



**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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*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 kualitas 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 <sub>2</sub> | Carbon dioxide                                               |
| CoA             | Coenzyme A                                                   |
| DFD             | Dark, firm and dry                                           |
| DMEM            | Dulbecco's Modified Eagle Medium                             |
| DMSO            | Dimethyl sulfoxide                                           |
| DNA             | Deoxyribonucleic acid                                        |
| DNL             | <i>De novo</i> 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                                            |

|               |                                                      |
|---------------|------------------------------------------------------|
| PPAR $\gamma$ | 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 PPAR $\gamma$  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).

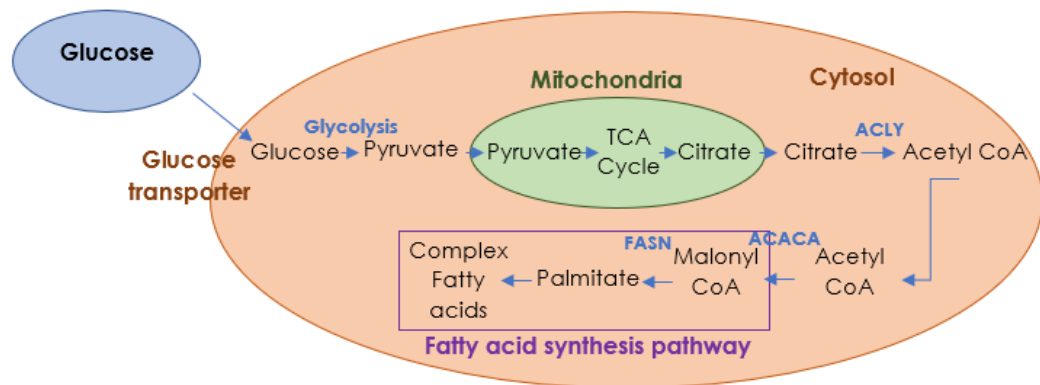


**Figure 2.1:** Fatty acids synthesis pathway (Ameer et al., 2014).

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  $\gamma$  (PPAR $\gamma$ ), 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).

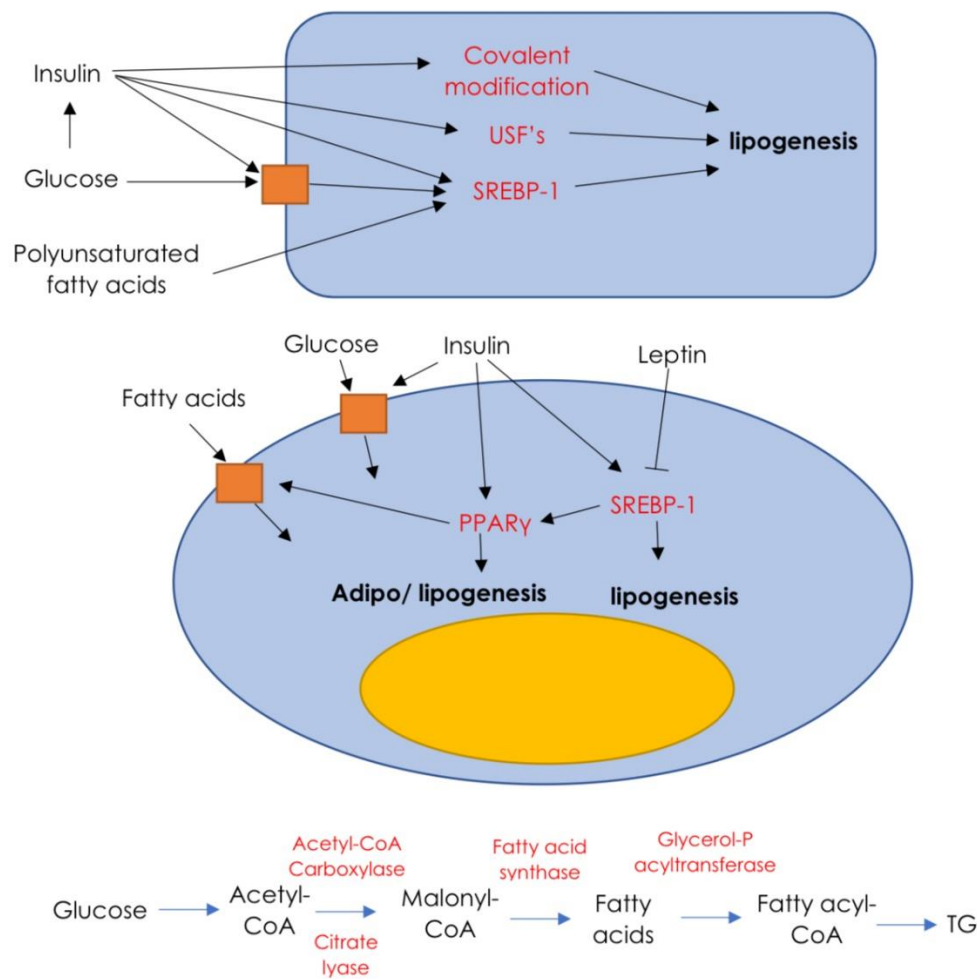


**Figure 2.2:** Lipogenesis regulation in hepatocytes (up) and adipocytes (down) (Kersten, 2001).

### 2.1.1 Dietary Regulation of Lipogenesis in Vertebrate

Dietary modifications have the greatest influence on lipogenesis. Consuming a high-carbohydrate meal could increase 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 (Niemenen 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

either rapid utilisation or deposition of fatty acids. Alternatively, VLDL-derived fatty acids can reach adipocytes and be re-synthesized into triacylglycerol, which is then stored in adipose tissue (Nematbakhsh et al., 2021).

### **2.1.2 Hormonal Regulation of Lipogenesis Pathway in Vertebrate**

Fasting causes major changes in the concentrations of numerous hormones in the bloodstream, such as decreased insulin and leptin levels and increased growth hormone and glucagon levels. Insulin, according to Smith and Kahn (2016), is critical in modulating lipogenesis. It stimulates glucose uptake in adipose cells by recruiting glucose transporters to the cell membrane and, through covalent modification, activates lipogenic and glycolytic enzymes. When insulin interacts with the insulin receptor on the cell surface, it causes tyrosine phosphorylation (Saltiel, 2021). Long-term effects of insulin on lipogenic gene expression are presumably mediated by the transcription factor sterol regulatory element binding protein-1 (SREBP-1) (Ruiz et al., 2014). Insulin stimulates SREBP-1 to generate and activate glucokinase, raising the concentration of a glucose metabolite which will then mediate glucose's effects on lipogenic gene expression (Foretz et al., 1999).

Another hormone that influences lipogenesis is growth hormone (GH) (Kopchick et al., 2020). Lipogenesis in adipose tissue is inhibited by GH, resulting in fat loss and muscle mass increase (Zhang et al., 2016b). The precise mechanics involved are still unknown, but two paths have been proposed. First, GH lowers insulin sensitivity, which leads to a decrease in fatty acid synthase expression in adipose tissue, possibly through interaction with post-receptor insulin signalling. Second, GH phosphorylates the transcription factors Stat5a and

5b, reducing fat deposition in adipose tissue, although the underlying mechanism is not fully studied too (Barclay et al., 2011).

Leptin may also play a role in lipogenesis regulation (Harris, 2014). It has been discovered to reduce fat storage by reducing food consumption and by altering metabolic pathways in adipose and other tissues. Leptin stimulates glycerol release from adipocytes by increasing fatty acid oxidation while decreasing lipogenesis (Baykal et al., 2020). Leptin achieves the latter effect by downregulating the expression of genes involved in fatty acid and triglyceride synthesis, according to an oligonucleotide microarray studies (Soukas et al., 2000). Notably, SREBP-1 is likely to be targeted by leptin, implying that it plays a role in mediating leptin's inhibitory effect on lipogenic gene expression (Kakuma et al., 2000; Soukas et al., 2000).

Acylation stimulating protein (ASP) is an endocrine/autocrine component that plays a role in the synthesis of triglycerides. ASP, which is identical to C3adesArg (a complement factor C3 product), is produced by adipose tissue and is thought to work in an autocrine loop (Saleh et al., 2011). ASP has been found *in vitro* to promote triglyceride production in adipose cells by decreasing lipolysis (Munkonda et al., 2012). Intraperitoneal ASP injection has similarly been shown to accelerate triglyceride clearance from plasma, indicating a similar impact *in vivo*. Despite increasing calorie consumption, studies utilising female ASP null mice revealed a considerable reduction in adipose tissue mass (Murray et al., 1999).

### **2.1.3 Transcription Regulation of Lipogenesis Pathway in Vertebrate**

According to the findings, SREBPs are critical in regulating the expression of lipogenic genes in response to various nutrients and hormones. SREBPs are transcription

factors that regulate gene expression in cholesterol and fatty acid metabolism (Xue et al., 2020). They belong to the basic helix-loop-helix (bHLH)-leucine zipper transcription factor family and are divided into three types: SREBP-2, SREBP-1a, and SREBP-1c. SREBP-1a and SREBP-1c are generated from the same gene but differ in their first exon (Liu et al., 2020).

The molecular mechanism of action of SREBP-2 has been thoroughly investigated (Xue et al., 2020). Under conditions of high cellular cholesterol levels, SREBP-2 appears as a large immature precursor attached to the endoplasmic reticulum. When there is a depletion of cholesterol, it is proteolytically destroyed, resulting in the release of a mature fragment that translocate into the nucleus. The mature SREBP-2 interacts with a sterol response element located in the promoter region of target genes within the nucleus, resulting in their transcription (Kersten, 2001). SREBP-2 overexpression in the liver of transgenic mice has been found to enhance the expression of cholesterol-metabolizing genes such as the LDL receptor, farnesyl pyrophosphate synthase, and HMG-CoA reductase (Ma et al., 2014).

Surprisingly, overexpression of SREBP-1a or SREBP-1c in the liver resulted in a significant increase in hepatic triglycerides as well as activation of lipogenic genes. SREBP-1 is known to activate genes involved in lipid synthesis in the liver (Shimomura et al., 1999; Horton et al., 2003). The phenotype of mice lacking SREBP-1 demonstrated that SREBP-1 plays a slightly distinct role in adipose tissue. Surprisingly, the lack of SREBP-1 had no effect on adipose tissue mass or the expression of fatty acid synthase and acetyl-CoA carboxylase in adipose tissue (Shimano et al., 1997). Studies employing transgenic mice that specifically overexpress SREBP-1c in adipose tissue via the aP2 promoter provide additional evidence for SREBP-1 protein's particular significance in adipose tissue. The expression of genes involved in cholesterol metabolism increased significantly in the white adipose tissue

of these mice, while the expression of genes involved in fatty acid and triglyceride synthesis remained unaffected (Shimomura et al., 1998). Surprisingly, these animals had a decreasing in adipose tissue mass that was less than half of that seen in wild-type mice. The cause of this decrease in fat mass is uncertain; however, it is possibly to be related to decreased expression of the adipogenic transcription factors peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and CCAAT enhancer binding protein (C/EBP). Some research shown that SREBP-1 mRNA expression is reduced, and the SREBP-1 precursor is proteolytically processed, which results in lipogenesis suppression (Thewke et al., 1998; Kim et al., 1999; Mater et al., 1999; Xu et al., 1999; Yahagi et al., 1999). Overall, these findings indicate that SREBP-1's roles in the liver and adipose tissue may differ.

The nuclear hormone receptor PPAR $\gamma$  is an important transcription factor in adipose tissue. PPAR $\gamma$  is activated not only by peroxisome proliferators, but also by fatty acids and their derivatives, as well as drugs of the thiazolidinedione class (Kersten et al., 2000). As part of the adipocyte differentiation programme, PPAR $\gamma$  plays a function in the differentiation of pre-adipocytes into mature adipocytes. Currently, only a small number of genes in adipose tissue have been identified as being controlled by PPAR $\gamma$ . These genes code for proteins like adipocyte fatty acid binding protein, lipoprotein lipase, fatty acid transport protein (FATP), acyl-CoA synthetase, phosphoenolpyruvate carboxykinase, and the fasting-induced adipose factor FIAF/PPAR $\gamma$  angiopoietin-related PGAR (Kersten et al., 2000; Yoon et al., 2000). Given the names of these genes and the fact that PPAR $\gamma$  expression is increased by insulin (Vidal-Puig et al., 1997) and SREBP-1 (Fajas et al., 1999), it is reasonable to assume that PPAR $\gamma$  has both adipogenic and lipogenic effects. Clinical research suggests that people who take synthetic PPAR $\gamma$  activators frequently acquire weight. Furthermore, heterozygous PPAR $\gamma$  mutant mice fed a high-fat diet exhibited lower fat reserves (Kubota et

al., 1999; Miles et al., 2000). The use of inducible and tissue specific PPAR $\gamma$  knockout animals will be beneficial in understanding the role of PPAR $\gamma$  in adult adipocytes. Although PPAR $\gamma$  is normally expressed at low levels in hepatocytes, an increase in PPAR $\gamma$  expression has been linked to increased hepatic triglyceride accumulation, indicating its possible role in lipogenesis stimulation (Chao et al., 2000).

#### **2.1.4 Cell Lines Used for Lipogenesis Studies**

There are several cell lines that have been utilised for the *in vitro* lipogenesis studies. Among them are 3T3-L1 and HepG2 cell lines.

The 3T3-L1 murine adipocyte cell line is one of the most useful cell lines which could be analysed on the differentiation process of preadipocytes to mature adipocytes. The formation of mature adipocytes could exhibit high similarity on their morphology and biochemical properties as compared to the adipose cells *in vivo* (Kowalska et al., 2014). *In vitro* cell differentiation is accompanied by the expression of lipogenic genes as well as receptors for lipogenic and lipolytic hormones (Ailhaud et al., 1992). They can also secrete biologically active molecules such as cytokines, enzymes, growth factors, and hormones, which are useful for lipogenesis research.

Rather than normal human hepatocytes, HepG2 cell line which differs from 3T3-L1 cell line, was shown to be more suitable for studying the impact of fat overaccumulation in the liver (Gomez-Lechon et al., 2007). As a result, it has mostly been employed in illness research, such as non-alcoholic fatty liver disease (NAFLD) studies (Chanda et al., 2011).

## 2.2 Overview of *Shorea macrophylla*

*Shorea macrophylla*, often known as the Engkabang tree by locals, belongs to the Dipterocarpaceae family, according to Chew et al. (2022). Dipterocarpaceae, which includes *S. macrophylla*, is found largely in Southeast Asia, where it usually grows in alluvial environments prone to floods and grows naturally along riverbanks. The locals refer to it as “butter in the forest” because of the high oil content of its fruit, known as the 'illipe nut.' The illipe nut provides food for a variety of species, including fish. *Tor tambroides*, a fish species, favoured the engkabang fruit for its high protein energy content, which contributes to its special flesh according to (Rahman et al., 2011). However, it is worth noting that dipterocarps, including *S. macrophylla*, exhibit irregular patterns of flowering and fruiting (Blicher-Mathiesen, 1994). Periodically, there are mass blossoming events, prompting locals to harvest the illipe nuts and dry them for future use (Chai, 1998). *Shorea gysbertsiana* Burck is an alternate name for *Shorea macrophylla* within the same species according to Chai (1998) and Sulistyawati and Widyatmoko (2018). Nurtjahjaningsih et al. (2012) discovered differences in allele frequencies between these two species, suggesting a relationship but not complete identity between them.

### 2.2.1 Distributions of *Shorea macrophylla*

*Shorea macrophylla* and other Dipterocarp species are plentiful in Borneo, Malaysia, Indonesia, and the Philippines, according to Chai (1998) and Ng et al. (2002). They thrive in wet environments, including periodically flooded alluvial areas and riverbanks, up to 1,500 metres above sea level. However, they are relatively rare and scattered on hillsides below 600 meters (Kanzaki et al., 1996; Lee et al., 1997; Ng et al., 2002). While *Shorea macrophylla* is flood tolerant, it is drought intolerant and should be planted in areas with

high soil moisture or clay-rich soils to reduce drought stress, according to Kenzo et al. (2007).

The soil type widespread in the places where *S. macrophylla* grows is classed as the Grey-White Podzolic soil group, which is characterised by a combination of sand, silt, clay, and loam textures, as detailed by Perumal et al. (2015) and Adanan et al. (2020). Although Grey-White Podzolic soil is generally unsuitable for most agricultural activities due to its high weathering and friable structure, which can lead to soil erosion, *Shorea* species exhibit extreme adaptability and can thrive in this soil type as well (Leysia, 2012; Perumal et al., 2013). Adanan et al. (2020) characterise Grey-White Podzolic soils as having a light colour and a mild to strong subangular blocky texture.

Adanan et al. (2020) discovered that the Grey-White Podzolic soils in Sarawak's Sabal Forest Reserve are very acidic, with high amounts of exchangeable aluminium and low levels of exchangeable bases with a clay content ranged from 9.3% to 27.9% in different plots. Clay contributes to low water retention, which may be linked to the occurrence of large floods in *S. macrophylla*'s habitat (Perumal et al., 2015).

### **2.2.2 Morphological Characteristics of *Shorea macrophylla***

*Shorea*, the largest member of the Dipterocarpaceae family, dominates the forest's emergent or primary canopy. According to Ashton (1982), the genus *Shorea* is divided into 11 parts, each with its own androecium. Sectional species are distinguished chiefly by leaf form and tomentum (Ashton et al., 1984). However, because to their similar looks, identifying various *Shorea* species based on morphology alone is difficult (Rosdayanti et al.,

2019). Several investigations on the morphology of *S. macrophylla* have been completed, but researchers in Malaysia and Indonesia have various identifications of physical features.

*S. macrophylla* is described as a tree with subsistent stipules, outer tomentose, pubescent leaf midribs, and a mature height of 15 to 20 metres by Hotta (1997), who performed research in West Kalimantan. *S. macrophylla*, on the other hand, was seen by Lee et al. (1997) in Sarawak, Malaysia, and reported that the tree size ranged from medium to large, reaching heights of up to 50 metres, with a stem girth at breast height of four metres and a buttress height of two metres. The description of Lee et al. (1997) corresponds to the observations of *S. macrophylla* in the planted plot at the Sarawak Forestry Corporation's Semenggoh Wildlife Centre (Chew et al., 2022) as illustrated in Figure 2.3. Large elliptic-oblong leaves with an obtuse or subcordate base, 13 to 18 pairs of prominent, well-spaced nerves, a hairy midrib on the upper surface, a stout petiole, smooth or shallowly scaly bark, and a short bole characterise the species (Ng et al., 2002). The leaves are 40 centimetres long and alternately arranged, with visible secondary and tertiary veins or a three-centimetre leaf stem (Riska and Manurung, 2018).

The bark of *S. macrophylla* is smooth and brownish green in the early stages of growth, but it gradually develops cracks and flakes as the tree ages (Chai, 1998; Riska and Manurung, 2018). Stipules of *S. macrophylla* are spear-shaped, blunt, or elliptic, measuring 5 cm in length and 1.3 cm in breadth, according to Windyarini and Hasnah (2015). They also discovered that a ten-month-old *Shorea* plant can achieve heights ranging from 67.19 to 88.79 cm, diameters ranging from 9.65 to 10.33 mm, and sturdiness ranging from 7.0 to 9.21 (Windyarini and Hasnah, 2015). Sidi et al. (2021) discovered that foliage damage was mostly caused by *Lymantriidae* hairy caterpillars during the early phases of tree development, with

"hole damage" being the most common type of foliage damage in their study on insect pest studies of *S. macrophylla*.

*Shorea* species classification is difficult due to their vast size, irregular flowering seasons, and the absence of reproductive organs for most of the time. Furthermore, the coexistence of numerous closely related species with comparable physical traits makes identification without the use of genetic tools even more difficult (Moura et al., 2019).



**Figure 2.3:** Diagram of *Shorea macrophylla* canopy (A) and adult *Shorea macrophylla* (B).

### 2.2.3 Flowering and Fruiting of *Shorea macrophylla*

The blooming and fruiting patterns of most Dipterocarps, including *S. macrophylla*, are irregular. *S. macrophylla* blossoms from September to October, with fruit ripening between January and March, according to Blicher-Mathiesen (1994) and Chai (1998). Flowering may commence at the age of 15 to 16 years; however it can take several years before the next flowering event (Chai, 1998). Even if the Dipterocarp trees flower every year, their flowering and seed production are limited. However, every few years, a huge

blossoming event will occur, resulting in a large number of seeds (Chai, 1998). This mass blossoming occurrence may be impacted by a mix of an aseasonal forest and Dipterocarp flowering systems (Appanah, 1993). Aseasonal forests lack a continuous annual dry season and receive an average annual rainfall of about 2,900 mm (Appanah, 1993; Chandler et al., 2021). Flowering, fruiting, and leaf changes are more predictable in seasonal regions like tropical America than they are in aseasonal zones like Southeast Asia (Appanah, 1993).

Ng et al. (2002) discovered evidence of considerable inbreeding in *S. macrophylla*, implying that mating occurs mostly among relatives or closely related species, most likely due to *S. macrophylla*'s higher fruit size. As evidenced by its habitat, which is prone to recurring floods, *S. macrophylla* is tolerant of soil acidity and well-adapted to difficult living conditions. *Shorea macrophylla* thrived in canopy gaps, according to Grubb (1977).

Perumal et al. (2021) investigated the growing performance of *S. macrophylla* seedlings of various ages and discovered that the survival rate decreased with seedling age. Three-month-old seedlings outperformed six-, nine-, and twenty-four-month-old seedlings throughout the experiment. As a result, they advise using three-, six-, and nine-month-old seedlings as planting material and saving twenty-four-month-old seedlings for planting during unexpected fruiting episodes (Perumal et al., 2021).

Due to the irregular flowering of *S. macrophylla*, numerous studies have been done to discover the optimal planting practises. For instance, Lo (1985) used a cutting method in which single-node leafy cuttings taken from the second node and treated with 1,200 ppm and 3,600 ppm IBA had superior root development than control samples.

Perumal et al. (2019) discovered that all types of fertilisers were useful for developing seedlings in a nursery in their fertiliser study. However, jellyfish fertiliser with a

nitrogen: phosphorus: potassium ratio of 13.1: 1.7: 0.03 outperformed the other two tested fertilisers, namely, chemical fertiliser (control) and controlled instant release fertiliser, with the exception of the root to shoot ratio, which was still achieved. Furthermore, the jellyfish fertiliser, which includes a large amount of organic matter (about 81%), has been shown to improve soil physical structure and moisture (Perumal et al., 2019).

#### **2.2.4 Uses of *Shorea macrophylla***

##### **2.2.4.1 *Shorea macrophylla* as Reforestation and Timber Sources**

*Shorea macrophylla*, often known as Light Red Meranti, is a popular timber source for light construction (Ng et al., 2002; Yunanta et al., 2014). However, because of the lack of exposure resistance and inability to be treated with preservatives of the wood, it is best suited for interior wall boarding in construction (Chai, 1998; Wong, 2009). Ismaili et al. (2016) classified engkabang, another name for *Shorea macrophylla*, as SG6, identical to *Acacia mangium*, which was classified as SG5. They concluded that engkabang is appropriate for furniture production and other non-structural uses.

*Shorea macrophylla* is good for reforestation due to its rapid growth and timber structure with a soft to medium texture. In plantations, the tree is a very fast grower, can achieve 50 to 60 cm diameter at breast height (dbh) in about 20 to 30 years and can continue regenerating after 15 to 16 years (Ng et al., 2002). The average height of *S. macrophylla* after 21 years of planting, beginning with the initial evaluation in May 1973, was 19 metres, according to Lee et al. (1997).

*Shorea macrophylla* has other favourable qualities for reforestation, such as its ability to adapt to severe conditions such as floods (Indriani et al., 2019). Fast-growing trees are

important for changing the microclimate and soil qualities, minimising temperature and humidity changes in the understory, and giving more light for seedling development (Nawar, 2012). As a result, *S. macrophylla* is an excellent candidate for reforestation. The biggest difficulty in employing *S. macrophylla* for reforestation is collecting a significant quantity of conveniently available seeds because their viability is restricted, they are usually located in woods, and seed collection is more difficult than in nursery settings (Chai, 1998).

