Faculty of Resource Science and Technology

In-silico and *in-vitro* analysis of Lipogenesis Effect of *Shorea macrophylla* Fruits' Crude Extract

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Lipogenesis is a mechanism that enhance lipid deposits in meat, and it can increase the market value of meat as increasing of its content in meat would improve meat quality. The fruit of *Shorea macrophylla* (engkabang), which is the illipe nut is rich in lipid contents. As the common feed of wild animal, its' composition is then an interested topic to be analysed. To identify if there are any lipogenesis-inducing factors in the engkabang fruit, phytochemical extraction is then carried out using various types of solvents. The isolated compound then was identified using GCMS approach and molecular docking between the lipogenesis-related proteins and identified compounds was carried out. *In-vitro* analysis was also carried out by treating the crude extracts on 3T3-L1 cells and MTT assay and adipogenesis assay was tested. Ethanol which has the highest polarity have the lowest extraction efficiency at 8.80% while acetone has the highest extraction efficiency at 38.30%. From the GCMS analysis of all the extracts, methyl stearate, methyl elaidate, methyl palmitate and methyl arachidate was found to be available in all the extracts. Methyl stearate is found to be the most abundant in most of the extracts except for acetone. Besides, the molecular docking results shown the four compounds achieved good docking score ranging from -6.7 kcal/mol to -7.1 kcal/mol with SREBP protein. By also considering on amino acid residues interaction, methyl elaidate is suggested to be the most potent lipogenesis-inducing factors which induce lipogenesis via regulation of SREBP protein. The suggested potent lipogenesis-inducing factors then can be included in diet of vertebrates hoping to increase the amount of fat stored in meat and improving its overall quality.

Keywords: 3T3-L1 cells, engkabang, molecular docking, phytochemical extraction, *Shorea macrophylla*

Analisis in-silico dan in-vitro atas Kesan Lipogenesis Ekstrak Mentah Buah Shorea macrophylla

ABSTRAK

Lipogenesis adalah mekanisme yang meningkatkan pemendapan lipid dalam daging dan dia boleh meningkatkan nilai pasaran daging kerana peningkatan kandungannya dalam daging akan meningkatkan kualiti daging. Buah Shorea macrophylla *(engkabang), iaitu kacang illipe kaya dengan kandungan lipid. Sebagai makanan biasa haiwan liar, komposisinya kemudiannya menjadi topik yang menarik untuk dianalisis. Untuk mengenal pasti sama ada terdapat faktor pengaruh lipogenesis dalam buah engkabang, pengekstrakan fitokimia telah dijalankan menggunakan pelbagai jenis pelarut. Sebatian yang dipencil telah dikenal pasti menggunakan pendekatan GCMS dan kajian dok molekul antara protein yang berkaitan dengan lipogenesis dengan sebatian yang dikenal pasti telah dijalankan. Analisis in-vitro juga telah dijalankan dengan merawat ekstrak mentah pada sel 3T3-L1 dan ujian MTT dan ujian adipogenesis telah dijalankan. Etanol yang mempunyai kekutuban yang tertinggi mempunyai kecekapan pengekstrakan yang paling rendah iaitu 8.80% manakala aseton mempunyai kecekapan pengekstrakan tertinggi iaitu 38.30%. Daripada analisis GCMS, metil stearat, metil elaidat, metil palmitat dan metil arakidat adalah didapati dalam semua ekstrak. Metil stearate didapati sebagai sebatian yang paling banyak dalam kebanyakan ekstrak kecuali aseton. Selain itu, keputusan dok molekul menunjukkan bahawa semua sebatian mencapai skor dok yang baik antara -6.7 kcal/mol hingga -7.1 kcal/mol dengan protein SREBP. Dengan juga mempertimbangkan interaksi sisa asid amino, metil elaidate dicadangkan sebagai faktor pencetus lipogenesis yang paling berkesan yang mendorong lipogenesis melalui pengawalan protein SREBP. Faktor pencetus lipogenesis yang dicadangkan kemudiannya boleh disertakan dalam diet vertebrata dengan harapan dapat*

meningkatkan jumlah lemak yang disimpan dalam daging dan meningkatkan kualiti keseluruhannya.

