $(NH_4)_6Mo_7O_{20}.4H_2O$, 1.57 g $CuSO_4.5H_2O$, 0.49 g $Co(NO_3)_2.6H_2O$), 11.42 g H_3BO_3
and 4.98 g $FeSO_4.7H_2O$.

The *Haematococcus pluvialis* was inoculated in 200mL Erlenmeyer shake flasks which contain 10 mL seed culture and 90 mL medium. The cultivation was performed at 21ºC pH6.8$\pm$0.2 and under a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 24:0 light:dark cycle for 5 days on the shaker. On the 5th day, 40 mL of culture was transferred into sterilized 500 mL Erlenmeyer flasks which contain 360 mL medium for microalgae growth observation for 14 days with continuous aeration. Each experiment was performed in duplicates to ensure reproducibility of result. After the growth profile was obtained and the stationary phase was identified, another set of experiment was performed to induce the astaxanthin production. A seed culture was prepared and inoculated into 6 500 mL Erlenmeyer flasks which contain 30 mL microalgae culture and 270 mL growth medium. All 6 flasks were made to grow under fluorescent light for 11 days before transferred to the induction rack to induce astaxanthin production under different LEDs illumination wavelength (red, blue and red-blue) at light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 24:0 light:dark cycle.

2.4 Growth Analyses and Quantification of Astaxanthin

Haematococcus pluvialis cultures OD readings were taken daily and observed using Shimadzu UVmini-1240 UV-vis spectrophotometer at 750 nm. Meanwhile the cell counting was determined by direct counting with haemacytometer using a 0.1 mm-depep Neubauer chamber (BOECO, Hamburg, Germany)

under a light microscope (Olympus CX31, Japan). Lastly, 10 mL of the culture were used for determining the CDW by filtering the sample through the Glass Microfiber Filters, GF/C (Whatmann) and then dried at 100 ºC to a constant weight in an oven (Model: UNB 500, Memmert GmbH, Schwabach, Germany). All CDW samples were cooled in desiccator before weigh.

Astaxanthin from samples were quantified to confirm astaxanthin accumulation in the cell. The cultures were sampled every two days (48 hours). 5 ml of sample was taken for every flask. Then, centrifuge for 5 min at 2500 rpm. The supernatant was removed. Resuspended the pellet in 5 ml solution of 5% KOH in 30% (v/v) methanol in water bath at 70 °C for 5 min. Mixture was centrifuged again at 2500 rpm for 5 min. Then, pellet was extracted with mixture 5 ml DMSO and 5 drops of acetic acid in water bath at 70 °C for 5 min. Mixture was centrifuged at 2500 rpm for 5 min and supernatant was collected to measure the absorbance at 490nm.The absorbance of the combined extracts was determined at 490 nm, and the per unit volume astaxanthin concentration (c) was calculated as:

$$c(\text{mg/L}) = 4.5 \times A_{490} \times V_a/V_b$$

where $V_a\ (L)$ was the volume of extracts, $V_b\ (L)$ is the volume of the culture sample, and A_{490} is the absorbance of extract at 490 nm [8].

3. RESULTS AND DISCUSSION

The growth profile of *Haematococcus pluvialis* in terms of optical density (OD) at 750 nm and cell count is depicted in Figure 1. The optical density for the biomass exhibits an exponential curve where a lag phase was observed for the first 2 days, followed by the growth phase after the 3rd day and finally reached the stationary phase after day 13. The maximum cell density and CDW were 3.0 x 10^7 cells/mL and 6.58 g/L respectively.

Figure 1. Optical density (OD) reading and cell count of *Haematococcus pluvialis* culture during 14 days of cultivation.

Specific growth rate is defined as the increase in cell mass per unit times. The growth rate of population is a measure by the increase in biomass over time and it is determined from the exponential phase. The specific growth rate and division time of *Haematococcus pluvialis* grown were, 0.272 day^{-1} and 0.392 respectively.

Table 1. Astaxanthin Accumulation (mg/L) Under Different LEDs Illumination

Day	Astaxanthin (mg/L)		
	Red LED	Blue LED	Red + Blue LED
2	1.222	0.5643	1.1219
4	1.2182	1.0382	1.4648
6	1.4459	1.5863	1.6839
8	1.6988	1.9143	2.2932
10	1.8077	1.8531	2.1987
12	2.1717	2.4633	2.3436
14	2.1744	2.4917	3.0407

The second part of the experiment is the induction phase which was carried out to investigate the effect of LED illumination light (red, blue and red-blue) on the astaxanthin accumulation within the cell structure when exposed to 24:0 hours light:dark cycle at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 14 days. After 14 days of induction, the flasks showed color changes demonstrating that the cell began to accumulate astaxanthin when subjected to stress, in this case the high light intensity. Table 1 showed that the culture flasks induced by red-blue LED light showed highest astaxanthin accumulation after 14 days of induction with the value of 3.0407 mg/L followed by red and blue LED, which revealed astaxanthin concentration of 2.4917 mg/L and 2.1744 mg/L respectively.

Previous work [5] reported that incorporating blue and red LEDs illumination as external light coupled with fluorescent internal illumination during induction process showed promising results in enhancing the astaxanthin accumulation in *Haematococcus pluvialis* culture compared to fluorescent illumination alone (both internal and external illumination) at 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. It is believed that LED illumination have effect on increased transcript levels of carotenoid biosynthesis genes under both blue and red-light conditions [9]. Based on current finding, the mixture of red-blue LEDs showed greater astaxanthin accumulation than monochromatic LED illumination.

4. CONCLUSION

Based on the results obtained, high intensity LED illumination is proven capable to create stress environment to the *Haematococcus pluvialis* culture to induce astaxanthin accumulation in the cell structure acting as self-defense mechanism to protect itself from the extreme environment changes. However, much study is needed as to further investigate the optimal and economical LED illumination strategy for faster and maximum astaxanthin accumulation in *Haematococcus pluvialis* cell structure.

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Subcritical CO_2 Extraction, Chemical Characterization and Biological Potential of Isolated Essential Oil from Stevia *Rebaudiana Bertoni*

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ABSTRACT

Many aromatic and medicinal plants are available in Malaysia from which the essential oil (EO) can be extracted and used for multipurpose. *Stevia rebaudiana* essential oil (EO) was extracted by using sub-critical carbon dioxide (SC-CO_2) method for the first time and their chemical compounds were characterized by using thin layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS) and fourier transform infrared (FT-IR) spectroscopy. The highest productivity yield of the extracted EO was found in the sample to ethanol ratio of 1:0.5 with 5.6%, followed by the ratio of 1:1 with 3.6% and 1:1.5 with 2.4%. About 33 up to 50 compounds were identified in EO through GC-MS method which representing 89.32%, 92.45%, and 81.42% of identified compound out of the total oil at different extraction ratio of 1:0.5, 1:1, and 1:1.5 respectively. The principal components in the EO were lupeol acetate, epiputranjivol, α-linoleic acid, hexadacanoic acid, and spathulenol. Interestingly, some new compounds were identified such as globulol, betulinic aldehyde, ionone, and γ-sitosterol which had not previously been reported in *Stevia rebaudiana* EO. In addition, FT-IR analysis showed a great abundance of alkynes, aldehydes, alkene, and alkanes were present in the EO. Antimicrobial activity of the EO was also tested against gram-positive and gram-negative bacteria and fungus as well, using a well diffusion method. Among tested microorganisms, two bands from EO of the ratio 1:1 (sample:ethanol) performed effective antimicrobial activity against gram-positive, *B. subtilis* at 10 mm and 11 mm, respectively. Furthermore, *Stevia rebaudiana* EO presented interesting radical scavenging activity against 1,1-diphenyl-2-picryl hydrazyl (DPPH) with IC_{50} values of 0.177 ± 1.04 mg/mL and 0.187 ± 0.94 mg/mL, respectively, performed by isolated EO (band 2 and 3). Results obtained indicated the efficiency of SC-CO_2 employed for extracting *Stevia rebaudiana* essential oil, which could be a good source of natural antimicrobial and antioxidant and may be beneficial as a functional biomaterial with possible applications in food and pharmaceutical. To the best of our knowledge, this is the first study of the composition, antioxidant and antimicrobial activities of EO from *Stevia rebaudiana* collected from Malaysia.

***Keywords**:* Essential oil, bioactive compounds, GC-MS, subcritical carbon dioxide, *Stevia rebaudiana*, FTIR, antioxidant, antimicrobial

1. INTRODUCTION

Stevia rebaudiana (bertoni) is a branded bushy shrub plant which belongs to Asteraceae family, widely distributed and cultivated in South and North America countries such as Paraguay and Brazil. The leaves are used as sweetener in food industry because of the sweetness properties present in the leaves. Besides that, they have been recognized for their medical properties such as antihyperglycemic, antihypertension, antioxidant, and antimicrobial (Basappa *et al.*, 2015). Many reports suggested the extract of the leaves have demonstrated a significant antioxidant activity in in vitro and in vivo studies. The leaves also possess certain antimicrobial activity against several bacteria and fungi. The bioactive compound of this plant is identified by extracting the leaves to produce the essential oil (EO) (Muanda *et*

al., 2011). It has abundant secondary metabolite compound which beneficial to the plant. The biological activity of this plant mostly comes from the EO. The EO have been isolated by using thin layer chromatography (TLC) method and identified its phytochemicals. Among the phytochemicals in the isolated EO of *S. rebaudiana*; terpenes, ester, and phenols is the predominant groups and are perhaps the most studied. The phytochemicals of ester (betulin, stearic acid) and phenol (linoleic acid, ionone) from the isolated EO showed a positive result in antimicrobial activity and also in antioxidant activity.

The extraction of EO from the leaves has been traditionally performed using hydrodistillation or soxhlet extraction. Traditional methods of extraction require large volume of solvent, time consuming and degradation of bioactive compounds. Subcritical carbon dioxide (SC-CO_2) has gained attention over traditional methods because it is green technology and generally recognized as safe (GRAS). CO_2 as a solvent is used for selective and mild extraction of sensitive natural products (Lemus *et al*., 2012). The efficiency of this method was shown by the identification of new ester of lupeol and other new compounds which never been reported before. To the best knowledge, this is the first time to find long chain hydroxyl fatty acid ester of lupeol as natural products.

Although there are a lot of researches on the plant EO, yet additional knowledge remains to be secured. Therefore, in recent years, the researcher has been conducting various experiment related to the EO and its benefits to the human body. To the best of our knowledge, the study of extracted and isolated EO from *S. rebaudiana* leaves are still insufficiently explored. Therefore, this experiment aims to give a new information and knowledge to the researcher to explore deeper regarding the EO in plants. The purposes of this study are to determine the productivity of EO from the extraction method using subcritical carbon dioxide method (SC-CO_2), to identify the bioactive compound from the isolated EO using TLC and GC-MS and to analyze their antimicrobial and antioxidant potential.

2. MATERIALS AND METHODS

2.1 Extraction of EO

Subcritical fluid extraction was carried out using pilot-plan SC-CO_2. The step of extraction was performed consequently at 70 MPa and at temperature of 28˚C to 29˚C. The samples were extracted with pure ethanol in different ratio of concentration (1:1, 1:0.5, and 1:1.5). The average duration of each extraction was 12 hours.

2.2 Gas Chromatography Mass Spectrophotometry (GC-MS)

The injector and mass transfer line temperature were set at 250˚C and 300˚C respectively. The oven temperature is heating from 50˚C to 200 ˚C at 8˚C /min, and keep isothermal for 20 min and finally raise it to 300˚C at 10˚C /min. Diluted samples of 0.2 microlitre were manually injected in splitless mode (Hossain, Siddique, & Rahman, 2010).

2.3 Thin Layer Chromatography (TLC)

Aluminum plates (20x20cm) covered with silica gel with 0.5mm thickness of stationary phase were used in this method. 10mg of sample, EO was diluted in 10ml of hexane for the method of separation. The solvent used in for EO separation was dichloromethane-toluene-ethyl acetate in proportions (70:28:2 v/v).

2.4 Antimicrobial Assay

The isolated EO is evaluated against S. *aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*, and *C. albicans* The microbial activity of isolated EO are determined by using well-diffusion method. The spreading volume of microbial inoculum over entire agar surface. Once a hole being made, 50µl of sample is introduced into the well. Agar plates are incubated for 24 hours and zone of inhibition is calculated after the incubation period is finished.

2.5 Antioxidant Assay

For each sample, different concentrations ranging from 0.6 to 500 μg/mL are prepare with methanol. The reaction mixtures in the 96-well plates consisted of sample (100 μL) and DPPH radical (100 μL, 0.2 mM) dissolved in methanol. The mixture is stirred and left to stand for 15 min in the dark. Then the absorbance is measure at 517 nm against a blank. All determinations are performed in triplicates. The percentage scavenging effect is calculated as:

$$\text{Scavenging rate} = [1 - (A_1 - A_2) / A_0] \times 100\%$$

where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample, A_2 is the absorbance of sample without DPPH radical. The scavenging ability of the samples is expressed as IC_{50} value, which is the effective concentration at which 50% of DPPH radicals are scavenged.

3. RESULTS AND DISCUSSION

3.1 Extraction of EO

Table 1. Yield Percentage of EO

Ratio (sample: ethanol)	Yield of EO (%)
1:0.5	5.6
1:1	3.6
1:1.5	2.4

3.2 Isolation of EO

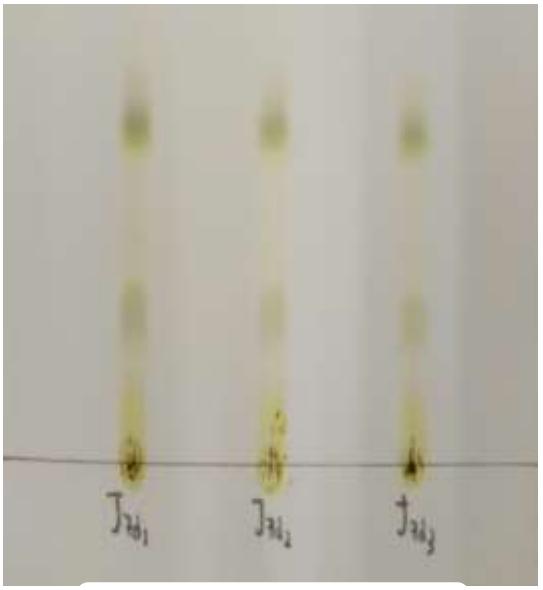

Figure 1. Bands on TLC plate

3.3 Antioxidant of EO

Table 2. IC_{50} of isolated EO

IC_{50} of isolated EO (µg/ml)			Control
Terpenes	Ester	Phenols	Trolox
-23.27±0.78	178.87±1.04	177.89±0.94	5.83±0.08

3.4 Antimicrobial of EO

Table 3. Antimicrobial activity of isolated EO

Extracts (10mg/ml)	Zone of inhibition (mm)				
	E. coli	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*C. albicans*
Terpenes	-	-	-	-	-
Ester	-	-	10±0.11	-	-
Phenols	-	-	11±0.88	-	-

4. CONCLUSION

EO produced by SC-CO_2 extraction method performed a high productivity of EO. The efficiency of this method can be proved by the identification of new compounds such as globulol, betulin, and lupeol acetate. Isolated EO (ester and phenol) have been found to perform antioxidant and antimicrobial activities. The finding and the knowledge from this study will hope can give new ideas to the researcher to explore a deeper understanding of EO in the plants.

5. ACKNOWLEDGEMENT

Thanks to my supervisor, Dr. Uswatun Hasanah for giving me opportunities to conduct this project. I want to express gratitude to the RMC for the grant research and also thank you to the Department of Chemistry, Faculty of Science and Supercritical Fluid Centre for providing a facility to conduct the research project.

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Optimization of *Swietenia Mahagoni* Seed in Supercritical Carbon Dioxide Extraction

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ABSTRACT

Supercritical carbon dioxide extraction has been used for extraction of essential oil from *Swietenia mahagoni* seed. The effect of different particle sizes on diffusivity coefficients, D (m^2/s) were studied by applying second Fick's law of diffusion proposed by Crank. Particle size of 710 μm showed the highest D of 3.1 x 10-12 m^2/s. Gas compression at pressure of 30 MPa and temperature of 60 ºC on seeds caused swelling thus allowed faster diffusion of carbon dioxide through porous structure of *Swietenia mahagoni* seed. For optimization of *Swietenia mahagoni* seed oil extraction, a three-level factorial design in response surface methodology was used to analysis the effect of pressure (20 – 30 MPa) and temperature (40 - 60 ºC) on extraction oil yield. The highest extraction oil yield of 29.70% was obtained at pressure of 30 MPa and temperature of 40 ºC. Qualitative phytochemical analysis showed the presence of alkaloid, saponin, triterpenoid, phenolic hydroquinone and tannin but absence of flavonoid.

Keywords:*Swietenia mahagoni*, Supercritical Fluid Extraction (SFE), optimization, diffusivity coefficients (D)

1. INTRODUCTION

Swietenia mahagoni, also known as 'tunjuk langit' in Malaysia is used traditionally to treat various diseases such as diabetes and high blood pressure [1]. In addition, *Swietenia. mahagoni* seeds have been reported to have various biological activities such as anti-inflammatory activity, anticancer and antitumor activity [2] as well as antidiabetic activity [3]. β-sitosterol is one of the diversified group of compounds in phytosterols. Phytosterols are well known as plant sterols, one of the vital components of plant membranes [4]. Several studies have suggested that phytosterols lowered the cholesterol level by reducing the adsorption of intestinal cholesterol [5], lowering the cholesterol [6] and may reduce the risk of heart disease by 40% [7]. The most ample compound in natural sterols is β-sitosterol [8] and it can be found in seeds, nuts, vegetables and fruits. The aim of this study is to determine the effect of pressure and temperature of supercritical carbon dioxide (SC-CO_2) extraction on oil yield and β-sitosterol content from *Swietenia mahagoni* seeds using a response surface methodology (RSM). Recently, attention on the importance of natural compounds from plants and herbs has been reassessed. As a matter of fact, bioactive compounds from plant sources are chemically sensitive and present in low concentration. Hence, supercritical carbon dioxide (SC-CO_2) extraction is the appropriate extraction method to be used. Application of SC-CO_2 has been applied in various fields due to the adjustable nature of the system. Therefore, for a successful SC-CO_2 extraction, several parameters must be considered to obtain an efficient extraction process such as temperature, pressure, flowrate, particle size and moisture content.

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

Figure 1.1 *Swietenia mahagoni* also known as 'tunjuk langit' in Malaysia (a) tree, (b) fruit, (c) winged seeds and (d) seeds

2. MATERIALS AND METHODS

2.1. Material

Commercial grade liquid carbon dioxide (purity 99.99%) used in supercritical carbon dioxide extraction was purchased from Kras, Instrument and Services, Johor, Malaysia. Acarbose, 1-deoxynojirimycin, p-nitrophenyl-α-D-glucopyranoside (pNPG), sodium dihydrogen phosphate, sodium phosphate tartrate, disodium hydrogen phosphate, sodium hydroxide, dimethyl sulfoxide (DMSO), potassium chloride, 3,5-dinitrosalicylic acid (DNS), starch (soluble), α-amylase from porcine pancreas and α-glucosidase from *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis MO, USA). Sodium chloride (Bendosen Laboratory Chemicals, Bendosen, Norway). Potassium phosphate monobasic (Merck, Millipore, Billerica, Massachusetss, USA), distilled water.

2.2 Sample Preparation

Swietenia mahagoni seeds were bought in the local market. The seeds were rinsed with tap water to remove any foreign particles and dirt prior to drying. Then, the cleaned seeds were cut into small pieces and dried by using oven (Memmert, Germany) at temperature of 50°C for a week to remove moistures. The seeds were ground by using a blender (Waring® Commercial blender, USA) and sieved to approximate 0.50 mm of particle size.

2.4 Supercritical Carbon Dioxide (SC-CO_2) Extraction

CLEAR supercritical fluid extraction (SFE) machine in Center of Lipids Engineering and Applied Research (CLEAR), Universiti Teknologi Malaysia consisted of CO_2 gas cylinder, CO_2 controller pump (Lab Alliance), co-solvent pump (Lab Alliance), oven (Memmert, Germany), 10 ml stainless steel extraction vessel, pressure gauge (Swagelockk, Germany), automatic back pressure regulator (Jasco BP 2080- Plus) and restrictor valve. A schematic diagram of CLEAR SFE apparatus is illustrated in Figure 2.1. The parameters and constant parameters used in extraction process are presented in Table 2.1. Five gram of sample was placed in 10 ml stainless steel extraction vessel and sealed tightly in the oven. Set all the parameters (temperature, pressure and flowrate of CO_2), the extraction process started after all the parameters were attained. Lastly, depressurized the system and the oil yields were collected after 120 minute extraction time.

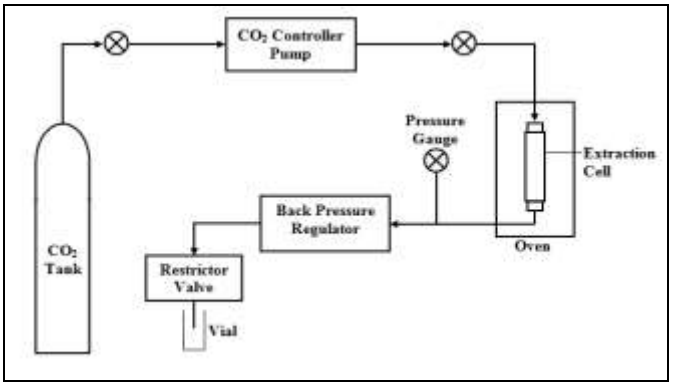

Figure 2.1 The schematic design of the SC-CO_2 unit

The parameters and constant parameters used in extraction process are presented in Table 2.1. Five gram of sample was placed in 10 ml stainless steel extraction vessel and sealed tightly in the oven. Set all the parameters (temperature, pressure and flowrate of CO_2), the extraction process started after all the parameters were attained. Lastly, depressurized the system and the oil yields were collected after 120 minute extraction time.

Table 2.1: The process parameters for SC-CO_2 extraction

Parameter	Range/ value
Temperature (°C)	40-60
Pressure (MPa)	20-30
Flowrate of CO_2 (ml/min)	2.00
Particle size (mm)	0.50
Mass of sample (g)	5.00
Extraction time (min)	120

Collected oil yield was calculated as percentage of oil yield by using equation below:

$$\text{Oil yield } (\%) = \frac{\text{Mass of oil extract (g)}}{\text{Mass of sample (g)}} \times 100 \qquad 2.2$$

3. RESULTS AND DISCUSSION

3.1. Determination of Extraction Time

The determination of the extraction time for the extraction of *S. mahagoni* seeds by using SC-CO_2 extraction was performed. The conditions used were pressure of 20, 25 and 30 MPa, with the temperature of 50°C. The flow rate used in this study was 2 ml/min. The yield of extract was collected every 20 minutes over 180 minutes of extraction time. The results of the best extraction time are presented in Figure 3.1

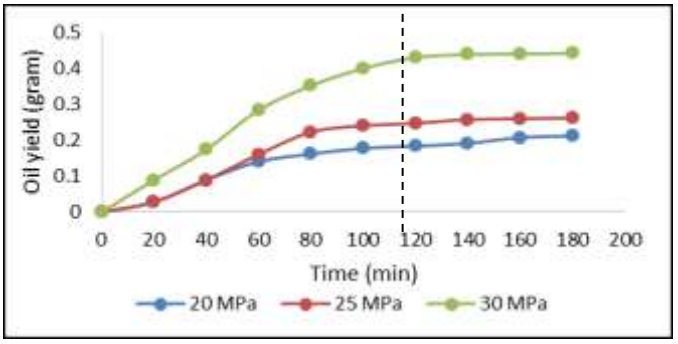

Figure 3.1 The effect of extraction time on extraction yield of SC-CO_2 at 20, 25 and 30 MPa and the temperature of 50 °C

Figure 3.1 displays the oil yield in percent versus the extraction time in minutes. At pressure of 20 MPa, it took more than 120 minutes to achieve the asymptomatic value. The highest yield (0.4306%) was at the highest pressure of 30 MPa within 120 minutes. Similar result with this study where the seed oil of *Microula sikkimensis* increased as extraction time increased [10]. The same trend was found in the study of saffron extract by [9]. In conclusion, the extraction time of *S. mahagoni* seeds was performed at 120 min constantly for each sample throughout this study in order to obtain asymptotic value as well as to ensure maximum extraction yield was extracted. Pressure influences both solvating power and intermolecular interaction strength. Oil yield increases with pressure from 20 MPa to 30 MPa. The increase in pressure will increase the density of solvent hence increase the solvating power of SC-CO_2. In the extraction *Vitex agnus*, the extraction rate increases with the pressure from 10 MPa to 45 MPa [11].