#### **2.2.4.2 Illipe Nut**

*Shorea* species, particularly *Shorea macrophylla*, produce illipe nuts, which are known in the forest as “butter” and are utilised as a substitute for cocoa butter in a variety of uses (Nesaretnam and Ali, 1992). The term “illipe” was coined in South India to characterise the nuts of *Bassia* species from the Sapotaceae family and the Mowrah nuts of *B. latifolia* (Nesaretnam and Ali, 1992). The name “illipe” has come to refer to any oil-bearing nut with similar qualities throughout time (Nesaretnam and Ali, 1992). Illipe nuts are huge and have an elongated calyx that resembles a badminton shuttlecock, as illustrated in Figure 2.4, and they have a high oil content ranging from 45% to 70% (Nesaretnam and Ali, 1992; Chai, 1998). Fig 2.4 is a drawing of illipe nut from Mr Victor Wong. Local communities plant these trees to meet their daily requirements as well as to provide raw materials for a variety of commercial products (Nesaretnam and Ali, 1992). However, large-scale production of illipe nuts is not prevalent among locals, and they are mostly found in forests, with some exported.



**Figure 2.4:** The fruit of *Shorea macrophylla*, the illipe nut. Illustration credited to Mr Victor Wong (Sketch, 2023).

The size and oil content of illipe nuts determine their value, with larger nuts carrying more oil being more valued (Chai, 1998). The oil derived from illipe nuts is yellow and solid (Roslan et al., 2019). The composition of the illipe nuts is based mostly on their fatty acid

contents, as they are known to have a high fat content. Initially, Kershaw (1987) discovered that illipe butter from Sarawak has free fatty acids (FFAs) ranging from 9.1% to 33.4%, whereas the oil content of the seed ranges between 45% and 55%. Nesaretnam and Ali (1992) discovered that the illipe nuts were high in C16, C18 and C18:1. Apart from fatty acids, no other phytochemical substances have been reported yet. Because the seeds are recalcitrant and have a short viability period, they must be collected as soon as they fall and placed in water (temporarily stored) to prevent immediate germination, which would use up the fat reserves in the seeds, reducing the oil content of the nuts (Chai, 1998). Immersion in water breaks the seed coat, yet it also protects the seeds from insect attack (Chai, 1998). To minimise moisture content, the seeds are dehusked and sun-dried (Nesaretnam and Ali, 1992; Chai, 1998). Dried seeds can be kept for longer periods of time and are commonly used for oil extraction (Lo, 1985).

Several studies have been done to assess the suitability of illipe nut oil, commonly known as “engkabang fat” for food industry application. Nesaretnam and Ali (1992) discovered that engkabang fat resembles cocoa butter fat and used it to manufacture chocolate with palm-mid fraction (PMF), a palm fraction high in palm olein (POP). They showed that engkabang fat is a good substitute for cocoa butter equivalent (CBE) fat, which is in high demand as a cocoa butter substitute. Because the liquid portion of engkabang fat contains more unsaturated triacylglycerides (TAGs), it can be used to make margarine or as a raw ingredient in cosmetic products (Yanty et al., 2013). Blends of canola oil and engkabang fat show thermophysical properties similar to lard, with palmitic and stearic acid proportions increasing and oleic and linoleic acid proportions decreasing, resulting in a lard-like blend (Illiyin et al., 2013). Roslan et al. (2019) conducted ongoing research to determine the composition of engkabang fat blends and discovered that the slip melting point (SMP)

value of blended canola oil and engkabang fat after six hours of interesterification closely approximated that of lard.

The biggest problem, given the high commercial value of illipe nuts in the food industry, is the limited seed yield caused by the unpredictable flowering and fruiting seasons of Dipterocarpaceae family trees. To promote future studies and utilisation, further research is needed to better understand the flowering and fruiting mechanisms of dipterocarps, as well as to investigate long-term storage solutions for nuts.

#### **2.2.4.3 Natural Feed for Animals**

Illipe nuts are consumed by wild creatures such as wild boar, woodpeckers, foxes, rats, freshwater fish, squirrels, and local reindeer due to their high oil content, which is rich in healthy unsaturated triglycerides (Chai, 1998). *Shorea* fruit is a natural food source for riverine carp species such as *Tor tambroides*, also known as mahseer or empurau. *Tor tambroides*, on the other hand, is an endangered species with significant value, with prices reaching up to RM800 per kilogramme depending on size, grade, and origin (Entri, 2013). Cooked empurau dishes can cost up to RM1,300 per person (Harith and Hassan, 2011). *T. tambroides*' distinct flavour may have resulted from consumption of engkabang fruit (Redhwan and Komilus, 2021). The usage of crude illipe oil from *S. macrophylla* as a dietary lipid source for *T. tambroides* was studied by Kamarudin et al. (2018). Their findings revealed that increasing the amount of illipe oil in the diet had no effect on *T. tambroides* juvenile survival, growth performance, feed utilisation, body indices, lean percentage, or whole-body composition. However, following consumption, saturated fatty acids (SFA) and

monounsaturated fatty acids (MUFA) in the fish increased dramatically, but polyunsaturated fatty acids (PUFA) dropped (Kamarudin et al., 2018).

Because of its availability in forest zones along riverbanks, the engkabang fruit is largely used as a feed for numerous fish species. However, information on its usefulness as a natural meal for other animals is lacking. Because the engkabang fruit may contribute to the unusual flavour of empurau, more research might be conducted to identify the components that contribute to its characteristic aroma or taste. Furthermore, the engkabang fruit, like *T. tambroides*, might be examined as a food source for other species to see if it evokes any unique preferences or modifications.

### **2.3 Plant Phytochemical Compound Extraction**

Throughout history, human have searching for natural products that can increase physical well-being, health, and longevity (Thakur et al., 2020). Plants have long been used for their nutritional and therapeutic properties. However, the expanding global health crises have posed significant hurdles to medical research, resulting in an increased interest in natural food-based treatments. As a result, nutraceuticals have emerged as a novel and valuable commodity for humanity (Thakur et al., 2020). Dr. Stephen de Felice invented the term "nutraceutical" to describe a new approach in food research by combining "nutrition" and "pharmaceutical" (Pandey et al., 2010). It includes food compounds or components that provide medicinal or health benefits, such as disease prevention and therapy. Isolated nutrients, dietary supplements, customised diets, genetically engineered designer foods, and herbal items are examples of nutraceuticals (Rishi, 2006).

Phytochemical study is required to assess a plant's potential therapeutic characteristics and identify the active chemicals responsible for its biological activity (Altemimi et al., 2017). It also serves as a foundation for more specific component extraction and investigation. The solvent used for phytochemical extraction from plant material is critical since it might influence the results (Thouri et al., 2017). Similarly, the phytochemical analysis procedures used evaluate whether a certain molecule is present in the sample. It is best to run two or more different tests to guarantee more reliable findings.

Indigenous tribes have traditionally relied on medicinal plants, and there is a wealth of literature on the ethnobotanical use of these plants. However, rising health concerns and lifestyle changes have prompted the use of bioactive chemicals as food additives. There has been an increasing demand for scientific evidence supporting the nutritional and medicinal value of phytochemicals in the prevention and treatment of numerous illnesses during the last decade (Kathirvel and Sujatha, 2016). While nutraceuticals can come from a variety of sources, plant-based bioactive components provide significant value as well as a wide range of health advantages. Despite their potential, many of these bioactive chemicals are underutilised and undiscovered, suggesting a fascinating study topic.

### **2.3.1 Conventional vs Green Extraction Techniques**

The process of isolating bioactive components from plants using selected solvents and traditional procedures is known as extraction (Jha and Sit, 2022). Plant extracts are complex mixtures of bioactive components that are available in liquid, semisolid, or dry powder form and are intended for oral or external application (Stéphane et al., 2021). Plant components are typically extracted via decoctions, infusions, fluid extracts, tinctures, or powdered extracts.

The key parameters that impact the quality of an extract are the plant part, solvent, and extraction procedure (Ncube et al., 2008). The bark, leaves, flowers, roots, fruits, seeds, and other sections of the plant contain bioactive components (Parekh et al., 2006). Fresh or dried plant parts can be utilised to extract phytochemicals. While fresh plant parts are frequently used, indigenous herbs are also preserved and conserved for later use (Rahman et al., 2022).

The solvent used for extraction has a large impact on the extraction of physiologically active chemicals from plant material. Low toxicity, simple evaporation at low temperatures, increased extract absorption, preservation action, high percentage yield, and other features make a suitable solvent for bioactive component extraction (Stéphane et al., 2021). Eloff (1998) examined aspects impacting solvent selection, such as extraction quantity and rate, compound diversity, inhibitory substances extracted, ease of handling, solvent toxicity, potential health dangers, and others. Because the final extracted product may contain traces of residual solvent, the solvent employed must be non-toxic and not interfere with the bioassay.

Several solvents have been used to extract phytochemicals from plants. Among the solvents often used to extract antioxidants from plant parts such as leaves, and seeds are methanol, hexane, and ethyl alcohol. To extract various phenolic chemicals from plants, several solvents with varied polarity are used. Solvents are chosen depending on the polarity of the solute of interest, as a solvent with polarity similar to the solute will dissolve it effectively (Zhang et al., 2018b). Multiple solvents can be employed in succession to reduce the number of identical compounds in the bioactive component's ultimate yield. Table 2.1 shows a summary of the phytochemicals screened by various types of solvents by Thakur et al. (2020).

**Table 2.1:** Phytochemical extracted by using different types of solvents with different polarity.

| Sr. No. | Name of solvents | Phytochemicals screened                                                                             |
|---------|------------------|-----------------------------------------------------------------------------------------------------|
| 1       | Water            | Anthocyanins, starches, tannins, terpenoids, saponins, polypeptides, lectins                        |
| 2       | Methanol         | Anthocyanins, tannins, terpenoids, saponins, polyphenols, totarol, lactones, flavones, xanthoxylins |
| 3       | Ethanol          | Polyacetylenes, flavonol, sterols, tannins, terpenoids, saponins, polyphenols                       |
| 4       | Acetone          | Phenols, flavanols                                                                                  |
| 5       | Chloroform       | Terpenoids, flavonoids                                                                              |
| 6       | Dichloromethanol | Anthocyanins, tannins, terpenoids, saponins, polypeptides                                           |
| 7       | Ether            | Alkaloids, terpenoids, coumarins, fatty acids                                                       |

Maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction using hydrofluorocarbon solvents are all general techniques for extracting phytochemicals from plant parts (Pandey and Tripathi, 2014). Specific extraction procedures are used for aromatic plants such as water distillation, steam distillation, and water-steam distillation are all popular hydrodistillation methods. Aromatic plant extraction can also be accomplished using hydrolytic maceration, distillation, expression, and enfleurage (cold fat extraction) (Handa et al., 2008). These techniques are especially useful at extracting aromatic chemicals from plants.

Green chemistry, in general, refers to the development, design and use of chemical products and processes that limit or eliminate the use and creation of hazardous compounds (Chemat et al., 2012). Major solutions have been established to design and demonstrate green extraction on laboratory and industrial scale to approach and optimise raw materials, solvents

and energy consumption (Chemat et al., 2012). Based on Putnik et al. (2018) and Mahgoub (2016), Table 2.2 have been listed on the advantages of the use of green technologies and disadvantages of using conventional techniques with minor modification.

**Table 2.2:** Disadvantages of conventional and advantages of green extraction techniques.

| Disadvantages of using conventional techniques    | Advantages of using green techniques |
|---------------------------------------------------|--------------------------------------|
| Usually not friendly for heat-sensitive compounds | Better isolation of compounds        |
| High energy consumption                           | Reduce energy consumption            |
| Large volumes of solvents were used               | Alternative solvents                 |
| Time consuming                                    | Renewable natural products           |
|                                                   | High quality extracts                |
|                                                   | More selectivity                     |
|                                                   | Safe products                        |
|                                                   | Low environmental impact             |

### 2.3.2 Types of Plant Phytochemical Compounds

Phytochemicals are often noted as bioactive compounds. The term bioactive has a broad meaning as well. Guaadaoui et al. (2014) defined bioactive substances as those that can interact with one or more components of a living tissue, potentially causing a variety of consequences. Phytochemicals have been divided into six broad categories based on their chemical structures and properties. The examples of these categories are carbohydrates, lipids, phenolics, terpenoids and alkaloids, and other nitrogen-containing chemicals (Huang et al., 2016). Within each category, more subgroups are formed depending on biogenesis or biosynthetic origin.

Phytochemicals are bioactive molecules found in fruits, vegetables, grains, and other plant foods that have been related to lower risk of major noncommunicable chronic diseases, according to Liu (2013). Phenolic chemicals, terpenoid compounds, and alkaloids are

examples of bioactive compounds. More than 5000 unique dietary phytochemicals with varied concentration and composition have been found in plant foods (i.e., fruits, vegetables, whole grains, legumes, and nuts) (Huang et al., 2016).

Polyphenols, flavonoids, isoflavonoids, anthocyanidins, phytoestrogens, terpenoids, carotenoids, limonoids, phytosterols, glucosinolates, and dietary fibres are among the phytochemicals that have been found as possibly beneficial to health (Thakur et al., 2020). As consumers become more health-conscious, the nutraceuticals sector is expanding rapidly, with billions of dollars at stake. The expanding of phytochemicals is due to their properties for having antibacterial, antifungal, anti-inflammatory, antiallergic, antispasmodic, chemopreventive, hepatoprotective, hypolipidemic, neuroprotective, hypotensive, immunomodulatory, and carminative activities. They have the potential to prevent ageing, diabetes, osteoporosis, cancer, and heart disease, as well as to induce apoptosis (cell death), act as diuretics, central nervous system stimulants, and analgesics, and protect against UVB-induced carcinogenesis (Dillard and German, 2000; Packer and Weber, 2001; Nichenametla et al., 2006).

Phytochemicals perform a variety of functions, including acting as biochemical reaction substrates, cofactors or inhibitors of enzymatic reactions, absorbents or sequestrants that bind and eliminate unwanted substances in the intestine, ligands that agonise or antagonise cell surface or intracellular receptors, and scavenging toxic chemicals (Rudzińska et al., 2023). They can also improve nutrient absorption and/or stability, act as selective growth factors for helpful bacteria, act as fermentation substrates for beneficial bacteria, and inhibit pathogenic gut bacteria selectively (Rudzińska et al., 2023).

## 2.4 Molecular Docking and its Application

Molecular docking is a structure-based drug design strategy that predicts receptor-ligand binding by simulating molecular interactions (Morris and Lim-Wilby, 2008). It has attracted a lot of attention in drug design research because of its capacity to screen compound databases for prospective pharmacophores, which makes it easier for researchers to obtain and synthesize compounds for further testing (Morris and Lim-Wilby, 2008). This strategy has also shown greater efficiency and lower research expenses as compared to in-vivo and in-vitro approaches. Furthermore, reverse molecular docking technology are useful in therapeutic target prediction and comprehend the molecular processes involved in drug design (Chen and Zhi, 2001).

The basic goal of molecular docking is to predict a ligand's binding affinity and interactive mode by simulating its ideal conformation (Morris and Lim-Wilby, 2008). The traditional “lock-and-key model” emphasizes geometric complementarity, in which the receptor and ligand dock tightly to discover the best orientation for their interaction (Morrison et al., 2006). However, in order to establish a good fit, the docking process requires conformational modifications of both the receptor and the ligand, leading to the development of the “induced fit model” (Koshland, 1994). This model considers geometric complementarity, energy complementarity, and pre-organization, resulting in a stable structure with low free energy (Audie and Scarlata, 2007). Figure 2.5 depicts the “lock-and-key model” and the “induced fit model”.

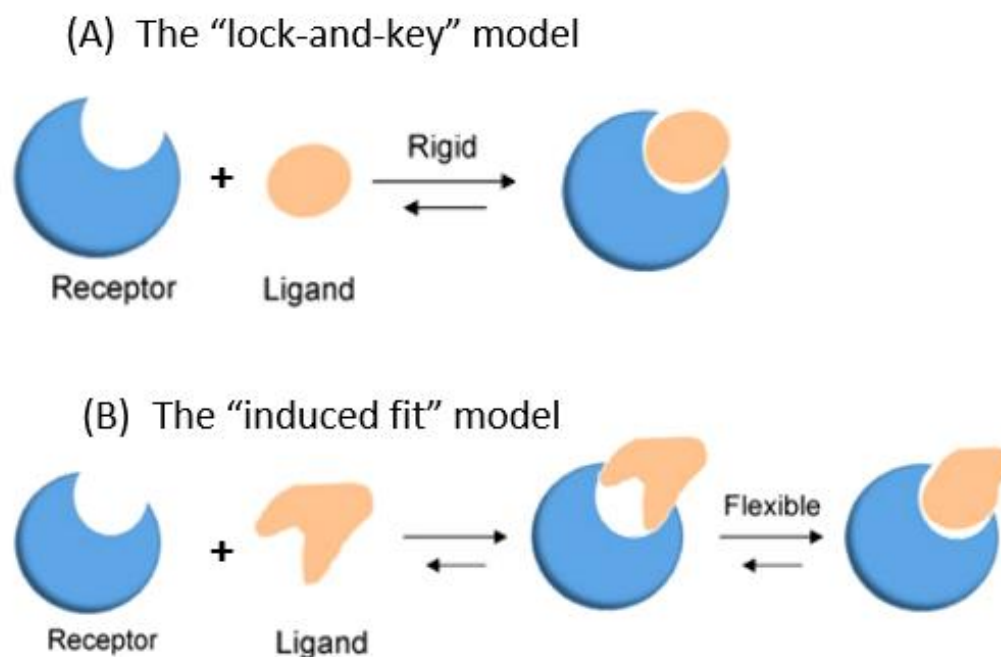


**Figure 2.5:** (A) The “lock-and-key model” and (B) “induced fit model” (Fan et al., 2019).

Molecular docking software employs particular algorithms to predict the optimum shape and orientation of ligand-receptor interactions (Trott and Olson, 2009). These software tools also use scoring methods to predict binding affinity and analyse the intermolecular interaction (Trott and Olson, 2009). There are various forms of molecular docking software available, including rigid docking, flexible docking and flexible-rigid docking with flexible-rigid docking being the most popular (Fan et al., 2019). Flexible docking is gaining popularity because to its higher precision (Trott and Olson, 2009). Figure 2.6 depicts the molecular docking procedure.

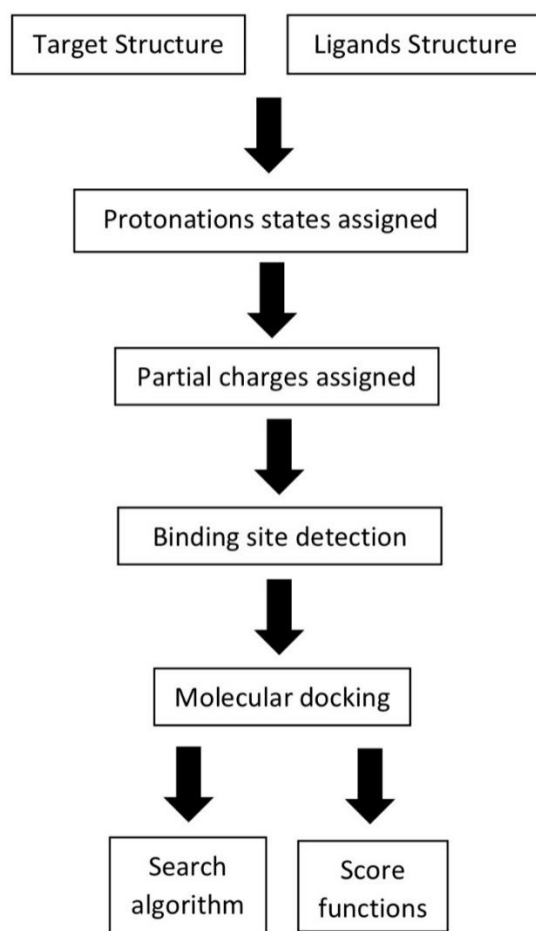


**Figure 2.6:** Diagram of molecular docking basic steps (Torres et al., 2019).

For molecular docking research, databases such as the Protein Data Bank (PDB), PubChem Compound Database, and ZINC are commonly used (Berman et al., 2000; Irwin and Shoichet, 2005; Kim et al., 2015). Commercial databases such as Compound Database and Cambridge Structural Database are also useful (Martin et al., 2002; Groom et al., 2016). Virtual screening, which uses a scoring system to find lead compounds from molecular databases, has dramatically improved screening efficiency over traditional procedures (Kitchen et al., 2004). The combination of high-throughput approaches, high-performance computing, machine learning, and deep learning has pushed virtual screening methods even

further (Melville et al., 2009; Ge et al., 2013; Gawehn et al., 2016). Examples of these integrated techniques are presented, emphasising their efficacy in extracting key information and predicting pharmaceutical targets (Pyzer-Knapp et al., 2015; Pereira et al., 2016).

While general docking methods focus on docking a receptor with several ligands from a database, reverse docking approaches use a different strategy. Chen and Zhi (2001) define reverse docking as the use of a single small-molecule ligand to dock with many receptors, finding possible binding sites and predicting pharmaceutical targets. This method has been used successfully in a number of studies by complement with in-vitro assay studies, including research into new target proteins and effective target fishing (Grinter et al., 2011; Chen et al., 2017). In addition, Fan et al. (2019) reveals that adding structural biology research, such as pocket analysis, can significantly improve creative drug design (Xie et al., 2013; Liu et al., 2017).

However, when using molecular docking, there are several limitations to consider. Inactive molecules may receive abnormally high scores from scoring functions, resulting in false positives (Ferreira et al., 2010; Zhang et al., 2016a; Zhang et al., 2017). Furthermore, if the physical properties of the actual medicine and the chemicals in the database change significantly, the docking scores may be incorrect (Enyedy and Egan, 2008). To examine the accuracy of affinity predictions, it is necessary to consider thermodynamic properties or undertake retrospective verification (Tame, 1999; Yabuuchi et al., 2011). The conformational changes that occur when the three-dimensional structure employed for docking is removed from its original environment might also affect docking results accuracy (Zhang and Zhang, 2017; Zhang et al., 2018a). Future advancements in scoring systems and search algorithms are projected to improve molecular docking's reliability as a drug design tool.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Phytochemical Extraction

##### 3.1.1 Plant Samples Collection and Preparation

The fruits of *Shorea macrophylla* (engkabang) were obtained in dried condition from LTT Aquaculture Sdn Bhd located at Asajaya (GPS coordinates: 1° 32' 53.647" N, 110° 32' 53.233" E). The samples were stored in a ziplock bag and kept in -20 °C fridge until further actions. The engkabang fruits were then chopped into smaller pieces and further grinded into powder form by using liquid nitrogen. The picture of grinded engkabang fruit was shown in Figure 3.1.



**Figure 3.1:** Grinded engkabang fruit samples.

##### 3.1.2 Soxhlet Extraction

The extraction was carried out by using Soxhlet extraction. The grinded engkabang fruit were weighed up to 20 g for each extraction. The 20 g of engkabang fruit powders were placed inside a thimble and a cotton wool were placed above the thimble to prevent leaking of the samples to the extractor chamber. Solvent such as hexane was used for the phytochemical extraction. 300 mL of hexane was prepared and poured into the round bottom flask and the round bottom flask was placed in the mantle to heat up the solvent until evaporation occurred. The evaporated hexane went through the extractor chamber and then to the condenser. The cold running water in the condenser condensed the hexane and it flow back to the extractor chamber to dissolve the phytochemicals in the engkabang fruit. Once the Soxhlet extractor reached its siphoning level, the extracts (phytochemicals) and solvent refluxed back to the round bottom flask and the process was continued until the extraction process being stopped after 8 hours. The solvents were then separated from the extract by using rotary evaporator and the extracts were finally stored in amber bottles and left in fume hood to further evaporate the solvent. The extraction was repeated for dichloromethane, methanol, acetone, ethanol, diethyl ether, isopropanol, and ethyl acetate and all of the concentrations was at 100%. The polarity index of all of the solvents used was listed in Table 3.1. The experiment was conducted in triplicates. The initial weight of the amber bottles and the final weight of amber bottle with extracts were weighed to obtain total extract weight and the extraction efficiency was calculated as stated in Eq. 3.1. The flowchart of the procedure was illustrated in Figure 3.2.