Kata kunci: *Sel 3T3, engkabang, dok molekul, pengekstrakan fitokimia, Shorea macrophylla*

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ASP	Acylation stimulating protein
ChREBP	Carbohydrate Responsive Element Binding Protein
CO_2	Carbon dioxide
CoA	Coenzyme A
DFD	Dark, firm and dry
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
EDTA	Ethylenediamine tetraacetic acid
FASN	Fatty Acid Synthase
GCMS	Gas chromatography-mass spectrometry
GH	Growth hormone
GLUT	Glucose Transporter
IMF	Intramuscular fat
MCD-fed	Methionine-choline-deficient-fed
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acids
PBS	Phosphate-buffered saline
PDB	Protein Data Bank

PPARγ	Peroxisome proliferator–activated receptor gamma
PSE	Pale, soft and exudative
RCSB	Research Collaboratory for Structural Bioinformatics
RFN	Red, firm and non-exudative
SCD	Stearoyl-CoA Desaturase
SFA	Saturated fatty acid
SREBP	Sterol regulatory element-binding proteins
USF	Upstream stimulatory factors
VLDL	Very low-density lipoproteins

CHAPTER 1

INTRODUCTION

1.1 Background

Besides from food safety, one of the most important factors of human's consumption is meat quality. Intramuscular fat (IMF) is the amount of fat found within muscles. It is distinct from intermuscular fat, which refers to fat between muscles in the same cut. The IMF is composed of cholesterol, triglycerides, and phospholipids, which are the primary sources of energy reserves. The marbling effect, which displays how much intramuscular fat is deposited in the beef slice, is a crucial factor of the selling price of meat such as beef. The more the marbling, the more expensive the beef is (Hocquette et al., 2010). IMF has a good impact on the flavour, juiciness, firmness, and overall acceptability of meat from numerous species. As a result, lipid deposits in the meat are significant to improve the market value of the meat.

To increase lipid deposits in meat, focus should be emphasized on a mechanism known as lipogenesis. Lipogenesis occurs in the liver and adipose tissue where fatty acid generates and will subsequently synthesis triglyceride. Although *de novo* lipogenesis (DNL) is required for cellular and overall body homeostasis, chronic elevations can possibly cause a variety of diseases and illnesses, including cardiovascular disease (CVD), non-alcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D), numerous cancers, viral infections, autoimmune disease, acne vulgaris, neurodegeneration and aging. This suggest that pharmacological inhibition of lipogenesis may be beneficial towards multiple disease areas (Batchuluun et al., 2022). However, the research on variety sickness and disease due to lipogenesis is still lacking in domesticated meat animals. Lipogenesis is the primary source

for fatty acids and triglycerides deposition in meat animals, which eventually leads to the tenderness and juiciness of meat upon consumption. Upon lacking such lipogenesis function, the resulting meat may suffer from Dark, Firm and Dry (DFD) condition.

To a certain extent, animal diet was also found to be critical in increasing intramuscular fat (IMF) in meat in addition to genetic factors. Lipogenesis process which will have IMF as final products is found to be upregulated mainly by high carbohydrate diet. Thus, animal feed is constantly considered to increase food safety and meat quality. Engkabang, also known as *Shorea macrophylla*, is mostly found in Southeast Asia (Chai, 1998; Ng et al., 2002). The illipe nut, also known as the engkabang fruit by the local, is tremendously high in fat content. The name illipe nut and engkabang fruit are interchangeable in this study. These fruits are usually collected by endogenous people as soon as it falls to the ground to be made into various food products since the fat will be immediately used for seed germination if left unharvested (Chai, 1998). The illipe nuts are consumed by most wild animals, including empurau, which is one of Malaysia's most costly fish (Kamarudin et al., 2018). Empurau are highly known for its distinct flavour and texture. However, the compounds responsible for empurau's distinct texture have yet to be identified (Chew et al., 2022). As a result, engkabang illipe nuts are a very valuable resource that deserves more consideration and research.

The Wildlife Protection Ordinance, 1998 in the Laws of Sarawak (Sarawak Government Gazette, 1998) lists *Shorea macrophylla* as a protected plant. The vast majority of illipe nuts are harvested locally and utilised for reforestation. Furthermore, *S. macrophylla* has irregular flowering and fruiting, making fruit harvesting increasingly more challenging. As a result, there will only be a limited supply of illipe nuts accessible for feeding in the future. As a result, determining whether *S. macrophylla* contains secondary metabolite or

biomolecule that would induce the lipogenesis pathway and eventually leads to increased intramuscular fat deposition in meat is critical. Hereby the research questions are: Does the illipe nuts contain compounds that can regulate lipogenesis? How do these compounds regulate lipogenesis? It is believed that through this study, lipogenesis-inducing molecules can be identified from the fruits of *S. macrophylla* which potentially activate the pathway through PPARγ or other lipogenesis related transcription factors.