3.2. Analysis of Response Surface

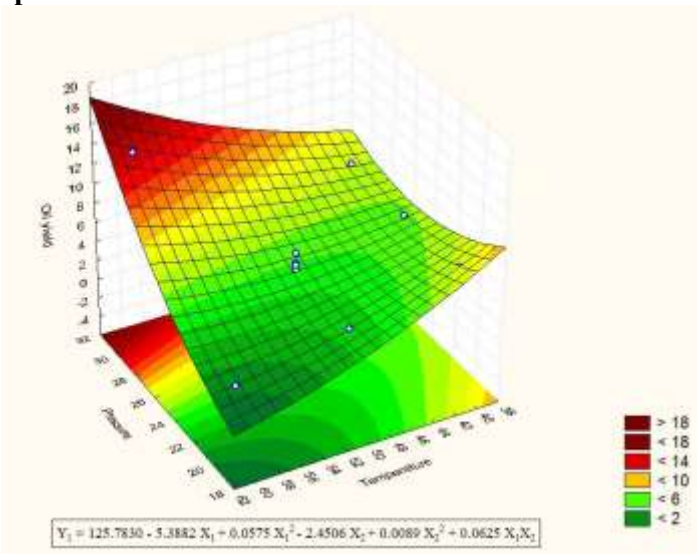

Figure 3.2 Surface plot of oil yield from *S. mahagoni* as a function of pressure and temperature

Figure 3.2 show the surface plot for the response of oil yield. When the temperature decreases from 60-40°C, the oil yield slightly increase while as pressure increase from 20-30 MPa, the oil yield increases. It is concluded that pressure is a dominant factor for the extraction of oil yield from *S. mahagoni* seeds whereas temperature has a minimal effect on the oil yield. The minimal effect of temperature in the extraction of *S. mahagoni* seeds as the drop of temperature from 60-40°C increases the extraction yield. This phenomenon can be explain by a phenomena where the solvent solubility increased at the lower temperature due to the changes in density. This is due to the increase in density of extraction fluid (SC-CO_2) when the temperature decreases from 100-40°C.

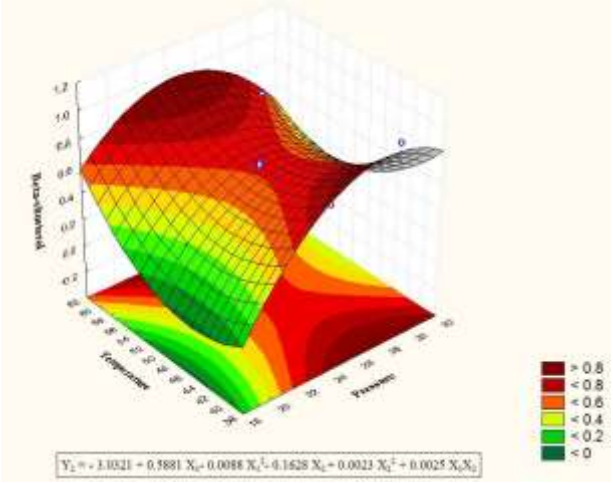

Figure 3.3 Surface plot of β-sitosterol content from *S. mahagoni* as a function of pressure and temperature

Meanwhile, the response surface plot of β-sitosterol content as a function of pressure and temperature is shown in Figure 4.7. The effect of pressure on the extraction of β-sitosterol shows a positive quadratic effect. As pressure increases from 20 to 25 MPa, the β-sitosterol content in extract increases as the solubility of β-sitosterol in the solvent increase but decrease as it reaching 30 MPa, shows the interaction of repulsive solute-solvent increases [12]. This may due to the compressed solvent at high pressure in the extractor.

3.3. β-sitosterol Content

The β-sitosterol content of *Swietenia mahagoni* seeds extract with different conditions in SC-CO_2 extraction were identified and quantified. The highest β-sitosterol content obtained was 9.2 mg/g at 30 MPa and 40°C meanwhile the lowest (3.12 mg/g) was obtained at 20 MPa and 50°C. Previous researches on the β-sitosterol content of other plants using SC-CO_2 extraction were compared with the result in this study in Table 4.4. Notably, the temperature of 40°C shows better extraction of β-sitosterol from plants since low temperature can avoid the degradation of compound. The temperature in SC-CO_2 extraction effect the yield of β-sitosterol because of the effect of solvent density. The solvent density increase with decreasing temperature hence the solubility of β-sitosterol increase by increasing the solvating power. On the other hand, high pressure also affect the solvent density.

4. CONCLUSION

The extraction of *Swietenia mahagoni* seeds using supercritical carbon dioxide (SC-CO_2) showed that the highest extraction yield obtained was at 30 MPa and 60°C while the highest β-sitosterol content was at 30 MPa and 40°C. The experimental data of SC-CO_2 extraction obtained were fitted to a second-order polynomial model and the obtained oil yields were 1.49-14.45%, while β-sitosterol content obtained were 3.12-9.20 mg/g. SC-CO_2 extraction of *Swietenia mahagoni* seeds also excellent for the extraction of β-sitosterol with the highest extracted yield was 9 mg/g of β-sitosterol in *Swietenia mahagoni* seeds. Hence the usage of SC-CO_2 extraction in enhancing the extraction of plant extract was proven in this study.

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The Potential of Edible Bird's Nest (EBN) Extract as Anti-Cancer Agent and Immunoadjuvant on Human Breast Cancer

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ABSTRACT

Edible Bird's Nest (EBN) is well regarded as a nutraceutical food especially among the Chinese. The aim of this study is to explore the potential of EBNE as an anti-cancer agent and immuno-adjuvant against human breast cancer (MCF-7). Primarily, EBNEs were tested on its cytotoxicity level against MCF-7 and human immune cytotoxic T cells (CD8+). Production of the key pro-apoptotic and anti-apoptotic molecules released in MCF-7 and CD8+ cells before and after EBN treatment were measured through mRNA expression level. Among the 3 types of extracts tested, HMG showed the best cytotoxic effect towards MCF-7 cells with IC50 of 0.06%. However, HMG showed no cytotoxic effect towards CD8+ cells with viability more than 90%. RTPCR results for activated and non-activated CD8+ cells showed increased pro-apoptotic gene expression while lower anti-apoptotic gene expression after treated with HMG for 72hr in single and co-culture. At the same time, supplementation of EBN down regulations anti-apoptotic genes and upregulates pro-apoptotic genes in MCF-7 cells. In conclusion, study showed that HMG is an effective anticancer agent and not cytotoxic to human immune cells. RTPCR tests also verified that HMG acts as an immuno-adjuvant by enhancing pro-apoptotic function in CD8+ cells.

Keywords: EBN, Immuno-adjuvant, Breast Cancer, Cytotoxicity, Pro apoptotic.

1. INTRODUCTION

Edible Bird's Nest (EBN) is produced by the salivary glands of swiftlets. It is regarded as a delicacy with medicinal values enjoyed mainly by the Chinese communities all over the world. The composition of EBN is about 35.8% protein, 4.5% ash and 1.5% fat while the carbohydrate content is 39.38% (1, 2). EBN is valued for its reputed health benefits such as improving digestion, increasing libido, alleviating asthmatic problems and slowing down the aging processes (1, 2). Edible Bird's Nest Extracts (EBNE) are also been reported for mitogenic effects on human blood monocytes after stimulation by Concanavalin A and Phytohemagglutinin A. Thus, EBNE could possess effects that help immune cells in division, therefore, exhibit immune enhancing potential (3). The immune enhancing properties of EBNE indicated that could treat human breast cancer (MCF-7) potentially. Study had also reported galactose or N-galactosamine and abundant contents of lectin-containing sugar chains have presence in EBNE could possess anti-cancer properties (4). Lectins from plants were shown to have anticancer properties in vitro, in vivo and in human case studies (5). On the other hand, some folk's tales indicated the linkage of protein rich food in helping cancer cells to grow. This obstruct the exploration of EBNE as an anti-cancer agent and immuno-adjuvant against human breast cancer. This has hindered the possible consumption of EBN and its related products in cancer patients where it was meant to rejuvenate cells.

2. MATERIALS AND METHODS

Materials: Dulbecco's Modified Eagle Medium (F12: DMEM), Fetal Bovine Serum (FBS), Histopaque, MAC's Microbeads Kit, PE Mouse Anti-Human CD8, APC Mouse Anti-Human CD3, Interferon gamma (IFNG), Granzyme B (GZMB), Perforin 1 (PRF-1) and Transforming growth factor beta 1 (TGF-β1), Caspase 7 (CASP-7), B-cell lymphoma 2 (BCL-2), Bcl-2-associated X protein (Bax), Estrogen receptor 1 (ESR-1) and Cytochrome c (CYCS).

EBNEs coded as HMG, EHMG and pHMG were prepared according to the standardized method innovated by Institute of Bioproduct Development (IBD). The breast cancer cell line (MCF-7) was obtained from American Type Culture Collection (ATCC) and cultured in F12: DMEM added with 10% FBS. The cytotoxic effects of three types of EBNEs on MCF-7 cells were tested on MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay (6). In this assay, 96 well plates were chosen to seed in 6000 cells per well. The cells were cultured in 100µL of F12: DMEM medium without serum. Besides, EBNE of concentrations 2%, 1%. 0.5%, 0.25%, 0.12% and 0% were added to the wells and treated for 72 hours. The most effective percentage of extract demonstrated with higher level of cytotoxicity in its lowest concentration towards MCF-7 cells was chosen to run in the subsequent test in this research. Apart from that, EBNE coded as HMG with 0.06% of concentration was tested upon CD8+ cells to check the cytotoxicity via MTT assay as well. Blood from healthy female individuals was collected and the leucocytes were separated from the whole blood by using Histopaque (density gradient medium). The obtained peripheral blood mononuclear cells were then isolated into CD8+ cells using MACs Microbeads Kit. The purity of isolated cells was checked using florescent antibodies (CD8 PE and CD3 APC) and flowcytometry. Besides, there are 4 different groups tested for CD8+ cells which are the non-activated and activated cells treated with HMG in single culture and co-culture with MCF-7 cells. This study tested both non-activated and activated CD8+ is to determine the effect of HMG in both naïve CD8+ cells and activated CD8+ cells after encountered with an antigen. The purpose of co-culture is mainly to measure the synergistic effect of HMG with CD8+ cells to kill MCF-7 cells. IFNG, GZMB, PRF-1 and TGF-β1 gene expression was measured in CD8+ cells while CASP-7, BCL-2, Bax, ESR-1 and CYCS were measured in MCF-7 by Real Time Polymerase Chain Reaction (RTPCR) (7).

3. RESULTS AND DISCUSSION

MTT assay measures cell proliferation rate. It assesses the reduction in cell viability when metabolic events lead to apoptosis or necrosis. IC_{50} is the inhibitory dose that induce 50% cell mortality (6). Figure 1, 2 and 3 shows IC_{50} for HMG, EHMG and pHMG which are 0.06% (15µg/mL), 0.08% (20µg/mL) and 1.30% (325µg/mL) respectively. Thus, HMG was chosen for the following test in this study as it shows the lowest IC_{50} concentration among the other two extracts. According to the American Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extract is an IC50< 20 µg/ml (8). On the other hand, HMG with various concentrations was also tested upon isolated CD8+ cells to verify that EBN is not cytotoxic to immune cells. The purity check from flowcytometry for isolated CD8+ is 97.5%. Results from Figure 4 for the MTT assay on CD8+ showed that even the highest concentration 0.5% of HMG is not cytotoxic to immune cells. The cell viability was more than 90%.

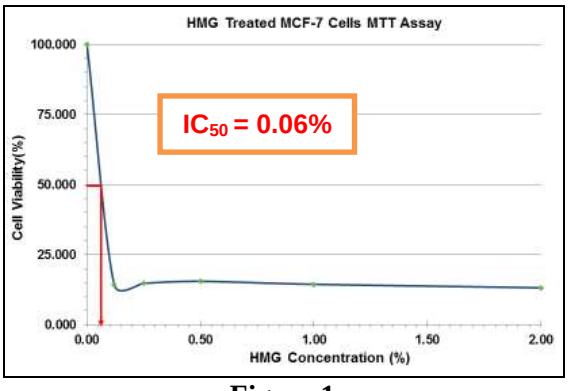

Figure 1

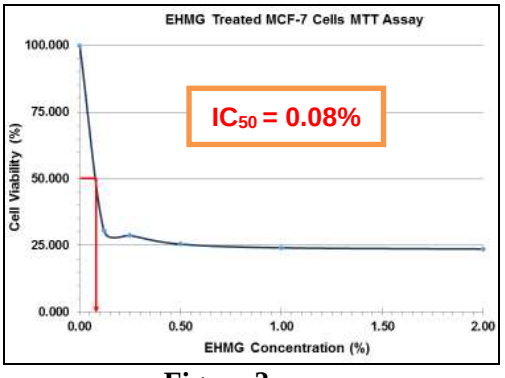

Figure 2

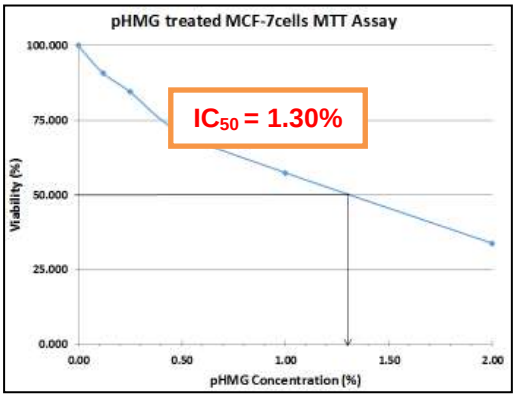

Figure 3

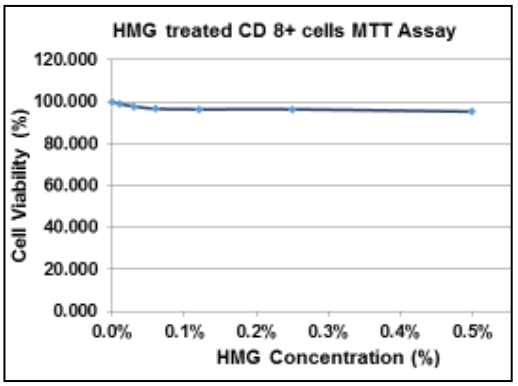

Figure 4

Figure Captions

Figure 1: MTT assay on MCF-7 cells treated with different HMG concentrations for 72 hours to determine the cell viability.

Figure 2: MTT assay on MCF-7 cells treated with different EHMG concentrations for 72 hours to determine the cell viability.

Figure 3: MTT assay on MCF-7 cells treated with different pHMG concentrations for 72 hours to determine the cell viability.

Figure 4: MTT assay on CD8+ cells treated with different HMG concentrations for 72 hours to determine the cell viability.

RT-PCR is a method commonly used in molecular biology to detect ribonucleic acid (RNA) expression (9). GZMB, PRF-1 and IFNG are chosen to be tested because they synthesized by cytotoxic CD8+ T cells to kill virally infected cells and tumors. They have the strongest pro-apoptotic functions. For an example, perforin forms a pore in the membrane of the target cell where the granzymes contained cytotoxic granules enter the malignant cell and cleave the proteins inside the cells. Thus, shutting down the production of viral protein and ultimately resulting in apoptosis of the target cell (10). Figure 5 and 6 shows that HMG treated CD8+ cells (non-activated and activated) upregulate the pro-apoptotic genes expression compared to the control group in single culture. At the same time, CD8+ cells (non-activated and activated) in co-culture with MCF-7 also demonstrates higher pro-apoptotic genes expression after treatment with HMG (Figure 7 and 9). Besides, non-activated CD8+ cells demonstrated increased TGF β1 expression while lower expression in activated CD8+ cells compared to control in both single and co-culture. This is due to TGF-β1 suppresses the activation of CD8+ t-cells when naive but promotes their survival and function once antigen experienced (11).

Tumor growth is the results of the deregulation of intrinsic proliferation (cell division) and cell death (apoptosis). Either inactivation of pro-apoptotic pathway or activation of anti-apoptotic pathway results in failure of apoptosis, thereby promoting tumor cell survival (12). BCL-2 and ESR-1 are anti-apoptotic and CASP-7, Bax, p53 and CYCS are pro-apoptotic genes. We hypothesize with supplementation of HMG can induce the apoptosis through downregulation of anti-apoptotic genes and upregulation of pro-apoptotic genes in MCF-7 cells. Figure 8 and 10 shows that CASP-7, Bax, p53 and CYCS are upregulated by HMG while downregulates BCL-2 and ESR-1 gene. The results are almost aligned with other study that have also showed lemon citrus extract induced apoptosis in MCF-7 breast cancer cells via upregulating the expression of Bax gene and downregulating the expression of BCL-2 gene (13). Furthermore, ethanolic extract of mango seed induced apoptosis in MCF-7, through increasing pro-apoptotic proteins (CYCS, Bax, CASP-7) and decreasing anti-apoptotic protein (BCL-2) (14).The higher expression of pro-apoptotic and lower expression of anti-apoptotic genes by HMG treated CD8+ and MCF-7 is important because when there is aberrant apoptosis, cells that should be killed instead become immortal, leading to the pathogenesis of many diseases including cancer (12).

Single Culture

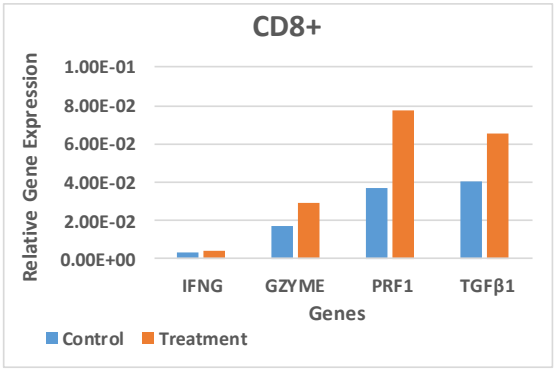

Figure 5

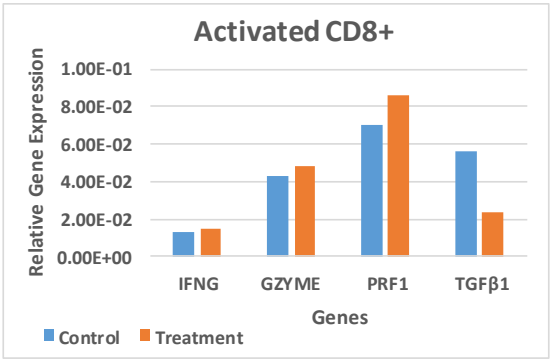

Figure 6

Co-culture (CD8+ and MCF-7)

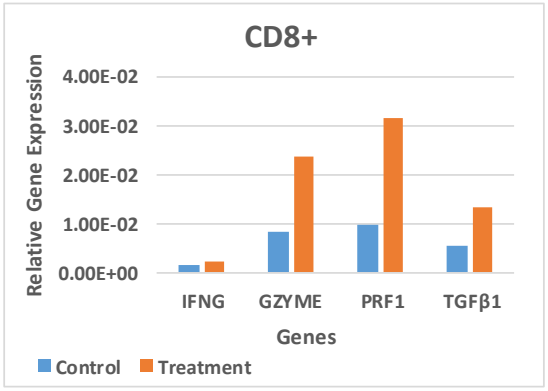

Figure 7

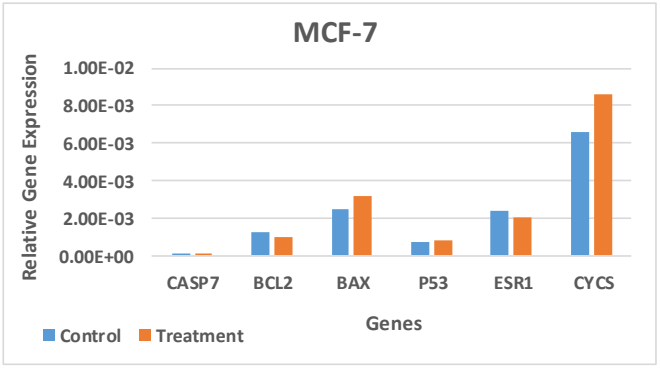

Figure 8

Co-culture (Activated CD8+ and MCF-7)

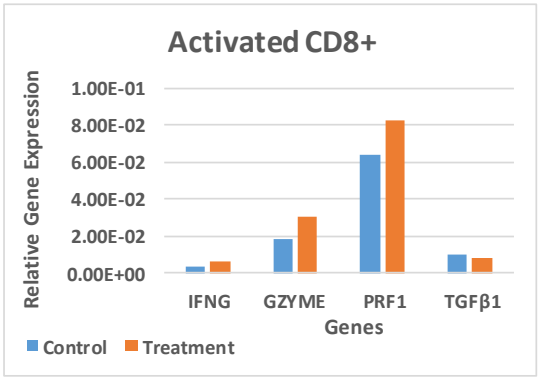

Figure 9

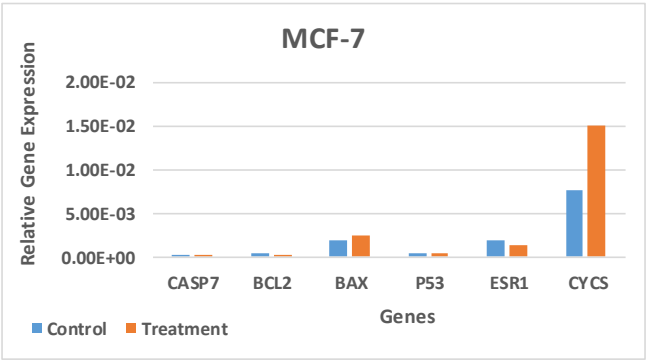

Figure 10

Figure Captions

Figure 5: Relative Gene Expression of IFNG, GZMB, PRF-1 and TGFβ-1 in non-activated CD8+ cells single culture before and after EBN treatment.

Figure 6: Relative Gene Expression of IFNG, GZMB, PRF-1 and TGFβ-1 in activated CD8+ cells single culture before and after EBN treatment.

Figure 7: Relative Gene Expression of IFNG, GZMB, PRF-1 and TGFβ-1 in non-activated CD8+ cells from co-culture with MCF-7 cells before and after EBN treatment.

Figure 8: Relative Gene Expression of CASP-7, BCL-2, BAX, p53, ESR-1 and CYCS in MCF-7 cells from co-culture with non-activated CD8+ cells before and after EBN treatment.

Figure 9: Relative Gene Expression of IFNG, GZMB, PRF-1 and TGFβ-1 in activated CD8+ cells from co-culture with MCF-7 cells before and after EBN treatment.
Figure 10: Relative Gene Expression of CASP-7, BCL-2, BAX, p53, ESR-1 and CYCS in MCF-7 cells from co-culture with activated CD8+ cells before and after EBN treatment.

4. CONCLUSION

The results of this study showed that HMG extract is an effective anti-cancer agent, and did not shows cytotoxic effect on human immune cells. In addition, the higher expression of pro-apoptotic genes by cytotoxic T cells after treatment with HMG proved that HMG can act as immune adjuvant in fighting breast cancer. Thus, we envisage that this study will contribute to the development of anti-cancer therapeutic modalities from EBN in future.

ACKNOWLEDGEMENTS

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Effect of *Momordica Charantia* Linn. On Human Skeletal Muscle Cell

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ABSTRACT

This study aimed to investigate the effect of *Momordica charantia* Linn. (MC) fruit juice extract on glucose uptake level in human skeletal muscle cell. The MC juice was extracted using juicer without additional water to obtain pure juice. Then, glucose concentration level in MC was determined to indicate the existed glucose content in the juice using D-Glucose R-Biopharm assay kit. The same batch of juice was used to further study the effect of glucose uptake level in human skeletal muscle cell when treated with and without MC juice. The data then was analyzed using statistic. In brief, 42 mL of juice can be extracted from each 100g of MC fruit. The glucose concentration level in MC fruit extract was 6.8241g/L. The glucose concentration level in cells treated with DMEM only was 4.422 g/L and significantly higher ($p < 0.001$) than cells treated with DMEM added with MC juice, 3.678 g/L. This result indicated that the cells treated with MC juice had increased glucose uptake and utilization, leading to a reduction of glucose concentration level in media. This study demonstrated that the beneficial effect of MC juice extract on human skeletal muscle cell glucose uptake.