**Table 3.1:** Polarity indexes of all solvents used.

| Solvent used    | Polarity index |
|-----------------|----------------|
| Hexane          | 0.1            |
| Diethyl ether   | 2.8            |
| Dichloromethane | 3.1            |
| Isopropanol     | 4.3            |
| Ethyl acetate   | 4.3            |

|          |     |
|----------|-----|
| Methanol | 5.1 |
| Acetone  | 5.1 |
| Ethanol  | 5.2 |

$$\frac{\text{Final weight of amber bottle with extract (g)} - \text{Initial weight of amber bottle (g)}}{\text{Weight of grinded engkabang fruit used for extraction (g)}} \times 100\% \quad (\text{Eq. 3.1})$$

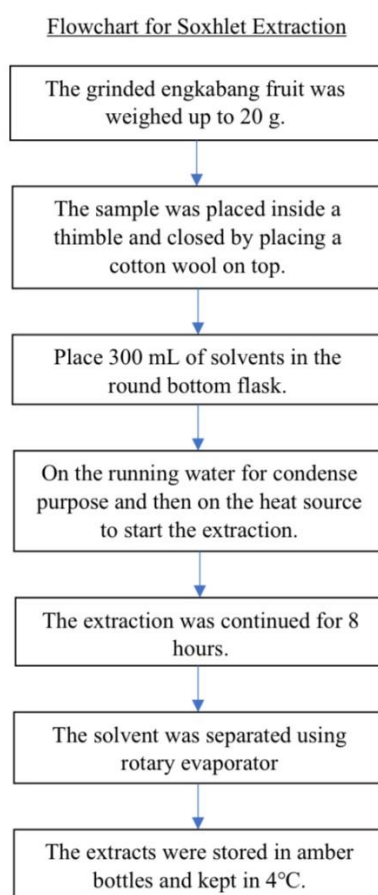


**Figure 3.2:** Flowchart of Soxhlet extraction.

## 3.2 Gas Chromatography-Mass Chromatography (GCMS)

A total of 1 mg of the crude extract was then redissolved back with 1 mL of their respective solvent. The crude extracts were subjected to GCMS analysis. The profiling for the potential agonists in engkabang extracts was performed using a GC-MS (Model: Shimadzu single quadrupole GCMS-QP2020 NX) equipped with a fused, non-polar silica column made of 5% phenyl polysilphenylene-siloxane named BPX-5 of 30 m length, 0.25 mm diameter and 0.25  $\mu$ m thickness. For GC-MS detection, an electron ionization system with an ionization energy of 70eV was used. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1 mL/min. A total of 1  $\mu$ L of the extract was injected into the instrument at 280 °C. The initial temperature of the column oven was set at 50 °C and held for 1 minute and was then increased to 260 °C with a speed of 4.5 °C/min throughout the process. The final temperature was set at 280 °C and held for another 10 minutes. The total run time of the GC-MS was 45 minutes. Interpretation of the mass spectrum of the plant extracts was conducted using the database of the National Institute of Standard and Technology (NIST) library, having more than 62,000 spectral patterns.

### **3.3 Molecular docking**

#### **3.3.1 Determination of Protein and Ligand**

The list of phytochemicals compounds identified from GCMS analysis were sorted by their abundance level. The compounds with 10% abundance were built as ligands to be dock with lipogenesis-related protein. The SMILES structure of the compounds was obtained in the PubChem database for the molecular docking analysis. Protein such as Acetyl-CoA Carboxylase (ACC) (pdb id: 3FF6), ATP-Citrate Lyase (ACLY) (pdb id: 6O0H), Carbohydrate Responsive Element Binding Protein (ChREBP) (pdb id: 5F74), Fatty

Acid Synthase (FASN) (pdb id: 4PIV), Glucose Transporter 1 (GLUT1) (pdb id: 5EQI), Peroxisome Proliferator-Activated Receptor  $\gamma$  (PPAR $\gamma$ ) (pdb id: 2PRG), Stearoyl-CoA Desaturase (SCD) (pdb id: 4YMK) and Sterol Regulatory Element Binding Protein (SREBP) (pdb id: 6K9M) were used in the docking analysis. The proteins were downloaded from the RCSB PDB website (<https://www.rcsb.org/>) in pdb format. The docking analysis was conducted via UCSF Chimera software v1.16 and AutoDock Vina program as shown in Figure 3.3. Python is Chimera's major programming language. Python is an interpreted, object-oriented programming language that is very simple to learn and comprehend (Pettersen et al., 2004). AutoDock Vina, achieves a two-order-of-magnitude speedup over the previous molecular docking software developed, while also significantly improving the accuracy of binding mode predictions (Trott and Olson, 2010).

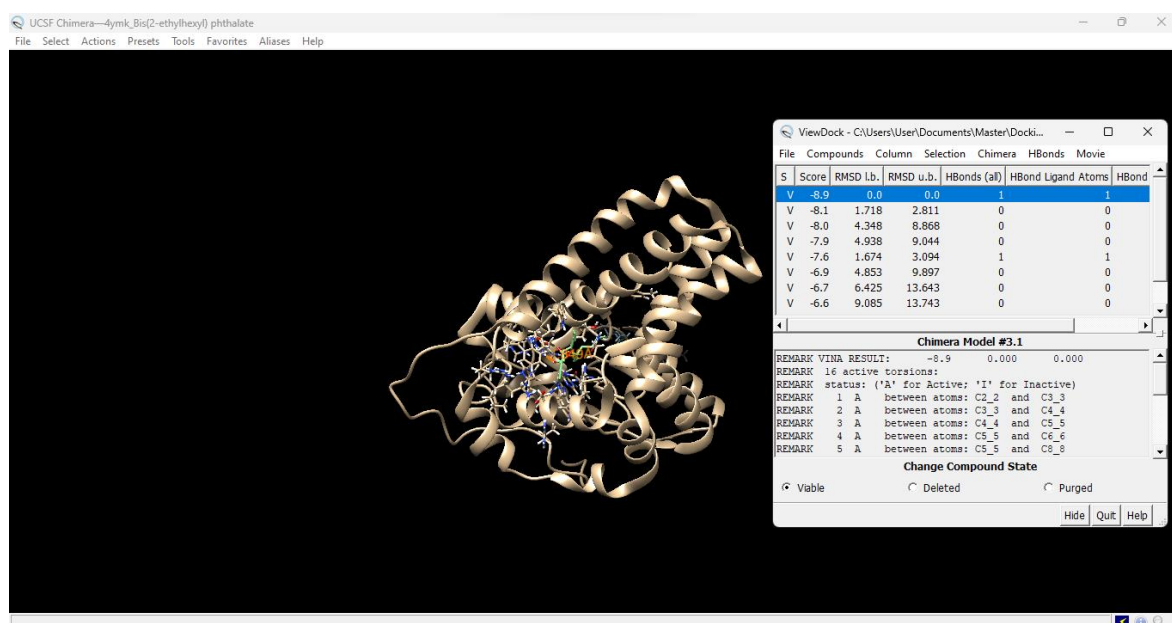


**Figure 3.3:** UCSF Chimera analysis of SCD protein with Bis(2-ethylhexyl) phthalate compound. Autodock Vina is utilised for the calculation and simulation of molecular docking analysis.

### 3.3.2 Three-dimensional (3D) Structural Model Validation of Proteins

The validation of each protein was analysed using Phyre2 software (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>). The proteins amino acid sequences were acquired from RCSB PDB database. The amino acid sequences for all the proteins were used for the Phyre2 job queries. The confidence level of protein was analysed and proteins with confidence level of higher or equal to 90% were used for the docking analysis.

### **3.3.3 Preparation of Proteins and Compounds**

The downloaded protein files were open at the USCF Chimera file and protein as receptors were prepared for docking as water molecules and ligands that were still attached to the receptors were removed and polar hydrogen atoms were added to the receptors. Each of the ligands were built by using SMILES structure. The compounds' structures were first energy minimized using the standard Tripos force field (Powell method and 0.05 kcal/mol Å energy gradient convergence criteria), and electrostatic charge was assigned by the Gasteiger–Hückel method. These conformations were used as starting conformations to perform docking.

### **3.3.4 Molecular Docking Analysis and Visualization**

After receptor and ligand preparation, the active site of protein was determined to identify the places for grid box settling. The grid box information was used for all docking analysis for the same protein. The details of grid box are listed in Appendices Table S1. Before starting with the docking analysis, the software was trained by using re-dock (also known as self-dock) method (Mateev et al., 2022). Rosiglitazone was used as a positive

control for the docking analysis. The docking result was saved as a .py (python file) for future analysis. The ligands' docking conformation was determined by selecting the pose with the highest affinity score (most negative Gibbs' free energy of binding). Besides, amino acid interactions among receptor and ligands were also be analysed.

### **3.4 Cell Culture Analysis**

#### **3.4.1 Recovery of Cells**

The frozen 3T3-L1 preadipocyte cells vial was warmed in a water bath at 37 °C. The thawed cells were then mixed with 10 mL pre-warmed preadipocyte medium (90% DMEM 4.5 g/L D-glucose, 10% Bovine Calf Serum (BCS), 1% Penicillin with Streptomycin antibiotics). The cell mixture was then centrifuged at 280 x g for 5 minutes, the supernatant was then discarded. The cell pellet was well resuspended with 1 mL preadipocyte medium. The T25 flask was first filled with 4 mL preadipocyte medium. The 1 mL resuspended cells were then added in the T25 flask. The T25 flask was then incubated in the 5% CO<sub>2</sub> incubator with 37 °C for 48 hours.

#### **3.4.2 Passaging of Cells**

After cells reaching 80-85% confluency, the cells were then passaged. The T25 flask was first removed from the CO<sub>2</sub> incubator and the attached cells condition were checked under microscope. The medium was then discarded, and 5 mL of 1X PBS were added to the flask to wash the cells. The PBS was removed from the flask after 5 minutes and the washing process was repeated once again. After the PBS being removed, 2 mL of 0.25% trypsin/

2.21mM EDTA solution was added to detach the cells from the flask. The T25 flask was incubated in 5% CO<sub>2</sub> and 37°C for 5 minutes. The condition of the cell detachment was checked under microscope. The flask was tapped gently to loosen the cells. After detachment, 3 mL of preadipocyte medium was added to stop the trypsinisation process. The mixture was then transferred into a 15 mL tube and was centrifuged at 280 x g for 8 minutes. The supernatant was then discarded, and the cell pellet were resuspended with 1 mL preadipocyte medium. A total of 25 µL of cell suspensions were then mixed with 25 µL of trypan blue solution for cell counting purposes.

### 3.4.3 Cell Counting

A total of 25 µL of cell suspensions were mixed with 25 µL of trypan blue solution. The cell number was then counted by using a hemacytometer that was covered with a cover slip. The cell numbers were observed and counted using a microscope and number counter. The number of cells in the shaded gridlines (Figure 3.4) was counted and average reading was taken. The cell number was then counted using the formula stated in Eq. 3.2.

$$\text{Cell number} = \frac{X}{5} \times 2 \times 10^4 \quad (\text{Eq. 3.2})$$

Where,

X is the number of cells counted in 5 grids

5 is the number of grids counted

2 is the dilution factor with trypan blue

10<sup>4</sup> is the multiplying factor for the hemacytometer.

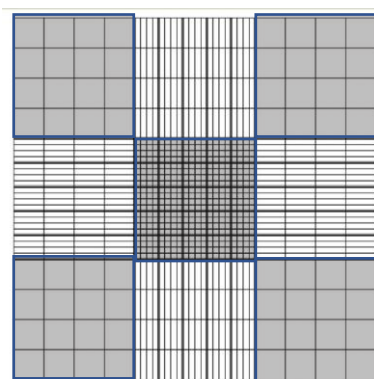


**Figure 3.4:** Shaded grid of hemacytometer that should be counted.

#### 3.4.4 Cell Seeding and Differentiation

After calculation,  $3.0 \times 10^4$  cells per well were seeded into the 96 well plate. The preadipocyte medium was added to a final total volume of 100  $\mu$ L per well. The cells in 96 well plate was then incubated in 5% CO<sub>2</sub> incubator at 37°C for 48 hours. After 48 hours, the cells were differentiated by changing the preadipocytes medium into 100  $\mu$ L of differentiation medium which contain Insulin, Dexamethasone and 3-Isobutyl-1-methylxanthine (IBMX). The culture was left for 72 hours in 5% CO<sub>2</sub> and 37°C.

#### 3.4.5 Maintenance of Cells

After 72 hours of differentiation, the mature adipocytes cells' condition was checked under microscope. The differentiation medium was then being replaced by 100  $\mu$ L of maintaining medium (contain only insulin). The maintaining process was continued for 5 days and cells will be rehydrated in every 2 days.

#### 3.4.6 Treatment of Extracts

The extracts of each solvent were weighed in 10 mg and 1 mL of dimethyl sulfoxide (DMSO) was added to make a concentration of 10 mg/mL. Each extract was then prepared, and serially diluted with maintaining medium to make a final concentration of 0.25, 0.025, 0.0025, 0.00025 and 0.000025 mg/mL in each well by referring to Hengpratom et al. (2018). A volume of 200  $\mu$ L of extracts and medium were seeded into each well accordingly. The experiment was conducted in triplicates. Rosiglitazone in same concentration was treated as positive control whereas 0.1% DMSO was used as vehicle/negative control. The extract was treated for 7 days, and cells will be rehydrated with extract and medium in every 2 days.

#### **3.4.7 Cell Viability Assay**

A volume of 100  $\mu$ L of  $3.0 \times 10^4$  preadipocytes cells with preadipocyte medium were seeded in 96 well plates for 48 hours at 5% CO<sub>2</sub> and 37°C. After 2 days of incubation, the 100  $\mu$ L of preadipocytes medium was changed with differentiation medium and incubated for 72 hours in 5% CO<sub>2</sub> and 37°C. After preadipocyte cells differentiated into mature adipocyte cells, the extract with final concentration of 0.25, 0.025, 0.0025, 0.00025 and 0.000025 mg/mL were prepared with maintaining medium and treated on each well and the final volume of extract with medium was at 200  $\mu$ L. The cell culture treatment was left incubated for 24 hours. After 24 hours, the medium was discarded, and the cells were washed with 100  $\mu$ L 1X PBS for twice and 100  $\mu$ L of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) stock solution (5 mg/mL in PBS) was added in each well and further incubated in 37°C for 4 hours. After 4 hours, the MTT solution was discarded and 100  $\mu$ L DMSO was added to each well to precipitate the salt and creating a purple colour precipitation. The 96 well plate was covered with aluminium and further incubate for 1 hour at room temperature. The relative cell viability was calculated using the

Eq. 3.3 by determining with a microplate reader with colorimetric absorbance at 570 nm. Reference wavelength is at 630 nm.

$$\text{Cell viability (\%)} = \frac{\text{Average OD}_{570} \text{ of treated cells}}{\text{Average OD}_{570} \text{ of negative control}} \times 100\% \quad (\text{Eq. 3.3})$$

#### 3.4.8 Oil Red O Assay

The Oil Red O assay was carried out as described by Fang et al. (2008) with minor amendment. The extract treatment was carried out as stated above, the cells were treated with fresh plant extracts in maintaining medium every other day from day 3 to day 7 from differentiation date. On day 7, the medium was removed, and the cells treated with and without plant extracts were rinsed with 1X PBS and fixed for 30 minutes in 4% paraformaldehyde. The cells were then washed with distilled water before being incubated for 20 minutes at room temperature with Oil Red O solution (0.5% w/v in 60% isopropanol). Finally, the dye was eluted from the 3T3-L1 cells using isopropanol and optical absorbance was measured at 540nm.

$$\text{Relative lipid accumulation (\%)} = \frac{\text{Average OD}_{540} \text{ of treated cells}}{\text{Average OD}_{540} \text{ of negative control}} \times 100\% \quad (\text{Eq. 3.4})$$

#### 3.4.9 Adipogenesis Analysis

The sample preparation steps were the same as the extract treatment steps stated above. The Adipogenesis Kit (Sigma) was used to measure the triglyceride levels according to the manufacturer's protocol. After 5 days, the medium was removed and wash once with 100  $\mu$ L of room temperature 1X PBS. Then, 100  $\mu$ L of Lipid Extraction Buffer was added in each well and the plate was seal and incubated for 30 minutes at 95°C. Next, the plate was

cool to room temperature and plate was shaken for 1 minute to homogenize the solution. 2  $\mu\text{L}$  of Lipase solution was added to each sample well and standard well and incubated for 10 minutes at room temperature to convert triglycerides to glycerol and fatty acids. The Master Reaction Mix was set up as stated in Table 3.2 and 50  $\mu\text{L}$  of the Master Reaction Mix was added to each well. The mixture was mixed using a horizontal shaker for 30 minutes at 37°C. The plate was covered with aluminium foil during the incubation. Finally, the colorimetric absorbance was measured at 570nm. The amount of triglycerides were determined from the standard curve and the concentration of triglyceride was calculated using the formula as stated in Eq. 3.5.

$$\frac{S_a}{S_v} = C \quad (\text{Eq. 3.5})$$

Where,

$S_a$  = Amount of Triglyceride in unknown sample (nmole) from standard curve

$S_v$  = Sample volume ( $\mu\text{L}$ ) added to reaction well

$C$  = Concentration of triglyceride in sample

**Table 3.2:** Master Reaction Mix for colorimetric analysis

| Reagent                   | Volume ( $\mu\text{L}$ ) |
|---------------------------|--------------------------|
| Adipogenesis Assay Buffer | 46                       |
| Adipogenesis Probe        | 2                        |
| Adipogenesis Enzyme Mix   | 2                        |

## CHAPTER 4

### RESULTS

#### 4.1 Phytochemical Extraction

In this study, Soxhlet extraction were conducted on grinded engkabang fruit using several solvents with different polarity. The solvents used in this research were hexane, diethyl ether, dichloromethane, ethyl acetate, acetone, isopropanol, methanol and ethanol, with hexane being the lowest polarity solvent and ethanol as the most polar solvent. For the calculation of extraction efficiency, the extract weight at the end of Soxhlet extraction was divided by the initial sample weight used. The extraction efficiency was illustrated in Figure 4.1. Among all the solvents, acetone has the highest extraction efficiency which is up to 38.30 %, while ethanol has the lowest extraction efficiency (8.80 %). Ethanol and methanol are the solvents with highest polarity among all the solvent used are observed to have lowest extraction efficiency. As for the other six solvents that have lower and middle polarity index have very high extraction efficiency. The details of the extraction efficiency have been listed in Appendices Table S2.

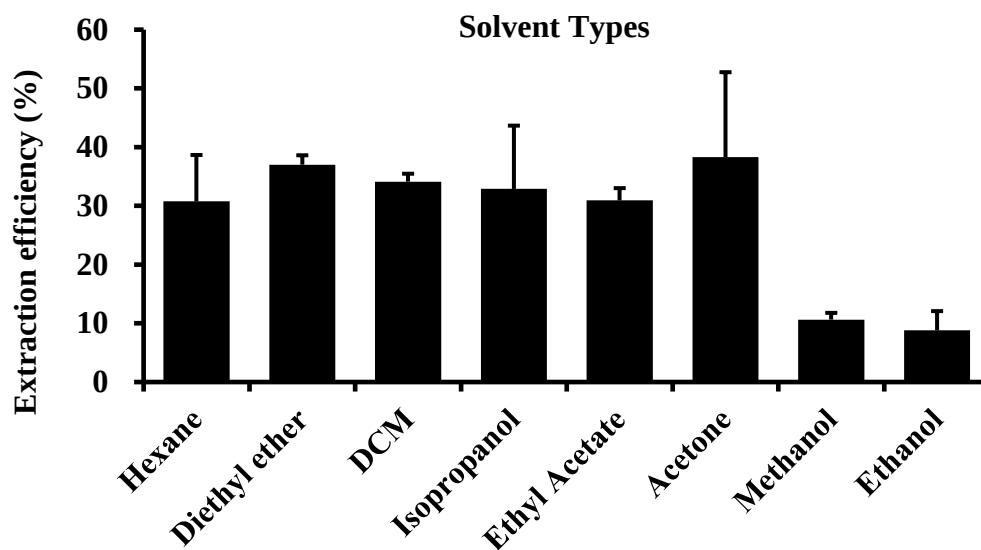


**Figure 4.1:** Extraction efficiency by different solvent.

#### 4.2 Gas Chromatography-Mass Spectrometry result

The crude extracts obtained from Soxhlet extraction using different solvents were subsequently subjected to gas chromatography-mass spectrometry (GCMS) analysis. Overall, the compounds detected through GCMS analysis of various extracts are listed in Table 4.1. Table 4.1 shows only compounds with an abundance of at least 1% fraction of the total extract volume, while the complete profile of GCMS for all extracts in different solvent is available in Appendices Table S3. The GCMS spectra for each solvent with relevant peaks related to compounds in the extracts is listed in Appendices Figure S1 to S8. Four compounds were consistently detected regardless of the polarity of the polarity of solvents, these are methyl stearate, methyl elaidate, methyl palmitate and methyl arachidate. Methyl stearate was found to have the highest abundance level in all solvents used, except for acetone at a range of 45.16% to 20.58%. From all the listed compounds, most of them are fatty acids, with all the extracts are having at least 60% of fatty acids, followed by phenolic compounds.

**Table 4.1:** List of compounds with abundance level in area (%). Compounds that are available in all extract fractions are highlighted.

| Solvent         | Compound                             | Pubchem CID | Abundance level (%) |
|-----------------|--------------------------------------|-------------|---------------------|
| Hexane          | Methyl stearate                      | 8201        | 24.69               |
|                 | Methyl elaidate                      | 5280590     | 16.92               |
|                 | Bis(2-ethylhexyl) adipate            | 7641        | 10.45               |
|                 | Hexacosane                           | 12407       | 10.44               |
|                 | Methyl palmitate                     | 8181        | 8.88                |
|                 | Bis(2-ethylhexyl) phthalate          | 8343        | 3.16                |
|                 | 3-Ethoxy-4-hydroxyphenylacetonitrile | 590922      | 1.14                |
|                 | Methyl arachidate                    | 14259       | 1.11                |
| Dichloromethane | Methyl stearate                      | 8201        | 42.68               |
|                 | Methyl elaidate                      | 5280590     | 28.09               |
|                 | Methyl palmitate                     | 8181        | 15.60               |
|                 | Methyl arachidate                    | 14259       | 1.94                |
|                 | Oxiran-2-ylmethyl tetradecanoate     | 346148      | 1.45                |
|                 | Methyl linoleate                     | 5284421     | 1.11                |
| Methanol        | Methyl stearate                      | 8201        | 36.25               |
|                 | Methyl elaidate                      | 5280590     | 35.50               |
|                 | Methyl palmitate                     | 8181        | 17.14               |
|                 | Methyl linoleate                     | 5284421     | 1.66                |
|                 | Methyl arachidate                    | 14259       | 1.50                |
| Acetone         | Glycidyl palmitate                   | 347736      | 23.78               |
|                 | Methyl stearate                      | 8201        | 20.58               |
|                 | Methyl elaidate                      | 5280590     | 13.74               |
|                 | Glycidyl oleate                      | 5354568     | 13.5                |
|                 | Methyl palmitate                     | 8181        | 8.06                |
|                 | Glycidyl stearate                    | 62642       | 2.98                |
|                 | Methyl arachidate                    | 14259       | 1.36                |
|                 | Methyl behenate                      | 13584       | 1.1                 |
| Isopropanol     | Methyl stearate                      | 8201        | 36.88               |
|                 | Methyl elaidate                      | 5280590     | 24.17               |
|                 | Methyl palmitate                     | 8181        | 13.83               |
|                 | Methyl arachidate                    | 14259       | 1.73                |
| Ethanol         | Methyl stearate                      | 8201        | 44.97               |
|                 | Methyl elaidate                      | 5280590     | 32.96               |

|               |                          |         |       |
|---------------|--------------------------|---------|-------|
|               | Methyl palmitate         | 8181    | 17.03 |
|               | Ethyl stearate           | 8122    | 1.40  |
|               | Methyl arachidate        | 14259   | 1.08  |
|               | Ethyl oleate             | 5363269 | 1.05  |
| Ethyl Acetate | Methyl stearate          | 8201    | 45.16 |
|               | Methyl elaidate          | 5280590 | 29.67 |
|               | Methyl palmitate         | 8181    | 21.81 |
|               | Methyl arachidate        | 14259   | 1.36  |
| Diethyl ether | Methyl stearate          | 8201    | 40.3  |
|               | Methyl elaidate          | 5280590 | 25.8  |
|               | Methyl palmitate         | 8181    | 14.58 |
|               | Butylated hydroxytoluene | 31404   | 2.52  |
|               | Methyl arachidate        | 14259   | 2.09  |

#### 4.3 Three-dimension (3D) Model Validation of Lipogenic Proteins

Prior to molecular docking analysis of the selected compounds, several lipogenesis proteins were selected. These proteins are Acetyl-CoA Carboxylase (ACC), ATP-Citrate Lyase (ACLY), Carbohydrate Responsive Element Binding Protein (ChREBP), Fatty Acid Synthase (FASN), Glucose Transporter 1 (GLUT1), Peroxisome Proliferator-Activated Receptor  $\gamma$  (PPAR $\gamma$ ), Stearoyl-CoA Desaturase (SCD) and Sterol Regulatory Element Binding Protein (SREBP) with EC number as listed in Table 4.2. Their involvement in lipogenesis pathway modulation have been verified by previous studies as listed in Table 4.2. In order to ensure that molecular docking analysis was accurately performed, these lipogenic factors were subjected to modelling using Phyre 2 software with normal modelling mode. Table 4.2 shows that all of the modelled protein has a 100% confidence level and a query coverage of 91% to 100%. Detailed of the predicted protein structure can be found in Appendices Table S4. with their respective ramachandran plot. Of these, ChREBP protein model showed a 100% query coverage which involved the whole open reading frame (ORF).

