UNIVERSITI PUTRA MALAYSIA

PHYSICOCHEMICAL, MICROBIOLOGICAL AND BIOLOGICAL PROPERTIES OF FERMENTED KEPAYANG (Pangium edule Reinw.) SEEDS

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PHYSICOCHEMICAL, MICROBIOLOGICAL AND BIOLOGICAL PROPERTIES OF FERMENTED KEPAYANG (*Pangium edule* Reinw.) SEEDS

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July 2020

Chairman : Yaya Rukayadi, PhD
Faculty : Food Science and Technology

Kepayang (*Pangium edule* Reinw.) is one of the indigenous fruits in Malaysia, Indonesia, Papua New Guinea, Vanuatu, and the Philippines. This *P. edule* Reinw. seeds can be developed as a functional food through fermentation. Unfortunately, the properties of fermented *P. edule* Reinw. seeds are not well reported yet. Therefore, the aims of this study were to analyse the physicochemical and microbiological characteristics of *P. edule* Reinw. seeds during fermentation process. Besides that, the objective of this study was to determine the antimicrobial and antioxidant activities of *P. edule* Reinw. seeds extract and to identify the presence of bioactive compounds in raw and fermented *P. edule* Reinw. seeds extract. These *P. edule* Reinw. seeds were obtained from Jerantut, Pahang in July 2018. These *P. edule* Reinw. seeds were undergone the spontaneous fermentation process which covered with charcoal for 40 days. The physicochemical properties were evaluated according to the Association of Analytical Communities (AOAC) methods while the microbiological analysis was determined using microbial selective media. Then, the raw and fermented *P. edule* Reinw. seeds were extracted using ethanol and water. The antimicrobial activity was following the Clinical and Laboratory Standard Institute (CLSI) methods. The antioxidant activity was measured using 2,2-diphenyl-1-dipicrylhydrazyl (DPPH) radical scavenging assay while total phenolic content was measured by following Folin-Ciocalteu methods. Other than that, the bioactive compounds present in extracts were identified using gas chromatography-mass spectrometry (GC-MS) profile and liquid chromatography-mass spectrometry (LC-MS) profile. In this study, the results showed that there were no significant differences in water activity while the pH value was decreased along fermentation days. The TSS was significantly increased from day 0 (11.00) to day 30 (19.50), however, it drastically decreased after 40 days fermentation which is 11.50. The value of lightness (L^*) parameter was decreased from 66.44 on day 0 to 25.28 on day 40. There were no significant differences for a^* and b^* parameter. For proximate analysis, the percentage of ash, crude protein, crude fat, and crude fibre were significantly increased while moisture content and carbohydrate were significantly decreased along the fermentation day. The microbiological analysis showed the reducing number of *B. cereus* from 2.15 Log_{10} CFU/g to not detected on day 40, while there were slightly increase in the number

of TPC, coliform, mould and yeast, *S. aureus*, *P. aeruginosa* and LAB. Interestingly, there was no *Salmonella* spp. and *E. coli* detected along fermentation process. The antimicrobial activity of raw and fermented seeds extract was evaluated against 16 types of pathogenic microorganisms. The disc diffusion assay (DDA) showed that the microorganisms were inhibited by extracts in the range of 6.25 mm and 9.88 mm. The lowest MIC and MBC values were at concentration 6.25 mg/mL and 12.50 mg/mL extract, respectively. Generally, the time-kill curve study showed that *B. cereus*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *C. albicans* can be killed using raw and fermented *P. edule* Reinw. seeds extract. Besides, the highest DPPH activity was observed in ferment-water extract at concentration 10 mg/mL which about 95.61% while the highest total phenolic content was obtained from the ferment-ethanol extract (173.79 mg GAE/100 mg). The GC-MS analysis revealed the volatile compounds in raw and fermented *P. edule* Reinw. seeds extract while LC-MS analysis was used to identify the non-volatile compounds in both positive and negative ion modes. In conclusion, there were some changes in the physicochemical properties of *P. edule* Reinw. seeds during fermentation, nonetheless, the fermented seeds showed a low level of microbial counts and potentially has the antimicrobial and antioxidant activities. The results of this study might be used as basic information to develop the fermented seeds as a functional food.