Keywords: *Momordica charantia* Linn.; glucose uptake; human skeletal muscle cell

1. INTRODUCTION

Momordica charantia Linn. (MC) or bitter gourd is known for its bitter taste comes from Cucurbitaceae family. This plant was used in Ayurveda therapeutic treatment as described in Ayurvedic texts written in Indian Sanskrit from 2000 to 200 BCE during Indo-Aryan culture [1] which indicates the early sign of MC cultivation in India [2]. In traditional medicinal practice, MC is used as anti-inflammatory, anti-ulcer, anti-leukemic, anti-HIV, anti-tumor, anti-diabetic, and anti-microbial [3], [4]. In diabetic studies, charantin, vicine and polypeptide-p extracted from fruit and seeds [5], [6] are the bioactive compounds in MC that have similar structure to insulin [7]. This similarities may contribute to MC abilities to increase insulin secretion, body tissue glucose uptake, glycogen synthesis in liver muscle and reduce glucose absorption [8]. These components and several type of extraction including pure juice, methanolic, ethanolic and chloroform extracts from the fruit, shows positive metabolic and hypoglycemic activity conducted on human, animal and cell culture research [9], [10].

Glucose uptake stimulation in insulin-sensitive tissues is one of crucial mechanism by which insulin controls blood glucose in human body [11]. In this study, human skeletal muscle cells were selected as it plays crucial role in energy homeostasis and insulin sensitivity. Skeletal muscle glucose uptake is the major site for insulin-regulated glucose clearance which mediated by GLUT-4 and GLUT-1 proteins, the major glucose transporter (GLUT) [12]. Skeletal muscle also responsible for the disposal of most of both oral and intravenous glucose loads for glucose homeostasis [12], [13]. In type 2 diabetes mellitus (T2DM), the insulin resistance occurred due to the decreased of insulin-stimulated glucose transport and impaired metabolism in adipocytes and skeletal muscle. Eventually contributed to the down-regulation GLUT-4 which acts as the major-insulin responsive [14]. Although there are lots of studies that links MC extract effect to glucose uptake, to the current knowledge, there are still limited reports on glucose uptake activity exerted by MC extract in vitro studies. Thus, this study aims to investigate the relationship between glucose uptake and *Momordica charantia* Linn. fruit juice extract treated in in human skeletal muscle cell.

2. MATERIALS AND METHODS

2.1 Plant material and juice extraction

The unripe MC fruit were obtained from the local market, washed thoroughly and the seeds were removed. The fresh juice was prepared on a juicer without adding water to extract pure MC juice. The 100% juice was stored in 4°C until further use.

2.2 Determination of glucose level in MC juice extract

The glucose level in MC juice extract was determined by using D-Glucose R-Biopharm assay kit. For the solutions preparation, Bottle 1 (consisting of triethanolamine buffer, pH 7.6; NADP, 110 mg; ATP, 260 mg; magnesium sulphate) was dissolved in 45 ml redistilled water and the Bottle 2 (consisting of 1.1 mL suspension containing hexokinase, 320 U; glucose-6-phosphate dehydrogenase, 160 U) was used undiluted. As for controls, D-glucose assay solution (Bottle 3) was used. Then, the solutions were pipetted into cuvettes as in Table 1. The absorbance reading was taken with Shimadzu UV-1800 spectrophotometer and the glucose concentration was calculated.

Table 1: Assay kit solutions preparation

Pipette into cuvettes	Blank (mL)	Sample (mL)
Solution 1	0.500	0.500
Sample solution*	-	0.050
Redistilled water	1.000	0.850
The solution* was mixed and the absorbance was read (A_1) approximately after 3 minutes. The reaction then was started by adding:		
Suspension 2	0.010	0.010
The solution* was mixed and waited for the action to stop approximately in 10-15 minutes and the absorbance of the solutions was read (A_2). However, if the reaction does not come to stop after 15 min, the absorbance read will be continued at 2 min intervals until the absorbance increases constantly for 2 min.		

2.3 Determination of MC juice extract on human skeletal muscle cell glucose level

Human skeletal muscle cells was cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin. The cells were sub-cultured every three days until the cells reached 70%-80% of confluent culture. 2-4 x 10^4cells/cm^2 were used as standard seeding density and then cultured in 75cm^2 flask. The cell culture was incubated in a humidified atmosphere that contained 5% CO^2 at 37°C and further maintained as stated before. For the differentiation induction, the cells were seeded at a density of 2-8 x 10^4cells/ml in 96-well plate and supplemented with Skeletal Muscle Cell Differentiation Medium (SKM-D). 100µL of cell solution were pipetted into each well. The cells were maintained until reaching the 70%-80% of confluent culture for about four to six days. Supporting growth medium was changed every other day and cells were washed with phosphate buffer saline (PBS). The cells confluence was monitored using an inverted microscope. To study the effect of MC juice extract on human skeletal muscle cell, the cells were treated with and without MC juice extract in 96 well-plate. The cells were discarded and the media was collected and further analyzed according to D-Glucose R-Biopharm glucose uptake assay protocol.

2.4 Statistical analysis

The obtained data were collected and analyzed using standard t-test where $p<0.05$ is considered significant. The data were presented in bar graph.

3. RESULTS AND DISCUSSION

3.1 Juice concentration

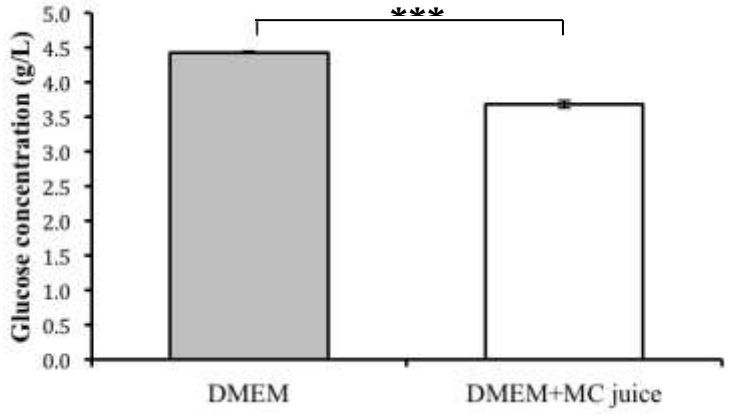
Concentration of the juice was expressed in relation to wet weight which is 42 mL obtained from 100g of MC fruits.

3.2 Glucose concentration level in MC juice extract

The glucose concentration of pure MC juice extract was determined using D-Glucose R-Biopharm assay kit with three replicates. From the experiment, we found out that the glucose concentration level in MC juice extract was 6.8241g/L.

3.3 Effect of MC juice extract on human skeletal muscle cell glucose level

In this study, an experiment was conducted on two different set human skeletal muscle cell treatments with three replications each. In Sample 1, the cells were treated with only DMEM media. Meanwhile in Sample 2, the cells were treated with DMEM added with MC juice. As presented in Graph 1, we found out that the glucose concentration level in cells treated with DMEM only was 4.422 g/L and significantly higher ($p<0.001$) than cells treated with DMEM added with MC juice, 3.678 g/L. This indicated that the cells treated with MC juice worked actively in glucose utilization that leads the cells to improve glucose uptake thus leads to the reduction of glucose concentration level in media in Sample 2. According to study by Lee et. al, they proposed two possible mechanism of MC extracts on blood; (1) MC extract has the abilities to regulate glucose content absorbed to the gut into the blood following a meal and; (2) the MC extracts has hypoglycemic effect and insulin-like properties which can stimulate the glucose uptake into skeletal muscle cell [15]. Isolated bioactive compound such as charantin, vicine and polypeptide-p extracted from fruit and seeds [5], [6] are responsible to exert potential effects in lowering blood glucose by increasing the glucose uptake and glycogen synthesis in the liver, muscles, and adipose tissue and activating insulin receptor substrate 1 (IRS1) in skeletal muscle by tyrosine phosphorylation [16]. There are also studies that reports the hypoglycemic effects of MC extracts were occurred due to the increase of beta cells number, glucose uptake by skeletal muscle and adipose tissue and AMPK pathway activation that contributes to insulin secretion stimulations [17].



Graph 1: Glucose concentration level in human skeletal muscle treated with and without MC fruit juice.
*** $p < 0.001$

4. CONCLUSION

From this study, it can be concluded that *Momordica charantia* Linn. fruit juice extract has shown anti-diabetic properties, which helped in increase glucose uptake into the cell thus reducing glucose level in blood glucose. For further studies, it is recommended to explore the optimum MC concentration intake for optimized the glucose uptake intake. Also, studying the effect of MC different varieties and its bioactive compound will help us to explore more on the potential of MC anti-diabetic activities. In addition, the ongoing work of this study includes the investigation of anti-inflammatory effect of *Momordica charantia* Linn.

ACKNOWLEDGEMENT

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Physicochemical Profile of *Stevia Rebaudiana* Undergone Microencapsulation using Ultrasonic Spray Drying

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ABSTRACT

This study aimed to determine the yield and physicochemical profile of microencapsulated *Stevia rebaudiana* prepared using ultrasonic spray drying at different percentage of gum arabic (0%, 2%, 4%, 6%, 8% and 10%) at two different temperatures (80°C and 90°C). Physical properties such as morphological evaluation and functional groups determination of microcapsules were carried out. Antioxidant profiles of microcapsules were analysed using DPPH radical scavenging activity, FTC analysis, TBA test, total phenolic content, total flavonoid content, and individual flavonoid analysis. Yield of microcapsules was in the range of 4.35% to 7.70%. Microstructure of all microcapsules was characterized as irregularly spherical shape and smooth surface, with cracks, pores, and breakage. Hydroxyl stretching, alkane stretching carbonyl groups, alkene stretching, ether groups and alkene bending were found in all microcapsules. This study also found that 6% gum arabic microcapsules exhibited the highest antioxidant profile. Rutin which was in the range of 2350.22 to 3987.94μg/100ml was found to be the highest in all microcapsules, followed by kaempferol (4.19 – 7.10μg/100ml and quercetin (0.16 – 60.59μg/100ml). This study found that microcapsules with 6% gum arabic at both 80°C and 90°C showed the best antioxidant activity and gave higher amount of phenolic and flavonoid content.

Keywords: Ultrasonic spray drying; microencapsulation; *Stevia rebaudiana*; gum arabic; antioxidant profile

1. INTRODUCTION

Aging and diseases such as inflammation, coronary heart disease, stroke, ischemia, failure in immunity and endocrine functions and certain cancers can be caused by over production of free radicals in the body [1, 7]. These free radicals can be suppressed by antioxidants. However, natural antioxidants are easily affected by the undesirable conditions such as extreme pH, light and oxygen level and lost during processing or storage [6, 13]. Stevia leaf was found to have significant potential as a natural source of antioxidants [7]. Ultrasonic spray drying is one of the microencapsulation techniques. Unlike spray drying method which requires high temperature during drying process, lower temperature is used in ultrasonic spray drying [10]. Therefore, it is a promising method to increase the shelf life of the product by reducing the loss of the sensitive core material to the outside environment. However, there is lack of study reported on the physicochemical properties of ultrasonic spray dried microencapsulated Stevia leaf extract. Thus, this study aimed to determine the yield and physicochemical profile of microencapsulated *S. rebaudiana* prepared using ultrasonic spray drying at different percentage of gum arabic (GA) at different temperatures.

2. MATERIALS AND METHODS

Dried Stevia leaves were purchased from Besut, Terengganu, Malaysia in August to September 2015.

2.1. Spray Dry Process

The leaves were ground with distilled water in ratio of 1:25 and filtered through 4 layers of muslin cloths. Filtered sample and different percentage of GA (0, 2, 4, 6, 8 and 10%) were mixed. The mixture was spray dried in an ultrasonic spray dryer (YKNTECH, Kulim, Malaysia) [11].

2.2. Properties of Microencapsulated Stevia

IR spectra of the samples were measured by the method [2] with some modifications using Fourier Transform Infrared (FTIR) spectrometer, (Nicolet iS10, Thermo Fisher Scientific, USA).

2.3. Morphological Evaluation of Microcapsules

Particle size and structure of microcapsules were evaluated by the method of [3] with some modifications using tabletop microscope (Hitachi 2000, Japan).

2.4. 2,2,-diphenyl 2-picryl hydrazyl (DPPH) Method

Antioxidant activity of Stevia was determined by the method of [14] with some modifications.

2.5. Ferric Thiocyanate Test and Thiobarbituric Acid Test

The test was conducted according to the method of Kikuzaki and Nakatani [8].

2.6. Determination of Total Phenolic Content

The test was conducted according to the method of Kim and coworkers [9].

2.7. Determination of Total Flavonoid Content

The test was conducted according to the method of Ebrahimzadeh and coworkers [4].

2.8. Individual Flavonoids Analysis

Individual flavonoid was detected using a slightly modified method from [12]. An aliquot of 10ml of samples or standards were injected in the HPLC (PerkinElmer Series 200 Autosampler, USA). Isocratic elution system was used with the flow rate of 1.0ml min^{-1}. Acetonitrile and acidified deionized water (pH 2.35) in the ratio of 60:40 as the mobile phase; whereas C_{18} column was the stationary phase. Measurements were done at 280 and 360nm.

2.9. Statistical Analysis

All experiments were conducted in replicates and statistical analysis were obtained using Minitab 17 with a significant level of $p < 0.05$ by analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1. Yield of Microcapsules

Table 1: Yield (%) of Stevia microcapsules prepared by different percentage of GA at different temperatures (80˚C and 90˚C) using ultrasonic spray dry technique

	Yield (%)					
	0 %	2 %	4 %	6 %	8 %	10 %
80˚C	4.88 ± 2.37[a]	4.35 ± 2.12[a]	4.60 ± 1.13[a]	4.45 ± 2.19[a]	5.33 ± 2.02[a]	5.83 ± 2.86[a]
90˚C	6.28 ± 0.95[a]	5.65 ± 1.06[a]	7.23 ± 2.30[a]	6.88 ± 2.79[a]	7.45 ± 1.34[a]	7.70 ± 0.00[a]

Note: [a] means sample with the same letters are not significantly different at p>0.05.

There was no significant interaction ($p > 0.05$) between temperature used in spray drying and percentage of GA. There was no significant difference between samples spray dried with different percentage of GA for both microencapsulation at 80˚C ($p > 0.05$) and 90˚C ($p > 0.05$) respectively.

3.2. Properties of Microencapsulated Stevia

The results show that there was no notable difference in the IR values of all the samples. Hydroxyl stretching was observed between the wavenumbers 3385.07 – 3385.91cm^{-1}; alkane stretching was observed between 2926.12 – 2927.68cm^{-1}; carbonyl groups between 1718.83 – 1724.98cm^{-1}; alkene stretching between 1636.50 – 1654.70cm^{-1}; ether groups between 1074.35 – 1076.92cm^{-1}; and alkene bending between 889.72 – 896.16cm^{-1}.

3.3. Morphological Evaluation of Microcapsules

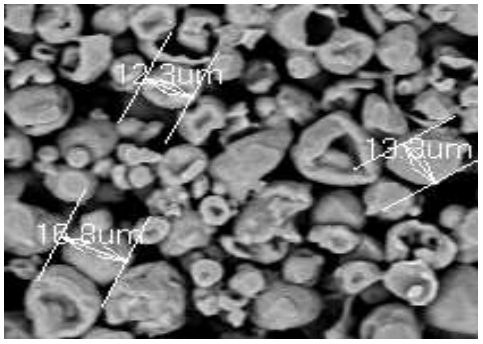

Figure 1: Morphology of Microcapsules

All the samples were characterized by irregularly spherical shape and smooth surface.

3.4. Antioxidant Profile

The results showed that microcapsules with 6% GA show the highest antioxidant activity in DPPH test and FTC test. High TPC and TFC were also found in microcapsules with 6% GA.

Table 2: Antioxidant Profiles of Microcapsules

Samples		Analysis				
Temperature (˚C)	% GA	DPPH	FTC	TBA	TPC	TFC
80	0	37.78 ± 0.19^{f}	56.28 ± 0.40^{de}	54.84 ± 0.04^{f}	58.94 ± 0.11^{AB}	15.34 ± 1.55^{A}
	2	57.67 ± 0.47^{e}	55.24 ± 0.03^{g}	46.69 ± 0.55^{g}	60.83 ± 2.82^{AB}	15.82 ± 2.45^{A}
	4	63.45 ± 0.26^{d}	55.95 ± 0.03^{ef}	9.20 ± 0.06^{l}	54.78 ± 0.01^{AB}	13.91 ± 1.05^{A}
	6	73.20 ± 0.40^{b}	59.04 ± 0.03^{c}	9.20 ± 0.06^{l}	66.72 ± 2.51^{A}	14.98 ± 3.40^{A}
	8	72.64 ± 0.42^{b}	51.53 ± 0.04^{i}	36.70 ± 0.03^{i}	53.87 ± 0.45^{AB}	13.90 ± 0.55^{A}
	10	67.42 ± 0.38^{c}	54.22 ± 0.06^{h}	39.12 ± 0.06^{h}	49.45 ± 8.58^{B}	14.63 ± 1.24^{A}
90	0	56.88 ± 0.45^{e}	55.66 ± 0.04^{fg}	90.96 ± 0.04^{a}	59.45 ± 0.35^{ab}	15.61 ± 3.74^{a}
	2	82.53 ± 2.20^{a}	54.03 ± 0.03^{h}	78.92 ± 0.00^{c}	59.48 ± 2.85^{ab}	16.59 ± 0.07^{a}
	4	59.11 ± 0.85^{e}	50.61 ± 0.04^{j}	73.68 ± 0.00^{e}	54.01 ± 2.39^{b}	15.90 ± 1.98^{a}
	6	82.64 ± 0.36^{a}	56.50 ± 0.04^{d}	77.71 ± 0.04^{d}	64.39 ± 4.48^{a}	17.39 ± 2.85^{a}
	8	71.13 ± 0.38^{b}	55.54 ± 0.15^{fg}	88.29 ± 0.03^{b}	60.94 ± 1.49^{ab}	15.74 ± 3.79^{a}
	10	63.53 ± 0.42^{d}	26.42 ± 0.08^{k}	78.74 ± 0.00^{c}	53.95 ± 0.44^{b}	16.89 ± 3.47^{a}
BHT		73.81 ± 0.28^{b}	61.76 ± 0.03^{a}	25.11 ± 0.04^{k}		
α-tocopherol		66.68 ± 0.09^{c}	60.55 ± 0.03^{b}	35.64 ± 0.03^{j}		

Note: $^{a-f}$ means samples with the same letters are not significantly different at $p > 0.05$ for the tests of DPPH, FTC and TBA respectively.
$^{a-b, A-B}$ means samples with the same letters are not statistically significant different at $p>0.05$ for the tests of TPC and TFC respectively.

In DPPH test, the percent inhibition of microcapsules spray dried with 6% GA at 90˚C (82.64 ± 0.36 %) was significantly higher than that of other samples. However, FTC test showed the inhibition of microcapsules spray dried with 6% GA at 80˚C (59.04 ± 0.03%) was significantly higher than that of microcapsules spray dried with 6% GA at 90˚C (56.50 ± 0.04%). In this study, Stevia microcapsules were made from the mixture of Stevia leaves and distilled water which is polar. Therefore, the amount of non-polar antioxidants in the microcapsules might be less. The TBA test results is in contrast with FTC test. The antioxidant activity of all the samples in FTC test was significantly lower than that of the positive controls. Higher antioxidant activities of samples in TBA test and higher antioxidant activities of positive controls in FTC test may indicate that the amount of peroxide in the initial stage of lipid peroxidation is greater than the amount of peroxide in the secondary stage [5]. The TPC test showed higher readings in microcapsules spray dried with 6% GA at both 80˚C and 90˚C. This result is in agreement with the result of DPPH and FTC which also show that microcapsules with 6% GA show the best antioxidant activity. In TFC test, Higher amount of flavonoid content was shown by the microcapsules spray dried with 6% GA at 90˚C (17.39 ± 2.85mg QE/g sample).

3.5. Determination and Quantification of Individual Flavonoids

Quercetin, kaempferol and rutin were present in the samples amounting 0.16 to 60.59µg/100ml, 4.19 to 7.10µg/100ml, and 2350.22 to 3987.94µg/100ml, repectively.

4. CONCLUSION

Temperature and GA have no significant effect on the yield and properties of microcapsules. All the samples were characterised by irregularly spherical shape and smooth surface. Microcapsules with 6% GA showed the best antioxidant profile at both 80°C and 90°C.

ACKNOWLEDGEMENT

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In Vivo Wound Healing Activity of Melaleuca Essential Oil-Based Cream

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ABSTRACT

Skin injury or wound is a normal event occurs in living organisms. In normal hygiene environment, wound would achieve full recovery within 14 days. Wound may be vulnerable to bacterial infection if not treated immediately, thus delaying the healing activity. Exisiting plant-derived medicines in the form of essential oils have shown encouraging effect in wound treatment. However, to our knowledge, there is limited report documented on the wound healing activity treated with melaleuca essential oil. In this study, we wish to determine the in vivo wound healing activity following topical application of melaleuca essential oil using murine model. Full thickness of mouse skin was excised and the wounded area was topically applied either with melaleuca essential oil-based cream, silver sulphadiazine (positive control) or cream base (negative control) for 10 days post-wounding. The percentage of wound contraction monitored by digital photographs and measured by using ImageJ software. It was observed that the mice treated with melaleuca essential oil showed significant acceleration healing activity as compared to the other groups as they achieved complete wound closure within 10 days post-excision. Future works focus on the combination of essential oils and the histological analysis of wound site at tissue level.

Keywords: Wound healing, wound contraction, essential oil, melaleuca

1. INTRODUCTION

Wound is an injury of the skin due to cut, tear or trauma that disrupt or damage the skin integrity. Wound healing is a complex biological process involving few stages including inflammatory phase, proliferative phase and remodelling of skin [1]. Natural remedies or herbal medicines have widely been used as wound healing agent due to its potential in wound care with optimal and faster healing property. These herbal remedies have been found to be effectively involved in the disinfection, removal of damaged tissues from wound site along with providing a moist environment as an effort to provide a conducive environment for natural wound healing [2]. It is common practice that plant-derived medicines come in the form of plant extract or essential oil (EO). Extensive researches have been done in discovering their role and potency in wound healing management, at which EOs are the current attention because of their pleasantness and inexpensiveness. In addition, a study demonstrated a faster absorption of EO into the blood stream following topical application of EOs on skin, which is within 10-30 minutes [3].

Melaleuca alternifolia or commonly known as tea tree plant is an Australian native plant that can be found in South Wales and Queensland. Notably, melaleuca EO and its main component, Terpinen-4-ol obtained from steam distillation of leaves or branches of this plant is widely and effectively used in dermatology to treat skin diseases and infections [4]. The antioxidant, anti-inflammatory and antimicrobial properties possessed by melaleuca contribute to wound renewal capacity as well [5]. For example, melaleuca EO has shown its effectiveness towards strains of *Staphylococcus aureus* isolated from normal wounds, surgical wounds as well as on methicillin-resistant and sensitive bacteria (MRSA

and MSSA) *in vitro* [6]. Meanwhile, in vivo study revealed its antifungal activity in a murine oral candidiasis model [7]. Similar to tap water, melaleuca EO accelerated the cooling and healing process of burn wound regardless immediate or delayed application [8].

However, to the best of our knowledge, there is limited report documented for the rate of wound healing activity treated with Melaleuca EO in mice model. The present study was conducted to determine and visualize the wound closure rate following topical application of melaleuca EO on excised wound model as well as to evaluate the epithelial gap on the wound site by H&E staining.

2. MATERIALS AND METHODS

2.1 Materials

Coconut oil, avocado oil, olive oil, beeswax and shea butter were purchased from Pure Nature, Auckland, New Zealand (http://purenature.co.nz/). Melaleuca EO was obtained from doTERRA International LLC, Utah, USA, while silver sulphadiazine was acquired from Ministry of Health, Malaysia. Healthy male Imprinting Control Region (ICR) mice (6-8 weeks old, 20-30g) were purchased from Universiti Kebangsaan Malaysia.

2.2 Preparation of topical ointment

A mixture of 38% w/w coconut oil, 21% w/w avocado oil, 21% w/w olive oil, 15% w/w beeswax and 5% w/w shea butter were melted at 40^{o}C and mixed well. The mixture was cooled down at room temperature until it reached 25^{o}C before adding 0.25ml EO of melaleuca. Topical ointment without the addition of EO was used as negative control (cream base).