**Table 4.2:** Lipogenesis-related proteins' confidence level analyse using Phyre 2.

| Protein Name                                                         | PDB id | EC number                             | Confidence level (%) | Coverage (%) | Previous literature                           |
|----------------------------------------------------------------------|--------|---------------------------------------|----------------------|--------------|-----------------------------------------------|
| Acetyl-CoA Carboxylase (ACC)                                         | 3FF6   | 6.4.1.2                               | 100                  | 96           | (Lawitz et al., 2018; Lally et al., 2019)     |
| ATP-Citrate Lyase (ACLY)                                             | 6O0H   | 2.3.3.8                               | 100                  | 93           | (Calejman et al., 2020; Feng et al., 2020)    |
| Carbohydrate Responsive Element Binding Protein (ChREBP)             | 5F74   | -<br>(Transcription factor)           | 100                  | 100          | (Uyeda et al., 2002; Dentin et al., 2005)     |
| Fatty Acid Synthase (FASN)                                           | 4PIV   | 2.3.1.85                              | 100                  | 91           | (Wang et al., 2004; Syed-Abdul et al., 2020)  |
| Glucose Transporter 1 (GLUT1)                                        | 5EQI   | -<br>(Membrane protein)               | 100                  | 91           | (Hajdich et al., 1992; DeBosch et al., 2014)  |
| Peroxisome Proliferator-Activated Receptor $\gamma$ (PPAR $\gamma$ ) | 2PRG   | -<br>(Nuclear receptor in regulation) | 100                  | 95           | (Jeong et al., 2012; Blanchard et al., 2016)  |
| Stearoyl-CoA Desaturase (SCD)                                        | 4YMK   | 1.14.19.1                             | 100                  | 95           | (Chong et al., 2008; Cheng et al., 2016)      |
| Sterol Regulatory Element Binding Protein (SREBP)                    | 6K9M   | -<br>(Transcription factor)           | 100                  | 92           | (Ferré and Foulfelle, 2010; Kim et al., 2010) |

#### 4.4 Molecular Docking Analysis

Molecular docking analysis between selected compounds identified from engkabang extract and lipogenic factors were conducted using Autodock Vina v.1.2.0 and UCSF Chimera v.1.16 at parameters as stated under Methodology section. The summarized

docking scores of all selected compounds against lipogenic proteins are listed in Table 4.3. The docking scores of positive controls, Rosiglitazone, a commercialized lipogenic inducing compound is also included in the docking analysis table. From Table 4.3, Bis (2-ethylhexyl) phthalate was found to be able to interact with four out of the eight lipogenic protein tested with score consistently above -7.5 which is higher than the positive control score in all cases. Both Bis(2-ethylhexyl) adipate and Butylated Hydroxytoluene interacted with three lipogenic factors respectively at a score higher than -7.0. Glycidyl oleate and Hexacosane were the two compounds that interacted with two lipogenic factors, respectively with scores above -7.0. Overall, 11 out of 16 compounds scored -7.0 and above with SREBP, a master regulator in lipogenesis while 50% of the compound tested scored -7.0 and above with SCD.

**Table 4.3:** Docking score of docking analysis of compounds with lipogenesis related protein. Bolded numbers are docking analysis with lower than -7.0 kcal/mol docking score. ACC: Acetyl-CoA Carboxylase; ACLY: ATP-Citrate Lyase; ChREBP: Carbohydrate Responsive Element Binding Protein; FASN: Fatty Acid Synthase; GLUT1: Glucose Transporter 1; PPAR $\gamma$ : Peroxisome Proliferator-Activated Receptor  $\gamma$ ; SCD: Stearoyl-CoA Desaturase; SREBP: Sterol Regulatory Element Binding Protein.

| Compounds / Protein                                                                        | ACC  | ACLY        | ChREBP | FASN        | GLUT 1      | PPAR $\gamma$ | SCD         | SREBP       |
|--------------------------------------------------------------------------------------------|------|-------------|--------|-------------|-------------|---------------|-------------|-------------|
| Rosiglitazone<br>SMILES:<br><chem>CN(CCOC1=CC=C(C=C1)CC2C(=O)NC(=O)S2)C3=CC=CC=N3</chem>   | -6.4 | <b>-8.0</b> | -6.9   | <b>-8.6</b> | <b>-8.6</b> | <b>-9.4</b>   | <b>-8.6</b> | <b>-9.0</b> |
| 3-Ethoxy-4-hydroxyphenylacetone nitrile<br>SMILES:<br><chem>CCOC1=C(C=CC(=C1)CC#N)O</chem> | -4.8 | -6.5        | -5.8   | -6.4        | -6.1        | -5.7          | <b>-7.3</b> | -6.5        |
| Bis(2-ethylhexyl) adipate<br>SMILES:<br><chem>CCCCC(CC)COC(=O)CCCCC(=O)OC(CCC)CCCC</chem>  | -4.3 | <b>-7.2</b> | -5.1   | <b>-7.4</b> | -6.4        | -6.2          | <b>-8.0</b> | <b>-7.5</b> |

|                                                                                                     |      |             |      |             |             |      |             |             |
|-----------------------------------------------------------------------------------------------------|------|-------------|------|-------------|-------------|------|-------------|-------------|
| Bis(2-ethylhexyl)<br>phthalate<br>SMILES:<br><chem>CCCCC(CC)COC(=O)C1=CC=CC=C1C(=O)OCC(CC)CC</chem> | -4.7 | <b>-8.4</b> | -5.8 | <b>-7.9</b> | -6.5        | -6.6 | <b>-8.9</b> | <b>-8.3</b> |
| Butylated<br>hydroxytoluene<br>SMILES:<br><chem>CC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C</chem>          | -4.6 | <b>-7.6</b> | -6.3 | <b>-7.4</b> | <b>-7.0</b> | -6.4 | -6.2        | <b>-7.8</b> |
| Ethyl Oleate<br>SMILES:<br><chem>CCCCCCCCC=CCCCCCCC(=O)OCC</chem>                                   | -3.9 | <b>-7.0</b> | -4.9 | -6.5        | -5.9        | -5.9 | -6.0        | <b>-7.1</b> |
| Ethyl stearate<br>SMILES:<br><chem>CCCCCCCCCCCCCCCCCCCC(=O)OCC</chem>                               | -3.5 | -6.6        | -4.5 | -6.2        | -5.4        | -6.1 | -6.5        | <b>-7.1</b> |
| Glycidyl oleate<br>SMILES:<br><chem>CCCCCCCCC=CCCCCCCC(=O)OCC1CO1</chem>                            | -4.0 | -6.8        | -5.0 | -6.4        | -5.9        | -6.1 | <b>-7.2</b> | <b>-7.0</b> |
| Glycidyl<br>palmitate<br>SMILES:<br><chem>CCCCCCCCCCCCCCCCCCCC(=O)OCC1CO1</chem>                    | -4.1 | -6.5        | -5.3 | -6.2        | -5.5        | -6.0 | -6.0        | -6.9        |
| Glycidyl stearate<br>SMILES:<br><chem>CCCCCCCCCCCCCCCCCCCCCCCC(=O)OCC1CO1</chem>                    | -3.4 | -6.5        | -5.0 | -6.1        | -5.7        | -5.9 | <b>-7.1</b> | -6.9        |
| Hexacosane<br>SMILES:<br><chem>CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC</chem>                              | -3.4 | -6.6        | -4.1 | -6.1        | -5.5        | -6.0 | <b>-7.6</b> | <b>-7.7</b> |
| Methyl arachidate<br>SMILES:<br><chem>CCCCCCCCCCCCCCCCCCCCCCCC(=O)OC</chem>                         | -3.4 | -6.6        | -4.7 | -6.3        | -5.5        | -5.7 | -6.1        | <b>-7.1</b> |
| Methyl behenate<br>SMILES:<br><chem>CCCCCCCCCCCCCCCCCCCCCCCCCCCC(=O)OC</chem>                       | -3.7 | -6.7        | -4.8 | -6.1        | -5.7        | -5.9 | -6.8        | <b>-7.2</b> |

|                                                                                                            |      |      |      |      |      |      |      |      |
|------------------------------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|
| Methyl elaidate<br>SMILES:<br><chem>CCCCCCCCC=CC</chem><br><chem>CCCCCCC(=O)OC</chem>                      | -3.6 | -6.6 | -4.8 | -6.2 | -5.5 | -6.3 | -7.1 | -7.0 |
| Methyl linoleate<br>SMILES:<br><chem>CCCCC=CCC=C</chem><br><chem>CCCCCCCC(=O)O</chem><br><chem>C</chem>    | -4.0 | -6.9 | -5.2 | -6.9 | -5.8 | -6.1 | -7.5 | -7.4 |
| Methyl palmitate<br>SMILES:<br><chem>CCCCCCCCCCCC</chem><br><chem>CCCC(=O)OC</chem>                        | -3.7 | -6.4 | -5.1 | -6.3 | -5.4 | -6.2 | -6.8 | -6.7 |
| Methyl stearate<br>SMILES:<br><chem>CCCCCCCCCCCCC</chem><br><chem>CCCCC(=O)OC</chem>                       | -3.3 | -6.7 | -4.6 | -6.2 | -5.5 | -5.6 | -6.3 | -7.0 |
| Oxiran-2-ylmethyl<br>tetradecanoate<br>SMILES:<br><chem>CCCCCCCCCCCCC</chem><br><chem>CC(=O)OCC1CO1</chem> | -3.7 | -6.3 | -5.2 | -6.1 | -5.3 | -5.9 | -6.1 | -6.7 |

In all cases, molecular docking must take into consideration of the amino acid interaction in the catalytic groove of the lipogenic protein and the selected compounds (Charoenjotivadhanakul et al., 2023). It was found that bis(2-ethylhexyl) adipate, glycidyl palmitate, glycidyl stearate and methyl elaidate are the compounds that have the most interactions with the list of lipogenic proteins which are found to interact with six out of eight lipogenic factors. Almost all compounds except for Butylated Hydroxytoluene and Hexacosane exhibited interactions with at least one lipogenic proteins. This is intriguing as both of these scored the highest in the docking analysis in Table 4.4. Seven compounds out of the 16 were found to interact with SREBP, master regulator of the lipogenesis pathway, all of them at HIS435 position, indicating the critical role of this amino acid in modulation of lipogenesis.

**Table 4.4:** Hydrogen bond formation between amino acid of proteins with ligands. ACC: Acetyl-CoA Carboxylase; ACLY: ATP-Citrate Lyase; ChREBP: Carbohydrate Responsive Element Binding Protein; FASN: Fatty Acid Synthase; GLUT1: Glucose Transporter 1; PPAR $\gamma$ : Peroxisome Proliferator-Activated Receptor  $\gamma$ ; SCD: Stearoyl-CoA Desaturase; SREBP: Sterol Regulatory Element Binding Protein.

| Compounds / Proteins                    | ACC                                  | ACLY                                         | ChREBP                                                                                                   | FASN                                                                                             | GLUT1                                                                                                         | PPAR $\gamma$                                         | SCD                                | SREBP           |
|-----------------------------------------|--------------------------------------|----------------------------------------------|----------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|------------------------------------|-----------------|
| Rosiglitazone                           | GLY2126 (2.192).                     | GLY309 (2.402),<br>GLY380 (2.240).           | ASN124 (2.434).                                                                                          | ILE2068 (1.994).                                                                                 | ASN288 (2.169),<br>GLN283 (2.604).                                                                            | TYR473 (2.361),<br>GLN286 (1.878),<br>SER289 (2.009). | TRP258 (2.071).                    | -               |
| 3-Ethoxy-4-hydroxyphenylacetone nitrile | -                                    | THR348 (2.290),<br>GLY380 (2.324).           | ASN123 (2.270),<br>ARG58 (2.021).                                                                        | TYR2034 (2.349),<br>LEU2069 (2.342),<br>GLY2061 (2.351),<br>LYS1995 (1.983),<br>GLY1897 (2.275). | GLN283 (2.469),<br>TRP388 (2.238).                                                                            | -                                                     | ASN144 (2.249),<br>HIS153 (2.454). | -               |
| Bis(2-ethylhexyl) adipate               | GLU2230 (2.056).                     | -                                            | ARG128 (2.175,<br>2.482), ARG129<br>(2.012), ASN175<br>(2.214), LYS122<br>(2.202).                       | ALA2062 (2.176).                                                                                 | ASN415 (2.536,<br>2.419),<br>ASN288 (1.964,<br>2.724, 2.324),<br>TRP388 (2.437,<br>2.453), GLN283<br>(2.595). | -                                                     | ASN261 (2.101),<br>HIS153 (2.454). | HIS435 (2.238). |
| Bis(2-ethylhexyl) phthalate             | GLY2202 (2.103).                     | -                                            | ARG58 (2.188),<br>LYS51 (2.193).                                                                         | -                                                                                                | GLN283 (2.278).                                                                                               | -                                                     | ASN261 (1.849).                    | -               |
| Butylated hydroxytoluene                | -                                    | -                                            | -                                                                                                        | -                                                                                                | -                                                                                                             | -                                                     | -                                  | -               |
| Ethyl Oleate                            | GLY2202 (2.098).                     | GLY342 (2.237,<br>2.584), GLY380<br>(2.266). | ARG58 (2.089),<br>LYS51 (2.169,<br>2.445), ARG128<br>(2.055, 2.528),<br>ARG129 (2.400,<br>2.333, 2.441). | SER2021 (2.234).                                                                                 | THR137 (2.721).                                                                                               | -                                                     | -                                  | -               |
| Ethyl stearate                          | GLY2162 (2.518),<br>GLY2163 (2.508). | THR348 (2.067),<br>ASN349 (2.019).           | ARG128 (2.223).                                                                                          | -                                                                                                | ASN415 (2.370,<br>2.652), ASN288<br>(2.272, 2.629,<br>2.628), GLN283<br>(2.690, 2.467).                       | -                                                     | -                                  | HIS435 (2.087). |
| Glycidyl oleate                         | PHE2160 (2.129).                     | THR348 (2.497),<br>ASN349 (2.428).           | ARG128 (2.100,<br>2.134), ARG129<br>(2.214), LYS51<br>(2.317).                                           | GLY1897 (2.210),<br>PHE1896 (2.324).                                                             | GLN283 (2.684),<br>ASN415 (2.467),<br>ASN288 (2.714).                                                         | SER289 (2.046).                                       | -                                  | -               |
| Glycidyl palmitate                      | -                                    | -                                            | ASN124 (2.737),<br>ARG128 (2.564),                                                                       | SER2021 (1.954).                                                                                 | TRP388 (2.439),<br>THR137 (2.377).                                                                            | HIS449 (2.358).                                       | ASN261 (2.858),<br>TRP258 (2.375). | -               |

|                                     |                                      |                                                       |                                                                                                                                                         |                                                                                     |                                                     |                 |   |                 |
|-------------------------------------|--------------------------------------|-------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------|-----------------|---|-----------------|
|                                     |                                      |                                                       | ARG129 (2.110,<br>2.370).                                                                                                                               |                                                                                     |                                                     |                 |   |                 |
| Glycidyl stearate                   | GLY2162 (1.917).                     | -                                                     | ARG129 (2.234).                                                                                                                                         | SER2021 (2.044),<br>GLY1895 (2.342).                                                | ASN415 (2.470),<br>ASN288 (2.417).                  | -               | - | HIS435 (2.266). |
| Hexacosane                          | -                                    | -                                                     | -                                                                                                                                                       | -                                                                                   | -                                                   | -               | - | -               |
| Methyl arachidate                   | GLY2202 (2.123).                     | GLY380 (2.134),<br>THR348 (1.908),<br>ASN349 (1.989). | ARG128 (2.718,<br>2.371, 2.332,<br>2.171), ASN124<br>(2.367, 2.566),<br>LYS51 (2.328),<br>TYR130 (2.453),<br>ARG129 (1.010,<br>2.491, 2.170,<br>2.356). | PHE1896 (1.868).                                                                    | ASN415 (2.574),<br>ASN288 (2.500).                  | -               | - | -               |
| Methyl behenate                     | GLY2162 (1.874),<br>GLY2163 (2.495). | GLY380 (2.199).                                       | ARG129 (2.289,<br>2.355, 2.332),<br>LYS51 (2.299).                                                                                                      | -                                                                                   | TRP388 (2.402).                                     | -               | - | -               |
| Methyl elaidate                     | GLY2162 (2.212),<br>GLY2163 (2.451). | GLY342 (2.175),<br>GLY380 (2.176).                    | LYS51 (2.328).                                                                                                                                          | SER2021 (2.314,<br>2.501), GLY1897<br>(2.276, 2.025),<br>PHE1896 (2.102,<br>2.203). | ASN415 (2.551),<br>ASN288 (2.393).                  | HIS323 (2.344). | - | HIS435 (2.155). |
| Methyl linoleate                    | -                                    | GLY380 (2.389,<br>2.284), GLY342<br>(2.366).          | ARG58 (2.293,<br>2.015), ARG128<br>(1.936).                                                                                                             | PHE1896 (2.196),<br>GLY1897 (2.076).                                                | ASN415 (2.540).                                     | SER289 (2.327). | - | HIS435 (2.346). |
| Methyl palmitate                    | -                                    | -                                                     | ARG128 (2.408),<br>ARG129 (2.327,<br>2.124), LYS51<br>(2.479).                                                                                          | GLY1897 (2.218),<br>PHE1896 (2.025).                                                | ASN415 (2.519,<br>2.353), ASN288<br>(2.292, 2.605). | -               | - | HIS435 (2.247). |
| Methyl stearate                     | GLY2162 (2.559).                     | GLY380 (2.272).                                       | ASN124 (2.302),<br>ARG128 (2.427).                                                                                                                      | GLY1897 (2.167),<br>PHE1896 (2.139).                                                | ASN288 (2.444,<br>2.390), ASN415<br>(2.394).        | -               | - | -               |
| Oxiran-2-ylmethyl<br>tetradecanoate | -                                    | -                                                     | ASN124 (2.259),<br>ARG128 (2.655),<br>ARG129 (2.269,<br>2.250).                                                                                         | -                                                                                   | TRP412 (2.450),<br>TRP388 (2.410).                  | -               | - | HIS435 (2.422). |

#### 4.5 Cytotoxicity Test

MTT assay was used to test on the cytotoxicity of the extract at different concentrations gradients towards adipocyte cells. The graph for all the extracts and positive control were plotted as shown in Figure 4.2. Figure 4.2(A) shows that positive control, Rosiglitazone, at 0.000025 mg/mL to 0.0025 mg/mL, does not cause a decrease in cell viability, however, when the concentration increased to 0.025 mg/mL and above, nearly 50% of cell loses its viability. As for hexane extract (Figure 4.2(B)), all 3T3-L1 cells managed to survive with 100% cell viability even at 0.25 mg/mL extract concentration. As for dichloromethane, methanol, acetone, isopropanol and diethyl ether extracts, cell survivability maintained at 100% viability for all concentrations except at 0.25 mg/mL. It should be noted that although at 0.25 mg/mL concentration, cell viability was lower than 10% for ethanolic extract, cell viability also fell below 90% at 0.025 mg/mL ( $p \leq 0.05$ ). As for ethyl acetate, the cell viability of 3T3-L1 adipocyte cells have cell viability lower than 100% at all tested concentration (from 0.000025 mg/mL to 0.25 mg/mL), however from 0.000025 mg/mL to 0.025 mg/mL concentration, the cell viability decreased in a lower extend in the range of 88 to 94%, and only at 0.25 mg/mL concentration, the cell viability drops to 38%. In conclusion, to avoid toxic effect to the adipocytes cells, the effective concentration of extracts was 0.025 mg/mL where no negative effect on cell survivability was observed.

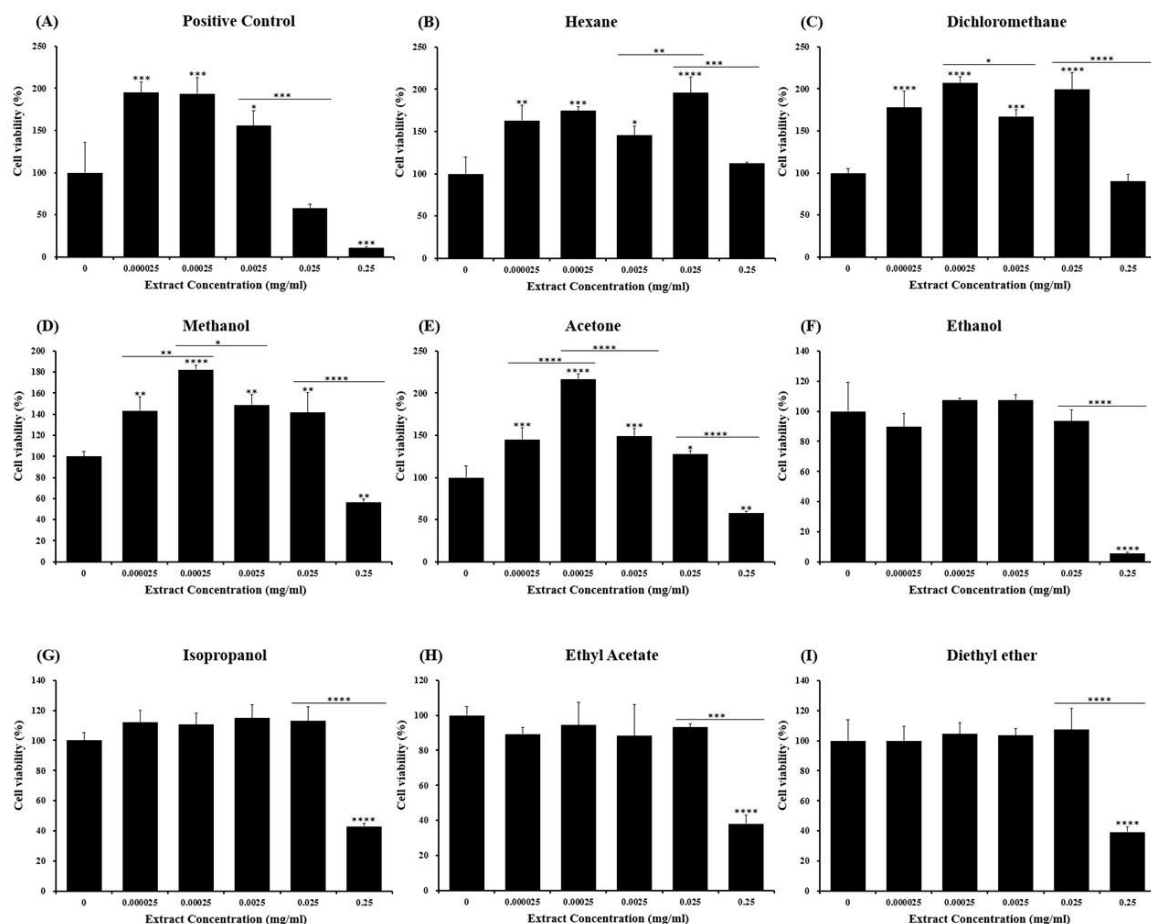


**Figure 4.2:** MTT assay of adipocyte cells treated with extracts that are extracted using different types of solvents. Data are means  $\pm$  S.D. of three replicates. \* $P \leq 0.1$ , \*\* $P \leq 0.001$ , \*\*\* $P \leq 0.0001$  and \*\*\*\* $P \leq 0.00001$  when compared with negative control.