1.2 Problem Statement

Meat is classified into numerous categories based on its quality. Dark, firm and dry (DFD), pale, soft and exudative (PSE) and red, firm and non-exudative (RFN) meat are among the types of meat. RFN meat is the best and most popular among these three forms of meat. PSE meat is described as watery pork with an exceptionally pale appearance and a high fluid loss when cut, whereas DFD meat is described as lean with a dark, purplish red to black colour and a high pH and water holding capacity. Consumers are discriminating against PSE and DFD meats because they are unappealing (Viljoen et al., 2002). When compared to regular meat, they have poor processing qualities, reduce yield and high risk of spoilage (Newton and Gill, 1981). Thus, it is critical to identify lipogenesis inducing factors to increase intramuscular fat in the meat of livestock and thus improve its quality. The hypothesis of this research is the crude extracts of engkabang fruit are found to have caused increasing lipid accumulation then can be introduced into the meals of livestock.

In this research, the engkabang fruit was collected from a local aquaculture farm and was grinded for the extraction. The phytochemical was carried out with eight types of solvents which are hexane, dichloromethane, methanol, acetone, ethanol, isopropanol, ethyl acetate and diethyl ether. The compounds that were identified using GCMS were then

docked with lipogenesis related proteins. The crude extracts were used to treat on mouse 3T3-L1 adipocyte cells and three assays were carried out which were MTT assay, oil red o assay and adipogenesis assay.

1.3 Objectives

i. To extract compounds from engkabang fruit using solvents with different polarity.

ii. To identify bioactive compounds present in the engkabang fruit.

iii. To evaluate the lipogenesis inducing potential of bioactive compounds through *in silico* and *in vitro* analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Lipogenesis in Vertebrate

Fatty acids are necessary for cell viability since they act as structural components of cell membranes as well as vital signalling chemicals. They are also the most calorie-dense form of energy storage, giving a higher energy reserve than glycogen during times of low nutrition (Batchuluun et al., 2022). Cells have developed strategies to maintain optimal fatty acid levels, including the ability to take in exogenous fatty acids and generate them from alternative carbon sources via a process known as *de novo* lipogenesis (DNL).

Fat accumulation in the body is determined by the balance of lipogenesis (fat synthesis) and lipolysis/fatty acid oxidation (fat breakdown) (Kersten, 2001). Lipogenesis involves the production of fatty acids followed by triglyceride synthesis mainly happened in the liver and adipose tissue (von Loeffelholz et al., 2021). Understanding the molecular regulation of triglyceride synthesis and looking into pharmaceutical approaches to minimise fat storage are two areas of significant interest nowadays (Kersten, 2001).

The metabolic process that turns extra carbohydrates into fatty acids, which are subsequently transformed into triglycerides for energy storage is known as *de novo* lipogenesis. This mechanism contributes only a tiny amount to serum triglyceride homeostasis, with dietary sources accounting for the majority of triglyceride levels in the body (Björntorp and Sjöström, 1978). According to studies, hepatic DNL may play a considerable influence in blood lipid levels, especially in people who eat a high carbohydrate diet (Schwarz et al., 2003). As shown in Figure 2.1, the pathway for fatty acid synthesis requires a coordinated set of enzyme activities, with the liver being more efficient than

adipose tissue in this process (Lodhi et al., 2011; Ameer et al., 2014). ATP-citrate lyase converts citrate to acetyl-CoA as the first step in this process. Acetyl-CoA carboxylase then converts acetyl-CoA to malonyl-CoA, which is further converted to palmitate (Abramson, 2011; Wang et al., 2015). Palmitate is then transformed into a variety of complex fatty acids. Palmitate is the main product of DNL, but it also generates stearate and shorter fatty acids (Abramson, 2011; Ameer et al., 2014).