2.3 Wound healing assays

All experimental procedures involved in this research is according to the approved protocols by UKM Animal Ethics Committee. A total of 16 healthy ICR mice were randomly assigned into four different groups; experimental, positive and negative control groups and test group with four mice each group.

The back of the mice were shaved and applied with depilatory cream to remove any remaining hair. Then, full-thickness excisional wounds were made using sterile 5-mm biopsy punch by picking up a fold of the skin [9]. One wound was generated on each side of the midline [10, 11]. Following excision, melaeuca EO-based cream, silver sulphadiazine (SSD) or cream base (CB) was applied gently onto the wound and this step was repeated daily until the end of timeline where mouse was healed completely. Mice in control group were left untreated (UN). Digital photographs were taken for each wound every day. Percentage of wound contraction was measured by calculating the area of wound using ImageJ software.

2.4 Statistical analysis

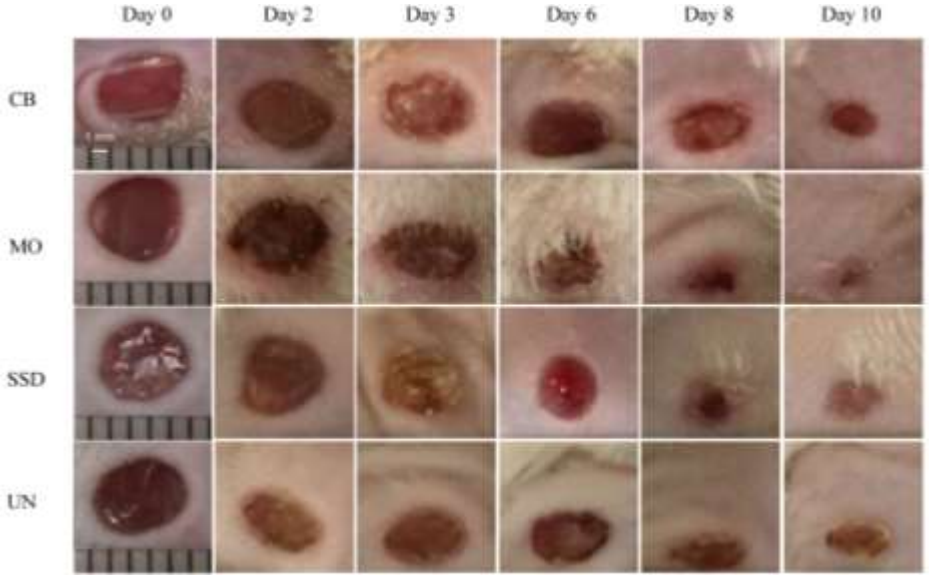
Student *t*-test was used to compare the significant difference between treatments. All statistical analysis was performed by GraphPad Prism version 7 software. Values were represented by mean ± S.E.M.

3. RESULTS AND DISCUSSION

3.1 Melaleuca EO-based cream accelerates wound closure

The wound healing progress was monitored throughout the experiment and is presented in Figure 1. Figure 1A showed the representative images of wound area following topical application of melaeuca EO-based cream, SSD and CB as well as untreated wound. From the images, it was clearly observed that wound treated with melaleuca EO-based cream reduced consistently and almost reached complete wound closure at day 10 post-wounding. The representation of wound contraction rate in Figure 1B supports the positive effect demonstrated by melaleuca EO-based cream. Partial wound closure was observed on wound treated with melaleuca EO-based cream at day 6 post-wounding (57.5%). More advanced wound closure was observed at day 8 (80.6%), at which wounds in control groups were not closed after the same period of time (CB; 67.6%, SSD; 68.2%, UN; 69.2%). Overall, the open wound area was significantly larger in control groups compared to melaleuca EO-based cream treated mice (*P<0.05 and **P<0.01). This favourable progression of skin renewal may be due to the anti-inflammatory, antioxidant and antimicrobial properties owned by melaeuca [12, 13].

A)



B)

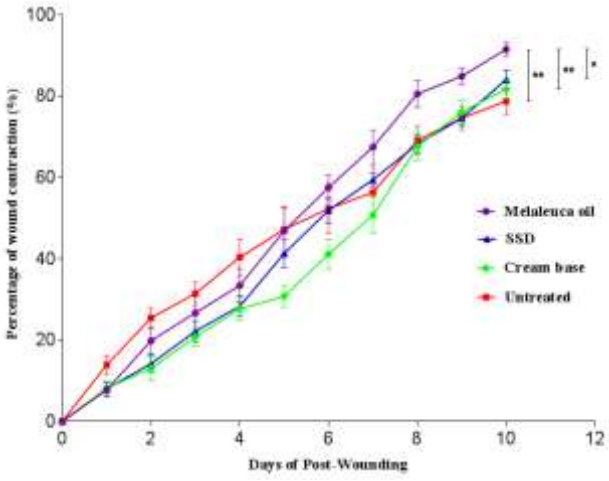


Figure 1. A) Excisional wound of group of CB, Melaleuca EO-based cream (MO), SSD and UN. **B)** Wound closure displayed as percentage as wound contraction. Values are expressed as mean ± SEM. n = 3 or 4 mice per group, *P<0.05 and **P<0.01

4. CONCLUSION

Melaleuca EO-based cream accelerates wound healing activity in excised wound model, suggesting it as an effective wound healing agent. However, future works are needed to evaluate the healing activity at tissue level including the tissue remodelling, re-ephithelization and collagen deposition to better understand the healing capacity of melaleuca EO. Combination of different EO in treating wound at pre-clinical setting could also be an important aspect for future study.

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Subacute Toxicity Study of *Clinacanthus Nutans* Ethanolic Extract *In Vivo*

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ABSTRACT

Clinacanthus nutans (Belalai Gajah) leaves extract has been used extensively as a traditional remedy in the Asian region. Despite of that, there was no standard measurement has been reported so far. Thus the application of the extract might be associated with risk of toxicity. The present study was designed to investigate the possible toxicity risk of *C. nutans* extract on the murine model *in vivo*. Sub-acute toxicity study was carried out following repeated oral administration of *C. nutans* ethanolic extract at daily doses of 50, 300, 2000 and 5000 mg/kg body weight for 28 days. From this study, the results showed that there were no significant differences in body weight, relative organs weight and physiological changes in control and treated groups. These results were further confirmed by observing histological changes on kidney and liver tissue where the results revealed that *C. nutans* extract exerts no toxic effect at concentration of 50 mg/kg body weight, whereas at the concentrations of 300, 2000 and 5000 mg/kg, a mild degree of toxicity was shown on both organs. These results indicated that *C. nutans* ethanolic extract at the concentration ranging from 300 to 5000 mg/kg body weight induced mild hepatic and renal toxicity in mice.

Keywords: *Clinacanthus nutans*; ethanolic extract; sub-acute toxicity.

1. INTRODUCTION

Recent trends in the application of Complementary and Alternative Medicine (CAM) therapies have led to a growing prevalence in US adults from approximately 36% in 2002 to 38.2% in 2007 [1]. One such practice is through the consumption of herbal based product, namely *Clinacanthus nutans.*

Scientifically named as *Clinacanthus nutans* (Burm. f) Lindau, it is commonly acknowledged as 'Belalai Gajah' in Malaysia. *C. nutans* fresh leaves have been used extensively as traditional remedies to treat various illnesses, namely skin rashes, insect and snake bite, herpes simplex virus (HSV), varicella-zoster virus (VZV) lesions and dysentery [2,3]. Its health promoting properties has successfully drawn public interest, leading to its commercialization into products such as capsule, herbal tea, and concentrated extract that is abundantly available in the market [4].

Despite of that, most of these products surprising do not come with standard measurement. This might expose consumers to a possible risk of toxicity with its prolonged use. Previous studies reported that the methanolic extract of *C. nutans* at the highest dose of 2500 mg/kg did not cause any toxic effect on the liver and kidney of mice, thus suggesting its no-observed-adverse-effect level (NOAEL) to be greater than the tested dose [5]. Therefore, the objective of this study is to investigate the possible subacute oral toxicity effect of *C. nutans* ethanolic extract in mice at dose up to 5000 mg/kg body weight for 28 days.

2. MATERIALS AND METHODS

2.1. Plant Material

The crude extract of *C. nutans* leaves was obtained from Dr Lim Vuanghao, Universiti Sains Malaysia, Penang. The extract was stored in the refrigerator at -20˚C for further use.

2.2. Experimental animals

Healthy female BALB/c mice at age of 8-10 weeks old were purchased from Universiti Kebangsaan Malaysia (UKM) and housed under standard environmental conditions. The experimental protocols of the animals were approved by the UKM Animal Ethics Committee [Ethical number: 72/2016].

2.3. Experimental design: Repeated dose 28-days oral toxicity study in mice

The study was conducted in accordance to OECD Guideline 420 by dividing the animals into groups of mice administered daily with 30% ethanol and 10% Tween 20 that served as positive control and delivery vehicle group respectively. This is followed with treated groups received *C. nutans* ethanolic extract at doses of 50, 300, 2000 and 5000 mg/kg body weight throughout the testing period. During the study, all animals were closely monitored for abnormal behavior and changes in body weight and feeding pattern.

2.4. Organs Weight and Histopathological analysis

After sacrification, vital organs like liver and kidney were harvested, blotted and weighed on analytical balance for absolute organ weight. They were then fixed in 10% neutral buffered formalin, processed, sectioned, stained with hematoxylin–eosin staining and examined under a light microscope for histological examinations. The relative organ weight (ROW) of each organ was calculated as follows [6].

$$\text{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Fasted body weight on sacrifice day}} \times 100$$

2.5. Statistical analysis

Statistical analysis was performed using Graphpad Prism version 7. Differences in parameters were evaluated using a One-way Analysis of Variance (ANOVA) followed with Dunnet's post hoc test.

3. RESULTS AND DISCUSSION

3.1. Body Weights and Feeding Pattern

Along with the increasing trend of *C. nutans* application as primary health care among consumers, there is an urge of interest to ascertain on its toxicity profile. Previous study on ethanol extraction has only been carried out in lower dose up to 250 mg/kg in 90 days of sub chronic testing period [7]. Considerable uncertainty still exists on the relationship between the dosage and course of treatment. Therefore, this study evaluates on much higher dosage with a shorter testing period of 28 days. Current study showed no mortality or physical signs of toxic effect were observed in treatment dose up to 5000 mg/kg and control groups. Similarly, there was no significant difference were observed on physical parameters like the mean of food and water intake (Table 1) and body weight gain (Figure 1) between the treated and control groups. Any alteration in body weight is believed to be associated with adverse effects of drugs and chemicals [8]. From the results obtained, it was appeared that all these parameters were not affected by the administration of *C. nutans* extract, thus evidencing a normal metabolism in the test animals.

Table 1. Weekly food and water intake of mice orally administered with *C. nutans* for 28-days. Results were expressed as mean ± SD, n=5.

Parameters	Groups (mg/ml)	Week 1	Week 2	Week 3	Week 4
Water intake (ml/day)	Positive control	3.60 ±1.75	3.29 ± 1.01	3.45 ± 1.09	4.14 ± 0.49
	Delivery	3.91 ±2.16	2.91 ± 1.03	2.54 ± 0.22	2.80 ± 0.28
	vehicle 50	2.66 ± 1.30	2.31 ± 0.84	1.91 ± 0.11	2.69 ± 0.47
	300	5.11 ± 3.56	2.43 ± 0.57	2.66 ± 0.50	2.69 ± 0.27
	2000	3.34 ± 2.13	3.74 ± 1.33	4.09 ± 0.55	4.20 ± 0.83
	5000	3.43 ± 1.89	3.91 ± 0.86	4.06 ± 0.28	3.63 ± 0.78
Food intake (g/day)	Positive control	2.23 ± 1.25	3.34 ± 1.51	3.77 ± 1.25	3.06 ± 1.36
	Delivery	2.51 ± 1.49	2.77 ± 0.47	2.54 ± 0.32	2.97 ± 0.54
	vehicle 50	2.17 ± 1.11	2.60 ± 0.16	2.74 ± 0.39	2.57 ± 0.76
	300	3.00 ± 1.37	3.06 ± 0.57	2.89 ± 0.23	2.13 ± 0.70
	2000	2.35 ± 1.61	2.54 ± 0.71	2.74 ± 1.55	2.31 ± 1.24
	5000	2.03 ± 1.16	3.40 ± 1.41	4.43 ± 1.20	4.23 ± 1.15

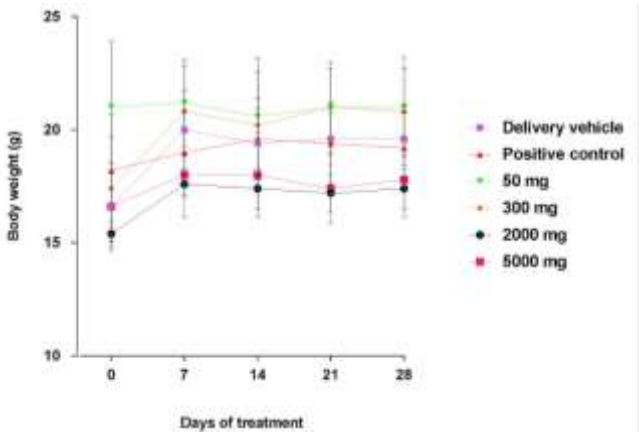

Figure 1. Weekly body weight measurements (g) of mice orally administered with *C. nutans* for 28-days. Results were expressed as the mean ± SD, n=5.

3.2. Organ Weights

Liver and kidney played critical roles in detoxification and excretion processes in biological system [9]. In this study, the results displayed non-significant changes for both organs in all treated groups as compared to the control (Table 2). In general, any change in the liver and kidney weight might be related to the organs injury, including swelling, atrophy or hypertrophy [10]. However, the results still need to be further validated by histological assessment to confirm on the findings.

Table 2. Relative organs weight of mice orally administered with *C. nutans* for 28-days. Results were expressed as mean ± SD, n=5.

Dosage administration (mg/kg)	Relative organ weight	
	Liver	Kidney
Positive control	5.915 ± 0.937	1.369 ± 0.255
Delivery vehicle	5.490 ± 0.756	1.252 ± 0.227
50 mg	5.802 ± 0.653	1.424 ± 0.173
300 mg	5.912 ± 0.730	1.672 ± 0.315
2000 mg	5.981 ±0.263	1.536 ±0.167
5000 mg	6.012 ± 0.762	1.400 ±0.110

3.3. Histopathological Examination

Histopathological examinations of the livers and kidneys had shown no signs of toxicity in the treatment group at a concentration of 50 mg/kg and control groups, but there were occasionally slight degeneration and necrosis in the cellular structure of both organs in treatment groups ranging from 300 to 5000 mg/kg of *C. nutans* extract (Images not shown). There were mild renal tubular lesions with the presence of congested blood vessel in the kidney, whereas droplets of fatty infiltration were displayed in hepatocellular of the liver. These findings are in line with previous studies that reported oral administration of *C. nutans* caused histopathological lesions to both organs [7].

4. CONCLUSION

It could be concluded that supplementation of *C. nutans* ethanol extract at doses of 300 to 5000 mg/kg body weight induced a mild degree of hepatic and renal toxicity in mice. Therefore, it may have potential health risk in a long run use. However additional studies in biochemical assays and hematological test are necessary to further support the findings.

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Evaluation of the Isoelectric Focusing (IEF) Method for Proteomic Analysis of *Orthosiphon stamineus* (var. Purple)

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ABSTRACT

Numerous studies on the remedial effects of the bioactive compound in *Orthosiphon stamineus* had been documented previously. Most of the studies were done on the white variety even though purple variety possess higher bioactive compound. However, protein separation study regardless of the colour is still absent due to the drawbacks such as complex time consuming procedure, poor reproducible result and demand for high human dexterity from two dimensional (2D) proteomic approach. Hence, study on the determination of the isoelectric focusing (IEF) method for 2D proteomic of purple variety of *O.stamineus* can serve as preliminary platform for protein identification and production for pharmaceutical industry thus saving time. The protein was first extracted using phenol extraction with three preliminary washes before its quality was checked via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Three different IEF methods applied were method 1 (2000V), method 2 (4000V), and method 3 (8000V). The second IEF method (4000V) exhibited the highest amount of distinct spots where 35 protein spots were observed from the gel replicates. IEF method 1 (2000V) showed that 17 protein spots were observed from the gel replicates whereas 11 protein spots were present on the gel replicates of IEF method 3 (8000V). Ultimately, method 2 (4000V) reflect difference of more than 2-fold spots count compared to the other two IEF method. Therefore, the IEF method 2 was obviously the best IEF method for purple *O. stamineus* according to the distinct and comparable protein spots amount.

Keywords: *Orthosiphon stamineus* purple variety**,** 2D proteomic; SDS-PAGE; IEF; protein spots

1. INTRODUCTION

Orthosiphon stamineus is a well-known perennial herbal plant that flourish tremendously in tropical climate countries including Africa, Australia and Southeast Asia (Hossain and Mizanur, 2011). Two variation of *O. stamineus* that have been identified are white and purple varieties according to its distinct floral and calyx colours (Ameer *et al.*, 2012). On the other hand, Lee (2004) reported that the purple variety *O. stamineus* possess higher bio-active compounds than its white variety. Nevertheless, most of the studies reported and published focused on white variety of *O. stamineus.* Various medicinal value that *O. stamineus* offers since decades ago has attracted the researchers' interest. Numerous studies were done on the pharmacological and phytochemical properties of its bioactive compound such as polyphenols. The leaves of the plant had been commercialized as java tea and capsule or tablets to promote diuretic activity, anti-fungal activity, antioxidative activity which lead to enhanced cytotoxicity activity, and antibacterial activity (Adam *et al*., 2009; Amzad *et al*., 2008; Chun-Hoong *et al*., 2010). However, few studies related to the herb's protein content were established. On the other hand, two dimensional (2D) proteomic analysis is a well-known approach in order to perform protein profiling. It has been widely applied in various plant species including Malaysian medicinal plant; *Piper sarmentosum* or "kadok", *Gynura procumbens* or "sambung nyawa", and *Phyllantus niruri* or "dukung anak" (Shim and Gam, 2012; Hew and Gam, 2011; Nail and Zin, 2015). Different sample require different voltage of isoelectric focusing (IEF) to separate the protein due to its isoelectric point (pI). Ultimately, this proteomic study on

O.stamineus was aimed to determine the isoelectric focusing (IEF) method for 2D proteomic of purple variety of *O.stamineus* thus can serve as preliminary platform for protein identification and production.

2. MATERIALS AND METHODS

2.1. Plant Material

The purple variety of *O. stamineus* was chosen in this study since it is pigmented hence it was expected to contain higher protein content compared to the white variety. The fresh young leaves were removed from the branches, weighed at 660mg and wrapped with aluminium foil before immediately frozen in liquid nitrogen. The leaves were then kept in the freezer at -80 ^{o}C. The liquid nitrogen was used even during grinding procedure to ensure that its protein was preserved.

2.2. Protein extraction

Previous studies to determine best extraction method for *O. stamineus* discovered that Phenol/SDS buffer with three solvent washes resulted in the highest protein amount (Ng, 2016). Hence, this method was used in this project.

2.3. Protein quantification

Protein concentration assay using Bradford reagent was done in order to determine the protein amount of the extracted protein.

2.4. Protein Separation

Two dimension of protein separation involved were first dimensional separation; isoelectric focusing (IEF) and second dimensional separation (SDS-PAGE). This procedure requires three consecutive days to obtain the result of interest.

2.4.1. Sample Preparation

During the first day, the sample preparation was done. Firstly, 50µg of the sonicated sample was topped up with 2% (v/v) IPG and rehydration buffer to a total of 125µL. The solution was then transferred into disposable tray and the pH4-7cm IPG strip (Bio-Rad ReadyStrip) was placed with its gel-surface facing downward. Finally, the strip was covered with mineral oil (Bio-Rad) and rehydrated overnight at room temperature.

2.4.2. Isoelectric Focusing (IEF)

On the second day, the isoelectric focusing (IEF) was run. Firstly, the rehydrated IPG strip was transferred into the IEF tray. Then, mineral oil (Bio-Rad) was applied to cover the strip to minimize evaporation. After that, 4 wicks soaked with extra pure water were placed on the electrodes of the IEF tray. Electrofocusing was then carried out at 20°C with gradually increasing voltage on the Bio-Rad Protean IEF Cell machine using three focusing protocol methods as follow; Method 1: 100 V for 30 minutes, 250 V for 30 minutes, 500 V for 1 hours, 1000 V for 1 hour, 1500 V for 1 hour, 2000 V for 1 hour, and hold at 100 V. Method 2: 250 V for 15min, 500 V for 1 hour, 1000 V for 1 hour, 4000 V for 1 hour, 4000 V for 6 hour, and hold at 100 V. Method 3: 200 V for 1 hour, 500 V for 1 hour,1000 V for 1 hour, 8000 V for 6 hour and finally hold at 100 V. After the electrofocusing was done, the strips were equilibrated in equilibration solution (20mL SDS equilibration buffer and 200mg DTT (Dithiothreitol)) for 15 minutes with gentle shaking. Then, the solution was discarded and replaced with the second equilibration solution (20mL SDS equilibration buffer and 500mg IAA (Iodoacetamide)) for 15 minutes

with gentle shaking. Finally, the solution was discarded before the strip was soaked in running buffer for second dimension SDS-PAGE.

2.4.3. SDS PAGE

On the third day, SDS-PAGE gels of 12% polyacrylamide were utilized. Next, the protein marker was loaded and the IPG strip was placed horizontally to the bottom of the well before the strip and well were sealed with agarose sealing solution. After that, the gel was run at 100V for 120 minutes in cold condition. The strip and gel was removed after SDS-PAGE and stained with staining solution and destained with destaining solution to remove the background. Finally, the spots formation on the gel were analysed before acetic acid was added to preserve gel and kept in 4°C.

3. RESULTS AND DISCUSSION

In this study, the best IEF method producing reproducible protein spots from purple variety of *O. stamineus* was determined by performing three different IEF methods. The 2D electrophoretic pattern of *O. stamineus* on 12% (w/v) SDS-PAGE gels for all 3 IEF methods were shown in Figure 1.

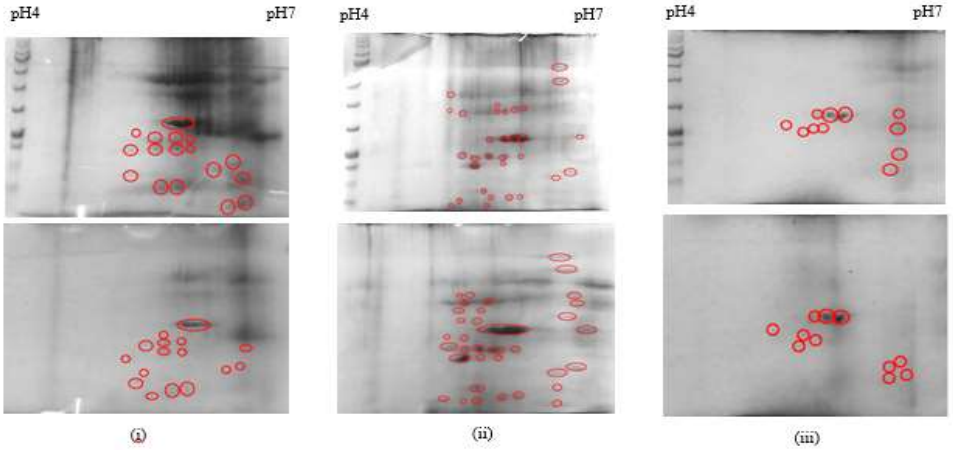

Figure 1. Protein spot distribution pattern of purple *O. stamineus* run on 7cm ipg strips using (i) IEF method 1 (2000 V), (ii) method 2 (4000 V)and (iii) method 3 (8000 V) on 12% (w/v) sds-page loaded at 50 µg.

The IEF method 1 (2000 V) produced 17 protein spots from the gel replicates whereas 11 protein spots were detected from the gel replicates of the IEF method 3 (8000V). Ultimately, the IEF method 2 (4000V) exhibited the highest amount of distinct spots where 35 protein spots were counted from the gel replicates. These reflect difference of more than 2-fold spots count compared to the other 2 IEF method. Hence, the IEF method 2 of 4000V was obviously the best IEF method for purple *O. stamineus* according to the distinct and comparable protein spots amount.