#### 4.6 Oil Red O Assay

Oil Red O assay was used as a fats accumulation indicator for verifying the potential of different extracts at varied concentration gradients in 3T3-L1 adipocytes cells. Some of the image of stained cells was provided in Appendices Figure S8 for reference. The graph obtained from Oil Red O staining on 3T3-L1 adipocytes cells treated with different extracts are listed in Figure 4.3. Figure 4.3 (A), the positive control, Rosiglitazone showed an increased lipid accumulation with the increasing extract concentration from 0.000025 mg/mL to 0.25 mg/mL. As for hexane, ethanol, isopropanol, and diethyl ether solvent

extracts, although all have shown slight decreasing trends in lipid accumulation with increasing concentration from 0.000025 mg/mL to 0.025 mg/mL, nonetheless there are significant increase in lipid accumulation level at 0.25 mg/mL if compared against non-treated control. For dichloromethane, acetone and ethyl acetate extracts, all did not show significant increase in lipid accumulation with increasing extract concentration and all tested concentration of ethyl acetate extracts have lower lipid accumulation as compared to the non-treated control. Increasing lipid accumulation was observed in methanol extract however, at the highest extract concentration (0.25 mg/mL) applied, the lipid accumulation decreased slightly, nonetheless still higher than the non-treated control.

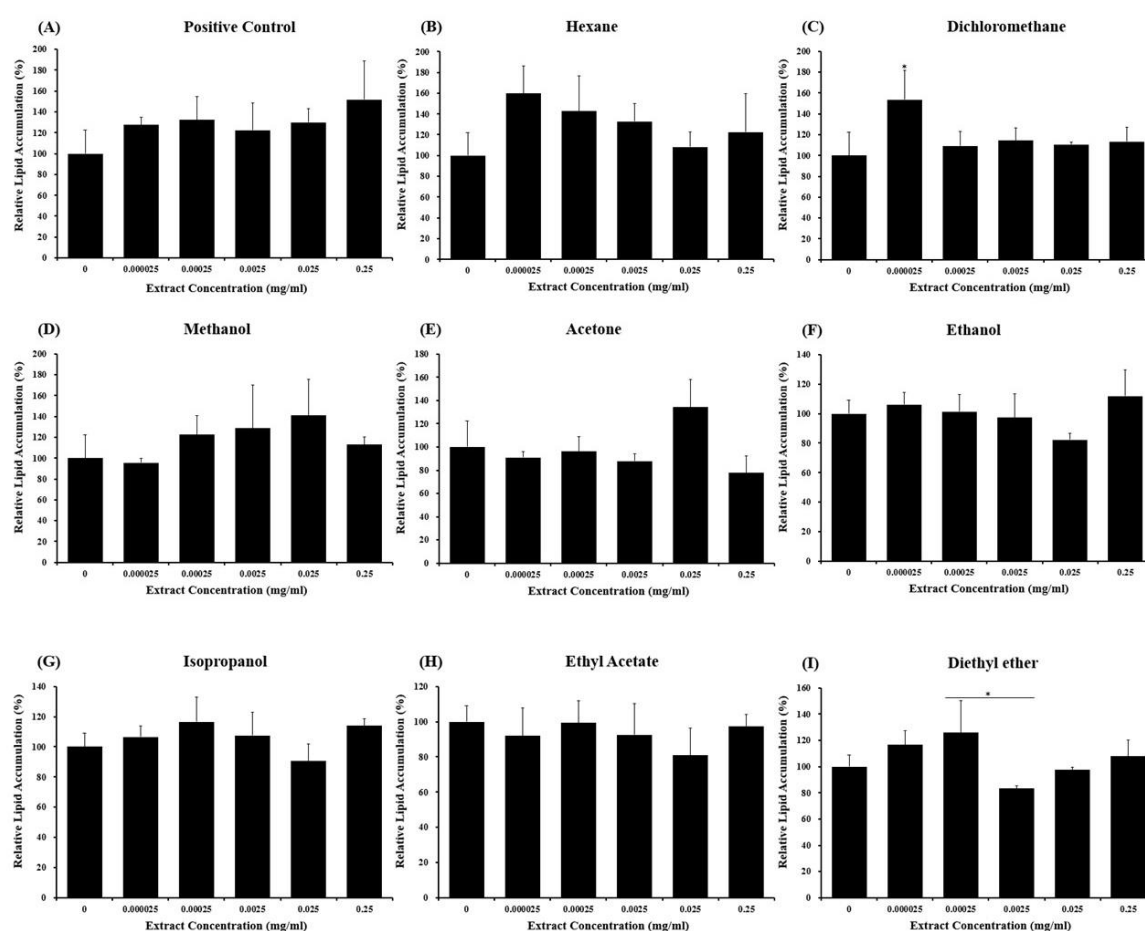


**Figure 4.3:** ORO assay indicating lipid accumulation of adipocyte cells treated with extracts that are extracted using different types of solvents. Data are means  $\pm$  S.D. of three replicates.

$*P \leq 0.1$ ,  $**P \leq 0.001$ ,  $***P \leq 0.0001$  and  $****P \leq 0.00001$  when compared with negative control.

#### **4.7 Adipogenesis Analysis**

By taking both the outcome of MTT and Oil Red O staining assay as reference, four extracts which are hexane, methanol, isopropanol, and diethyl ether were selected for adipogenesis analysis. As shown in Figure 4.4 (A), the positive control showed an increase of triglyceride content with an increasing extract concentration from 0.000025 mg/mL to 0.25 mg/mL. Although generally, both hexane and isopropanol extract do not show any increase in adipogenesis level in 3T3-L1 cells, methanol and diethyl ether extracts have shown an increase of triglyceride accumulation with an increase in extract concentration from 0.000025 mg/mL and peaks at 0.25 mg/mL.

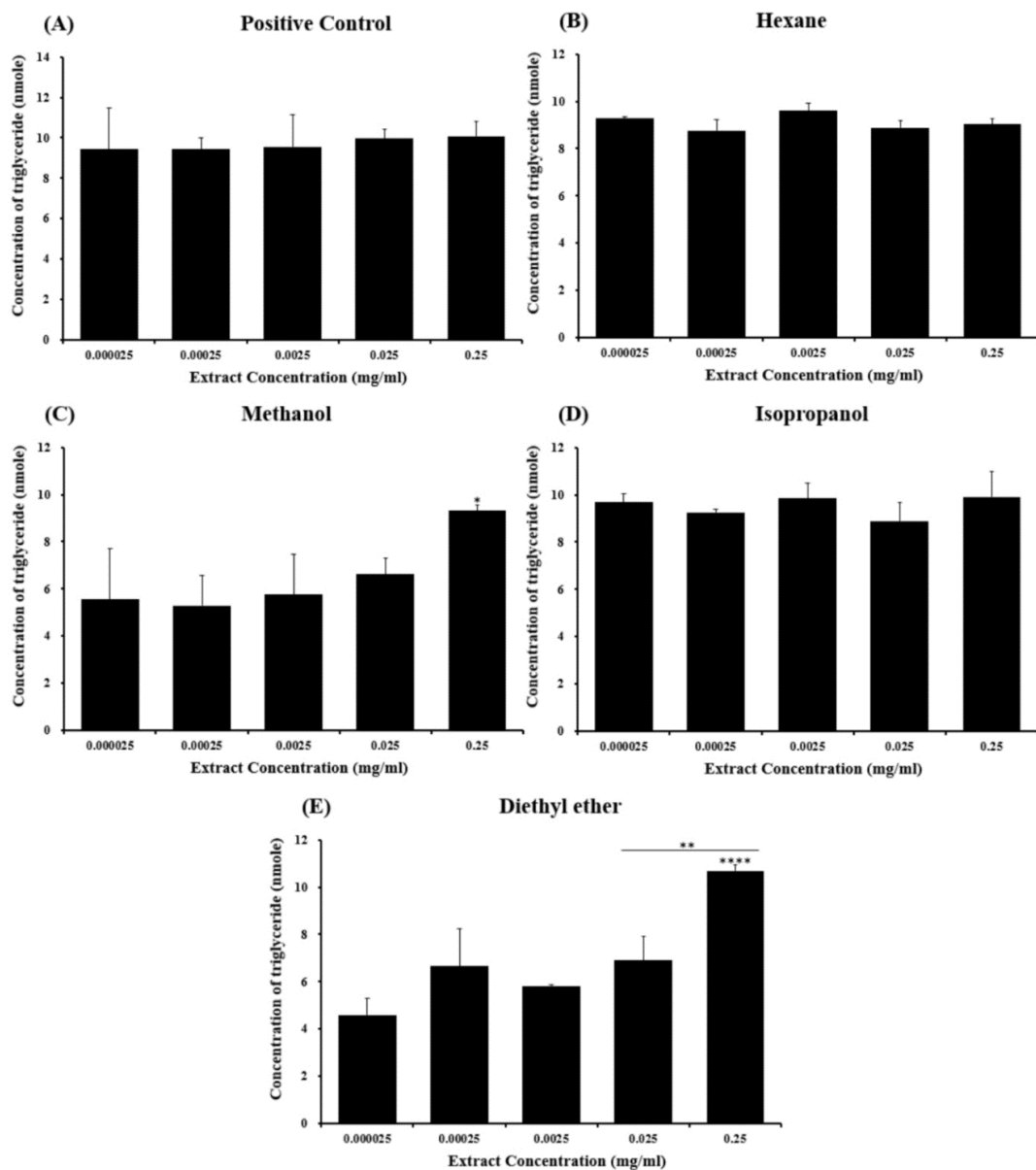


**Figure 4.4:** Adipogenesis assay indicating concentration of lipid accumulation of adipocyte cells treated with extracts that are extracted using different types of solvents. Data are means  $\pm$  S.D. of three replicates. \* $P \leq 0.1$ , \*\* $P \leq 0.001$ , \*\*\* $P \leq 0.0001$  and \*\*\*\* $P \leq 0.00001$  when compared with negative control.

## CHAPTER 5

### DISCUSSION

#### 5.1 Extraction and Identification of Bioactive Compounds

In this study, it was observed that different solvents have shown different extraction efficiency to one another due to difference in polarity index, chemical properties, and functional groups. It is obvious that overall, acetone as extraction solvent yielded highest extraction efficiency. Among all the eight solvents, both alcohol which are methanol (10.64%), and ethanol (8.80%) are having rather low extraction efficiency as compared with other six solvents which have extraction efficiency ranging from 30.77 to 38.30%. By listing the solvents used according to their polarity index in ascending order, hexane, diethyl ether, dichloromethane, isopropanol, ethyl acetate, acetone, methanol, and ethanol, with hexane as the lowest polarity and ethanol as the most polar solvents (Sadek, 1996). The results shown that different solvents with different polarity index resulting in different extraction efficiency (Karseno et al., 2022). The differences in solvent sequence for extraction yield could attribute to the variation in plant biochemical composition. Viscosity, surface tension and polarity are among the factors which influence the extraction efficiency (Alshammari et al., 2021).

Phytochemicals have been categorised in the form of carbohydrates, lipids, terpenoids, phenolic acids and alkaloids with other nitrogen containing metabolites (Huang et al., 2016). By looking into the gas chromatography mass spectrometry results, the result shown that most of the compounds such as methyl stearate, methyl elaidate, methyl palmitate and methyl arachidate are all fatty acids compounds, which are with non-polar properties. It is then corresponded to our results that low-polarity solvents such as hexane, diethyl ether

having very high extraction efficiency compared to the high-polarity solvents such as methanol and ethanol. By referring to Rguez et al. (2023), flavonoids and alkaloids are found to be extracted from solvent with broad range of polarity index, however, the extracted flavonoids from high polarity solvents are found to be quantitatively more than the low polarity solvents. However, for terpenes, glycosides terpenes and steroids, they are absent in the diethyl ether fraction, but they are detected in the high polarity solvents. Besides from the above examples, Selvamuthukumaran and Shi (2017) stated that ethanol and methanol have relatively higher polarity and according to their study, tannins, polyphenols, flavonol, terpenoids and alkaloids are examples of compounds extracted from ethanol, whereas anthocyanin, terpenoids and saponins are from methanol extractions. Flavonoids can be detected from low to middle-range polarity solvents such as acetone and chloroform (Selvamuthukumaran and Shi, 2017).

As stated before, the phytochemicals identified are mostly comprised of fatty acid molecules. Besides from the four compounds that are identified generally from all of the extraction, there are also some other compounds identified. In hexane fractions, bis(2-ethylhexyl) adipate, hexacosane, bis(2-ethylhexyl) phthalate and 3-Ethoxy-4-hydroxyphenylacetonitrile are found to reach abundance level of 10.45%, 10.44%, 3.16 % and 1.14% respectively in the extracts. Bis(2-ethylhexyl) adipate and bis(2-ethylhexyl) phthalate are also being isolated from *Benincasa hispida* (Du et al., 2006). However, these two compounds are plasticizer that is usually adapted from environmental pollution. Since they are synthetic compounds, the discovery of these phthalate in plants are very rare. It has been postulated by Du et al., (2006) that their accumulation in plant might be due to the presence of the former in aquatic environment due to leaching, which were subsequently taken up by the plant and stored in their fruit.

Hexacosene is also found available in other plant species as well that are then found to have analgesic and antinociceptive activity (Githinji et al., 2011; Githinji et al., 2012). Whereas 3-Ethoxy-4-hydroxyphenylacetone nitrile is previously isolated from Frankincense by Alharbi et al. (2022), but it is not further discussed on its properties.

Using dichloromethane as solvent, oxiran-2-ylmethyl tetradecanoate and methyl linoleate was identified at abundances of 1.45% and 1.11% respectively while in methanol extract, methyl linoleate is identified at 1.66%. Oxiran-2-ylmethyl tetradecanoate, also known as myristic acid glycidyl ester in the GCMS database is reported to have antioxidant capacity by Prasath et al. (2019) and Xia et al. (2020). The acetone extract, which exhibits the highest crude extraction efficiency, contains glycidyl palmitate as the most abundant compound at 23.78%, followed by glycidyl oleate, glycidyl stearate, and methyl behenate at abundances of 13.5%, 2.98%, and 1.10% respectively. Methyl linoleate, glycidyl palmitate, glycidyl oleate, glycidyl stearate, and methyl behenate are all fatty acid methyl esters and fatty acid derivatives that are commonly extracted from lipid-rich resources (Zhang et al., 2021). Research done by Jahan et al. (2022) proposed glycidyl oleate as effective analgesic medication through *in-silico* studies.

As for isopropanol and ethyl acetate extracts, apart from the four compounds mentioned earlier, other detected compounds are all below than 1% abundance level, thus are not listed in the Table 4.1. Ethyl stearate and ethyl oleate are found in ethanol extracts at 1.40% and 1.05% abundance respectively. Ethyl oleate had been found to have antimicrobial activity (Akin-Osanaiye et al., 2011). As for diethyl ether extract, butylated hydroxytoluene (2.52%) is the only compound identified apart from the four main compounds. Butylated hydroxytoluene is a type of synthetic compounds that used to prevent the oxidation problems

in meat products (Devatkal et al., 2012). Detection of butylated hydroxytoluene in the extracts might be due to the contamination of the extracts.

However, the available references are mostly on properties and biological active activity of crude extracts instead of compounds. Thus, further exploration on molecules is advisable to target the compounds that cause such effect.

## **5.2 *In-silico* Analysis of Compounds in Engkabang Fruit Extracts**

To determine the presence of lipogenesis inducing factor in the engkabang extracts, molecular docking analysis were performed with eight lipogenesis-related proteins against the GCMS compound list from different solvent extraction. These lipogenesis-related proteins were acetyl-CoA carboxylase, ATP-citrate lyase (ACLY), carbohydrate-responsive element-binding protein (ChREBP), fatty acid synthase (FASN), glucose transporters (GLUTs), peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), stearyl-CoA desaturase-1 (SCD-1) and sterol regulatory element binding protein (SREBP) protein.

The reason acetyl-CoA carboxylase was chosen was because it lies in the junction of lipid synthesis and oxidative metabolic pathway (Jones et al., 2017). Inhibition of acetyl-CoA Carboxylase (ACC) can reduce hepatic de novo lipogenesis (DNL) and could effectively reduce steatosis, inflammation, and fibrosis in animal models of fatty liver disease (Stiede et al., 2017). The available docking references of ACC are suggesting potent ACC inhibitors that would cause reducing lipogenesis effect. By comparing to Bhadauriya et al. (2013) which have ACC inhibitors docking score at -7.0 kcal/mol, all of the analysed compounds in this study have subsequently low binding affinity with scoring of only -3.3

kcal/mol to -4.8 kcal/mol. This is then suggesting that all of the tested compounds may not be potential ACC inhibitors.

By looking into the amino acid residues, three compounds which are ethyl stearate, methyl behenate and methyl elaidate have interactions with ACC at two amino acids (GLY2162 and GLY2163) with scoring of -3.5 to -3.7 kcal/mol. Formation of hydrogen bonds with GLY2162 are also observed in Bhadauriya et al. (2013), Huang et al. (2015) and Wu et al. (2021) research but no interactions are found for GLY2163 in previous studies. Besides, seven compounds which are bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) phthalate, ethyl oleate, glycidyl oleate, glycidyl stearate, methyl arachidate and methyl stearate have only one interaction with ACC proteins at amino acid PHE2160, GLY2162, GLY2202, GLU2230. All of the amino acid residues are found in previous docking study except for GLY2202 (Bhadauriya et al., 2013; Huang et al., 2015; Wu et al., 2021). The compounds that are not mentioned have no hydrogen bond found with ACC proteins.

Second lipogenesis-inducing factor chosen for docking analysis was ATP-citrate lyase. The main reason was that upregulation of ACLY enzymatic activity have been found to increase lipid droplet formation in the adipose tissue which subsequently supports cell growth (Lucenay et al., 2016). ACLY catalyses a key regulatory step in *de novo* lipogenesis in mammalian cells by cleaving mitochondrially derived citrate to generate cytosolic acetyl CoA plus oxaloacetate, where both were found to be key factors in driving Krebs cycle (Dufort et al., 2014). Looking at the docking score, there are only four compounds that achieved scoring of lower than -7.0 kcal/mol, which are ethyl oleate (-7.0 kcal/mol), bis(2-ethylhexyl) adipate (-7.2 kcal/mol), butylated hydroxytoluene (-7.6 kcal/mol) and bis(2-ethylhexyl) phthalate (-8.4 kcal/mol). As compared to Wang et al. (2022) which have

docking score of -7.1 to -9.9 kcal/mol, compounds listed above have higher docking score which is not as stably docked comparatively.

However, by comparing the four compounds mentioned above on their hydrogen bonds formation, no hydrogen bonds interactions are found on three of them except ethyl oleate which have three hydrogen bonds forming with amino acid residues GLY342 and GLY380. According to Miladiyah and Nuryadi (2022) and Wang et al. (2022), amino acid interactions are usually formed with residues GLY342, THR348, ASN349, and GLY380, which are important in ATP citrate lyase inhibitory activity in lipid metabolism. Apart from butylated hydroxytoluene and hexacosene which have no hydrogen bonding found, other compounds have three to eight interactions with the ACLY proteins with all the above-mentioned amino acid residues.

The activation of carbohydrate-responsive element-binding protein (ChREBP), which is a crucial regulator of lipogenesis, is triggered by an increase in glucose flow. As a result, it is closely associated with the functioning of GLUT4 in adipocytes. In cases where GLUT4 expression is defective, leading to reduced ChREBP activation and possibly hindered GLUT4 translocation, the diminished ChREBP activity might be responsible for the decreased expression of genes involved in the synthesis of new fats in obesity (Eissing et al., 2013). Carbohydrate response element-binding protein (ChREBP) is necessary for both the baseline and carbohydrate-induced expression of various liver enzymes that play a vital role in the coordinated control of glucose metabolism, fatty acid metabolism, and the production of fatty acids and triglycerides in living organisms (Iizuka et al., 2004). Additionally, Witte et al. (2015) also discovered that ChREBP connects *de novo* lipogenesis to PPAR $\gamma$  activity and the differentiation of adipocytes. The docking scores of all the compounds with ChREBP are around -4.1 to -6.3 kcal/mol which are relatively low as

compared to Gillella and Sumithra (2022) which have docking score ranging from -8.1 to -9.2 kcal/mol.

In this study, the interaction between compounds and ChREBP protein included amino acid residues LYS51, ARG58, LYS122, ASN123, ASN124, ARG128, ARG129, TYR130, ASN175. All the amino acids are found to be present in other studies (Hou et al., 2017; Sijbesma et al., 2020; Gillella and Sumithra, 2022), except ASN175. For docking analysis with ChREBP protein, only butylated hydroxytoluene, hexacosane, methyl palmitate and oxiran-2-ylmethyl tetradecanoate have no interactions found. Other thirteen analysed compounds have different number of hydrogen bonding forming between the protein-ligand complex ranging from one to twelve hydrogen bonding with methyl arachidate having 12 as the highest number of bonding formed among all the thirteen compounds.

Fatty acid synthase (FASN) is the central enzyme in *de novo* lipogenesis, catalysing all of the enzymatic steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FASN activity measurements and FASN mRNA levels are frequently used as markers of DNL, and changes in FASN activity are due primarily to alterations in the transcription rate of the FASN gene (Wang et al., 2004). Bis(2-ethylhexyl) adipate (-7.4 kcal/mol), butylated hydroxytoluene (-7.4 kcal/mol) and bis(2-ethylhexyl) phthalate (-7.9 kcal/mol) are the three compounds that have a strong binding to FASN protein among all of the tested compounds.

However, only Bis(2-ethylhexyl) adipate have one interaction with amino acid ALA2062 by forming a 2.176Å hydrogen bonding with it and no amino acid residues interactions have been found between the other two compounds with FASN protein. By referring to John et al. (2015), Angeles and Hudkins (2016) and Pulla and Ahil (2021), the catalytic amino acids of FASN protein are SER2021 and TYR2034 which are both found

interacting with some of the compounds analysed namely ethyl oleate, glycidyl palmitate, glycidyl stearate, methyl elaidate and 3-ethoxy-4-hydroxyphenylacetonitrile. LYS1995 amino acid interaction are also found at another research as well as the active cavity residue (John et al., 2015; Pulla and Ahil, 2021). Other amino acid residues interaction found such as GLY1895, PHE1896, GLY1897, GLY2061, ALA2062, LEU2069 have not been mentioned and found in previous studies. Among all the compounds, methyl elaidate with docking score of -6.2 kcal/mol have up to six hydrogen bonding interaction. Previous study by Nuiyen et al. (2022), is showing that increasing level of methyl elaidate in AX-024 (a small molecule) that inhibit Nck-CD3 cells upon activation correlated with an increase in lipid content and lipid droplets.