Keywords: Antimicrobial activity, antioxidant activity, bioactive compounds, fermented *Pangium edule* Reinw. seed, microbiological analysis, physicochemical properties

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Master Sains

KARAKTERISTIK FISIKOKIMIA, ANALISA MIKROBIOLOGI DAN AKTIVITI BIOLOGI BIJI BUAH *KEPAYANG* (*Pangium edule* Reinw.) YANG DIPERAM

Oleh

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Kepayang (*Pangium edule* Reinw.) adalah salah satu daripada buah-buahan asli yang terdapat di Malaysia, Indonesia, Papua New Guinea, Vanuatu dan Filipina. Biji *P. edule* Reinw. ini boleh dikembangkan sebagai makanan fungsional melalui pemeraman. Malangnya, sifat biji *P. edule* Reinw. yang telah diperam ini masih belum diketahui sepenuhnya. Oleh itu, matlamat kajian ini adalah untuk menganalisis ciri-ciri fisikokimia dan mikrobiologi biji buah *P. edule* Reinw. semasa proses pemeraman. Selain itu, objektif kajian ini adalah untuk menentukan aktiviti antimikrobial dan antioxidant bagi ekstrak biji *P. edule* Reinw. dan untuk mengenalpasti sebatian aktif yang terdapat dalam ekstrak biji *P. edule* Reinw. ini. Buah *P. edule* Reinw. ini diperoleh dari Jerantut, Pahang pada Julai 2018. Biji buah *P. edule* Reinw. ini telah menjalani proses pemeraman spontan yang ditutupi dengan arang selama 40 hari. Ciri-ciri fizikokimia telah dinilai mengunakan kaedah Persatuan Komuniti Analitik (AOAC) manakala analisis mikrobiologi telah ditentukan menggunakan media selektif mikroba. Kemudian, biji *P. edule* Reinw. yang mentah dan sudah diperam ini diekstrak menggunakan etanol dan air suling. Aktiviti antimikrobial ditentukan mengikuti kaedah Institut Standard Klinikal dan Makmal (CLSI). Kegiatan antioksidan diukur dengan menggunakan ujian memerangkap radikal DPPH manakala jumlah kandungan fenolik diukur mengikuti kaedah Folin-Ciocalteu. Selain itu, sebatian bioaktif yang terdapat dalam ekstrak telah dikenalpasti menggunakan kaedah analisa GC-MS dan LC-MS profil. Dalam kajian ini, hasil menunjukkan bahawa tidak ada perbezaan yang ketara untuk aktiviti air manakala nilai pH menurun sepanjang hari pemeraman. TSS meningkat dengan ketara dari hari 0 (11.00) hingga hari 30 (19.50), tetapi ia menurun secara drastik selepas pemeraman selama 40 hari (11.50). Nilai parameter *L** menurun dari 66.44 pada hari ke 0 hingga 25.28 pada hari ke-40. Tidak ada perbezaan yang ketara untuk parameter *a** dan *b**. Untuk analisis jarak dekat, peratusan abu, protein mentah, lemak mentah, dan serat kasar meningkat dengan ketara manakala kandungan kelembapan dan karbohidrat menurun sepanjang hari pemeraman. Analisis mikrobiologi menunjukkan penurunan jumlah *B. cereus* dari 2.15 Log_{10} CFU/g kepada tidak ditemui pada hari ke-40, manakala, terdapat sedikit peningkatan dalam jumlah TPC, *P. aeruginosa*, *S. aureus*, LAB, koliform, kulat