Proteomic study involving 2DGE on medicinal plant in Malaysia is currently on trend since the researchers are highly attracted to the medicinal value potential offers by those plant. Proteomic analysis on *Piper sarmentosum* and *Gynura procumbens* show that IEF method utilizing 8000V to focus the proteins pI was the best method (Shim and Gam, 2012; Hew and Gam, 2011) whereas *Phyllantus niruri* applied IEF method utilizing 10000V to focus the proteins (Nail and Zin, 2015).

After all, the IEF method or voltage required to separate different protein sample according to their pI values varies in every species since different sample possess different component. *O. stamineus* for instance is highly abundance in polyphenolic compound in which the polyphenols itself is another type of protein (Saidan *et al.,* 2015). The polyphenols can also interact with other protein and affect the sample composition. Indeed, the extraction method also capable of influenced the sample constituent (Shaw and Riederer, 2003). In other words, proteomic analysis is a complex field since protein itself is very complex in nature and the steps involve in analysing protein are highly correlated with each other. A part of that,

severe horizontal streaking and smearing on the SDS gel were clearly observed especially in the gels of IEF method 1 and 2 while fewer horizontal streaking and smearing were observed in the gel replicates of IEF method 3. There are several factors contributing to the horizontal streaking and smearing on 2D gel. According to Saravanan and Rose (2004), the crude extract of the protein sample can be contaminated with protease, lipid, polypeptides and nucleic acids. In addition, nucleic acids interference may cause horizontal streaking in the acidic region of the gel (L᾽opez, 2007). The aggregation of protein in the first dimension and incomplete protein solubilization and separation in the second dimension will also contribute to the same problem (Shaw and Riederer, 2003). Prominently, the high phenolic content in the leaf extract of *O. stamineus* may interact with protein and result in horizontal streaks on the 2D gel (Saidan *et al.*, 2015; Gorg *et al.*, 2004).

4. CONCLUSION

This proteomic analysis of purple variety *O. stamineus* revealed that 4000V was the best IEF method for this plant with more than two-fold increase in number of protein spots detected compared to 2000V and 8000V IEF methods. Future study can be conducted in identifying the expected proteins, reveal the effect of external stimuli such as salinity, drought, and infection to the protein expression and to analyze the mechanism of action of the compound present in the plant.

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Antibacterial Potency of *Heterotrigona Itama* Honey against Five Clinically Isolated Pathogenic Bacteria

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ABSTRACT

The antibacterial activity of Malaysian *Heterotrigona itama* honey (pH of 2.73 ± 0.02 and water activity at 32.09 ± 0.05) at different concentrations (undiluted, 75%, 50% 25% (w/v)) was assessed against five pathogenic bacteria using agar well diffusion method. Our results revealed that *H. itama* honey inhibited the growth of all pathogenic bacteria even at the lowest concentration (25%), displaying the smallest average inhibition zone of 14.67±1.52 mm. As expected, the largest inhibition zone, at 29±1.00 mm was obtained using undiluted honey. Analysis of one-way ANOVA followed by post-hoc Tukey test showed that the antibacterial activity in 25% (w/v) of *H. itama* honey was significantly different ($p < 0.05$) to that of 50% (w/v) for all pathogenic bacteria, except for *E. coli.* Our results also conveyed the insignificant difference ($p > 0.05$) in antibacterial activity for concentrations, 75% and 100% for all pathogenic strains. Hence, it was evident that the broad spectrum of antimicrobial activity shown by *H. itama* honey suggests its potential application as a beneficial food alternative.

Keywords: *Heterotrigona itama* honey; antibacterial activity; agar well diffusion method.

1. INTRODUCTION

Heterotrigona itama (*H. itama)* or locally named as 'Kelulut' is a common stingless bee species practiced in beekeeping (Meliponiculture) in Malaysia (Kelly *et al.*, 2014). Honey produced by stingless bees is highly valued due their therapeutic profiles such as rich in antioxidants, having anti-inflammatory properties and recently, moisturizing effects to facilitate wound healing (Souza *et al.*, 2006). Unlike honey bees which store honey in hexagonal shaped honey combs, stingless bees store the collected honey in horizontal cerumen pots made up of wax and propolis. Honey produced by stingless bee is also considerably less as compared to sting bees and, the former is well-known for its uniquely sweet and yet acidic flavour (Souza *et al.*, 2006).

The emergence of diseases caused by pathogenic microbial strains and their growing threat on global health is a serious public concern. According to the Malaysian Society of Intensive Care (2012), bacterial strains *viz. Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* are among the most frequently reported healthcare-associated strains isolated from patients. Also, the excessive use of antibiotics have led to recent unsavoury development of resistant bacteria, which has restricted efficient use of existing antibiotics. In this milieu, the quest for alternative antimicrobial compounds derived from natural products, i.e. honey, to replace conventional antibiotic therapy may prove beneficial. Herein, the study is focused on exploring and investigating the antibacterial potential of undiluted and diluted *H. itama* honey against five clinically isolated pathogenic strains. It is hypothesized that high concentrations of *H. itama* honey were effective in inhibiting these pathogenic bacteria and such effect is decreased with further dilution of the honey.

2. MATERIALS AND METHODS

2.1 Collection of *H. itama* honey

H. itama honey was collected from Bukit, Khor (Terengganu, Malaysia) in December, 2016. Samples were withdrawn from the pots of stingless bee using a sterilized 25 ml syringe. The collected honey sample was stored at room temperature and analysed within one week.

2.2. Analysis of pH and Water Content

The pH of *H. itama* honey was determined by dissolving 10 g of honey in 75 ml of Milli-Q ultrapure water and measured using a pH meter (Mettler Toledo,Greinfensee, Switzerland). The measurements were carried in triplicate to ensure accuracy and the result was presented as mean ± standard deviation (SD). Moisture content was determined based on refractometric method whereby the refractive index (RI) increases with the increase in the solid content of a sample. RI of *H. itama* honey was measured at 20°C using an Atago refractometer (ATAGO RX-5000X, Hamburg, Germany). Measurements of moisture content were performed in triplicate and the percentage of moisture content which corresponds to the value of RI was estimated using the Wedmore equation (Wedmore, 1955) (Equation 1):

$$W_{RI}(\%) = [-0.2681 - \log(RI-1)]/\ 0.002243 \qquad \text{(Equation 1)}$$

2.3 Microorganism and culture conditions

Bacterial cultures, *P. aeruginosa* (ATCC 27853), *E. coli (*ATCC11775), *B. subtilis* (ATCC21332) and *S. aureus* (ATCC 25923) were purchased from ATCC (American Type Culture Collection). *K. pneumonia* was obtained from the culture collection of Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia. Each bacterium was routinely cultured on Mueller Hinton (MH) agar (Merck, Germany) and the agar plates were incubated at 37°C for 18−24 h. Several colonies of each organism were then chosen and suspended in 0.85% saline water and the cell suspensions were adjusted to a concentration of 10^8 CFU/mL.

2.4 Agar well diffusion assay

The antibacterial activity of *H. itama* honey was assessed against five pathogenic bacterial strains using the agar well diffusion assay. Briefly, 100 uL (10^8 CFU/mL) of the pathogenic bacterial suspension was spread over the entire of Mueller Hinton (MH) agar surface. Using the reverse end of a sterile P1000 tip, a 7-mm diameter well was punched aseptically onto the MH agar. An aliquot of 100 uL of different concentrations of *H. itama* honey (undiluted, 75%, 50% 25% (w/v)) were seeded into the well. The plates were incubated at 37°C for 24 h before the diameter of the growth inhibition zone was measured. The obtained results were calculated from three independent experiments.

2.5 Statistical analysis

Any significant difference between the concentrations of honey was determined by one-way Anova followed by post-hoc Tukey test ($p < 0.05$) comparison using the IBM SPSS version 20.0 software. Unless indicated otherwise, the data meant for statistical inference are presented as mean ± SD.

3. RESULTS AND DISCUSSION

Table I: Mean zones of inhibition sizes (mm) determined by agar well diffusion method

Concentration of Honey (w/v)	Test Pathogenic Bacteria				
	P. aeruginosa	***B. subtilis***	***E. coli***	***K. pneumonia***	***S. aureus***

Undiluted	27±2.00	29.33±2.08	28.33±2.08	27.67±2.08	29±1.00
75%	25.67±1.15	27±1.00	25.67±2.52	26±1.00	27.33±1.15
50%	20.67±0.57	23.67±1.52	22.67±2.30	21.67±2.08	23±1.00
25%	14.67±1.52	17.67±1.52	19±2.00	15±2.00	17.33±2.30

Our results demonstrate that *H. itama* honey has an average pH of 2.72 ± 0.02 and water activity of 32.09 % ± 0.05. We found out that the pH of the Malaysian *H. itama* was slightly lower than previously reported for stingless bee honey from other parts of the world (Boorn *et al.,* 2010). The low pH value seen here might be due to the production of gluconic acid by lactic acid bacteria, which is the natural flora in *H. itama* honey. This factor presumably contributes to the antibacterial activity in the honey since the optimal pH for most pathogenic bacteria is within pH 4.0 to 4.5 (Manyi-Loh *et al.,* 2011).

H. itama honey has been described to demonstrate a wide spectrum of antimicrobial activity. The findings in this study corroborates previous assessments, whereby the honey sample exhibited inhibitory effect against five pathogenic bacteria even at the lowest concentration (25%) with the minimum inhibition zone of 14.67 ± 1.52 mm (*P. aeruginosa*), 17.67 ± 1.52 (*B. subtilis*) 19 ± 2.00 (*E. coli)* 15 ± 2.00 (*K. pneumonia*) and 17.33 ± 2.30 (*S. aureus*). The reasoning for such observation could be attributed to the liberation of hydrogen peroxide, H_2O_2 when honey was diluted. Most types of honey tend to liberate H_2O_2 when diluted, as the introduction of water into *H. itama* honey induces the expression of carbohydrates-metabolizing enzyme, glucose oxidase. The enzyme is produced by the *H. itama* bees during nectar collection, oxidases glucose to gluconic acid and H_2O_2 (Bogdanov *et al.,* 2008) resulting in oxidative stresses and production of highly reactive free radicals which beneficially regulates bacterial colonization (Iurlina and Fritz, 2005). Accumulation of H_2O_2 is reported to reach the maximum for honey concentrations between 30-50% and rapidly declines when the concentration falls below 30%. This is associated with the relatively low affinity of *H. itama* glucose oxidase for glucose (Kwakman and Zaat, 2012). This factor was speculated to be the reason that contributes to the statistically significant differences in antibacterial activity of the *H. itama* honey samples at the assessed concentrations of 25% and 50% (w/v). Aside from glucose oxidase that originated from bees, catalase from flowers is also one of the main contributing enzymes to impart antibacterial activity in honey (Zainol *et al.,* 2013).

The maximum inhibition zone for antibacterial activity of *H. itama* honey was found to be 29.33 ± 2.08 mm from assay samples that contained the undiluted honey. The larger zone of inhibition can be accounted to the undiluted honey containing fully functional glucose oxidase, which is not inactivated by the presence of water (due to dilution). Therefore, the deactivating effects of the liberated H_2O_2 on glucose oxidase is minimal. Moreover, the possibility of the combined action of floral origin catalase with that of glucose oxidase, the high osmotic pressure and high acidity as well as the presence of other types of antioxidants might have contributed to the exceptional antibacterial properties displayed by the undiluted *H. itama* honey. Variations in the chemical composition and volatile compounds, too, could have influenced the antibacterial activity of *H. itama* honey. Further analysis on the antibacterial compounds present in this honey, is therefore necessary and would constitute an interesting future study.

It was observed that the Gram-positive *B. subtilis* showed the largest inhibitory effect against the pathogenic bacteria, to afford the largest inhibition zone for a 50% concentration of the undiluted *H. itama* honey. Conversely, the Gram-negative *P. aeruginosa* was the least efficient in inhibiting growth of the pathogenic bacteria and resulted in the smallest inhibition zones for concentrations, 25%, 50%, 75% (w/v) and undiluted *H. itama* assay samples. The results in this study mirrors the global problem of the Gram-negative bacteria presenting serious therapeutic challenges associated with antibiotic resistance. This is largely due to the excellent adaptive mechanism of such bacteria against current available antibiotics (Arias and Murray, 2009). It is also revealed that there is no significant difference in antibacterial activity of *H. itama* honey at 75% (w/v) and undiluted. Hence, it is suggested that consumption of *H. itama* honey at 75% (w/v) confers the same protective effect on the consumer as that of consuming the undiluted honey.

4. CONCLUSION

The study found that *H. itama* honey has a broad spectrum of antibacterial activities. The significant difference in the *H. itama* honey samples at 25% and 50% (w/v) were likely due to the liberation of H_2O_2. Conversely, the insignificant difference observed for the 75% (w/v) and undiluted honey could be attributed to presence of other phytochemical components in the samples.

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Short-Term Hypoglycemic Effect of *Momordica Charantia*

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by elevated blood glucose level caused by insulin insensitivity. The treatment of T2DM relies on oral antidiabetic agent for strict blood glucose level control to avoid the complications such as cardiovascular diseases, kidney failure and diabetic retinopathy. However, the undeniable side effects caused by the consumption of antidiabetic drugs such as weight gain, kidney complication and liver diseases as well as the high expenses have became an issue in the management of this disease. *Momordica charantia* (bitter melon) has become the subject of biochemical and clinical studies through the rising interest in natural herb that aids blood glucose management. However, the short-term hypoglycemic effect of the bitter melon consumption in human body is yet to be studied. In this study, the fasting blood glucose level of volunteers will be recorded and act as a base line for the following measurements. In brief, the volunteers will be subjected to the consumption of 300 ml raw bitter melon juice following overnight fast. Their blood glucose level will be monitored at time interval of every 15 minutes for the duration of 90 minutes using a glucometer. The short-term metabolic impact of bitter melon on blood glucose level may contribute towards the application of bitter melon on blood glucose management.

Keywords: diabetes, bitter melon, blood glucose level

1. INTRODUCTION

Diabetes Mellitus is a chronic, progressive disease which is estimated to affect approximately 422 million people in the world (5.6 % of the world population) in the year of 2014 [1]. Diabetes is an important public health problem and it is targeted for action by world leaders. The majority of people with diabetes are affected by type 2 diabetes mellitus (T2DM). Malaysia has one of the highest number of T2DM patients in South East Asia region. In fact, the 2015 National Health and Morbidity Survey [2] reported that 17.5 percent of Malaysian population suffered from this disease, which means one out of six Malaysian has T2DM. Out of this 17.5 percent of Malaysian population (3.5 million), only 1.66 millions of them know they have the disease, while the remaining 1.84 millions are undiagnosed.

A number of risk factors associated with the development of T2DM have been identified. These include, amongst others, aging, genetics, lifestyle, nutrition, and lack of physical activity. Metabolic disorder that leads to hyperglycemia, which occur in isolation or in combination, has been proposed for eight pathophysiological mechanisms underlying T2DM [3]. These include (i) reduced insulin secretion from pancreatic β-cells, (ii) elevated glucagon secretion from pancreatic α cells, (iii) increased production of

glucose in liver, (iv) neurotransmitter dysfunction and insulin resistance in the brain, (v) enhanced lipolysis, (vi) increased renal glucose reabsorption, (vii) reduced incretin effect in the small intestine, and (viii) impaired or diminished glucose uptake in peripheral tissues such as skeletal muscle, liver, and adipose tissue. Currently available glucose-lowering therapies target one or more of these key pathways.

T2DM has been associated with fatal complication such as cardiovascular diseases, kidney failure, blindness, nerves system damage etc. To date, there is no definitive cure for this particular progressive and dangerous disease. T2DM requires good management on diet and life style of the patients as well as continuous medications to maintain the blood glucose level of the patients. If T2DM is not well managed, complications develop will threaten health and endanger life. Acute complications are a significant contributor to mortality, costs and poor quality of life. T2DM patients have to rely on oral antidiabetic drugs such as metformin and sulphonylureas or even insulin injection to lower the blood sugar level and prevent complications.

As the expenses of the drugs can be an economic burden to most of the patients, it is very difficult to manage T2DM. Insulin and oral hypoglycaemic agents are reported as generally available in only a minority of low-income countries [1]. In fact, in many parts of the world, especially the poor countries, their people cannot afford the oral antidiabetic agents and only rely on plant-based remedy which is cost-effective and easily available [4]. Moreover, the oral anti diabetic drugs are found to be the culprit of certain side effects such as weight gain, kidney complications, liver disease etc [5]. Therefore, there is raising interests in investigating a natural diet supplement to aid with the management of blood glucose level throughout the last decades.

Bitter melon (*Momordica charantia*) is a fruit that is widely cultivated and available in Asia, India, East Africa, and South America. It has been recognized as a herbal medicine which possessed hypoglycemic effect through in vitro, animal studies and human studies [6,7,8]. It has been proposed that bitter melon may reduce the blood glucose levels by stimulate insulin secretion, improve glycogen storage, increase peripheral and skeletal muscle glucose uptake, and inhibit adipocyte differentiation and suppress gluconeogenic enzymes that catalyzes the breakdown of disaccharides into monosaccharaides [9,10]. The active component with hypoglycemic properties in bitter melon have been identified as saponins, charantins, polypeptide-p, and vicine, lectin, triterpenes, and alkaloids [10,11].

This study is designed to discover the potential of *Momordica charantia* in the treatment process of Type 2 diabetes. The main objective is to compare the short term blood glucose reducing activity of *Momordica charantia* juice on T2DM patients with healthy individuals. It is a clinical approach employing T2DM patients and healthy individual in investigating the hypoglycemic effect of *Momordica charantia* through monitoring blood glucose level using glucometer. Furthermore, the result of the study will suggest a new potential prognosis procedure for T2DM as the hypothesis of the study proposed that T2DM patient will give different biological respond to *Momordica charantia* treatment compared to healthy individual.

2. MATERIALS AND METHODS

2.1. Preparation of fresh bitter melon juice

Fresh bitter melon (peria katak) will be purchased from local farmer market. The fruit will be washed and cleaned and the seed will be removed. The conventional juicer will be used to process the bitter melon with most of the pulp removed. The raw bitter melon juice is then ready to use.

2.2. Process flow of the experiment

The candidates of the experiment will be recruited in UTM Bioprocessing Centre. A total of 10 candidates will join the experiment. They will perform overnight fasting before the experiment and their fasting blood glucose level will be measured using OneCheck glucometer. The fasting blood glucose level will act as a base line for the perturbation after the consumption of 300ml of raw bitter melon juice by each candidate. A fasting blood sugar level less than 100 mg/dL (5.6 mmol/L) is normal. A fasting blood sugar level from 100 to 125 mg/dL (5.6 to 6.9 mmol/L) is considered prediabetes. If it's 126 mg/dL (7 mmol/L) or higher, the person is said to has diabetes [12]. The blood glucose level of candidates will be monitored throughout the 90 minutes experiment with the data collection point at 0 minutes (fasting glucose level), 15minutes, 30 minutes, 45 minutes, 60 minutes and 90 minutes.

2.3. Data analysis

Statistical analysis will be carried out to determine the significance of the data and quantify the effectiveness of hypoglycemic action of bitter melon on healthy individual, T2DM patients and prediabetes.

3. RESULTS AND DISCUSSION

The hypothesis of the study expected T2DM patients to react with fresh bitter melon juice differently from healthy individual. At data collection point 1, which the time is 0, represent the fasting glucose level of the subjects. T2DM patients will have approximately 8.0mmol/L of blood glucose concentration as they will stop their daily oral anti diabetic drug one day before taking part in this study. The prohibition of oral anti diabetic drugs is due to the prevention of hypoglycaemia which may occur if the patients took the drugs and fresh bitter melon juice simultaneously and the synergism effect of this combination of intake. Meanwhile, the normal or healthy fasting blood glucose concentration will be approximately 5.6mmol/L. There are group of subjects which are possible undiagnosed of T2DM or prediabetes will have their blood glucose level with 6.0mmol/L as their fasting glucose concentration.

T2DM patients will take longer time to reestablish their base line blood glucose level due to the metabolic disorder. Normal healthy individual will perform the negative feedback mechanism of the body to restore and stabilize the lowering blood glucose level and bounce back to their original glucose level almost immediately. The prediabetes will have difficulties to restore the normal glucose level but not as slow as T2DM patient.

4. CONCLUSION

Fresh bitter melon juice has immediate result in lowering blood glucose level. T2DM patients and healthy individuals show different respond towards the hypoglycemic effect of *Momordica charantia*. However, further study on the mechanistic pathway of the action of bitter melon should be done to provide new insight for current medical practice.

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Determination of the Total Phenolic Content of different Medicinal Plant Extracts (*Moringa olifera*, *Erythrinia senegalensis* and *Leptadenia hastata*)

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ABSTRACT

Three different medicinal plant extracts *Moringa olifera, Erythrinia senegalensis and Laptadenia hastata*, were analyzed for total phenolic content, which is a determinant for antioxidant activity, the amount of phenolic content was analyzed using the Folin-Ciocalteu method with Gallic acid as standard, the total phenolic content were expressed as mg/g Gallic acid equivalents curve equation: (y = 0.0206x-0.2393) R_2 =0.9818).The value of Total phenolic content varied from (15.86 ± 0.06 to 66.89 ± 0.68 mg/g) in the extracts, .The maximum concentration was found in the Erythrinia senegalensis extract (66.89 ± 0.68 mg/g)mg/g). All the plant extracts were found to contain antioxidant activity.

Keywords: *Moringa olifera, Erythrinia senegalensis, Laptadenia hastate*, Total phenolic content: Gallic acid; Folin-Ciocalteau

1. INTRODUCTION

The use of herbal plants in traditional treatment of aliments is well documented [1], medicinal plants have been used in traditional treatment by nearly every culture, all over the world [2], [3] ,[4]. It is not surprising to estimate that about 90% of people have tried medicinal plants, at least once in their life, due to limited availability or the inability to afford pharmaceutical drugs [5], between 70 and 95% of individuals in developing countries consider traditional medicine as their primary health care and most of this is in the aqueous form [6]. *Moringa olifera* a multi-purpose tree with a variety of potentials uses, it is regarded by people as ' the Miracle Tree', its leaves, pods, seeds, flowers, and roots are edible, and have different nutritional and medicinal values [7]. The common names include horseradish tree, radish tree, drumstick, West India Ben, and Benzoline in French. The tree is known as Zogale in Hausa, Gawara in Fulfulde, Okwe oyibo in Igbo, and Ewe igbale in Yoruba, it is known for its nutritional and traditional value in the northern part of Nigeria [8]. Typically, Moringa can be found in every part of the Nigeria and beyond. This plant has long been identified traditional herbalists as a cure for many diseases, it used extensively as a food and nutrition supplement, but it is only of recent that modern scientists began to reap out its benefits. *Erythrina senegalensis* a thorny shrub or small with common names coral tree in English and Mínjíryáá in Hausa, Nigeria), *E. senegalensis.* The leaves are used to treat malaria, gastrointestinal disorders, fever, dizziness, secondary sterility, diarrhoea, jaundice, nose bleeding and pain [9]. The leaf has also been shown to contain many phytochemicals [10]. The stem and root bark are used by traditional healers to cure wide range of illnesses [9], [11]. The stem bark extract have also been shown to have antimicrobial activity [12]. *Leptadenia hastata* commonly known as *Yààdííyáá in Hausa*, is a voluble edible vegetable herb with creeping latex stems, glabescent leaves glomerulus and racemus

flowers as well as follicle fruits, it is collected wild throughout Africa *L. hastata* is used medicinally in many areas and often traditionally to treat hypertension, catarrh, fever and diabetes. Literature survey and ethano-botanic investigations from traditional healers revealed that consumption of leaf extracts and leaf stems of L. hastata could be used in the management of Diabetes mellitus, [13]. Throughout these last few years there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world. A detailed knowledge of these medicinal plant is important for the preservation and utilization of biological resources.

2.0 MATERIALS AND METHODS

2.1 Collection and Identification of Medicinal Plants

The plants of interest in this study were collected from a natural population in Kano, Nigeria. The plants were botanically identified by a botanist at the department of Biological Science; Voucher specimens were deposited for future reference and reference code were given Bayero University, Kano Herbarium Accession Number (BUKHAN); *Moringa olifera* (0011), *Erythinia senegalensis* (0310) *and Leptadania hastata* and stored at the herbarium for future reference.