GLUT1 protein as glucose transporter certainly stand an important role in lipogenesis (Macotella et al., 2009). A family of facilitative glucose transporters (GLUTs) is involved in regulating tissue-specific glucose uptake and metabolism in the liver, skeletal muscle, and adipose tissue to ensure homeostatic control of blood glucose levels (Chadt and Al-Hasani, 2020). For GLUT1 protein, only butylated hydroxytoluene have scoring at -7.0 kcal/mol but with no hydrogen bonding interaction. The rest of the compounds have docking score of -5.3 to -6.5 kcal/mol, which is only about intermediate of binding affinity (Almahmoud et al., 2019).

The amino acid residues found interacting are mostly consistent with studies by Almahmoud et al. (2019) and Sun et al. (2023) which are THR137, GLN283, ASN288, TRP388, TRP412, except for ASN415. As mentioned in Almahmoud et al. (2019), PHE379, TRP88 and TRP412 have important roles in GLUT1 function and glucose uptake. However, PHE379 was not found in all GLUT1-compounds interactions. Butylated hydroxytoluene

and hexacosane are the only two compounds that have no interactions at all with GLUT1 protein.

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is critical for the regulation of lipogenesis and promotes lipid storage (Rosen et al., 1999). Studies indicated that lipogenic genes in the liver could be induced by PPAR $\gamma$  (Kersten, 2001). According to the study of Kadegowda et al. (2009), activation of PPAR $\gamma$  in bovine mammary cells would upregulate mRNA expression of lipogenic target genes identified primarily in rodent studies. By referring to docking studies did on PPAR $\gamma$  by Stalin et al. (2016); Penas et al. (2017) and Prasad et al. (2018), their docking score are at the range of -7.6 to -10.3 kcal/mol. Whereas for this research, none of the analysed compounds achieved binding affinity of lower than -7.0 kcal/mol, thus indicating intermediate binding effect of all the compounds with PPAR $\gamma$  protein.

Different from previously discussed proteins, most of the compounds did not have any interactions with the protein, only four compounds have hydrogen bonding which are glycidyl oleate, glycidyl palmitate, methyl elaidate and methyl linoleate. Their interactions involved SER289, HIS449 and HIS323 amino acid residues which are consistent with research by Penas et al. (2017) and Prasad et al. (2018). Stalin et al. (2016) have stated that amino acid residues GLN286, ARG288, SER289, HIS323, HIS449 and TYR473 present in PPAR $\gamma$  are the key residues for their interactions.

In the research of Rizki et al. (2006), the loss of SCD-1 mRNA from the liver, caused the expression of upstream lipogenic genes to be reduced significantly while downregulation of lipogenic gene expression in MCD-fed mice could be attributable in part to the MCD-mediated suppression of SREBP-1 mRNA, which regulates a number of target genes involved in the lipogenic pathway. Stearoyl-CoA desaturase-1 (SCD-1), a key enzyme in

lipogenesis, catalyzes the conversion of SFA to MUFA, and inhibition of its activity impairs lipid synthesis (Cheng et al., 2016). By looking at the docking scores in this analysis, eight out of seventeen compounds did achieve docking score of lower than -7.0 kcal/mol. Among them, bis(2-ethylhexyl) phthalate have the most negative binding affinity (-8.9 kcal/mol) and it also have one amino acid interaction with amino acid residue ASN261 by forming a 1.849Å hydrogen bond.

Although there are half of the compounds having good binding affinity with SCD-1, which is slightly better as compared to previously discussed proteins, the compounds having hydrogen bonding interactions among them are limited. 3-Ethoxy-4-hydroxyphenylacetonitrile, bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) phthalate and glycidyl palmitate are the only four compounds that have amino acid interactions found with amino acid ASN144, HIS153, TRP258 and ASN261 at docking score of -7.3, -8.0, -8.9 and -6.0 kcal/mol respectively. All of the amino acid residues identified are also found in the study of Huang et al. (2020) which docking are conducted between SCD-1 protein with compounds derived from natural product databases.

Moving on to the next protein, SREBP protein have essential role in hepatic triglyceride and cholesterol syntheses (Ji et al., 2006). Three SREBPs with overlapping functions control the lipid synthesis in the liver and other tissues of mammals. SREBP-1c regulate genes involved in triglyceride synthesis such as activation of glucokinase, acetyl-CoA synthetase and carboxylase, and stearoyl-CoA desaturase and SREBP2 regulates genes involved in cholesterol synthesis such as activation of HMG-CoA synthase and reductase, while SREBP-1a regulates both triglyceride and cholesterol syntheses (Horton et al., 2003). SREBP protein as compared to other proteins have the most compounds achieved docking

score at -7.0 kcal/mol and above. There are a total of twelve compounds having good docking score and bis(2-ethylhexyl) phthalate at -8.3 kcal/mol is the best among all compounds.

Different from the docking analysis with SCD protein, the docking result with SREBP protein have a total of seven compounds that have hydrogen bond interactions. All the seven compounds interact with the same amino acid which is HIS435 at different hydrogen bond length. The findings of Chen et al. (2020) stated that HIS435 is one of the key bindings of SREBP protein which is related to its function in triglyceride and cholesterol syntheses.

Besides from active site analysed in the domain, there are also allosteric site which sometimes would likely to regulate the protein activity. Allosteric regulation of protein activity occurs when the regulatory trigger, such as the binding of a small-molecule effector or inhibitor, happens at a distance from the active region of the protein or protein complex (Laskowski et al. 2009). Different stimuli, such as ligand binding, protein modification, or light absorption, might cause changes in the distant region (Fauser et al. 2022). These changes are communicated to the active site via an extensive network of essential amino acids in the protein structure and can either increase or hinder protein function (Dokholyan, 2016).

In this study, molecular docking analysis is conducted at their active sites and interactive locations where other small molecules are found to regulate lipogenesis, including their active sites and their allosteric site (Huang et al., 2015; Angeles and Hudkins, 2016; Prasad et al., 2018; Almahmoud et al., 2019; Chen et al., 2020; Huang et al., 2020; Sijbesma et al., 2020; Wang et al. 2022). However, small molecules as inhibitor or activator which would up-regulate or down-regulate a process are not able to be identified by using molecular docking analysis alone. Thus, this is a limitation of molecular docking, and it can

only stand as an indicator on how well the small molecules bind and interacts with those proteins.

In conclusion, this result suggested that the list of compounds which are mainly fatty acids works better with SCD and SREBP proteins based on their docking score. Among all the docking analysis, bis(2-ethylhexyl) phthalate docked into Stearoyl-CoA Desaturase (SCD) have the best docking score at -8.9 kcal/mol with one amino acid interaction at amino acid residue, ASN261. Bis(2-ethylhexyl) phthalate are also the highest scorer for five proteins (ACLY, FASN, PPAR $\gamma$ , SCD and SREBP) compared to all the compounds. Among all the compounds, bis(2-ethylhexyl) phthalate, bis(2-ethylhexyl) adipate and butylated hydroxytoluene have four docking with lipogenic proteins with docking score more negative than -7.0 kcal/mol. However, butylated hydroxytoluene have no interaction with all analysed lipogenic proteins. According to the docking analysis conducted in this study, bis(2-ethylhexyl) phthalate is a potential candidate that can affect lipogenesis process.

### **5.3 Cytotoxicity Test**

Cytotoxicity is important to make sure the correct and safe dosage to be used especially when it comes to drugs. The cytotoxicity assay is usually being carried out by measuring the effect of a drug on the growth of a population of cells and the endpoint is an estimate of cell number (Plumb, 2004). For this research, the concentrations of extracts treated on the 3T3-L1 cells are set in the range of 0.000025 to 0.25 mg/mL. Although different types of solvents are being used to carry out the extraction of phytochemicals in the fruit, the cell viability of each extract after one week of treatment are showing similar patterns. Most of the extracts including positive control, methanol, acetone, ethanol, isopropanol, ethyl acetate and diethyl ether at 0.25 mg/ml concentration have a typically low

cell viability from the range of 5.75 % to 58.14%, which decrease about 50 to 95% of cell survivability. For positive control Rosiglitazone, at 0.025 mg/ml, the cell viability is up to 57.70% only which is relatively low as compared to the tested extracts. The extracts all achieved cell viability of above than 90% at 0.025 mg/mL. Thus, the recommended dose is at 0.025 mg/mL to avoid toxicity effect to cells and organism.

As compared to existing research regarding different types of extracts and compounds towards cytotoxicity of 3T3-L1 cells, Rhyu et al. (2014) tested the pear pomace aqueous extracts and Mohan et al. (2020) tested on *Artabotrys suaveolens* methanolic extracts, both of the results are showing relatively high cell viability (above 90%) even at a concentration of 400 µg/mL and 250 µg/mL which is the highest concentration tested on the 3T3-L1 cells. However, the extracts in this research tested at 0.25 mg/mL, have low cell viability as mentioned above, except for hexane and dichloromethane which both achieved cell viability of above 90% at 250 µg/mL.

However, by comparing to Nishina et al. (2017) result, the cell viability of 3T3-L1 after treatment on hexane, ethyl acetate and methanol extracts of *Vitex trifolia* L., are showing a different result. The cell viability decreased gradually with the increasing concentration of extracts treated. As for hexane extracts, there is an obvious decrease of cell viability at 100 µg/mL extract concentration compared to 30 µg/mL extract concentration which aligned which aligned with the result in this study.

Thus, the toxicity of drug is important and will be different among compounds. So, it is crucial to detect the cytotoxicity of an unknown drugs before proceeding to *in-vivo* and *in-vitro* analysis as toxicity of cells could disrupt cell functions and would then affect the accuracy of results.

#### 5.4 Lipogenesis Effect of Extracts

Lipogenesis is a hot topic as tons of research are carried out on it to identify potential anti-diabetic drug for the treatment on obesity, cardiac and liver diseases. The process of lipogenesis involves the synthesis of fatty acids from glucose, ultimately leading to the accumulation of fat within an organism (Ameer et al., 2014). Lipogenesis poses a detrimental impact on human health due to the deposition of fat, which can lead to obesity, cardiovascular disease, and various other health complications. However, fat accumulation might not be bad for animals where meat that is rich in fat deposition are proven to come out with a good texture which is favoured by consumers (Schumacher et al., 2022).

By taking the MTT results as reference, hexane, methanol, isopropanol, and diethyl ether are the four solvents that are chosen for further lipogenesis analysis. In the oil red o staining analysis, hexane, isopropanol, and diethyl ether are showing a slight decrease followed by a drastic increase in relative lipid accumulation level of the cells. Besides, all of the tested concentration of the solvents' extract on 3T3-L1 cells have managed to accumulate lipid at a higher level as compared to the non-treated cells. The increasing of triglyceride content after treated with crude extracts of *Torreya nucifera* seed oil are also being observed in Koh et al. (2021) analysis. In the case of the methanol extract, the relative lipid accumulation exhibits a gradual increase as the concentration of the treated extract increases. However, a decrease is observed once the extract concentration reaches 0.25 mg/ml. This decline could be attributed to the potential toxicity of cells, which may have led to inaccuracies in the results.

When subjecting hexane, isopropanol, methanol and diethyl ether extracts to an additional analysis using an adipogenesis kit, it was observed that both hexane and isopropanol consistently achieved triglyceride levels of nine nmole across all tested

concentrations. On the other hand, both methanol and diethyl ether exhibited a notable and dose-dependent increase in triglyceride levels. These findings provide further evidence that all the extracts possess favourable effects on fat accumulation, ultimately leading to an increase in triglyceride content within the adipocyte cells.

The current body of research exploring the augmentation and stimulation of lipogenesis is limited, as most studies predominantly concentrate on the opposing objective of reducing and inhibiting lipogenesis, targeting potential drugs for the treatment of diabetes and obesity-related health issues. Nevertheless, there are still studies being conducted on lipid accumulation investigations. Vahmani et al. (2015) conducted a study where different fatty acids were administered to cells, resulting in a significant increase in total fatty acid content compared to control cells, which aligns with our findings. Additionally, other compounds such as Orexin-A (Skrzypski et al., 2011) and tetrabromobisphenol-A (Chappell et al., 2018) have been discovered to promote lipogenesis. These research endeavors primarily focus on the expression of lipogenic genes, including ACC, FAS, PPAR $\gamma$ , and GLUT4 proteins, which are also included in the molecular docking analysis conducted in this research.

To relate the *in-silico* analysis with the *in-vitro* analysis of this research, all of the tested extracts (hexane, methanol, isopropanol, diethyl ether) have high triglyceride concentration as compared to positive control, and methanol and diethyl ether are further showing an increasing triglyceride content with a higher concentration of extracts applied. The compounds that the four extracts have in common might be the target compounds that have caused lipogenesis to occur. As explained in GCMS results above, only four compounds that have in all the extracts in common which are methyl stearate, methyl elaidate, methyl palmitate and methyl arachidate. By referring to molecular docking results,

they are all achieving good docking score ranging from -6.7 kcal/mol to -7.1 kcal/mol by docking into SREBP protein. Besides, the docking analysis are showing hydrogen bond interaction of methyl elaidate and methyl palmitate with the HIS435 critical amino acid residues of the SREBP protein. As methyl elaidate have a better docking score as compared to methyl palmitate, hence it is suggested that methyl elaidate is the potential lipogenesis inducing factors which induce lipogenesis via the regulation of SREBP protein. Methyl elaidate also known as 9-octadecenoic acid, methyl ester is a type of fatty acid methyl ester which possess antioxidant and anti-cancer properties (Hagr et al., 2021). Methyl elaidate was reported to inhibit cancer cell proliferation by induction of apoptosis (Adebayo et al., 2018). However, no lipogenesis related effect of methyl elaidate have been reported yet.

As crude extracts are used in this experiment, there might be a synergistic or cancelling effect among compounds which may affected the overall results. Thus, it is recommended to purify and test the pure compounds on the cells to have a much clearer insight on the effect of each compound on lipogenesis. Besides, the docking analysis are carried out on the compounds that achieved 1% abundancy level in the GCMS analysis of the extracts. The lipogenesis effects might be triggered and stimulated by minor components instead of major components (Jugreet et al., 2020).

## CHAPTER 6

### CONCLUSIONS AND RECOMMENDATIONS

#### 6.1 Conclusions

To study set forth to extracted and identify compounds from engkabang fruit. Then next step was directed towards the *in-silico* study of the identified compounds with lipogenesis related protein. Finally, the *in-vitro* assay was carried out to measure the lipogenesis effect of the extracts by treated them on 3T3-L1 cell line. From the results obtained, methanol and ethanol which are solvents with higher polarity index has the lowest extraction efficiency. Besides, the bioactive compounds are identified by using GCMS approaches. Most of the bioactive compounds identified are fatty acids where there are four compounds that are available and dominant in all the extracts. The four compounds are methyl stearate, methyl elaidate, methyl palmitate and methyl arachidate.

The lipogenic activity of the compounds are first successfully identified through *in-silico* analysis. Methyl stearate, methyl elaidate, methyl palmitate and methyl arachidate are showing promising results at their docking analysis with SREBP protein and they achieved docking score of -6.7 kcal/mol to -7.1 kcal/mol. SREBP is one of lipogenesis regulating protein where it regulates genes that involved in triglyceride synthesis such as activation of glucokinase, ACC and SCD.

The crude extracts of engkabang extracts are then being used to test on cytotoxicity and lipogenic effect on 3T3-L1 cells. Generally, the 3T3-L1 cells viability maintained above 90% at 0.025 mg/mL and below which is recommended as the safe dose to be used for future *in-vivo* studies. Besides, the treatment of crude extracts at various concentrations on 3T3-L1 cells were tested to check on lipogenic activity of the crude extracts. Both methanol and

diethyl ether extracts are showing significant increasing lipid accumulation compared to non-treated group which indicates increasing lipogenesis occurrence. By looking into the bioactive compounds available in both extracts, only the four compounds that are available in all of the extracts are found. However, as the four compounds are available in all the extracts but only methanol and diethyl ether extracts are showing lipogenic events. Thus, the lipogenic events might be due to the minor component such as nomelidine and cis-verbenol available in the extracts. It might also be due to synergistic or antagonistic events between compounds that altered the lipogenic activity.

## **6.2 Recommendations**

This study had evaluated the crude extracts of engkabang on their lipogenic activity on adipocyte cells. However, as compounds might have interaction among each other, such as synergistic or antagonistic event, further studies up to molecule levels are needed to give a much clearer insight on what is causing the lipogenic activity in the engkabang extracts. Besides, the compounds analysed through GCMS is a very big database and it had been sorting down to analyse compounds with 1% abundance level in the whole extract composition in this study. Nevertheless, the biological activity might be due to minor constituent and more analysis could be done on the minor component analysed.

Furthermore, the freshness of samples is recommended to be taken into considerations. As the fruit of engkabang is limited and it can only be obtained in bulk numbers once in several years, the phytochemicals available in the samples might have lost or degraded along with time. It is advisable to collect fresher samples to carried out for future studies.

As mentioned before, there is limitation of molecular docking analysis where it can only indicate how well is the interaction and binding of molecules to the protein and it will not indicate the upregulation or downregulation of the expression of the protein. Thus, further experiment such as animal studies and further proteomic analysis is suggested to be carried out.

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## APPENDICES

**Table S1:** Grid box details for molecular docking analysis.

| Protein       | Pdb id | Center of grid box |        |        | Size of grid box |
|---------------|--------|--------------------|--------|--------|------------------|
|               |        | x                  | y      | z      |                  |
| ACC           | 3FF6   | 5.94               | 23.72  | 25.73  | 22 x 22 x 22     |
| ACLY          | 6O0H   | 129.81             | 157.48 | 179.7  | 32 x 32 x 32     |
| ChREBP        | 5F74   | -39.51             | 11.11  | 12.35  | 25 x 25 x 25     |
| FAS           | 4PIV   | 10.47              | 6.29   | -33.47 | 30 x 30 x 30     |
| GLUT1         | 5EQI   | -39.51             | 11.11  | 12.35  | 25 x 25 x 25     |
| PPAR $\gamma$ | 2PRG   | 50.01              | -36.89 | 19.48  | 22 x 22 x 22     |
| SCD           | 4YMK   | -31.17             | -27.57 | 10.93  | 35 x 35 x 35     |
| SREBP         | 6K9M   | 11.59              | -10.03 | -35.42 | 22 x 22 x 22     |

**Table S2:** Extraction efficiency.

| Solvent  | Replicate | Initial engkabang fruit weight (g) | Final extract weight (g) | Extraction efficiency (%) | Average of extraction efficiency (%) |
|----------|-----------|------------------------------------|--------------------------|---------------------------|--------------------------------------|
| Hexane   | 1         | 20.08                              | 5.231                    | 26.05                     | 30.77                                |
|          | 2         | 20.057                             | 5.288                    | 26.36                     |                                      |
|          | 3         | 20.006                             | 7.979                    | 39.88                     |                                      |
| DCM      | 1         | 20.148                             | 6.716                    | 33.33                     | 34.12                                |
|          | 2         | 20.039                             | 6.684                    | 33.35                     |                                      |
|          | 3         | 20.016                             | 7.142                    | 35.68                     |                                      |
| Methanol | 1         | 20.062                             | 2.091                    | 10.42                     | 10.64                                |
|          | 2         | 20.026                             | 2.377                    | 11.87                     |                                      |
|          | 3         | 20.096                             | 1.933                    | 9.62                      |                                      |
| Acetone  | 1         | 20.065                             | 5.155                    | 25.69                     | 38.30                                |
|          | 2         | 20.036                             | 10.836                   | 54.08                     |                                      |
|          | 3         | 20.007                             | 7.029                    | 35.13                     |                                      |

|               |   |        |       |       |       |
|---------------|---|--------|-------|-------|-------|
| Isopropanol   | 1 | 20.092 | 4.353 | 21.67 | 32.88 |
|               | 2 | 20.016 | 6.768 | 33.81 |       |
|               | 3 | 20.009 | 8.637 | 43.17 |       |
| Ethanol       | 1 | 20.114 | 1.575 | 7.83  | 8.80  |
|               | 2 | 20.005 | 2.491 | 12.45 |       |
|               | 3 | 20.01  | 1.222 | 6.11  |       |
| Ethyl Acetate | 1 | 20.158 | 6.307 | 31.29 | 30.94 |
|               | 2 | 20.02  | 6.571 | 32.82 |       |
|               | 3 | 20.01  | 5.744 | 28.71 |       |
| Diethyl ether | 1 | 20.033 | 7.35  | 36.69 | 37.02 |
|               | 2 | 20.016 | 7.129 | 35.62 |       |
|               | 3 | 20.016 | 7.756 | 38.75 |       |