dan yis. Menariknya, tiada *E. coli*, dan *Salmonella* spp. ditemui sepanjang proses pemeraman. Kegiatan antimikrobial ekstrak biji yang mentah dan yang diperam telah dinilai terhadap 16 jenis mikroorganisma pathogen. Ujian DDA pula menunjukkan bahawa mikroorganisma berjaya direncatkan oleh ekstrak dengan lingkungan antara 6.25 mm dan 9.88 mm. Nilai MIC dan MBC terendah masing-masing berada pada kepekatan 6.25 mg/mL dan 12.50 mg/mL ekstrak. Secara amnya, analisis keluk-masa pembunuhan menunjukkan bahawa, *B. cereus*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* dan *C. albicans* boleh dibunuh dengan menggunakan ekstrak biji *P. edule* Reinw. yang mentah dan yang telah diperam. Selain itu, aktiviti DPPH tertinggi dikenalpasti dalam ekstrak yang diperam dengan air pada kepekatan 10 mg/mL yang mana kira-kira 95.61% manakala jumlah kandungan fenolik tertinggi diperoleh dari ekstrak biji diperam-etanol (173.79 mg GAE/100 mg). Analisis GC-MS menunjukkan sebatian yang mudah meruap dalam ekstrak biji *P. edule* Reinw. yang mentah dan diperam manakala analisis LC-MS telah digunakan untuk mengenal pasti sebatian tidak mudah menguap dalam mod ion positif dan negatif. Sebagai kesimpulan, terdapat beberapa perubahan sifat fisikokimia biji *P. edule* Reinw. semasa proses pemeraman, walaubagaimanapun, biji yang diperam ini menunjukkan tahap kiraan mikrob yang rendah dan berpotensi mempunyai aktiviti antimikrob dan antioksida. Hasil kajian ini mungkin dapat digunakan sebagai maklumat asas untuk menjadikan biji yang diperam ini sebagai makanan yang berfungsi.

Kata kunci: Aktiviti antimikroba, aktiviti antioksida, sebatian bioaktif, pemeraman biji buah *Pangium edule* Reinw., keselamatan mikroba, ciri-ciri fisikokimia,

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ASE	Accelerated solvent extraction
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
a_w	Water Activity
*a**	Red/greenness
*b**	Yellow/blueness
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	*Bacillus cereus* Selective Agar
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DDA	Disc Diffusion Assay
DMSO	Dimethylsulfoxide
DPPH	1-diphenyl-2-dipicrylhydrazyl
EMB	Eosin-methylene blue
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography – Mass Spectrometry
GRAS	Generally Regarded as Safe
h	Hour
H_2SO_4	Sulphuric acids
HPLC	High-Performance Liquid chromatography
ICMSF	International Commission on Microbiological Specifications for Foods
*L**	Lightness
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography-Mass Spectrometry
MAE	Microwave-assisted extraction
MBC	Minimum Bactericidal Concentration
MF	Molecular Formula
MFC	Minimum Fungicidal Concentration
MHA	Mueller-Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
MRS	De Man, Rogosa and Sharpe
MSA	Mannitol Salt Agar
MW	Molecular weight
m/z	Mass/charge ratio
N.A	No activity
NaOH	Sodium hydroxide
NAD	Nicotinamide Adenine Dinucleotide
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline

PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PEME	*P. edule* methyl ester
PROSEA	Plant Resources of South-East Asia
Ppm	Parts per million
Rpm	Revolutions per minute
Rt	Retention time
SD	Standard deviation
SFE	Supercritical fluid extraction
spp.	Species
TPC	Total Plate count
TSS	Total soluble solid
UAE	Ultrasound-assisted extraction
UPM	Universiti Putra Malaysia
XLD	Xylose lysine deoxylate

CHAPTER 1

INTRODUCTION

1.1 Background

The tropical rainforest offers excellent sources of indigenous fruits and vegetables that have a valuable effect and one of them is *kepayang* fruit or the scientific name is *Pangium edule* Reinw. This *P. edule* Reinw. tree is mainly grown in South-East Asia region including Malaysia, Indonesia, Papua New Guinea, Vanuatu, and the Philippines. The fruit is commonly consumed due to its taste and it is widely used as a preservative agent. *P. edule* Reinw. seeds have been used as an alternative approach to preserve and maintain the quality of raw fish, shrimp, and meat when the supply of ice and cooling supplies were limited (Kasim and David, 2013; Heruwati et al., 2009; Heruwati et al., 2007). Traditionally, this fruit can be used to treat the infections and it also has an anthelmintic antiseptic and antibacterial property.