2.2 Preparation of Crude Aqueous Extract

The leaves of the fresh plants were separated from undesirable plant parts and air dried under the shade. The dried leaves were then grounded into a fine powder with the help of a suitable grinder, the traditional mortar and pestle, then passed through a meshed sieve, 100g of the dried powder of each plant was then soaked in 1000 ml sterilized tap water in a conical flask, the mixture is then homogenized and left to stand overnight at room temperature, the mixture is then filtered using a cheese cloth and the extract collected, the mixture of each preparation is then filtered using Whitman filter paper and suction pump.

2.3 Preparation of Hexane Extract

The powdered leaves (100 g) were weighed and soaked in 350 ml of methanol in a conical flask. The flask containing the leaves were shaken, corked and left to stand for 48 hours at room temperature. The mixture is then filtered and the extract collected and concentrated by evaporation to dryness in evaporating dish.

2.4 Chemicals and Instruments

Gallic acid, Na_2CO_3, Folin-Ciocalteau's reagent (50%), Methanol and Spectrophotometer.

2.5 Folin-coicaleteau Assay

The total phenolic content was determined using a published procedure slightly modified, 1 ml of sample added into a tube to which 1 ml 50% of Folin coicalteau reagent solution was added and 1 ml of 10% (Na_2CO_3) sodium carbonate was added to the mixture, the sample was vortexed for 30 s and kept at room temperature for 2 hours. The absorbance was read at 765 nm, with calibration curves gallic acid which was used as standards, the results are described as mean values of milligram of gallic acid equivalent per gram of sample [14].

Fig 1 Standard Gallic acid curve

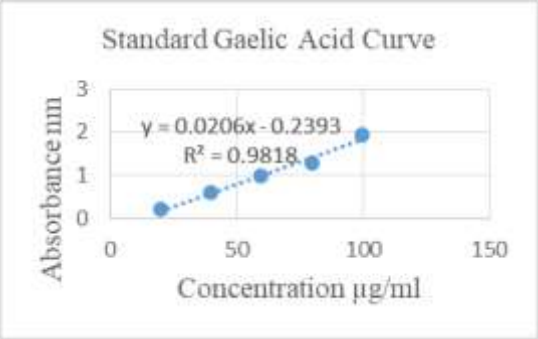

Table 1: Standard Gallic acid equivalent curve

Concentration (µg/ml)	**Absorbance(nm) 765nm**
Concentration (µg/ml)	Absorbance ($\lambda_{max)}$ 765(nm)
20	0.1250
40	0.5913
60	0.9640
80	1.2795
100	1.9295

The samples were measured in triplicates and the average taken

Table 2: Total Phenolic Content of Aqueous Samples of Extracts

`Extracts	**Concentration µg/ml**	**Mean ± SD (mg/g)**
Moringa olifera	1000	55.63 ± 6.76
Erythrinia senegalensis	1000	66.89 ± 0.68
Laptadenia hastata	1000	54.22 ± 1.57

The samples were measured in triplicates and the average taken

Table 3: Total Phenolic Content of Hexane Samples of Extracts

Extracts	**Concentration µg/ml**	**Mean ± SD (mg/g)**
Moringa olifera	1000	16.00 ± 0.12
Erythrinia senegalensis	1000	16.99 ± 0.09
Laptadenia hastata	1000	15.86 ± 0.06

3. RESULTS AND DISCUSSION

The total phenolic content can be determined using the Folin ciocalteau assay which is sensitive to reducing compounds, on reacting with polyphenols it changes to a blue colour, this colour is measured spectrophotometrically. Gallic acid was used as standard, from the standard curve equation the y = 0.0206x-0.2393 with R_2 =0.9818) where y is the absorbance and x is the concentration to be determined from the curve, phenolic compounds are free radical scavengers. Table 1 shows the concentration of standard gallic acid equivalent, used in determining the concentration of aqueous and hexane extracts of samples, table 2 shows the concentration of extracts (54.22 ± 1.57 to 66.89 ± 0.68), the highest concentration is found in aqueous extract of *Erythrinia senegalensis.* Table 3 shows the concentration phenolic content of hexane extracts (15.86 ± 0.06 to 16.99 ± 0.09), with highest concentration in *Erythrinia senegalensis* and lowest in *Laptadenia hastata*.

4.0 CONCLUSION

The phenolic content was determined using the Folin ciocaltaeau assay, with Gallic acid equivalent as standard, the total phenols were expressed as Gallic acid equivalents in mg/g, the highest antioxidant activity was seen in the aqueous extract of *Erythrinia senegalensis* (66.89 ± 0.68), with the lowest concentration observed in the *Leptadenia hastata* plant extract (15.86 ± 0.06) , the results indicate that these plant extract have anti-oxidant activity, can be used in opposing and inhibiting oxidation and peroxidation of free radicals.

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Antioxidant activity of Pigmented and Non-Pigmented Malaysian Upland Rice

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ABSTRACT

The antioxidant activities of two upland rice cultivars *(Oryza sativa)* variety Hitam (pigmented), and Wai (non-pigmented) from Sibu, Sarawak were evaluated and compared with MR220, a wetland non-pigmented rice. The raw and cooked samples were used to assess their potential antioxidative properties using Ferric reducing antioxidant power (FRAP) and α,α-diphenyl-β-picrylhydrazyl (DPPH) assays. It was found that the Hitam variety possessed the highest antioxidant capacities in both raw and cooked condition from both methods compared to other rice cultivars with FRAP value of 96.326-29.22 mmol Fe^{2+}/100g and also percentage of radical scavenging activity (RSA) at around 78-55% respectively, and thus suggest its potential as a source of natural antioxidants. Interestingly, the findings showed that higher antioxidant activity was also found in the raw rice extracts compared to the cooked ones across all cultivars which suggest the decomposition of the bioactive compounds by heating. The overall order of the antioxidant activity was Hitam>Wai>MRR220. The strong antioxidant activity of upland rice extracts reflects to the phenolic compounds present in upland rice which could also be associated with the presence of many stress-response enzymes in it.

Keywords: Upland Rice**,** Antioxidant activity; FRAP; DPPH; percentage of radical scavenging activity (RSA)

1. INTRODUCTION

Rice *(Oryza sativa)* is known as important staple food crops throughout Asia and also several other Western and Eastern countries in the world. Besides providing carbohydrates to the consumers, rice also rich in other nutrients such as vitamins, minerals, fibres and other bioactive compounds [1]. Normally in Malaysia rice can be found in two types of growing conditions, they are upland rice as well as irrigated rice which also known as wetland rice. Upland rice thrive in minimum rain-fed level, naturally well drained soil without surface water accumulation and with the ability to survive under dry condition. It is cultivated in mountainous side with low requirement of fertilizer [2]. Meanwhile, wetland rice survived in high water requirement usually in the forms of rain fed irrigated on normal flatland which accommodates over 50% of types of rice planted in Asian countries and make the most productive rice yield [3]. In terms of taste and scents, upland rice possess long shaped, softer texture and more aromatic over wetland rice. Despite the tastier properties, this type of rice can also be found in interesting pigment colour usually brown, red as well as black coloured. Several studies on rice variants claimed that pigmented rice is a potent antioxidant sources and has been proposed as one of a natural source of antioxidants for human consumption alongside vegetables and fruits [4].

Pigmented rice has been reported to have a remarkable amount of phenolic compounds with significant free radical scavenging activity [5]. Coumaric acid, gallic acid, salicylic acid are part of phenolic compounds that can be commonly found in rice as reported by [6]. In 2000, a positive correlation between the lower incidence of cancers and heart disease in Asian population and rice consumption were found which boost research interest of antioxidants in rice variants especially in pigmented variants [7]. These shows the importance on discovering antioxidant in our local upland rice as it ensure huge number of benefits towards human health being. To date, there is limited study of antioxidant in our local upland rice. Therefore, the objectives of this study were to determine the total antioxidants activity of several upland rice cultivars by using FRAP and DPPH assays respectively. Considering the consumption of rice is primarily taken in cooked form, the study on the effect of cooking temperature on rice antioxidant activity was also compared with the raw rice to obtain a clear information on the effect of heat towards its antioxidant activity.

2.0 MATERIALS AND METHODS

2.1. Plant materials

Several upland rice seed is directly obtained from farmers in rural area in Bintulu, Sarawak Malaysia. Two types of upland rice were chosen to be assayed. They were Hitam, and Wai cultivars. Additionally, one irrigated normal land variety was used as control varieties namely MR220.

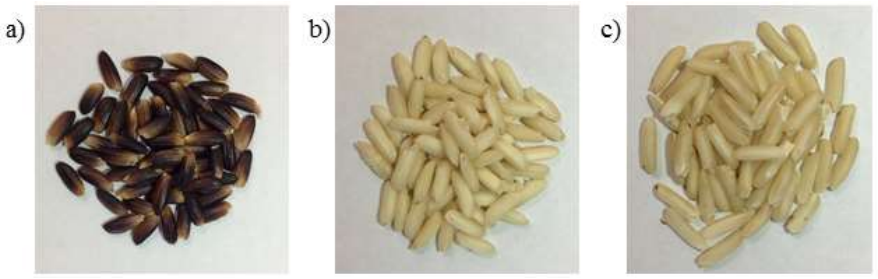

Figure 1. Types of upland rice cultivars used a) Hitam b) Wai and a normal land variety c) MR220

2.2. Sample preparation

Rice seeds were prepared in the conventional way by de-coating the seed from the outermost layer without milling and polishing steps. For cooked rice, rice seeds were cooked by using distilled water at 100^{o}C for 30 minutes and ground using mortar and pestle.

2.3. Sample extraction

Extraction was performed by using 80% methanol a polar solvent, that is commonly be used in extracting bioactive compound in plants. Due to the lower boiling point of methanol (64.7^{o}C) , it can be evaporated readily thus minimizing the risk of thermal damage to the bioactive compounds[8]. First, 10g of grounded raw rice sample was soaked overnight in 80% methanol (v/v) and agitated, at ambient temperature before being separated from the residue by filtration through Whatman No. 1 filter paper. The remaining residue was re-extracted twice, and the two extracts were combined. For cooked rice, same amount of cooked rice was soaked in 80% (v/v) methanol. The mixture were then incubated, agitated for 4 hours at room temperature followed by filtration. Then, the residue was re-extracted again for overnight agitation at ambient temperature and finally combined. The residual solvent of methanolic extract for all samples were removed under reduced pressure at 40^{o}C using rotary evaporator. This crude extracts were stored at -80^{o}C prior to the antioxidant and radical scavenging activity determination by using FRAP and DPPH assays. The methanolic extract was produced in triplicates.

2.4. Determination of total antioxidant activity by FRAP assay

The method is a slight adaptation of that described by Benzie and Strain (1996) with some modifications. FRAP reagent containing 0.3M acetate buffer pH3.6, TPTZ solution and ferric chloride. The assay performed by mixing 30µl plant extracts and 90µl of distilled water into 900µL of the FRAP reagent into a cuvette, then the cuvette was inverted to mix the solutions. The changes in absorbance was recorded at 593nm at exactly 30 minutes using a UV-Vis spectrophotometer against a blank. In vitro antioxidant power results were expressed as mmol Fe^{2+}/100g using a standard curve.

2.5. Radical scavenging activity via DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

The RSA of raw and cooked rice extracts were done according to procedure described by [9] with few modifications. 150µl of sample was mixed with 2.85ml of freshly made 0.1Mm DPPH solution in methanol. The preparation was done under minimal light condition at room temperature. The reaction was allowed to stand for 30 minutes before the absorbance was read at 517nm against methanol. The

percentage radical scavenging activity (%RSA) was calculated based on the given formula; % RSA = $((A_0 - A_S) / A_0) \times 100$; where; A_0: Absorbance of positive control and A_S: Absorbance of sample.

2.6. Statistical analysis

Findings were analysed using ANOVA for mean differences among rice cultivars. The independent *t*-test was used to analyse differences between raw and cooked rice.

3.0 RESULTS AND DISCUSSION

3.1. FRAP reducing antioxidant power

Antioxidants can be defined as organic molecules that promotes health by protecting body's cell from damage caused by free radicals and reactive oxygen species (ROS) that may eventually exerts harmful metabolic effects. It can undergo redox reaction which includes a reaction species (oxidants) is reduced at the expense of the oxidations of another antioxidant. The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. This reduction will form an intense blue complex with an absorption maximum at 593nm [10]. Among the studied rice cultivars, Hitam exhibited the greatest FRAP value, followed by Wai and MR220. The comparison of mean total antioxidants activity of raw and cooked rice is represented in descending order in Table 1. Means FRAP values of Hitam, Wai and MR220 were 96.326 ± 5.16, 25.48 ± 0.55 and 21.21 ± 2.06 Fe^{2+}/100g respectively. Results of ANOVA analysis indicated that Hitam had the highest significant FRAP value ($p<0.05$) than the rest of rice cultivars suggest that colour shows significant effect on reducing ability. This high reducing power was associated with the antioxidant activity of bioactive compounds (mainly low and high molecular phenolic) present in the rice seed extracts that specifically assist in scavenging free radicals[11].

Heat treatment has been known to affect antioxidant activity [12].As shown in Table 1, the FRAP value for both conditions were reduced significantly ($p<0.05$) after heat treatment. Pigmented Hitam cultivar compared to non-pigmented Wai and MR220 which reduced over 69.67% compared to 57% and 44% respectively after cooked. This might be due to the deterioration of bioactive compounds during the heat treatment that caused extensive hydrolysis degrading the phenolic compounds[13][14].

Table 1. The antioxidant activity by FRAP and DPPH assays

Rice cultivars	FRAP (mmol Fe^{2+}/100g)	
	Raw	Cooked
Hitam	96.326 ± 5.16	29.22 ± 1.89
Wai	25.48 ± 0.55	10.97 ± 0.87
MR220	21.21 ± 2.06	11.84± 2.38

* Data are means of three duplicate experiments ± SEM

3.2. Radical scavenging activity of rice extracts

The total antioxidant DPPH activities ranged from 34.08 to 78.34% and 16.36 to 55.34% in raw and cooked rice extracts respectively (Fig. 2). In general, the scavenging activity in both conditions were significantly differed at $p<0.05$ among all rice cultivars evaluated in this study. Results revealed that two upland rice cultivars served better scavenging effect than the wetland rice cultivar either raw or cooked as similarly reported in a previous study[15]. The order of %RSA of the cultivars was as follows: Hitam> Wai> MR220. This trends was in line with the present FRAP study in which pigmented rice holds the highest scavenging effects compared to the non-pigmented one due to the high phenolic compounds it contained. Meanwhile, the reduction in %RSA in cooked samples might also be

due to the heat treatment on rice bioactive compounds during the cooking process. Nevertheless, Hitam and Wai possess low percentage reduction when compared to MR220 after cooked with 29.36, 40.71 and 52% respectively.

In general, upland rice cultivars possess high reducing ability and radical scavenging activity than MR220 in both conditions and assays. Though the value for the Wai and MR220 is insignificant, it may also suggest the association of stress responsive mechanism, environmental factors, such as climatic growth conditions, temperature, duration of storage and thermal treatment may have influenced the antioxidant activity in upland rice cultivars [16].

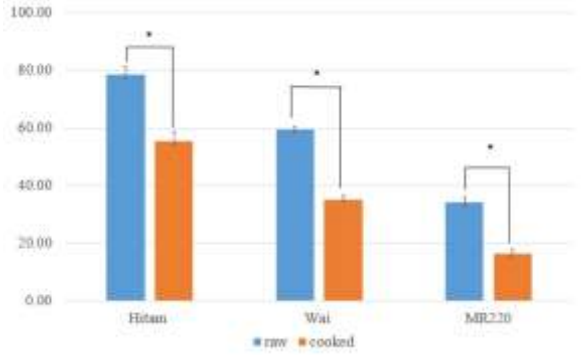

Figure 2. Means of percentage free radical scavenging (%RSA) effect of three rice cultivars in raw and cooked conditions. Asterisk (*) indicates a significant difference at the level p<0.05 between raw and cooked rice.

4. CONCLUSION

This study described the antioxidant property of the two selected upland rice in comparison with the wetland rice. In general upland rice is a potent antioxidant source over normal wetland rice. Even though cooking process may affect the nutritional value, upland rice is still a better option compared to the wetland rice which reflects considerable percentage reduction of antioxidant activity after being cooked. This study could suggest that upland rice is a healthier choice for human daily consumptions. Thus, the effort in cultivating and commercializing upland rice need to be revised in the future due to the health benefits that it can provides. With regards of scarcity of wetland space to accommodate rice demands, manoeuvring to upland rice commercialization is always a good alternative.

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Usage of Human Scalp Hair (HSH) as Potential Repellent Material for Wild Hog Invasive Activity

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ABSTRACT

In the recent time, large amount of Human Scalp Hair (HSH) waste is being generated and dumped in municipal solid waste and to soil without any recovery process. The improper management of this waste resource may leads to many problems such as blockage in drainage system and some of chemical used and stick on the hair are hazardous to environment. In poor countries, HSH has been used as a low cost wild hog repellent. It is believed that the texture itself can cause severe respiratory problem as they sniff the material which will affect their tracking system. However, the direct disposal of HSH onto land without prior adequate treatment may leads to unpleasant odor and pathogenic breeding. This study aims to provide hair waste management through exploring the efficacy of HSH as potential repellent for wild hog. For this study, HSH was collected from nearby hair salons and washed using non-ionic detergent-acetone method to remove the impurities such as sweat, and inorganic materials before oven-dried overnight. The field test were conducted by varying the weigh of HSH used throughout the 5 days test along with evaluation on HSH efficacy through reduction on corn feed used as feed bait.

***Keywords**:* Human Scalp Hair (HSH), wild hog, repellent

1. INTRODUCTION

Wild hog is an invasive mammals species that always cause problem in human daily life especially in agricultural sector. Despite known as destructive species, the contribution of this animal in seed dispersal for plantation species is undeniable (Schupp, 1993). However, the inclination of the need between this species and human has sparked the human-wildlife conflict (HWC). This conflict exacerbated with the ability of the species in adapting with new environment and excellent in restoring their population which led their activities spread into agricultural land (Massei, 2004) and finally led to HWC (Schlageter, *et al.*,2012). HWC happened for so many years and has led to several negative impact such as deterioration and damage of crop (Habib, 2015). Some minor cases involve fatality and injury. The worst case of this species attack on agricultural land has been reported on 1973 by Diong in Changkat Cermin, Perak. Normally, wild hog use their snout and sense of smell that has been developed since piglet phase in order for them to survive (Kittawornrat et al., 2011). They can be found in group for female, consisting of sow and her litter, while for the adult male, most of them companionless as shown in Figure 1. The traditional method shown that, human scalp hair (HSH) has become the alternative way for most of small farmer to reduce the problem of wild hog attack on agricultural as they are cheap and easy to obtain. Murty (2011) in his review stated that, the usage of HSH will disturb the tracking system of that species by causing respiratory irritation to the pests as they sniff of it. Potential of HSH in agricultural may open further research and expand their potential in agricultural which will significantly contribute to the hair salon waste management and reduce problem of blockage in draining system.

Figure 1. Image of wild hog from footage taken in November 2016. a) Female wild hog usually move in a group, consisting of sow and her litter and b) The mature male wild hog normally companionless.

A study conducted by Vasudeva in 2015 has proven the effectiveness of HSH usage in reducing the nuisance by reduction from 70% up to 80% (Agrawal et al., 2016). However, until now, the usage of HSH on land didn't emphasis on the method of processing prior its usage on agricultural. Figure 2 show the undertaking of HSH research and until now, the commercialization of HSH as repellent are still far behind from Gupta (2014) review.

New uses/areas of research	Countries where research is undergoing
Liquid fertilizers	India, USA, Korea, and Bangladesh
Concrete reinforcement	Canada, India
Pollution control	Canada, Singapore, India, Iran, Korea, Egypt, and Jordan
Molded furniture and objects	UK
Engineering polymers	Singapore, China, Japan, and India
Follicle cell cultures/tissue regeneration	Switzerland, UK, Korea, and France
Composites for superconducting systems	India, Greece, and The Netherlands
Flexible microelectrodes	China

Figure 2. List of countries undertaking new research on human scalp hair (HSH)
Reference: Gupta, 2014

2. MATERIALS AND METHODS

2.1. Hair Segregation, Hair Washing Process and Procedure

HSH collected from hair salon will undergo segregation and washed with detergent as in Figure 3. Hair that has been separated from impurities were placed in a clean polyethylene. HSH is subsequently washed using non-ionic detergent-acetone washing method (Cheng et al., 1992; Sen J *et. al.*, 2001). HSH is rinsed using distilled water and oven-dried at 90ºC overnight. The dried hair is then washed in 5% dilution of non-ionic detergent. Tween 80 has been used as the non-ionic detergent. Hair is washed for 30min and rinsed with deionized water. This process is important to remove the impurities of all form contaminant such as oil, dust, organic matter from the human hair that might cause pathogen breeding..

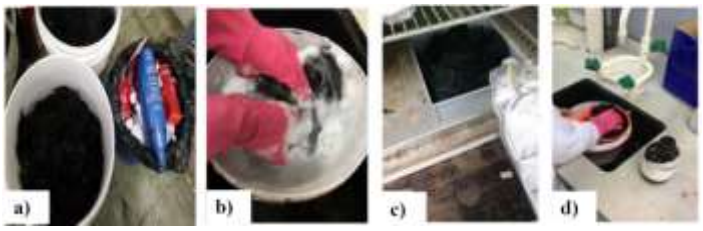

Figure 3. During the washing process, HSH undergo the segregation process and washed using mild detergent and non-ionic detergent to remove the impurities such as oil and dirt.

Hair were subsequently washed with 1:1 of acetone and deionized water in a clean polyethylene container for 30 minutes and rinsed repetitively using deionized water before oven-dried at 100^{o}C overnight. The step is simplified as in Figure 4. This step is essential to remove the inorganic pollutant on the hair strands (Sen J *et. al.,* 2001).

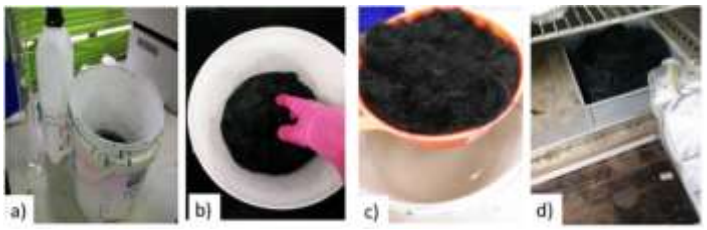

Figure 4. The acetone wash is essential to remove the inorganic pollutant that come from hair product on hair strand.

2.3 Repellency Test on the Field

This study on effectiveness of human scalp hair (HSH) as repellent has been conducted between 25th April 2017 until 29th April 2017 at private orchard located in Kampung Parit Mohamad, Bukit Bakri Muar. The effectiveness of human hair as repellent material is observed through measuring the weight for remaining corn feed along the test period. This feeding test is conducted within 5 days. The use of corn feed as bait is remain constant along the study which is 5kg of corn feed for each samples per trial day, while the usage of human hair is varies; 20 grams (g) ; 40 grams (g); and 60 grams (g). The test of HSH as repellent is categorized into; (a) corn feed; (b) corn feed mixed with 20g of HSH; (c) corn feed mixed with 40g of HSH; and (d) corn feed mixed with 60g of HSH. The process of test is simplified in Figure 5. The weight before and after for bait feed is measured.

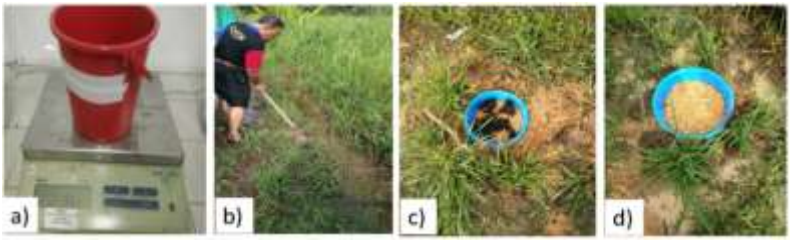

Figure 5. The feed corn mixture is weighed before and after the repellency test on the wild hog. The results is observed and recorded.

3. RESULT AND DISCUSSION

3.1. Repellency Test on Day-1 and Day-2

The remaining of corn feed reading (in kg) shown slightly change in day-1 and day-2 as in Figure 6 and Figure 7. For day-1, sample A showed 4.47kg remaining which means about 89% remained uneaten. While for sample B, and sample D only 0.10kg is eaten, and 98% remained uneaten. Meanwhile, for sampel C is remained uneaten. For day-2, the control sample A showed 89% of corn feed remained uneaten, while 97% of sample B is remained uneaten. Both sample sample C and D, showed 99% of corn feed remained uneaten. The reading for remaining of corn feed on first day and second day of test show there is no wild hog activities occurs during the test. However, the study does not deny the possibilities

for the presence of other small invasive animals such as birds that may contribute to the slightly change in reading.