**Table S3:** All compounds in engkabang extracts identified by GCMS.

| No | Name in GCMS data file                                           | Pubchem id | Pubchem name              | Solvent                                                                            |
|----|------------------------------------------------------------------|------------|---------------------------|------------------------------------------------------------------------------------|
| 1  | Naphthalene                                                      | 931        | Naphthalene               | Acetone                                                                            |
| 2  | n-Hexadecanoic acid                                              | 985        | Palmitic Acid             | Acetone                                                                            |
| 3  | Benzyl Benzoate                                                  | 2345       | Benzyl Benzoate           | Acetone                                                                            |
| 4  | Dibutyl phthalate                                                | 3026       | Dibutyl Phthalate         | Acetone, Hexane                                                                    |
| 5  | 3-Methyl-1-(5-oxohexyl)-7-propyl-3,7-dihydro-1H-purine-2,6-dione | 4938       | Propentofylline           | Diethyl ether                                                                      |
| 6  | Octadecanoic acid                                                | 5281       | Stearic Acid              | Acetone                                                                            |
| 7  | Benzene, pentachloronitro                                        | 6720       | Pentachloronitrobenzene   | DCM                                                                                |
| 8  | 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester          | 6782       | Diisobutyl phthalate      | Acetone, Hexane                                                                    |
| 9  | Bis(2-ethylhexyl) adipate                                        | 7641       | Bis(2-ethylhexyl) adipate | DCM, Hexane, Isopropanol                                                           |
| 10 | Isopropyl myristate                                              | 8042       | Isopropyl Myristate       | Acetone                                                                            |
| 11 | Decanedioic acid, diethyl ester                                  | 8049       | Diethyl sebacate          | DCM                                                                                |
| 12 | Octadecanoic acid, ethyl ester                                   | 8122       | Ethyl stearate            | Ethanol                                                                            |
| 13 | Isopropyl stearate                                               | 8162       | Isopropyl stearate        | Isopropanol                                                                        |
| 14 | Hexadecanoate <methyl->                                          | 8181       | Methyl palmitate          | Acetone, DCM, Diethyl ether, Ethanol, Ethyl Acetate, Hexane, Isopropanol, Methanol |
| 15 | Dodec-1-ene                                                      | 8183       | 1-Dodecene                | Acetone                                                                            |
| 16 | Methyl stearate                                                  | 8201       | Methyl Stearate           | Acetone, DCM, Diethyl ether, Ethanol, Ethyl Acetate, Hexane,                       |

|    |                                                                               |       |                                    |                                                                                                   |
|----|-------------------------------------------------------------------------------|-------|------------------------------------|---------------------------------------------------------------------------------------------------|
|    |                                                                               |       |                                    | Isopropanol,<br>Methanol                                                                          |
| 17 | Palmitoyl chloride                                                            | 8206  | Palmitoyl chloride                 | Acetone                                                                                           |
| 18 | Tetradecanol <n->                                                             | 8209  | 1-Tetradecanol                     | Acetone, Hexane                                                                                   |
| 19 | Docosanoic acid                                                               | 8215  | Docosanoic acid                    | DCM                                                                                               |
| 20 | Bis(2-ethylhexyl) phthalate                                                   | 8343  | Bis(2-ethylhexyl) phthalate        | DCM, Hexane                                                                                       |
| 21 | Isopropyl palmitate                                                           | 8907  | Isopropyl Palmitate                | Hexane                                                                                            |
| 22 | Hexadecanoic acid, ethyl ester                                                | 12366 | Ethyl palmitate                    | Ethanol                                                                                           |
| 23 | Heneicosane                                                                   | 12403 | Heneicosane                        | Acetone                                                                                           |
| 24 | Hexacosane <n->                                                               | 12407 | Hexacosane                         | Hexane                                                                                            |
| 25 | Docosanoic acid, methyl ester                                                 | 13584 | Methyl behenate                    | Acetone                                                                                           |
| 26 | Eicosanoate <methyl->                                                         | 14259 | Methyl arachidate                  | Acetone, DCM,<br>Diethyl ether,<br>Ethanol, Ethyl<br>Acetate, Hexane,<br>Isopropanol,<br>Methanol |
| 27 | Dodecanoic acid, 2,3-dihydroxypropyl ester                                    | 14871 | 2,3-Dihydroxypropyl dodecanoate    | Diethyl ether                                                                                     |
| 28 | Heptadecanoic acid, methyl ester                                              | 15609 | Methyl heptadecanoate              | Acetone, Ethanol                                                                                  |
| 29 | Eicosene                                                                      | 18936 | 1-Eicosene                         | Acetone                                                                                           |
| 30 | Benzoic acid, 2,3,5,6-tetrachloro-4-<br>[(methylthio)carbonyl]-, methyl ester | 19583 | Glenbar                            | DCM                                                                                               |
| 31 | Benzene, 1,3,5-trichloro-2,4-dinitro                                          | 22696 | 1,3,5-Trichloro-2,4-dinitrobenzene | Methanol                                                                                          |
| 32 | 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl)<br>ester                      | 22932 | Bis(2-ethylhexyl) terephthalate    | Hexane                                                                                            |
| 33 | 2-Hydroxy-3,5,6-trichloropyridine                                             | 23017 | 3,5,6-Trichloro-2-pyridinol        | Diethyl ether                                                                                     |
| 34 | 1-Nonadecene                                                                  | 29075 | 1-Nonadecene                       | Acetone, Hexane                                                                                   |
| 35 | Tetrachloroethylene                                                           | 31373 | Tetrachloroethylene                | Isopropanol                                                                                       |
| 36 | Butylated Hydroxytoluene                                                      | 31404 | Butylated Hydroxytoluene           | Acetone, Diethyl<br>ether                                                                         |

|    |                                                                        |        |                                           |                         |
|----|------------------------------------------------------------------------|--------|-------------------------------------------|-------------------------|
| 37 | Pentanoate <butyl->                                                    | 61137  | Butyl valerate                            | DCM                     |
| 38 | 3-Pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-nitroso-, methyl ester | 62103  | N-Nitrosoguvacoline                       | Methanol                |
| 39 | Glycidol stearate                                                      | 62642  | Glycidyl stearate                         | Acetone, Hexane         |
| 40 | Octadecanoic acid, ethenyl ester                                       | 66077  | Vinyl stearate                            | Acetone                 |
| 41 | Myristoyl chloride                                                     | 66986  | Myristoyl chloride                        | Diethyl ether           |
| 42 | 1-Hexacosanol                                                          | 68171  | 1-Hexacosanol                             | Acetone                 |
| 43 | Androst-4-en-3-one, 4-chloro-17-hydroxy-, (17.beta.)-                  | 68947  | Clostebol                                 | Diethyl ether           |
| 44 | Stearic anhydride                                                      | 69485  | Stearic anhydride                         | Acetone, DCM            |
| 45 | Decyl sulfide                                                          | 69662  | Decyl sulfide                             | Acetone                 |
| 46 | Cyclododecanecarboxylic acid                                           | 70165  | Cyclododecanecarboxylic acid              | Diethyl ether           |
| 47 | 1-Tetracosene                                                          | 82436  | 1-Tetracosene                             | Acetone                 |
| 48 | Nickel, bis(dimethylcarbomodithioato-S,S')-, (SP-4-1)-                 | 84966  | Nickel dimethyldithiocarbamate            | Diethyl ether, Methanol |
| 49 | Ethyl 2-bromotetradecanoate                                            | 85791  | Ethyl 2-bromotetradecanoate               | DCM                     |
| 50 | 17.alpha.-Hydroxypregnenolone                                          | 91451  | 17alpha-Hydroxypregnenolone               | Hexane                  |
| 51 | Pregnane-3,20-dione, (5.beta.)-                                        | 92745  | 5beta-Pregnane-3,20-dione                 | DCM                     |
| 52 | Endosulfan ether                                                       | 97768  | Endosulfan ether                          | Diethyl ether           |
| 53 | Nonadecanenitrile                                                      | 98566  | Nonadecanenitrile                         | Isopropanol             |
| 54 | 21-Acetoxy-pregn-4-ene-3,11,20-trione                                  | 102006 | 11-Dehydrocorticosterone acetate          | Diethyl ether           |
| 55 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester              | 123409 | 2-Palmitoylglycerol                       | DCM                     |
| 56 | trans-4-Hydroxycyclohexanecarboxylic acid                              | 151138 | trans-4-Hydroxycyclohexanecarboxylic acid | DCM                     |
| 57 | 2-Dodecylcyclobutanone                                                 | 161875 | 2-Dodecylcyclobutanone                    | Acetone                 |
| 58 | Verbenol <cis->                                                        | 164888 | cis-Verbenol                              | Methanol                |
| 59 | Phthalic acid, monocyclohexyl ester                                    | 165618 | Monocyclohexyl phthalate                  | Hexane                  |
| 60 | Acetamide, N,N-dicyclohexyl                                            | 222401 | N,N-Dicyclohexylacetamide                 | Diethyl ether           |
| 61 | Heptane, 1,1,1,3-tetrachloro                                           | 231032 | 1,1,1,3-Tetrachloroheptane                | Diethyl ether           |

|    |                                                                        |        |                                                                                                                           |               |
|----|------------------------------------------------------------------------|--------|---------------------------------------------------------------------------------------------------------------------------|---------------|
| 62 | 2-Chloro-3-morpholino-1,4-naphthoquinone                               | 236652 | 2-Chloro-3-morpholino-1,4-naphthoquinone                                                                                  | Isopropanol   |
| 63 | Cholest-5-en-3-ol, 4,4-dimethyl-, (3.beta.)-                           | 280427 | Cholest-5-en-3beta-ol, 4,4-dimethyl-                                                                                      | Isopropanol   |
| 64 | Phenylacetic acid, 2,4-dimethoxycarbonyl-3,5-dihydroxy-, methyl(ester) | 287226 | Dimethyl 2,4-dihydroxy-6-(2-methoxy-2-oxoethyl)isophthalate                                                               | Methanol      |
| 65 | 3.beta.-Hydroxyetioallocholanolic acid                                 | 301461 | 3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17-carboxylic acid | Diethyl ether |
| 66 | Cholest-5-en-3-ol (3.beta.)-, propanoate                               | 313255 | Cholesteryl propionate                                                                                                    | Ethyl acetate |
| 67 | 2,4,6-Trimethyl-3'-methoxydiphenylsulfone                              | 345608 | 2-(3-Methoxyphenyl)sulfonyl-1,3,5-trimethylbenzene                                                                        | Diethyl ether |
| 68 | Myristic acid glycidyl ester                                           | 346148 | Oxiran-2-ylmethyl tetradecanoate                                                                                          | Acetone, DCM  |
| 69 | Glycidyl palmitate                                                     | 347736 | Glycidyl Palmitate                                                                                                        | Acetone       |
| 70 | 16,23-Cyclocholesta-5,16(23)-dien-22-one, 3,26-dihydroxy-, (3.beta.)-  | 417706 | Fesogenin                                                                                                                 | Methanol      |
| 71 | cis-Thujopsene                                                         | 442402 | Thujopsene                                                                                                                | Methanol      |
| 72 | Oleic Acid                                                             | 445639 | Oleic Acid                                                                                                                | Acetone       |
| 73 | N-(4-Methyl-1-piperazinyl)-2-(2-pyridinyl)quinoline                    | 453063 | 4-(4-Methylpiperazino)-2-(2-pyridyl)quinoline                                                                             | DCM           |
| 74 | Phthalic acid, butyl hexyl ester                                       | 526381 | Butyl hexyl phthalate                                                                                                     | Hexane        |
| 75 | Aromadendrene oxide-(1)                                                | 528759 | decahydro-1,1,7-trimethylspiro[4H-cycloprop[e]azulene-4,2'-oxirane]                                                       | DCM           |
| 76 | Trichloroacetic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester             | 531065 | Trichloroacetic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester                                                                | Methanol      |
| 77 | Ethyl bis(2,4-dinitrophenyl) acetate                                   | 533653 | Ethyl bis(2,4-dinitrophenyl) acetate                                                                                      | Methanol      |
| 78 | 1,3-Didodecylurea                                                      | 533921 | 1,3-Didodecylurea                                                                                                         | Isopropanol   |
| 79 | 1,1':4',1''-Terphenyl, 2,2''-dinitro                                   | 533948 | 1,1':4',1''-Terphenyl, 2,2''-dinitro-                                                                                     | Diethyl ether |
| 80 | 3a,9-Dimethyldodecahydrocyclohepta[d]inden-3-one                       | 534730 | 3a,9-Dimethyldodecahydrocyclohepta[d]inden-3-one                                                                          | DCM           |
| 81 | 3,7-Diazabicyclo[3.3.1]nonane, 9,9-dimethyl                            | 534923 | 9,9-Dimethyl-3,7-diazabicyclo[3.3.1]nonane                                                                                | Isopropanol   |
| 82 | Cyclopentaneundecanoic acid, methyl ester                              | 535041 | Cyclopentaneundecanoic acid, methyl ester                                                                                 | DCM           |

|    |                                                                                                                                                 |        |                                                                                                                                                               |               |
|----|-------------------------------------------------------------------------------------------------------------------------------------------------|--------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| 83 | (-)-Thujylideneglycine, ethyl ester                                                                                                             | 535093 | (-)-Thujylideneglycine, ethyl ester                                                                                                                           | Isopropanol   |
| 84 | Pyrimidine-5-carboxylic acid, 1,2,3,4-tetrahydro-4-(2,5-dimethoxyphenyl)-6-methyl-2-oxo-, methyl ester                                          | 535974 | Methyl 4-(2,5-dimethoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate                                                                      | DCM           |
| 85 | [2]Benzopyrano[7,6-f]indazole-8-acetic acid, 2,4,6,8,9,11-hexahydro-5,10-dihydroxy-2,6-dimethyl-4,11-dioxo-, methyl ester                       | 536807 | Methyl 2-(11-hydroxy-2,6-dimethyl-4,5,10-trioxo-1,6,8,9-tetrahydroisochromeno[7,6-f]indazol-8-yl)acetate                                                      | Hexane        |
| 86 | 6.beta.-Hydroxyfluoxymesterone                                                                                                                  | 537089 | 6beta-Hydroxyfluoxymesterone                                                                                                                                  | DCM           |
| 87 | Methyl 9-acetoxyleneicosanoate                                                                                                                  | 537181 | Methyl 9-acetoxyleneicosanoate                                                                                                                                | Acetone       |
| 88 | 1-Diphenylmethylsilyloxy-2-methylhexan-5-ol-3-one                                                                                               | 538388 | 1-Diphenylmethylsilyloxy-2-methylhexan-5-ol-3-one                                                                                                             | Isopropanol   |
| 89 | 3.beta.,4.alpha.,9.alpha.,11-Diepoxyuurolan-10-ol                                                                                               | 538855 | 3beta,4alpha,9alpha,11-Diepoxyuurolan-10-ol                                                                                                                   | Isopropanol   |
| 90 | 1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol, 3-[(acetyloxy)methyl]-1a,1b,4,4a,5,7a,8,9-octahydro-1,1,6,8-tetramethyl-, 9,9a-diacetate | 541019 | 9a-(Acetyloxy)-3-[(acetyloxy)methyl]-5,7b-dihydroxy-1,1,6,8-tetramethyl-1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-1H-cyclopropa[3,4]benzo[1,2-E]azulen-9-yl acetate | Isopropanol   |
| 91 | 4-[4-Chlorophenylmercapto]-6-methoxy-8-acetaminoquinoline                                                                                       | 541099 | 4-[4-Chlorophenylmercapto]-6-methoxy-8-acetaminoquinoline                                                                                                     | Isopropanol   |
| 92 | 5,6-Dihydro-2-(4-nitrophenyl)-4H-1,3-oxazin-5-one                                                                                               | 541588 | 2-(4-Nitrophenyl)-4H-1,3-oxazin-5(6H)-one                                                                                                                     | Isopropanol   |
| 93 | Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)-                                                                                             | 542384 | Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)-                                                                                                           | DCM           |
| 94 | 1,3-Xylyl-15-crown-4, 2,3-.pinanedioxyboryl                                                                                                     | 542562 | 1,3-Xylyl-15-crown-4, 2,3-.pinanedioxyboryl                                                                                                                   | Methanol      |
| 95 | N2-Isopropyl-5-methyl-1,3,4-thiadiazol-2-amine                                                                                                  | 544670 | 5-methyl-N-(propan-2-yl)-1,3,4-thiadiazol-2-amine                                                                                                             | Acetone       |
| 96 | 3-Pyridinecarboxylic acid, 1,4,5,6-tetrahydro-1,2-dimethyl-6-oxo-, ethyl ester                                                                  | 544936 | Nicotinic acid, 1,4,5,6-tetrahydro-1,2-dimethyl-6-oxo-, ethyl ester                                                                                           | Hexane        |
| 97 | Octadecanoic acid, 2-(hexadecyloxy)ethyl ester                                                                                                  | 545952 | Octadecanoic acid, 2-(hexadecyloxy)ethyl ester                                                                                                                | Isopropanol   |
| 98 | Cyclopentane, 1-pentyl-2-propyl-                                                                                                                | 549948 | 1-Pentyl-2-propylcyclopentane                                                                                                                                 | Acetone       |
| 99 | 1,2-Dimethyl-4-heptafluorobutyryloxycyclohexane                                                                                                 | 550201 | 1,2-Dimethyl-4-heptafluorobutyryloxycyclohexane                                                                                                               | Diethyl ether |

|     |                                                                                       |        |                                                                                    |               |
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| 100 | Pyrrolo[1,2-a]pyrazine, perhydro-2-(2,4-dinitrophenyl)-                               | 550933 | 2-(2,4-Dinitrophenyl)octahydropyrrolo[1,2-a]pyrazine                               | Hexane        |
| 101 | Glycerol 3-phospho(dimethyl)ethanolamine, 3TMS derivative                             | 551440 | 2,3-Bis[(trimethylsilyl)oxy]propyl 2-(dimethylamino)ethyl trimethylsilyl phosphate | Isopropanol   |
| 102 | alpha.-D-Glucofuranose, 6-chloro-6-deoxy-1,2-O-trichloroethylidene                    | 552339 | alpha.-D-Glucofuranose, 6-chloro-6-deoxy-1,2-O-trichloroethylidene-                | Isopropanol   |
| 103 | 3,7,11,15,19-Pentaoxa-2,20-disilaheneicosane, 2,2,20,20-tetramethyl                   | 552945 | 3,7,11,15,19-Pentaoxa-2,20-disilaheneicosane, 2,2,20,20-tetramethyl-               | Hexane        |
| 104 | Glycerol monostearate, 2TMS derivative                                                | 553936 | 2,3-Bis[(trimethylsilyl)oxy]propyl stearate                                        | Acetone       |
| 105 | 2-Chloro-4-(diphenylamino)-6-morpholino-1,3,5-triazine                                | 555930 | 4-chloro-6-(morpholin-4-yl)-N,N-diphenyl-1,3,5-triazin-2-amine                     | Diethyl ether |
| 106 | Trichothec-9-ene-3,4,7,8,15-pentol, 12,13-epoxy-, 4,15-diacetate, (3.alpha.,4.beta.)- | 557498 | Trichothec-9-ene-3,4,7,8,15-pentol, 12,13-epoxy-, 4,15-diacetate, (3alpha,4beta)-  | Hexane        |
| 107 | 4-Heptanone, 2-methyl-6-(4-methyl-3-cyclohexen-1-yl)-, [S-(R*,S*)]-                   | 564737 | 4-Heptanone, 2-methyl-6-(4-methyl-3-cyclohexen-1-yl)-, [S-(R*,S*)]-                | Hexane        |
| 108 | Bicyclo[2.2.1]heptane-7-carboxylic acid-2-(methoxycarbonyl)                           | 564809 | Bicyclo[2.2.1]heptane-7-carboxylic acid-2-(methoxycarbonyl)                        | Isopropanol   |
| 109 | 9,19-Cyclocholestan-3-ol, 14-methyl-, (3.beta.,5.alpha.)-                             | 565570 | Pollinasterol                                                                      | Methanol      |
| 110 | 2-Butanone, 3-(2,5-dihydro-4,5,5-trimethyl-2-pyrimidinyl)-3-methyl-, (.+/-.)-         | 565948 | 2-Butanone, 3-(2,5-dihydro-4,5,5-trimethyl-2-pyrimidinyl)-3-methyl-, (+/-)-        | Ethyl acetate |
| 111 | Benzoic acid, 3-(2,3-dimethoxy-4,5-methylenedioxyphenyl)propyl ester                  | 570355 | Benzoic acid, 3-(2,3-dimethoxy-4,5-methylenedioxyphenyl)propyl ester               | Diethyl ether |
| 112 | 1-N-Phenyl-4-(trichlorovinyl)benzamide                                                | 571186 | 1-N-Phenyl-4-(trichlorovinyl)benzamide                                             | Diethyl ether |
| 113 | 1-Chloro-1-ethyl-2-methyl-1-germacyclopent-3-ene                                      | 573256 | 1-Chloro-1-ethyl-2-methyl-1-germacyclopent-3-ene                                   | DCM           |
| 114 | 1-Ethyl-1-(1-cyclopentylethyloxy)-1-silacyclohexane                                   | 575307 | 1-Ethyl-1-(1-cyclopentylethyloxy)-1-silacyclohexane                                | Isopropanol   |
| 115 | 2H-Benzotriazole, 2-[3-[4-(2-methoxyphenyl)-1-piperazinyl]propyl]-                    | 577725 | 2H-Benzotriazole, 2-[3-[4-(2-methoxyphenyl)-1-piperazinyl]propyl]-                 | Methanol      |
| 116 | O-(5-Chloro-3-cyano-4,6-dimethyl-2-pyridinyl) O,O-dimethyl thiophosphate              | 580789 | 5-Chloro-2-[(dimethoxyphosphorothioyl)oxy]-4,6-dimethylnicotinonitrile             | Diethyl ether |

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| 117 | S-2-[Tetrahydrothiopyran-4-ylamino]ethyl thiosulfuric acid                                                      | 582482 | S-2-[Tetrahydrothiopyran-4-ylamino]ethyl thiosulfuric acid                                                      | Methanol      |
| 118 | 1-Cyclohexyldimethylsilyloxybutane                                                                              | 582893 | 1-Cyclohexyldimethylsilyloxybutane                                                                              | Acetone       |
| 119 | 3-Ethoxy-4-hydroxyphenylacetonitrile                                                                            | 590922 | 3-Ethoxy-4-hydroxyphenylacetonitrile                                                                            | Hexane        |
| 120 | 2-Ethylsulfanyl-benzimidazole-1-carboxylic acid, phenyl ester                                                   | 591039 | Phenyl 2-(ethylsulfanyl)-1H-benzimidazole-1-carboxylate                                                         | Hexane        |
| 121 | Tartronic acid, (p-ethoxyphenyl)-, diethyl ester                                                                | 591180 | Tartronic acid, (p-ethoxyphenyl)-, diethyl ester                                                                | Hexane        |
| 122 | 3-(3a,6-Dimethyl-3,8-dioxotetradecahydrocyclohepta[e]inden-6-yl)propionic acid, methyl ester                    | 591197 | 3-(3a,6-Dimethyl-3,8-dioxotetradecahydrocyclohepta[e]inden-6-yl)propionic acid, methyl ester                    | Diethyl ether |
| 123 | Octahydroquinolin-10-ol                                                                                         | 592618 | Octahydroquinolin-10-ol                                                                                         | Isopropanol   |
| 124 | Phosphorothioic acid, O,O-diethyl O-[2-(1-hydroxy-1-methylethyl)-6-methyl-4-pyrimidinyl] ester                  | 592775 | Phosphorothioic acid, O,O-diethyl O-(2-(1-hydroxy-1-methylethyl)-6-methyl-4-pyrimidinyl) ester                  | Methanol      |
| 125 | Quinoline, 1,2,3,4-tetrahydro-4-methyl-2,4'-spiro(1,2,5-trimethylpiperidine)-                                   | 594574 | 1,2,4',5-tetramethyl-3',4'-dihydro-1'H-spiro[piperidine-4,2'-quinoline]                                         | Isopropanol   |
| 126 | 6-Bromo-3-t-butyl-6-(1-hydroxy-1-methyl-ethyl)-2-oxa-4-aza-bicyclo[3.1.0]hexane-4-carboxylic acid, methyl ester | 598368 | 6-Bromo-3-t-butyl-6-(1-hydroxy-1-methyl-ethyl)-2-oxa-4-aza-bicyclo[3.1.0]hexane-4-carboxylic acid, methyl ester | Diethyl ether |
| 127 | Trichothec-9-ene-4,8-diol, 12,13-epoxy-3,15-bis[(trimethylsilyl)oxy]-, diacetate, (3.alpha.,4.beta.,8.alpha.)-  | 603968 | Trichothec-9-ene-4,8-diol, 12,13-epoxy-3,15-bis[(trimethylsilyl)oxy]-, diacetate, (3alpha,4beta,8alpha)-        | Isopropanol   |
| 128 | Olean-12-en-28-oic acid, 3.beta.-hydroxy-21-oxo-, methyl ester                                                  | 605790 | Methyl 3-hydroxy-21-oxoolean-12-en-28-oate                                                                      | Methanol      |
| 129 | Isoxazolo[3,4-a]benzo[d]cyclohepten-3-one, 3a,4,9,10-tetrahydro-4-phenyl                                        | 608354 | Isoxazolo[3,4-a]benzo[d]cyclohepten-3-one, 3a,4,9,10-tetrahydro-4-phenyl                                        | Hexane        |
| 130 | Dodecanoic acid, undecyl ester                                                                                  | 608380 | Dodecanoic acid, undecyl ester                                                                                  | Hexane        |
| 131 | 8,11-Bis(heptafluorobutyryl)culmorin                                                                            | 608778 | 8,11-Bis(heptafluorobutyryl)culmorin                                                                            | Diethyl ether |
| 132 | 2,9-Dimethyl-2,3,4,5,6,7-hexahydro-1H-2-benzazonine                                                             | 608917 | 2,9-Dimethyl-2,3,4,5,6,7-hexahydro-1H-2-benzazonine                                                             | Isopropanol   |
| 133 | 1-(4-Bromophenyl)ethyl isocyanate                                                                               | 610983 | 1-Bromo-4-(1-isocyanatoethyl)benzene                                                                            | Hexane        |