Interestingly, *P. edule* Reinw. seeds are edible in raw after undergoing some treatment such as boiling or soaking in water. These seeds usually called as "*dage*" which can be eaten and commonly being utilized as a vegetable especially in West Java, Indonesia. Other than that, these *P. edule* Reinw. seeds also can undergo the fermentation process and it commercially called as "*keluwak*" (Hoe and Siong, 1999). According to Andarwulan et al. (1999a), fermentation process can make these seeds edible as it can get rid of the cyanide content and at the same time can increase their nutritional value and flavour. Besides that, this process also can change the colour of the seeds from milky white to brown or black and the texture became softer.

In general, fermentation process is one of the common methods that can be used to process and preserved the foods. Fermentation is a metabolic process that produces chemical changes in organic substrates through the action of enzymes. It can convert the complex compound of carbohydrates, such as starch or a sugar, into a simpler compound such as alcohol or acid (Chojnacka, 2006). This fermentation process enhances the freeing up water molecules and indirectly produced edible food products by removing or destroying the undesirable or anti-nutritional factors that might present in the food to ensure their food safety (Steinkraus, 2018). Other than that, fermentation also can improve their taste, flavour and appearance thereby increasing their acceptability (Ejoh, 2007). Based on previous studies, it is confirmed that fermentation process can enhance the nutrition content while increasing the food digestibility (Hasan et al., 2014; Tamang et al., 2016).

Traditionally, the process to ferment the *P. edule* Reinw. seeds were done manually using spontaneous fermentation. Hence, good hygiene practices should be emphasized during fermentation to lower or discards especially from microorganisms that dangerous for health. Previous studies had shown major microorganisms usually involved in this fermented food are bacilli family-like *Bacillus subtilis* and *B. cereus* (Steinkraus, 2018).

Besides, as studied by Gadaga et al. (2007) and Capozzi et al. (2017), *Bacillus cereus*, *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Aeromonas* are the most commonly encountered pathogens in African fermented foods. Steinkraus (2018) also stated that *tempe bongkrek*, which is also one of fermented dregs as it is produced from coconut dregs, has been reported to be contaminated by *Pseudomonas* species such as *Pseudomonas aeruginosa* and *P. cocovenenans* if no proper handling during process of fermentation. Other than that, the processing of foods such as fermentation processes can give some effect on the chemistry of these foods. During fermentation process, the microorganism might be present and can change the physical characteristics and biological of the food product. The evaporation and metabolism of the microorganism cause the water activity, a_w might vary (Tsao, 1999) while the pH value may drop along the with fermentation day.

From the best knowledge, there are still scares of studies regarding physicochemical characteristics and microbiological safety of *P. edule* Reinw. seeds during fermentation and these properties are not well reported yet. Furthermore, there are also lacks studies on the determination of bioactivities and the identification of bioactive compounds of the raw and fermented *P. edule* Reinw. seeds extracts. Therefore, this present study was conducted to analyse physicochemical characteristics and microbiological safety of *P. edule* Reinw. seeds during fermentation, to determine the antimicrobial and antioxidant activities of the raw and fermented seed extracts and to identify the bioactive compounds present in the extracts using Liquid Chromatography Mass-Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS).

1.2 Problem Statements

There were some problems that were faced in this study. According to Steinkraus (2002), the physicochemical properties of the food product might be changed during the fermentation process. Besides that, fermentation process has also involved the microorganisms which may be caused by contamination (Caplice and Fitzgerald, 1999). Generally, spontaneous fermentation is an uncontrolled process, involving the succession of different types of microorganisms including pathogenic and non-pathogenic bacteria that results in a variety of end products of fermented food. Besides that, fermentation process also involved some chemical reactions which could give effects on food properties and bioactive compounds including the compound that can enhance the antioxidant and antimicrobial activity inside food product (Mehta et al., 2012). Antioxidants are one of the essential components that can be found in plant extracts that have the ability to scavenging free radical inside the biological system (Anjaneyulu et al., 2003). Moreover, the biological products derived from plant sources exhibited a wide range of antimicrobial properties against disease and food spoilage causing microorganisms. which including the pathogenic microbes and candida species. Several recent studies on antimicrobial elements in food products were able to purge the microbes responsible for causing food spoilage, thereby extending the expiry date of food products (Tajkarimi *et al*., 2010).