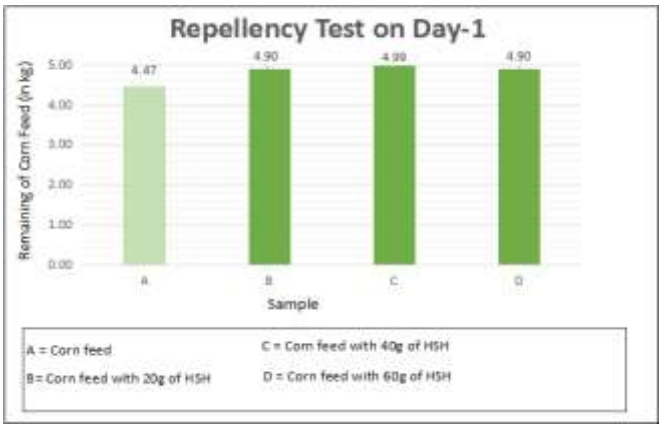

Figure 6. Remaining corn feed (in kg) of wild hog consumption following various samples for day-1 of field test.

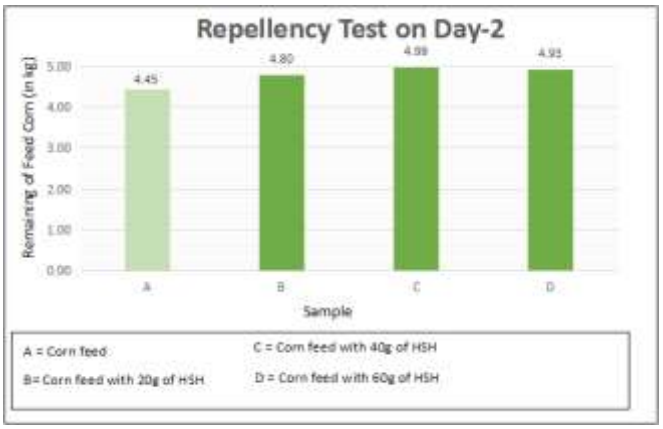

Figure 7. Remaining corn feed (in kg) of wild hog consumption following various samples for day-2 of field test.

3.2. Repellency Test on Day-3 and Day-4

On day-3 and day-4, there is a major changes in reading for remaining feed corn as in Figure 8 and Figure 9 which indicates the possibility of wild hog browsing activity at the study area. The day-3 reading for control sample A showed, 41% of corn feed remained uneaten with 2.95kg of corn feed is eaten. Sample B showed slightly losses with 4.96kg of corn feed remaining. Sample C and D are remain uneaten. For day-4 test, control sample A showed, only 39% out of total corn feed remain uneaten. Meanwhile, sample B, sample C and sample D are remain uneaten through the test day. Wild hog depend entirely on their olfactory sense as they has poor mechanism for both hearing and vision (Agrawal et al., 2016). In order to find location and territory, food and prey, these mammal used their snout to sniff from one place to another. The use of hair will cause irritation to their respiratory tract as they inhale the hair during sniffing and create fear or sense of aware

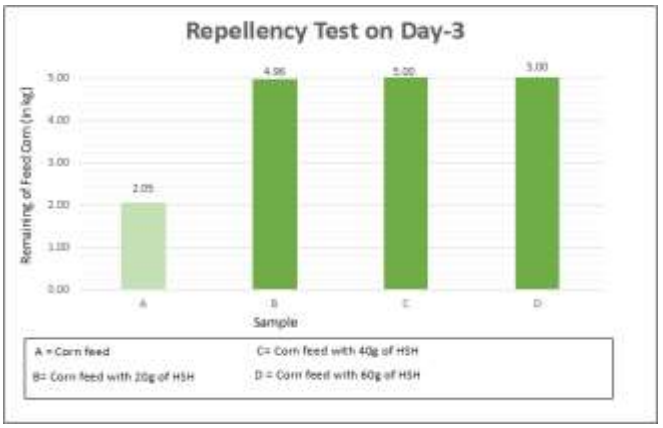

Figure 8. Remaining corn feed (in kg) of wild hog consumption following various samples for day-3 of field test.

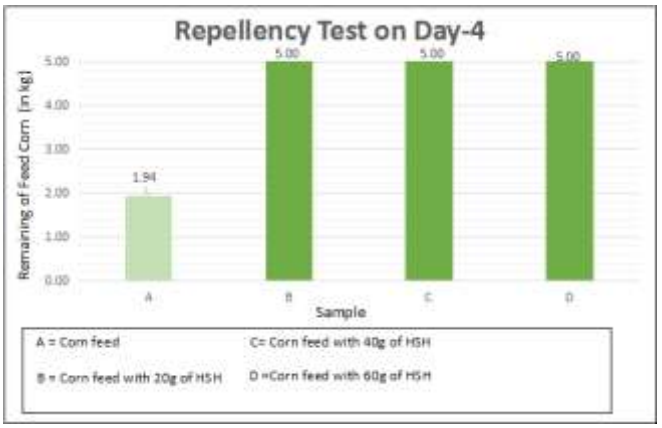

Figure 9. Remaining corn feed (in kg) of wild hog consumption following various samples for day-4 of field test.

3.3. Repellency Test on Day-5

The day-5 reading test showed the sensitiveness of wild hog towards usage of hair as repellent. The usage of HSH has triggered the effect to the reading. For control sample A, 60% corn feed remain uneaten and sample B, C and D remain constant. There are various possibilities, namely; whether the use of HSH have been impacted fear and respiratory irritation to the wild hog or they roam elsewhere for source of food.

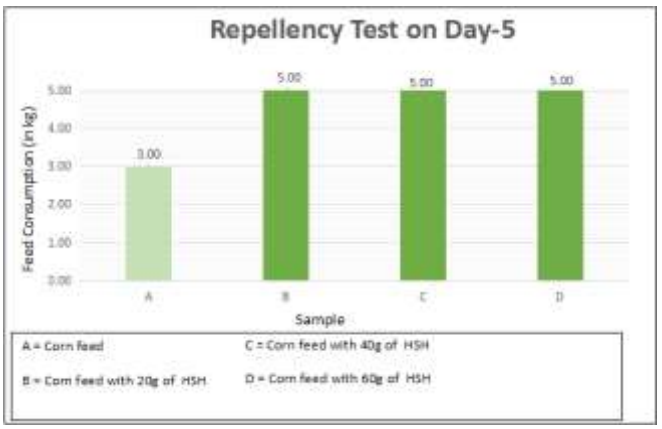

Figure 10. Remaining corn feed (in kg) of wild hog consumption following various samples for day-5 of field test.

4. CONCLUSION

In producing animal repellent, several factors need to be taken into account for the purpose of producing animal repellent. Repellent should be; user friendly; provide temporary protection from over-grazing; low-cost; easy to apply; time saving and safe for both animals and human (Stephens, 2005). Result from 5 days test indicates the potential of HSH as temporary repellent from wild hog browsing activity. This study is not just intended to prove the effectiveness of human hair as repellent, but also at the same time to promote the usage of HSH and value proposition of hair waste in agricultural. The proposition value of HSH will significantly contribute in eco hair salon waste management and to environmental for a safe disposal through prior disposal treatment.

5. ACKNOWLEDGEMENT

The authors express sincere thank and gratitude to farmers of Kampung Parit Mohamad for letting us to conduct the field study and for technical assistant, as well as to the technician staff from Environmental Engineering Laboratory UTM who was willing to help and provide equipment for the purpose of this study. This study comply the animal act 1953 and no animal harmed, abused or involved in cruelty as this research only to provide temporarily natural repellent that safe not just for human or plantation, but also for wild hog.

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Effect of Solvent and Ultrasonic Extraction on Hydroxycitric Acid (HCA) in *Garcinia Cambogia*

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ABSTRACT

This study evaluates the effect of solvent and ultrasonic application in the extraction process on Hydroxycitric Acid (HCA) content of *Garcinia cambogia*. In this study, the fruit was extracted with methanol, ethanol, water and acetone by using two types of ultrasonic assisted extraction which are Ultrasonic probe and water-bath treatment. The HCA content was analysed by using HPLC-PDA. Ultrasonic probe treatment showed significantly higher yield of HCA for all solvent compared to water bath extraction.The HCA content extracted in methanol, ethanol, water and acetone by using probe was 12.59, 18.58, 26.69 and 13.78 %w/w, respectively meanwhile by using Ultrasonic Water Bath treatment, the HCA content was 11.36, 15.89, 22.11 and 12.43 %w/, respectively. The results obtained showed that the suitable solvent for extraction of HCA was water for both extraction methods that give the highest HCA content. Thus, extraction method by using Ultrasonic probe with water is more efficient in producing higher yield of HCA.

Keywords: Hydroxycitric acid, *Garcinia cambogia*, ultrasonic extraction

1. INTRODUCTION

Garcinia cambogia (Malabar tamarind) had been valued for variety of benefits traditionally to treat constipation, rheumatism, oedema, and intestinal parasites in many Asian countries [1]. Previous reported that Garcinia cambogia contains many different phytochemical including organic acids [2], benzophenones and xanthones [3]. However, HCA, an α-,β-dihydroxy tricarboxylic acid, is the key component present in the fruit rind which exhibited anti-obesity activity including reduced food intake and body fat gain by regulating the serotonin levels related to satiety, increased fat oxidation and decreased *de novo* lipogenesis. The crude extract from the plant also exerted antimicrobial and antioxidant properties [4].

There are many techniques to recover phytochemicals from plants, such as Soxhlet extraction, maceration, supercritical fluid extraction and subcritical water extraction. However, extraction yield not only depend on the extraction method but also on the solvent used for extraction. The presence of various compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent [5]. Polar solvents are frequently used for recovering polyphenols from plant matrices. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption. Methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols, whereas aqueous acetone is good for extraction of higher molecular weight flavanols [6]. In research involving plants, the efficiency of ultrasound-assisted extraction methods have been developed for the extraction of phytochemicals in order to increase the extraction yield, shorten the extraction time as well as enhance the quality of extracts. It is simple, inexpensive and efficient alternative to conventional extraction techniques [7]. There are two types of ultrasonic treatment which are ultrasonic probe treatment and ultrasonic bath treatment. A study from Cheok *et al.*, (2013), the optimum direct ultrasonic pre-treatment conditions using the probe for maximum total monomeric

anthocyanin (TMA) and total phenolic content (TPC) extractions from mangosteen were compared with an in-direct pre-treatment using a bath and the conventional magnetic stirring extraction as a control [8]. The efficacy of ultrasonic-assisted extraction is not only shown through the improved bioactive compound yield recovery but also helps in reducing the extraction time. This study is carried out to evaluate the usage of ultrasound in overcoming the limitation of conventional extraction of *Garcinia cambogia* as well as its effect on the Hydrocitric acid content of the fruit using different solvent extraction.

2. MATERIALS AND METHODS

2.1. Plant material

The raw material was used for extraction process is the fruits of *Garcinia cambogia* collected from orchard in Perak. All fruits were cleaned and inspected to remove damage, disease or pest infected fruits. The fruits were cut into small pieces and undergo drying in a hot oven at 45℃. Then, the tray dried samples were ground into powder and stored at 4℃ until use.

2.2. Chemical and Reagents

(-)-Hydroxycitric Acid Calcium Salt Standard was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Ethanol (99%), acetone, methanol AR grade were obtained from Merck (Massachusetts, USA). Dowex 50WX8, mesh size 100-200 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphoric Acid (85%) and acetonitrile HPLC grade from Sigma Chemical Co. (St. Louis, MO, USA) were used for HPLC analysis. Deionized water was prepared using a Millipore water purification system (Barnstead, California, USA).

2.3. Extraction methods of *Garcinia cambogia*

A 700 WATT Sonic Dismembrator, 220V (FB-705, Fisher Scientific, Loughborough, UK) with a 1/2" probe with replaceable tip and 1/8" micro tip with amplitude of 50% was used. *G.cambogia* powder (10 g) was placed in a 250 mL beaker and extracted with 100 ml distilled water, acetone, methanol and ethanol with 10 minutes extraction time. The samples were submerged to a depth of 25 mm in sonicator probe. Meanwhile, the extractions with ultrasonic bath treatment were carried out in a WUC-D06H water-bath sonicator (Daihan Scientific, South Korea). *G.cambogia* powder (10 g) was sonicated in 100 mL of distilled water, acetone, methanol and ethanol in 10 min. The aqueous of extraction was filtered and concentrated by using rotary evaporator to 30 ml and treated with 120 ml ethanol to remove pectinaceous material, then centrifuged at 2000 rpm for 15 min. The supernatant is concentrated under reduced pressure to 25 ml and stored at 4℃ until further HPLC analysis.

2.4. HPLC-PDA Analysis of Hydroxycitric Acid

Waters e2695 Alliance Separation Module liquid chromatography system comprising of vacuum degasser, quaternary pump, auto-sampler and Waters 2998 photodiode array detector (Millford, MA, USA) will be used. Empower software is used to control the HPLC system and data processing. Agilent Hi-Plex H, (7.7 × 300 mm, 8 µm) will be used as stationary phase. The isocratic system will be used for the separation is 0.005N Sulphuric Acid with the flow rate of 0.6 ml/min and detection wavelength is

210 nm. Sample injection volume is 20 µl. The total running time for HPLC analysis for (-)-HCA is 10 minutes. The chromatographic peaks of the analytes are confirmed by comparing their retention times and UV spectra with the reference standards.

3. RESULTS AND DISCUSSIONS

Solvents such as methanol, acetone and water have commonly been used for the extraction of phytochemical from Garcinia species [9,10,11]. In this research, distilled water, methanol, acetone and ethanol were used for solvents extraction. In determining HCA content of G.cambogia rinds, the fine powder of dried material was prepared. The HCA content of the extract were estimated using High Performance Liquid Chromatography (HPLC). The effect of solvent extraction on the release of HCA content extracted by water, acetone, methanol and ethanol as a solvent is shown in Table 1. The yield percentage HCA content extract using probe sonicator by water, ethanol, acetone and methanol was 26.67, 18.59, 13.78 and 12.59 %w/w, respectively. Meanwhile the water-bath sonication gave slightly lower HCA yield than probe sonicator as shown in Table 1. The water extraction gave the highest percentage yield because HCA was dependent on the type of solvent used, its polarity and the solubility of organic acid in the extraction solvents. Jayaprakasha *et al*. (2003) studied the extractability of organic acids from the rinds of *G.pedunculata* with different solvents; water, ethanol and methanol. Among the 3 solvents, water extracted maximum organic acid, which may due to higher polarity of waters than other solvents

Table 1: Composition of HCA in different solvent extraction of dried rinds *Garcinia cambogia*

Extraction solvent	**Probe Sonicator**	**Water-bath Sonicator**
	LC Method (g/100g)	
Water	26.67	22.11
Methanol	12.59	11.36
Ethanol	18.58	15.89
Acetone	13.78	12.43

Thus, it was concluded that HCA was obtained highest with water extraction by using probe sonicator method of extraction. This condition gave the best result and it was taken as the best condition to perform further extraction.

The study for the effect of ultrasonic types was performed by maintaining the time and solid liquid ratio. The optimized direct ultrasonic probe conditions in giving the highest extracted HCA yields with respect to ultrasonic time and amplitude were compared to an indirect ultrasonic bath system. The result shows that HCA extracted with probe ultrasonic was significantly higher than the water-bath ultrasonic for all solvents. A probe sonicator makes direct contact with the sample. This can be an advantage since a more concentrated energy can be added to the sample meanwhile bath sonicators isolate the sample from the energy source in an energized bath of water. They require significantly more energy, are unpredictable, and often over-heat the sample [12]. The improvement of HCA extractions from *Garcinia cambogia* powder with direct ultrasonic pre-treatment over the indirect method of using bath has proven. The

advantage of using the probe compared to the bath has also been reported in the extraction of oleuropein from olive fruit [13]. They revealed that the optimized ultrasonic probe conditions of the three extraction step of 20 min, 44 ◦C and pure methanol was more efficient in comparison to the ultrasonic bath and agitation, with up to 33% and 80% enhancement in the extraction of oleuropein from olive fruit.

4. CONCLUSION

The HCA content of *Garcinia cambogia* rind extracted with two types application of ultrasonic were compared in this study. The results clearly indicated that the values of extraction with probe sonicator were significantly higher when water was used as solvent extraction. This study showed that *Garcinia cambogia* extraction can benefit from UAE especially by reducing the extraction time. The use of water as a solvent for extraction can reduced environmental impact, inexpensive and environmentally benign but it is also non-flammable and nontoxic [14] and save for making foods and supplements.

5. ACKNOWLEDGEMENT

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Effect of Temperature and Initial pH on the Growth and Viability of Direct-Fed Microbial Microorganism *Lactobacillus casei* in Rice Bran Extract

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ABSTRACT

The aim of this study was to observe the effect of incubation temperature and initial pH on the cell viability and biomass of direct-fed microbial microorganism *L. casei* ATCC 393 in rice bran extract. The fermentations were carried out in single step and under microaerophilic conditions, without growth supplementation. Batch fermentations of rice bran extract by *L. casei* were studied at various initial pH and temperature, ranging from 5.0 to 7.0 and 30°C to 42°C, respectively. The cell viability and biomass were measured after 30 hours fermentation. It was noted that, incubation temperature had distinguished effect on the cell viability but not on the biomass. Meanwhile, the initial pH was observed affecting both cell viability and biomass.

***Keywords**:* Direct-fed microbial, *Lactobacillus casei*, rice bran, temperature, initial pH

1. INTRODUCTION

Direct-fed microbial sector is currently on the rising, due to the consumer awareness increasing on food safety. Direct-fed microbial is also known as probiotics; is defined as live microorganisms consume for their benefits in regulating and enhancing the host immune response towards pathogen and balancing the microflora in the host, especially in gastrointestinal tract. Common species used as direct-fed microbial Lactic acid bacteria such as *Lactobacillus* sp., *Bifidobacterium* sp. are the most bacteria used as direct-fed microbial and *Lactobacillus casei* is one of the most commonly consumed and best characterized probiotics species [1, 2]. As a direct effort in minimizing cost, there are studies of *L. casei* cultivated in some lignocellulose medium such as in cabbage juice [3], in cantaloupe juice [4], and in pineapple juice [5]. Physicochemical properties such as incubation temperature and initial pH are known to exert significant effect in the fermentation process in terms of microbial development especially in the production of direct-fed microbial [6]. In previous study [7], the cultivation of *L. casei* in different initial rice bran concentration was conducted with the aim to investigated the effect of initial rice bran concentration has on the growth of *L. casei*. In that work, it was proposed that the initial rice bran concentration has certain effect on the growth of *L. casei* with highest viability obtained in 20% (w/v) initial rice bran concentrations. As continuation of the previous work [7], the effect of incubation temperature and initial pH were investigated in this study. Incubation temperature and initial pH are two of the significant physicochemical properties that have significant effect on the fermentation performance. Therefore, it is important to determine the incubation temperature and initial pH at which the optimal growth is achieved [8].

2. MATERIAL AND METHODS

2.1 Bacterial Strain and Rice Bran Extract

Lactobacillus casei ATCC 393 was obtained from American Type Cell Culture (ATCC, Virginia, USA) in freeze dried form and re-activated by spread plate method in MRS agar with Tween 80 (Biolife, Milano,Italia) [7]. A colony of *L. casei* was transferred in 10 mL MRS broth with Tween 80 and incubated at 37°C for 20 hours without agitation. This culture was being used as inoculum in the fermentation. Meanwhile, 20% (w/v) of rice bran was added into distilled water and boiled for 15 minutes and the extract was collected through centrifugation at 5000 rpm for 20 minutes. This step was taken to eliminate any interference for cell density measurement [9].

2.2 Fermentation Procedure

In this study, five different temperatures, 30°C, 35°C, 37°C, 40°C and 42°C; were used to investigate the effect of temperature on the growth and survival of *L. casei* in rice bran extract. The fermentations were carried out for 30 hours in 100 mL conical flask with 100 mL working volume in order to minimize the oxygen concentration during fermentation. The initial pH was adjusted to 6.5 using 1 M NaOH. Twenty hours old inoculum of 1% (v/v) was used for the fermentations. Once the optimum temperature had been determined, the study of initial pH was conducted by varying the initial pH to the value of 5.0, 5.5, 6.0, 6.5 and 7.0 using 1 M NaOH. The culture were inoculated with 1% (v/v) of 20 hours old inoculum culture and was incubated statically for 30 hours.

2.3 Samples and Analyses

Samples of 5 mL was collected prior and at the end of the fermentation for cell viability (log cfu/mL and cell dry weight (cdw g/L) measurement. Cell viability was measured by spread plate method in MRS agar with Tween 80 and incubated at 37°C for 72 hours. The remaining sample was used for cell dry weight measurement. The culture was centrifuged at 10 000 rpm for 5 minutes, and the pellet was separated from the supernatant. After that, the pellet was washed using 0.85% saline and dried in an oven until a constant weight was obtained. All the analyses were conducted in triplicate.

3. RESULTS AND DISCUSSION

3.1 Incubation Temperature

Figure 1 and 2 show the cell viability and biomass of *L. casei* incubated in rice bran extract at different incubation temperatures. It was observed that the highest viability; 8.5 log cfu/ml was achieved when the culture was incubated at 37°C for 30 hours. However, there was no distinguished difference in biomass when incubated in different temperatures. The highest cell biomass, 25.17±2.58 g cdw/L was produced when the culture was incubated at 40°C. Noted that, the optimum temperature for the optimum cell viability and biomass are different. The same findings were also observed in the study conducted by Fonteles *et al*., [4] and Costa *et al*., [5]. From the results, it can be observed that the cell viability was affected by the incubation temperature (Figure 1). However, cell biomass was only marginally affected by the incubation temperature as shown in Figure 2.

3.2 Initial pH

Figure 3 and 4 show the cell viability and biomass of *L. casei* incubated in different initial pH. Cell viability was recorded to be the highest, 8.76±0.03 log cfu/mL when the initial pH was 5.5. And it was observed that, the viability of *L. casei* were decreasing when the initial pH value increased. Interestingly, the opposite can be observed in regards of biomass, when the initial pH increased, the biomass was decreasing and the highest biomass, 19.0±1.1 g cdw/L was obtained when the culture was incubated at initial pH value of 7.0. It can be conclude that, initial pH affects cell viability and biomass. However, Costa *et al*., [5] reported that the initial pH did not exert any significant effect on the cell viability and cell biomass. Meanwhile, according to Fonteles *et al*., [4], the initial pH was demonstrated to have significant effect on cell biomass but not on cell viability.