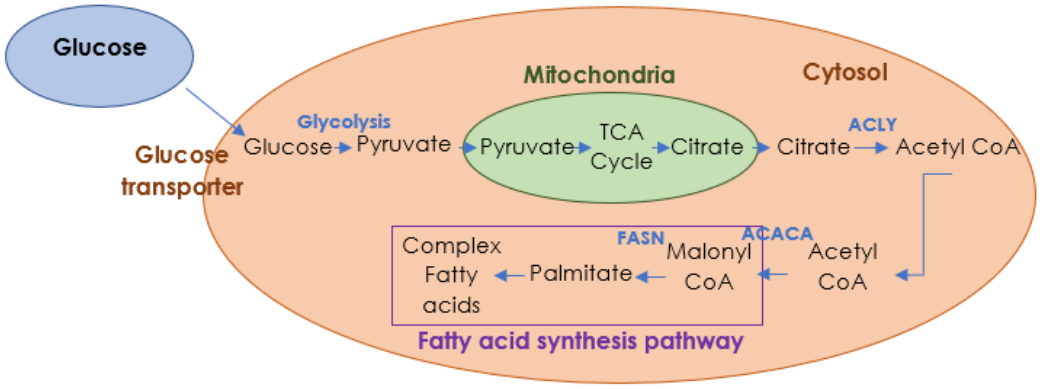

Figure 2.1: Fatty acids synthesis pathway (Ameer et al., 2014).

Lipogenesis is stimulated by a high carbohydrate diet, but it can be inhibited by polyunsaturated fatty acids and fasting (Kersten, 2001; Wang et al., 2015). Growth hormone, leptin, and insulin all play a role in lipogenesis regulation (Cordoba-Chacon et al., 2015; Song et al., 2018). This process also involves transcription factors such as sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor γ (PPARγ), which are potential targets for pharmaceutical interventions (Shao and

Espenshade, 2012; Ladeira et al., 2016; Sun et al., 2020; Xiong et al., 2022). The details of factors that influence lipogenesis are addressed further below and illustrated in Figure 2.2.

Because of their high energy content and hydrophobic nature, triacylglycerols as produced from fatty acids, are an efficient form of energy storage. Adipose tissue has evolved as a specialised storage site for triacylglycerols in animals and human, allowing us to live in habitats with varying food availability and promoting migration (Vernon et al., 1999; Azeez et al., 2014). The ability to synthesise and maintain triacylglycerols is also particularly important for mammalian reproduction, as lipid deposition during pregnancy supplies energy to the growing foetus (Hansen et al., 2013). However, DNL dysregulation has been linked to a variety of metabolic disorders, including insulin resistance, non-alcoholic fatty liver disease, and obesity, as well as cardiovascular disease, malignancies, viral infections, autoimmune diseases, acne vulgaris, neurodegeneration, and ageing (Vernon et al., 1999; Diraison et al., 2002; Strable and Ntambi, 2010; Ma et al., 2014; Zhao et al., 2018). Thus, understanding the regulatory mechanisms of transcription factors, microRNAs, and fat metabolism-related genes, as well as their interactions that affect fat metabolism and deposition, is crucial (Nematbakhsh et al., 2021).