In general, there are a lot of studies were conducted on the characteristic of raw and fermented *P. edule* Reinw. seeds. However, no systematic study had been reported

regarding the changes in physicochemical properties and microbiological population of *P. edule* Reinw. seeds during fermentation process. Besides that, the properties of antimicrobial and antioxidant activities had potentially present in water and ethanol extract of raw and fermented *P. edule* Reinw. seeds extracts. Although the effect of these plant extracts against some microorganisms had been studied, the information on the effectiveness of the plant extracts against certain microorganisms was scarce. Thus, the physicochemical properties and microbiological analysis of *P. edule* Reinw. seeds during fermentation need to be analysed. Besides that, the antimicrobial and antioxidant of raw and fermented *P. edule* Reinw. seeds extracts need to be determined. Other than that, the volatile and non-volatile bioactive compounds that contributed to the antimicrobial and antioxidant activities might be present in the raw and fermented *P. edule* Reinw. seeds extracts. These bioactive compounds also can be identified using Gas Chromatography Mass-Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).

1.3 Research Objectives

The objectives of this study are:

1. To analyse the physicochemical and microbiological characteristics of *Pangium edule* Reinw. seeds during fermentation process.
2. To determine the antimicrobial and antioxidant activities of raw and fermented *Pangium edule* Reinw. seeds extract.
3. To identify the presence of bioactive compounds of raw and fermented *Pangium edule* Reinw. seeds extract.

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BIODATA OF STUDENT

The student, Nur Hafizah Binti Mustaffer was born on 22nd March 1994 at Alor Setar, Kedah. She is the daughter of Mustaffer Bin Abdullah and Zubaidah Binti Mohd Yusoff. She started her primary education in Sekolah Kebangsaan Langgar, Kedah and continued her secondary education at Sekolah Menengah Kebangsaan Agama Yan. During form 4, she changed to Sekolah Menengah Sains Pokok Sena, Kedah. Then, she continued her foundation study at UiTM Puncak Alam study in the Science stream. After that, she furthered her study at Universiti Putra Malaysia (UPM) by taking Bachelor of Food Science and Technology and graduated in 2017. Currently, she is continued her study in Master of Science under the Dual-Degree program between UPM and Kasetsart University (KU), Thailand. She is taking master in the field of Food Security and Climate Change under the supervision of Assoc. Prof. Dr. Yaya Rukayadi at Faculty of Food Science and Technology, UPM and master in the Food Science and Technology under the supervision of Dr. Kullanart Tongkhao in Department Food Science and Technology at Faculty of Agro-Industry, KU. The following are the list of publication and thesis involved during her course of study.

Thesis:

1. Bachelor of Food Science and Technology:
Quality Evaluation of Physical Properties, Nutritional Composition and Antioxidant Activity of Six Different Palm Dates (*Phoenix Dactylifera* L.) Varieties from Saudi Arabia

2. Master of Food Security and Climate Change:
Physicochemical, Microbiological and Biological Properties of Fermented Kepayang (*Pangium Edule* Reinw.) Seeds

LIST OF PUBLICATIONS

Abdul-Hamid, N. A., Mustaffer, N. H., Maulidiani, M., Mediani, A., Ismail, I. S., Tham, C. L., Shadid, K. and Abas, F. (2018). Quality evaluation of the physical properties, phytochemicals, biological activities and proximate analysis of nine Saudi date palm fruit varieties. *Journal of the Saudi Society of Agricultural Sciences*.

Mustaffer, N. H., Rosni, N. K., Ramli, N. S., Rukayadi, Y. (2020). Changes in physicochemical properties and antioxidant activity of fermented Kepayang (*Pangium edule* R.) seeds. *Food Research.* (Manuscript in preparation for publication).

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