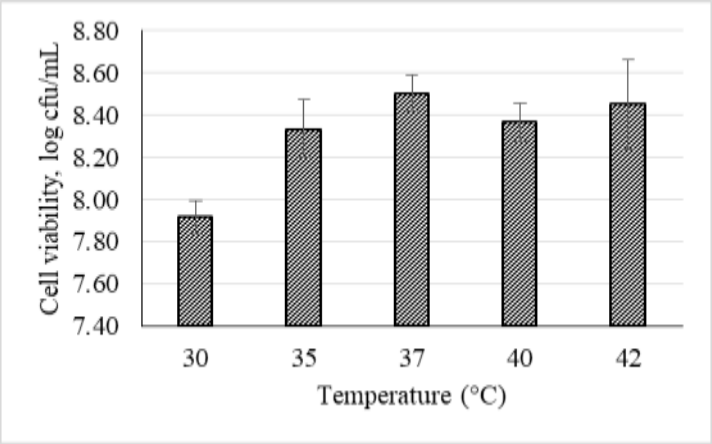

Figure 1. Viability of *L. casei* grown in different incubation temperature, initial pH of 6.5 and inoculum size of 15 (v/v)

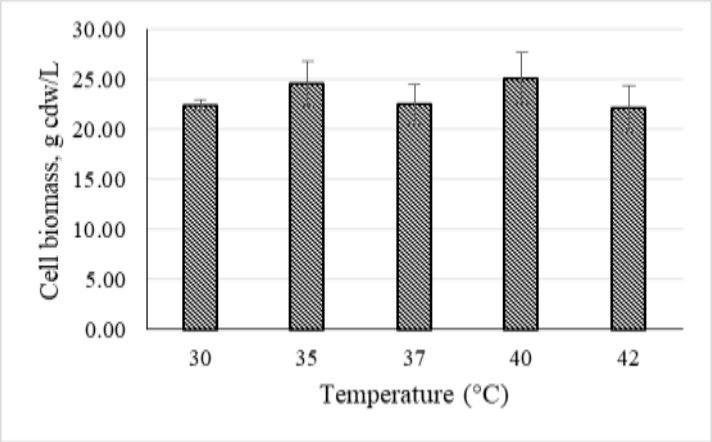

Figure 2. Biomass of *L. casei* grown in different incubation temperature, initial pH of 6.5 and inoculum size of 15 (v/v)

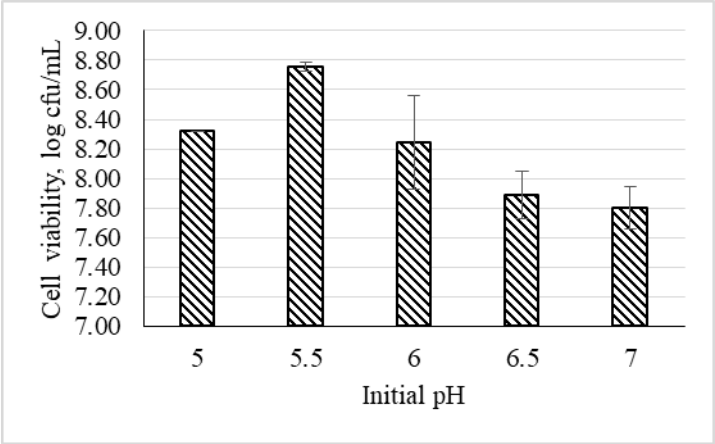

Figure 3. Cell viability of *L. casei* grown in different initial pH, incubation temperature of 37°C and inoculum size of 15 (v/v)

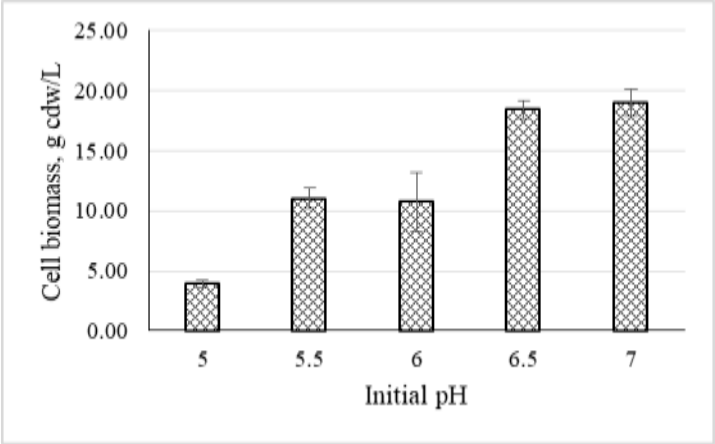

Figure 4. Biomass of *L. casei* grown in different initial pH, incubation temperature of 37°C and inoculum size of 15 (v/v)

4. CONCLUSION

The aim of this study was to observe the effect of incubation temperature and initial pH on the biomass and cell viability of *L. casei* grown in rice bran extract. It was noted that, the incubation temperature had a distinguished effect on the cell viability but not on the biomass. Meanwhile, the initial pH was demonstrated to display distinguished effect on both cell viability and biomass.

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Delignification of Lignocellulosic Biomass by Immobilized Laccase: A Mini Review

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ABSTRACT

The production of energy from lignocellulosic biomass has become common interest among the researchers to fully utilize the waste by converting it into valuable energy sources. The main problem is that the lignocellulosic biomass is difficult to hydrolyse due to their recalcitrant and heterogeneous structure. Therefore, a pretreatment or delignification process is required to remove or modify the lignin structure in order to make it more susceptible to the hydrolysis stage. The first part of this paper reviews the current method used in delignification and emphasizes more on the biological treatment which is more eco-friendly and less energy consuming. While the second part focuses on ligninolytic enzymes, laccase which is considered to have great potential in delignification. Due to the limitations of the enzyme in term of operational stability and reusability, the improvements of enzyme after immobilization are discussed briefly. Finally, the future outlooks and challenges on the implementation of immobilized laccase in delignification process are also covered in this review.

Keywords: Delignification; Lignocellulosic biomass; Laccase; Immobilization

1. INTRODUCTION

The development of technology and civilization nowadays leads to an increasing demand in energy source which is mainly dependent on the non-renewable supply of fossil fuels. An alternative supply from a renewable source such as lignocellulosic biomass gives a promising solution in order to fulfill the energy demand in the future. Lignocellulosic biomass is referred as plant biomass that is composed of three major components which are cellulose, hemicellulose and lignin. It is the most abundant organic component on earth which can be used as the feedstock for biofuel and fine chemical productions.

Degradation of lignocellulose material is the major focus prior to its application as the feedstock in biofuels production. Lignocellulose is a complicated natural composite with three main biopolymers which are cellulose, hemicellulose and lignin. The presence of lignin hinders the enzymatic hydrolysis during the production of biofuel. The functions of lignin in lignocelluloses are for structural rigidity, integrity and prevention from swelling. Lignin holds the cellulose and hemicellulose together and it act as a physical barrier which hinders the enzymatic hydrolysis (Alvira *et al.*, 2010). Thus, its presence and distribution in the lignocellulosic biomass is one of the factors which is responsible for the recalcitrance of lignocellulosic biomass. Since, cellulose and hemicellulose are closely associated with lignin, it needs to be modified or removed to allow hydrolysis of hemicellulose and cellulose (Singh *et al.*, 2014).

2. PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

A pretreatment process such as delignification is one of the most important processes in the production of biofuel from the lignocellulose material in order to tackle the limiting factors in enzymatic hydrolysis. There are several factors that hinder the enzymatic action during the hydrolysis such as cellulose crystallinity, cellulose degree of polymerization, available surface area of the substrate, lignin content, hemicellulose content and feedstock particle size (Alvira *et al.,* 2010). Several requirements suggested by Sun and Cheng (2002) on the delignification process are: (i) enhance the formation of sugar during enzymatic hydrolysis; (ii) avoid the degradation of carbohydrate; (iii) avoid the byproducts formation that inhibit hydrolysis and fermentation process; (iv) cost effective.

There are many methods that have been developed and used in the delignification process including physical, chemical and physicochemical methods. However, most of the methods require the usage of harmful chemical and perform in harsh condition which will cause problems to the environment. On the other hand, a promising method which is more environmentally friendly and less energy consumption is being offered by the biological pretreatment. Regardless on its limitations- low efficiency and long residence time (Liguori and Faraco., 2016), this method shows advantages in term of energy, environment and safety. It does not require high amount of energy as the process can be carried out under normal conditions, no toxic material is generated as no harmful and severe substances are used in the process, does not require corrosion-resistant reactors and reduce or no inhibitor to fermentation (Wan and Li, 2012). In biological pretreatment, two different methods are being used which are fungal pretreatment and enzyme pretreatment. The fungal pretreatment required longer residence times (minimum of 3 to 7 weeks) than enzymatic pretreatment which make it less favorable to be used in the industry. Besides shorter residence times, enzymatic pretreatment also does not require nutrient supplementation and no sugar consumptions (Liguori and Faraco, 2016). The advantages offer by enzymatic pretreatment shows that it is a great alternative way to be used in biological pretreatment.

3. LACCASE

According to Bilal *et al.,* (2017), there are three main ligninolytic enzymes that are usually found in lignin degrading rot fungi which are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase microorganisms. Among those rot fungi, white rot fungi is the most effective to be used in delignification due to their unique ligninolytic system in producing various extracellular ligninolytic enzymes mainly peroxidase and laccase (Meehnian et al., 2017). Even though there are three main enzymes involved in the modifying or degrading lignin, not all white rot fungi produce all three enzymes (Pinto *et al.,*2012). It shows that every ligninolytic enzyme have their own ways in degrading the lignin structure. The advantage of laccase over peroxidase is that it only requires oxygen as it co-substrate which is present in the surroundings (Arora and Sharma, 2010). However due to its low redox potential, laccase cannot directly attack the substrate with a large size or high redox potential such as non-phenolic lignin. The presence of redox mediators in the process has been proven to provide a key to solve the limitation of laccase by expanding its catalytic activity towards high redox substrates (Fillat *et al.,* 2017).

4. IMMOBILIZATION OF ENZYME

The application of laccase in delignification process shows a positive alternative as it offers green technology with low energy consumption and little or no waste generation. However, enzyme is sensitive to denaturant agent and difficult to recycle which restricts its application in the industry. Therefore, enzyme immobilization technology helps to enhance the characteristics of the enzyme by holding it in a distinct support or matrix using method as shown in Figure 1. The purpose of immobilization is to make the enzyme immobile during the reaction which contributes to a more efficient process and easy separation from the product so that it is reusable. Finally, immobilization also improves the thermal stability of the enzyme, and it resistance to degradation, denaturation and aggregation (Bilal *et al.*, 2017).

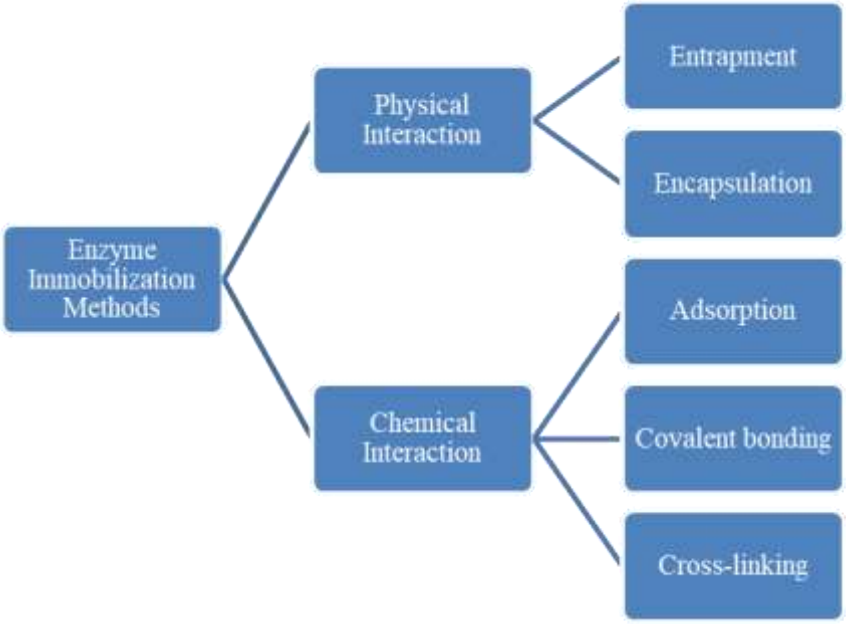

Figure 1. Enzyme Immobilization Methods (Adapted from: Fernández-Fernández *et al.*, 2013)

5. FUTURE DIRECTION AND CHALLENGES OF IMMOBILIZED LACCASE FOR THE APPLICATION IN LIGNOCELLULOSIC BIOMASS DELIGNIFICATION

The enhancement of laccase after immobilization had opened up its usage to be used widely in the industry as enzyme-based process offers notable features such as mild reaction condition, less power and energy consumption and environmentally friendly process. However, there were only a few researches conducted to study on the application of immobilized laccase in delignification of lignocellulosic biomass. Since not all types of support and immobilization techniques are suitable for each industrial application,

the study on immobilized laccase which focused on this application should be conducted in depth to create an efficient system.

Besides, laccase is only able to catalyze the oxidation of phenolic subtrates due to it relatively low redox potential. The presence of redox mediators in the system enhances the oxidation capabilities of laccase towards the non-phenolic subtrates. From the research conducted by Sun *et al.*, (2016), they have successfully immobilized both laccase and a mediator (acetylacetone) into a hydrogel to be used in conversion of malachite green. This finding helps to reduce the cost of the system since both laccase and mediator could be reused, a more effective biocatalyst is produced. For the future study, the finding should be implemented in the application of lignocellulosic biomass delignification as only less than 20% of lignin polymer is phenolic substrate (Fillat *et al.,* 2017). A suitable mediator and support system should be studied further for better result in the co immobilization of both laccase and mediator.

Finally, the search for a new support or matrix from nanosized material such as nanotube, nanoparticle, magnetic nanoparticle, nanofiber and so on had become common interest to the researchers recently due to the large surface area features. However, due to the complex preparation and high cost of the material for large scale synthesis, their applications in industries could be challenging.

6. CONCLUSION

There is a huge potential of immobilized laccase to be used in the biological delignification of lignocellulosic biomass. By using the immobilized laccase, the reaction time could be reduced compared to the fungal or microbial treatment and enzyme stability will be further enhanced. Deciding and selecting the suitable support system and immobilized method for the delignification should be the main concern in order to apply the system in this industry.

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Effect of Oil Palm Frond Leaves Particle Size on Cellulase and Xylanse Production by Trichoderma Asperellum UC1 under Solid State

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ABSTRACT

Cellulases and xylanase are highly required for the efficient hydrolysis of lignocellulosic (LGC) biomass. Cellulase, xylanase and pectinase contribute almost 20% to the world enzyme market. Microbes including fungi and bacteria have been demonstrated to produce these enzymes. Filamentous fungi, usually degrade plant cell constituents better because they produce their enzymes extracellular and possess hyphal penetration power, mostly forming the commercial sources of these enzymes. In order to increase hydrolytic enzyme production and improve LGC biomass degradation, researchers are constantly working on the optimization of solid-state fermentation parameters such as optimum temperature, pH, moisture content, microbial inoculum size, substrate pretreatment etc. For the purpose of this study, focus was on the pretreatment of oil palm leaves (OPFL) through change in substrate size. Direct utilization of untreated OPFL for the production of cellulases and xylanase by *Trichoderma asperellum* UC 1was conducted under solid state fermentation (SSF). The maximum activities of the enzymes cellulases-endoglucanase (83.53 U/g), and exoglucanase (15.59 U/g),were recorded at initial pH 5.0, 80% moisture level, 2×10^8spore/g (inoculum) with 250 µm of OPFL as sole carbon source. The β-glucosidase and xylanase maximum activities were recorded with 600 µm and 900 µm OPFL size having activities 20.31 and 152.77 U/g respectively.

Keywords: Cellulase, xylanase, solid-state fermentation, oil palm frond leaves, *Trichoderma asperellum* UC 1.

1. INTRODUCTION

With the level of agricultural lignocellulosic waste currently produced, enzymatic degradation has become the most efficient and sustainable method for their removal as well as their conversion to other value-added products such as bioethanol, organic acids, food additives, compost for agricultural use etc. [1,2]. There is increasing demand for microbial enzymes because of their numerous uses in industries due to their relative ease of production and optimization than plant and animal enzymes, catalytic activity and stability [3]. However, the production cost of these enzymes remains a bottleneck in their large scale production for both lignocelluloses (LGC) biomass degradation and for use in other relevant industries. Reduction in production cost remains top in the agenda of researchers thus several options such as use of cheap raw materials for enzyme production and the use of unpurified concentrated enzymes for biomass breakdown, on-site production, cleaner/more sustainable pretreatment methods and more efficient fermentation methods are being investigated [4]. Solid state fermentation (SSF) is generally defined as the growth of microorganisms on solid material in the absence or near absence of free water [5]. It has been well established from previous studies that enzymes produced by filamentous fungi in SSF systems are several folds higher than in submerged fermentation (SmF) systems [6].

Pretreatment is usually done to break the lignocellulosic matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose, a preferred form for enzymatic breakdown [7], making it susceptible to fast hydrolysis leading to increased yield of monomeric sugars [8]. The methods generally fall into two major categories; physical and chemical pretreatment. The later involves the use of chemicals, usually accompanied by the formation of chemical side products that may have suppressing effects on microbial growth and respiration. This study aims at optimizing a pretreatment method with minimal to zero pollution to life and the environment. The choice of OPFL as enzyme production substrate was guided by the fact that oil palm frond is the largest produced oil palm waste biomass. Forms about 62% of the total wastes produced through pruning and replanting [9]. So far no study has been reported on the optimization of cellulase and xylanase production using different particle sizes of OPFL as substrate by *Trichoderma asperellum* UC 1, through solid state fermentation.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals used in this work was produced by Sigma-Aldrich, USA and EMD Chemicals Germany. The fungal strain used was isolated from decaying OPEFB and molecularly identified as *Trichoderma asperellum* UC 1.

2.2. Preparation of Inoculum

The inoculum was prepared by maintaining the fungus on PDA plates at 30˚C for 7 days. *T.asperellum* UC 1 spores were harvested using 1% (v/v) Tween-80 and collected by centrifuging the suspension at 4000 rpm for 20 min [10]. The spores were then diluted to obtain the spore inoculum of 2.0×10^8 spores/g of OPF.

2.3. Production of Cellulolytic and Xylanolytic Enzymes by Solid-State Fermentation using Various Sizes of OPFL as Substrate

The OPFL was ground into fine particle sizes using a table blender (NL9206AD-4, Philips UK), particles were sieved to different sizes using standard steel sifters, Endecottes Test Sieve of size 250, 500, 600, and 900 μm, respectively. The solid-state fermentation (SSF) was prepared by wetting the sieved OPF with appropriate volumes of the production medium and spore suspension of *T. Asperellum* UC 1 (2.0×10^8 spores/g of OPF) until a final moisture level of 80% was achieved. The final moisture level of the OPF substrates was determined using a commercial moisture analyser (MX50, A&D Weighing Co., Ltd., Japan). The flasks containing the OPF substrates plus the production medium were autoclaved at 121 ˚C and 20 psi for 20 mins followed by inoculation with the fungal spores in a laminar flow bench. The production medium consisted of a modified Mendel medium containing $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2.0 g/L, urea 0.3 g/L, yeast 1.25g/L, $CaCl_2$ 0.3g/L, $MgSO_4$ 0.3 g/L, $FeSO_4$ 0.005 g/L, $MnSO_4.2H_2O$ 0.0016 g/L, $ZnSO_4.7H_2O$ 0.0014 g/L, $CoCl_2$ 0.002 g/L, peptone 1.0 g/L and 2mL of Tween 80 [11]. All inoculated flasks were incubated at 30 ˚C for 12 days and 4 g of the fermented OPF substrates were drawn every 24 h intervals for cellulases and xylanase analysis.

2.4. Extraction of Crude Enzymes and Analysis of Cellulase and Xylanase Activity

Fermented substrate of approximately 4 g was transferred into a 250 mL Erlenmeyer flask containing 100 mL of cold 0.05M sodium acetate buffer. The suspension was vortexed at maximum speed for 1 min to extract the cellulases and xylanase enzymes. The mixture was centrifuged at 4000 rpm for 20 min and the supernatant which contained the crude enzymes was decanted. The mixture of crude cellulases and xylanase were stored at -20 ℃ prior to assay to prevent enzyme degradation. The EnG, ExG and BGL activities were considered to represent the cellulase activity of the crude enzyme. A glucose calibration curve was used to estimate the (carboxymethylcellulase, CMCase), and ExG (filter paper, FPase) activities, a ρ-nitrophenol (ρNP) calibration curve was used to estimate the βGL activity while a xylose calibration curve was used to estimate xylanase activity. All calibration curves were determined using a set of triplicate preparations to minimize error in the respective assays. EnG (CMCase), ExG activity (FPase) and xylanase activities were assayed in triplicates and the activity of each type of enzyme was estimated based on standard procedure recommended by IUPAC [12]. Xylanase activity was assayed under the same conditions as above except birchwood xylan 1% (w/v) was used as the substrate [12]. One unit of exoglucanase activity is expressed as 1 μmole of glucose liberated per ml of enzymes per minute and one unit of xylanase activity is expressed as 1μmole of xylose liberated per ml of enzyme per minute. All reaction mixtures above were boiled with 1 mL 3,5-dinitrosalicylic acid (DNS) solution and 2 drops of 0.1 M sodium hydroxide to measure the reducing sugars released [14]. BGL production was done according to a modified of Takashima *et al.*, 2007. One unit of β-glucosidase activity is expressed as 1μmole of ρ-nitrophenol liberated per ml of enzyme, per minute.

3. RESULTS AND DISCUSSION

3.1. Cellulases and Xylanase Production Profile

Cellulases productions were significantly affected by particle size of substrates (Fig. 3.2). Cellulase activities increased dramatically as the particle size decreased for CMCase and FPase, but β-glucosidase activity increased with increase in particle size from (500, 600 and 900 μm) after a 9.5% decrease in activity from OPFL size 250 μm to 500 μm. The maximum CMCase (83.53 U/g), FPase (15.59 U/g), β-glucosidase (20.31 U/g) activities were observed at day 2, day 2 and day 7 respectively, using minimum OPFL size of 250 μm, for CMCase and FPase and 900 μm OPFL size for BGL (Fig 3.1). The increase in BGL activities was due to larger particle size that may have induced the development of intra-particle network of hypha and hence stimulated the enzymes production [15]. β-glucosidase activity increased exponentially and reached maximum on day 7 using 900 μm OPFL particles. This increment could be explained by accumulation of large quantities of cellobioses, which were produced from hydrolysis of cellulose by CMCase and FPase on the same day that accelerated the production of BGL to convert cellobiose (substrate) to glucose [15]. For xylanase, OPFL with particle size of 600 μm significantly induced xylanase production. The maximum xylanase production was detected at 152.77 U/g and increased 1.06 fold compared to activity on substrate particle size 600 μm (Fig. 3.1). Generally the reduction of lignocellulosic material into smaller fractions would increase the exposure of both cellulose and hemicellulose's fragments and in turn substrate's nutrients to the microbes. The size, shape and manner of packing of substrate particles would interfere with the binding as well as the inter-particle spaces and thus affect overall enzymes production yield. In general, xylanase activity was 1.8, 9.8 and 7.8-fold higher than EnG, ExG and BGL respectively.

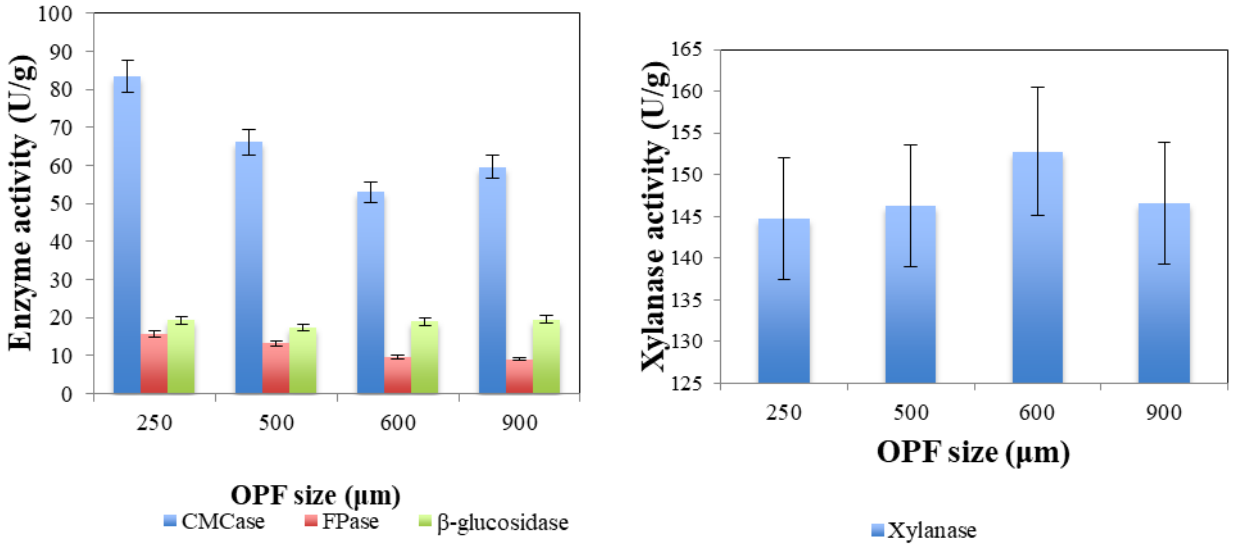

Fig 3.1 Highest cellulase and xylanase activities on different OPFL particles sizes *T.asperellum* **UC 1**

4. CONCLUSION

From the results of the study we conclude that for the production of cellulase and xylanase enzymes using *T.asperellum* UC 1 in SSF, optimum particle size for EnG and ExG is 250 µm, BGL 900 µm and xylanase 600 µm and that untreated OPFL is a promising substrate for the enzymes production. Future works include investigation of other SSF parameters, biochemical characterization of the enzymes, optimization using Response Surface Methodology (RSM) and assessment of degradation/saccharification of OPFL.

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