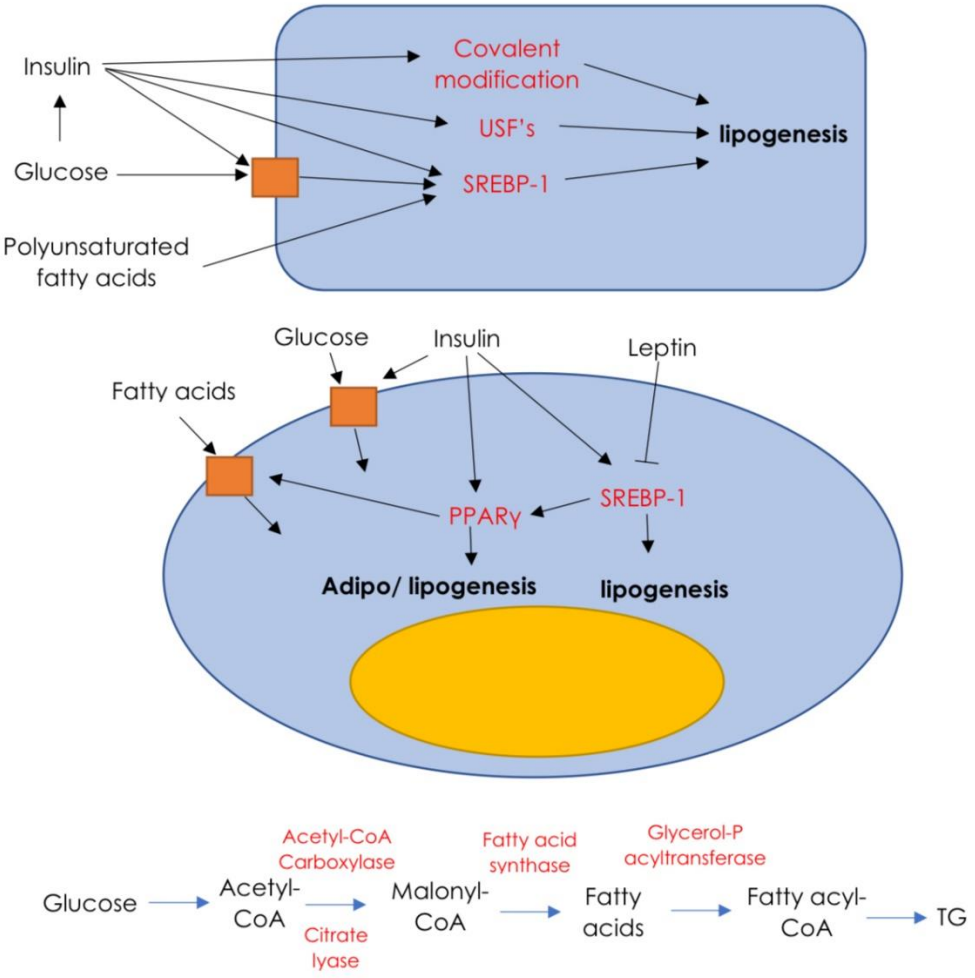

Figure 2.2: Lipogenesis regulation in hepatocytes (up) and adipocytes (down) (Kersten, 2001).

2.1.1 Dietary Regulation of Lipogenesis in Vertebrate

Dietary modifications have the greatest influence on lipogenesis. Consuming a high-carbohydrate meal could increases lipogenesis in both the liver and adipose tissue, resulting in greater levels of postprandial plasma triglyceride levels. Fasting, on the other hand, reduces adipose tissue lipogenesis, resulting in a net loss of triglycerides from fat cells due to an increase in lipolysis (Nieminen et al., 2016). Besides from fasting, polyunsaturated fatty acids have been shown to reduce lipogenesis in the liver by downregulating the

expression of certain genes involved in fatty acid synthesis (Yao et al., 2016). When there is a slower rate of fatty acid synthesis and decreased expression of lipogenic genes, it will then trigger the higher influx of free fatty acids from adipose tissue causes increase triglyceride synthesis in the liver but will eventually resulting in hepatosteatosis (Ress and Kaser, 2016).

Glucose stimulates lipogenesis through a variety of methods. Firstly, glycolysis converts glucose to acetyl-CoA, which is then used as a substrate for fatty acid synthesis (Krycer et al., 2020). Secondly, glucose stimulates the synthesis of lipogenic genes, and finally, glucose promotes lipogenesis via boosting insulin release and lowering pancreatic glucagon release (Mann et al., 2020). High-carbohydrate diets trigger a lipogenic response in the liver, increasing the synthesis and release of very low-density lipoproteins (VLDL) (Alves-Bezerra and Cohen, 2017). However, hepatic DNL could contributes to hypertriglyceridemia development (Schwarz et al., 2003).

It has been demonstrated that the type and quantity of dietary carbohydrates could influence the rate of DNL. Fructose affects DNL significantly, demonstrating that simple sugars are more effective than complex carbohydrates in promoting hepatic DNL (Schwarz et al., 2015; Geidl-Flueck and Gerber, 2023). When compared to hepatic DNL, lipogenesis in adipose tissue is less impacted by acute or prolonged carbohydrate overfeeding (Diraison et al., 2003).

Research did by Nematbakhsh et al. (2021) recently shown that most of the fatty acid synthesis in chickens occurs in hepatocytes via lipogenesis, accounting for 70% of the process, while just 5% occurs in chicken adipose tissue. Diet provides the remaining of fatty acid requirements. The liver then creates lipids in the form of very low-density lipoproteins (VLDL), which are delivered through the bloodstream to tissues such as adipose tissue. Lipoprotein lipase (LPL) subsequently hydrolyses lipoproteins in adipose tissue, allowing