|     |                                                                                                                           |        |                                                                                                                                                |                                |
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| 134 | 1,10a-Phenanthrenediol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl                                                       | 615029 | 1,10a-Phenanthrenediol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl                                                                            | Isopropanol                    |
| 135 | (3-Hydroxy-6-oxo-2,3,3a,9a-tetrahydro-6H-furo[2',3':4,5][1,3]oxazolo[3,2-a]pyrimidin-2-yl)methyl pivalate, TMS derivative | 617362 | Propanoic acid, 2,2-dimethyl-, [2,3,3a,9a-tetrahydro-6-oxo-3-[(trimethylsilyl)oxy]-6H-furo[2',3':4,5]oxazolo[3,2-a]pyrimidin-2-yl]methyl ester | Methanol                       |
| 136 | Quinazoline, 2,4-dimorpholino                                                                                             | 622868 | 2,4-Di(morpholin-4-yl)quinazoline                                                                                                              | Isopropanol                    |
| 137 | Isoquinoline, 2-(2,2-dimethyl-1-oxopropyl)-1,2,3,4-tetrahydro-6,7-dimethoxy                                               | 624005 | 1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-2,2-dimethyl-propan-1-one                                                                    | DCM                            |
| 138 | 3,9-Ethylene-1,4,6,8,11,13-hexaazatetracyclo[9.3.0.0(3,10).0(4,8)]tetradeca-5,7,12,14-tetraone, 2,6,13,trimethyl-2-phenyl | 624429 | 3,9-Ethylene-1,4,6,8,11,13-hexaazatetracyclo[9.3.0.0(3,10).0(4,8)]tetradeca-5,7,12,14-tetraone, 2,6,13,trimethyl-2-phenyl                      | Isopropanol                    |
| 139 | 7-Bromo-1-methyl-5-phenyl-1,3-dihydro-benzo[e][1,4]diazepin-2-one                                                         | 627432 | 7-Bromo-1-methyl-5-phenyl-1,3-dihydro-benzo[e][1,4]diazepin-2-one                                                                              | Methanol                       |
| 140 | 4-Amino-4'-isopropylidenehydrazino diphenyl sulfone                                                                       | 627508 | 4-Amino-4'-isopropylidenehydrazino diphenyl sulfone                                                                                            | Methanol                       |
| 141 | Cyclopenta[d]anthracene-8,11-dione, 1,2,3,3a,4,5,6,6a,7,12-decahydro-3-isopropyl-6-methylene                              | 628236 | Cyclopenta[d]anthracene-8,11-dione, 1,2,3,3a,4,5,6,6a,7,12-decahydro-3-isopropyl-6-methylene-                                                  | Diethyl ether                  |
| 142 | 1,4-Benzodiazepin-2(1H,3H)-one, 7-chloro-5-phenyl-3-propyl                                                                | 628497 | 1,4-Benzodiazepin-2(1H,3H)-one, 7-chloro-5-phenyl-3-propyl-                                                                                    | Hexane                         |
| 143 | Butanedioic acid, mono[7-bromo-5-(2-chlorophenyl)-2,3-dihydro-2-oxo-1H-1,4-benzodiazepin-3-yl] ester                      | 629281 | Cinazepam                                                                                                                                      | Hexane                         |
| 144 | 5-Methoxy-6-trimethylsilyloxy-2-indolecarboxylic acid, trimethylsilyl ester                                               | 631696 | 5-Methoxy-6-trimethylsilyloxy-2-indolecarboxylic acid, trimethylsilyl ester                                                                    | Diethyl ether                  |
| 145 | Benzamide, N-(7-diethylamino-2-oxo-2H-chromenyl)-4-nitro                                                                  | 632621 | N-(7-Diethylamino-2-oxo-2H-chromen-3-yl)-4-nitro-benzamide                                                                                     | Hexane                         |
| 146 | 3-Demethylthiocolchicine, N-decarbonyl                                                                                    | 632983 | 3-Demethylthiocolchicine, N-decarbonyl-                                                                                                        | Diethyl ether                  |
| 147 | Propyl 5,8-diethoxyquinoxaline-2,3-dicarboxylate                                                                          | 633698 | Propyl 5,8-diethoxyquinoxaline-2,3-dicarboxylate                                                                                               | Methanol                       |
| 148 | Squalene                                                                                                                  | 638072 | Squalene                                                                                                                                       | Acetone, diethyl ether, hexane |

|     |                                                                                       |         |                                                                                        |                                                                                    |
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| 149 | 9-Hexadecenoic acid, methyl ester, (Z)-                                               | 643801  | Methyl palmitoleate                                                                    | DCM                                                                                |
| 150 | 5-Phenyl-7-(trifluoromethyl)pyrazolo[1,5-a]pyrimidine-2-carboxylic acid               | 655493  | 5-Phenyl-7-(trifluoromethyl)pyrazolo[1,5-a]pyrimidine-2-carboxylic acid                | Isopropanol                                                                        |
| 151 | 1H-Benzoimidazole, 1-(4-fluorobenzyl)-2-(4-methoxyphenyl)-                            | 712188  | 1H-Benzoimidazole, 1-(4-fluorobenzyl)-2-(4-methoxyphenyl)-                             | Isopropanol                                                                        |
| 152 | Butyric acid, 4-(4-chloro-5-methyl-3-nitropyrazol-1-yl)-                              | 776524  | 4-(4-chloro-5-methyl-3-nitro-1H-pyrazol-1-yl)butanoic acid                             | DCM                                                                                |
| 153 | 1H-Pyrazole-1-acetamide, N-[(3-methoxyphenyl)methyl]-3-nitro                          | 2978699 | N-(3-methoxybenzyl)-2-(3-nitro-1H-pyrazol-1-yl)acetamide                               | DCM                                                                                |
| 154 | 2-Thioxo-imidazolidin-4-one-5-propanolic acid                                         | 3145946 | 3-(5-Oxo-2-thioxoimidazolidin-4-yl)propanoic acid                                      | DCM                                                                                |
| 155 | Piperidin-4-ol, 4-(4-ethylbenzyl)-1,2,5-trimethyl                                     | 3579174 | 4-[(4-Ethylphenyl)methyl]-1,2,5-trimethylpiperidin-4-ol                                | Diethyl ether                                                                      |
| 156 | 2-(4-Methoxyphenyl)-4-propyl-1, 9b-dihydro-5-oxa-3,3a-diazacyclopenta[a]naphthalene   | 4140612 | Methyl 4-(5-propyl-1,10B-dihydropyrazolo(1,5-C)(1,3)benzoxazin-2-YL)phenyl ether       | Diethyl ether                                                                      |
| 157 | 2-Chloro-3-[(4-methoxyphenyl)amino]naphthalene-1,4-dione                              | 4184980 | 2-Chloro-3-[(4-methoxyphenyl)amino]naphthalene-1,4-dione                               | Methanol                                                                           |
| 158 | 2H-Benzo[d][1,3]oxazine, 2-[2-(5-methylfuran-2-yl)ethyl]-4,4-diphenyl-1,4-dihydro     | 4663836 | 2H-Benzo[d][1,3]oxazine, 2-[2-(5-methylfuran-2-yl)ethyl]-4,4-diphenyl-1,4-dihydro-     | Diethyl ether                                                                      |
| 159 | Bicyclo[2.2.1]heptane-1-carboxamide, N-(2,5-difluorophenyl)-4,7,7-trimethyl-2,3-dioxo | 4904236 | Bicyclo[2.2.1]heptane-1-carboxamide, N-(2,5-difluorophenyl)-4,7,7-trimethyl-2,3-dioxo- | Diethyl ether                                                                      |
| 160 | 9-Octadecenoic acid, methyl ester, (E)-                                               | 5280590 | Methyl elaidate                                                                        | Acetone, DCM, Diethyl ether, Ethanol, Ethyl Acetate, Hexane, Isopropanol, Methanol |
| 161 | 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester                                   | 5283468 | Glyceryl monooleate                                                                    | Acetone, Ethyl acetate                                                             |
| 162 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester                                        | 5284421 | Methyl linoleate                                                                       | Acetone, DCM, Diethyl ether, Ethanol, Ethyl Acetate, Hexane,                       |

|     |                                                                                           |         |                                                              |                          |
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|     |                                                                                           |         |                                                              | Isopropanol,<br>Methanol |
| 163 | 3-Thiophenecarboxylic acid, 5-methoxy                                                     | 5300915 | 5-Methoxythiophene-3-carboxylic acid                         | Diethyl ether            |
| 164 | 1,8,11-Heptadecatriene, (Z,Z)-                                                            | 5352709 | (Z,Z)-Heptadeca-1,8,11-triene                                | Acetone                  |
| 165 | 9-Octadecenoic acid (Z)-, oxiranylmethyl ester                                            | 5354568 | Glycidyl oleate                                              | Acetone                  |
| 166 | 2,5-Furandione, 3-(2E)-2-dodecenyldihydro                                                 | 5362708 | 2-Dodecen-1-ylsuccinic anhydride                             | Methanol                 |
| 167 | 2,5-Furandione, dihydro-3-(1-octenyl)-                                                    | 5362721 | 1-Octenylsuccinic anhydride                                  | DCM                      |
| 168 | Decyl oleate                                                                              | 5363234 | Decyl oleate                                                 | Isopropanol              |
| 169 | Ethyl Oleate                                                                              | 5363269 | Ethyl oleate                                                 | Acetone, Ethanol         |
| 170 | 8-Octadecenoic acid, methyl ester                                                         | 5364422 | 8-Octadecenoic acid, methyl ester                            | Acetone                  |
| 171 | 9-Octadecenoic acid (Z)-, methyl ester                                                    | 5364509 | Methyl oleate                                                | DCM                      |
| 172 | Oleoyl chloride                                                                           | 5364783 | Oleoyl chloride                                              | DCM                      |
| 173 | 4,4-Difluororetinoic acid (all-trans)                                                     | 5366642 | 4,4-Difluororetinoic acid                                    | Methanol                 |
| 174 | 5,8,11,14-Eicosatetraenoic acid, ethyl ester, (all-Z)-                                    | 5367369 | Ethyl arachidonate                                           | DCM                      |
| 175 | Methyl eicosa-5,8,11,14,17-pentaenoate                                                    | 5367433 | Methyl eicosa-5,8,11,14,17-pentaenoate                       | DCM                      |
| 176 | Stearic acid, 9-hexadecenyl ester, (Z)-                                                   | 5369024 | Palmitoleyl stearate                                         | Acetone                  |
| 177 | Z-7-Pentadecenol                                                                          | 5369066 | Z-7-Pentadecenol                                             | Acetone                  |
| 178 | Corynan-16-carboxylic acid, 16,17-didehydro-9,17-dimethoxy-, methyl ester, (3.beta.,16E)- | 5376107 | Speciocilatine                                               | Diethyl ether            |
| 179 | (E)-2-Fluoro-3-(3-methoxyphenyl)-propenoic acid, ethyl ester                              | 5376445 | (E)-2-Fluoro-3-(3-methoxyphenyl)-propenoic acid, ethyl ester | Diethyl ether            |
| 180 | 2-Propen-1-one, 1-(4-cyclopropylphenyl)-3-phenyl)-                                        | 5377173 | 1-(4-Cyclopropylphenyl)-3-phenylprop-2-en-1-one              | Isopropanol              |
| 181 | Nomelidine                                                                                | 5378630 | Nomelidine                                                   | Methanol                 |
| 182 | trans-4'-Bromo-4-(methylthio)chalcone                                                     | 5379142 | trans-4'-Bromo-4-(methylthio)chalcone                        | Methanol                 |
| 183 | Benzhydrazide, 4-nitro-N2-(1-phenylhexylidene)-                                           | 6390560 | Benzhydrazide, 4-nitro-N2-(1-phenylhexylidene)-              | Methanol                 |
| 184 | Oxalic acid, isohexyl propyl ester                                                        | 6420409 | Oxalic acid, isohexyl propyl ester                           | Ethanol                  |
| 185 | Phthalic acid, 4-methoxybenzyl isohexyl ester                                             | 6423729 | Phthalic acid, 4-methoxybenzyl isohexyl ester                | Hexane                   |

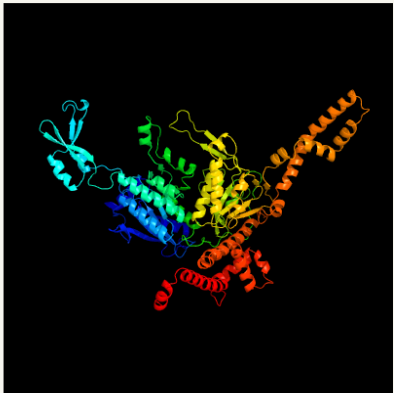
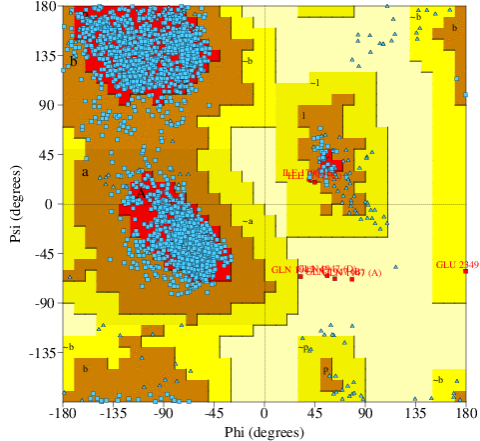
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| 186 | Phthalic acid, butyl phenyl ester                                                                                                            | 6424003  | Phthalic acid, butyl phenyl ester                                                                                                                                                                                                                                    | Hexane        |
| 187 | Phthalic acid, 2-bromo-4-fluorophenyl butyl ester                                                                                            | 6424009  | Phthalic acid, 2-bromo-4-fluorophenyl butyl ester                                                                                                                                                                                                                    | Hexane        |
| 188 | 3-Phenyl-5-p-tolylamino-cyclopentane-1,1,2,2-tetracarbonitrile                                                                               | 6425496  | 3-Phenyl-5-p-tolylamino-cyclopentane-1,1,2,2-tetracarbonitrile                                                                                                                                                                                                       | DCM           |
| 189 | 2,4,6,8-Tetradecatetraenoic acid, 9a-(acetyloxy)-1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5 | 6437362  | 2,4,6,8-Tetradecatetraenoic acid, 9a-(acetyloxy)-1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1H-cyclopropa(3,4)benz(1,2-e)azulen-9-yl ester, (1aR-(1aalpha,1bbeta,4abeta,7aalpha,7balpha,8alpha,9beta,9aalpha))- | Methanol      |
| 190 | 4H-Pyrimido[5,4-b]indol-4-one, 3-[3-(dipropylamino)propyl]-8-fluoro-3,5-dihydro                                                              | 6623619  | 4H-Pyrimido[5,4-b]indol-4-one, 3-[3-(dipropylamino)propyl]-8-fluoro-3,5-dihydro                                                                                                                                                                                      | Methanol      |
| 191 | Propanal, 2-methyl-, (2,4-dinitrophenyl)hydrazone                                                                                            | 9568083  | Propanal, 2-methyl-, (2,4-dinitrophenyl)hydrazone                                                                                                                                                                                                                    | Isopropanol   |
| 192 | 2-[p-Chlorophenyl]tetrahydrofuran-2-carboxaldehyde semicarbazone                                                                             | 9602173  | 2-[p-Chlorophenyl]tetrahydrofuran-2-carboxaldehyde semicarbazone                                                                                                                                                                                                     | DCM           |
| 193 | Butanehydrazide, 2-hydroxy-2-ethyl-N2-(3-phenylpropenylideno)-                                                                               | 9602957  | Butanehydrazide, 2-hydroxy-2-ethyl-N2-(3-phenylpropenylideno)-                                                                                                                                                                                                       | Isopropanol   |
| 194 | 1-[4-Chloro-6-methyl-2-pyrimidyl]-3-[3,4-dichlorophenyl]guanidine                                                                            | 9604214  | 1-[4-Chloro-6-methyl-2-pyrimidyl]-3-[3,4-dichlorophenyl]guanidine                                                                                                                                                                                                    | DCM           |
| 195 | Ferrocene, 1,2,3,4-tetrachloro                                                                                                               | 11986378 | Ferrocene, 1,2,3,4-tetrachloro                                                                                                                                                                                                                                       | Methanol      |
| 196 | 2H-1-benzopyran-2-one, 5,7-dimethoxy-3-[(2-oxo-2H-1-benzopyran-3-yl)carbonyl]-                                                               | 12777082 | 5,7-dimethoxy-3-[(2-oxo-2H-chromen-3-yl)carbonyl]-2H-chromen-2-one                                                                                                                                                                                                   | Methanol      |
| 197 | Methyl 3-undecenoate                                                                                                                         | 12957097 | 3-Undecenoic acid methyl ester                                                                                                                                                                                                                                       | Ethyl acetate |
| 198 | Stigmasta-3,5-diene                                                                                                                          | 13783149 | Stigmasta-3,5-diene                                                                                                                                                                                                                                                  | Isopropanol   |
| 199 | (1R,3aR,5aR,9aS)-1,4,4,7-Tetramethyl-1,2,3,3a,4,5a,8,9-octahydrocyclopenta[c]benzofuran                                                      | 14105916 | Italicene ether                                                                                                                                                                                                                                                      | Hexane        |
| 200 | .alpha.,.beta.-D-Glucopyranoside, 1-deoxy-1-undecylthio                                                                                      | 14775991 | Undecyl 1-thiohexopyranoside                                                                                                                                                                                                                                         | Isopropanol   |
| 201 | Stannane, (acetyloxy)triphenyl                                                                                                               | 16682804 | Fentin acetate                                                                                                                                                                                                                                                       | DCM           |

|     |                                                                                                                                 |          |                                                                                                  |               |
|-----|---------------------------------------------------------------------------------------------------------------------------------|----------|--------------------------------------------------------------------------------------------------|---------------|
| 202 | 3,6,9,12-Tetraoxatetradecane-1,14-diyl dibenzoate                                                                               | 19391910 | 3,6,9,12-Tetraoxatetradecane-1,14-diyl dibenzoate                                                | Hexane        |
| 203 | Pregnan-20-one, 3,11,21-tris[(trimethylsilyl)oxy]-, O-(phenylmethyl)oxime, (3.beta.,5.beta.,11.beta.)-                          | 20845377 | Pregnan-20-one, 3,11,21-tris[(trimethylsilyl)oxy]-, O-(phenylmethyl)oxime, (3beta,5beta,11beta)- | Methanol      |
| 204 | [1-(4-Fluorophenyl)pyrazol-4-yl]methanol                                                                                        | 21770104 | [1-(4-fluorophenyl)-1H-pyrazol-4-yl]methanol                                                     | Diethyl ether |
| 205 | Pregnane-3,20-dione, 16-methylene-11,17,21-tris[(trifluoroacetyl)oxy]-, (5.alpha.,11.alpha.)-                                   | 22213406 | Pregnane-3,20-dione, 16-methylene-11,17,21-tris[(trifluoroacetyl)oxy]-, (5alpha,11alpha)-        | DCM           |
| 206 | 11-Hydroxyandrosterone (3.alpha.,5.alpha.,11.beta.)-, TMS derivative                                                            | 22214186 | 5alpha-Androstan-17-one, 11beta-hydroxy-3alpha-(trimethylsiloxy)-                                | Diethyl ether |
| 207 | Pregn-4-ene-3,20-dione, 19-chloro-17,21-dihydroxy                                                                               | 22296531 | 19-Chloro-17,21-dihydroxypregn-4-ene-3,20-dione                                                  | Hexane        |
| 208 | Methyl 3-(3,4-diethoxyphenyl)prop-2-enoate                                                                                      | 22987080 | Methyl 3-(3,4-diethoxyphenyl)prop-2-enoate                                                       | DCM           |
| 209 | Ethyl 11-oxo-14-thia-8,12,17-triazatetracyclo[8.7.0.0(2,7).0(13,17)]heptadeca-1(10),2,4,6,8,12,15-heptaene-4-carboxylate        | 24220867 | ethyl 5-oxo-5H-[1,3]thiazolo[2',3':2,3]pyrimido[5,4-c]quinoline-10-carboxylate                   | DCM           |
| 210 | 2-(Ethylamino)-7-(4-hydroxy-3-methoxyphenyl)-4H,6H,7H-[1,3]thiazolo[4,5-b]pyridin-5-one                                         | 42647670 | 2-(ethylamino)-7-(4-hydroxy-3-methoxyphenyl)-6,7-dihydro[1,3]thiazolo[4,5-b]pyridin-5(4H)-one    | Diethyl ether |
| 211 | 5-Chloro-2-fluoro-N-(1-hydroxy-3-phenylpropan-2-yl)benzenesulfonamide                                                           | 45479954 | 5-chloro-2-fluoro-N-(1-hydroxy-3-phenylpropan-2-yl)benzenesulfonamide                            | Methanol      |
| 212 | Tungsten, tris(.pi.-allyl)(.eta.-3-acetato)-                                                                                    | 51238316 | CID540937                                                                                        | Isopropanol   |
| 213 | Menthol, 1'-(butyn-3-one-1-yl)-, (1R,2S,5R)-                                                                                    | 52191418 | CID536442                                                                                        | Methanol      |
| 214 | Tungsten, tris(.pi.-allyl)(.pi.-1,3-dimethylallyl)-                                                                             | 52946293 | CID534339                                                                                        | Diethyl ether |
| 215 | Benzene <para-dichloro->                                                                                                        | 53786885 | 4-dichlorobenzene                                                                                | Acetone       |
| 216 | 2,20-Cycloaspidospermidine-3-carboxylic acid, 6,7-didehydro-, methyl ester, (2.alpha.,3.beta.,5.alpha.,12.beta.,19.alpha.,20R)- | 78358491 | Vindoline dihydrochloride                                                                        | Methanol      |
| 217 | N,N,1-Trimethylbenzo[g]indol-9-amine                                                                                            | 91691308 | N,N,1-Trimethylbenzo[g]indol-9-amine                                                             | Methanol      |
| 218 | 2,3-Anhydro-d-galactosan                                                                                                        | 91691622 | 2,3-Anhydro-d-galactosan                                                                         | Hexane        |
| 219 | Dimethylmalonic acid, pentadecyl 2-pentyl ester                                                                                 | 91691632 | Dimethylmalonic acid, pentadecyl 2-pentyl ester                                                  | Hexane        |

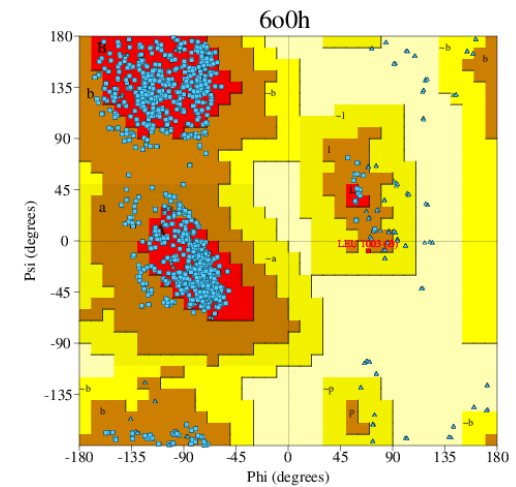
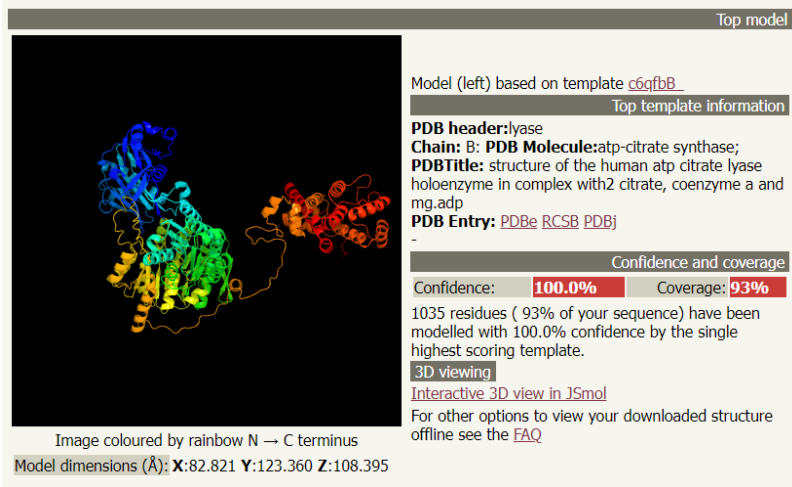
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|-----|----------------------------------------------------------------------------------------------------------------------------------------------|----------|---------------------------------------------------------------------------------------------------|---------------|
| 220 | Gonane-12,17-dione, 3-(acetyloxy)-5,14-dimethyl-, (3.beta.,5.beta.,8.alpha.,9.beta.,10.alpha.,13.xi.,14.beta.)-                              | 91692200 | Gonane-12,17-dione, 3-(acetyloxy)-5,14-dimethyl-, (3beta,5beta,8alpha,9beta,10alpha,13xi,14beta)- | Isopropanol   |
| 221 | 1,3-Dipropyl 5-(methoxyimino)-4,6-dimethyl-2-oxocyclohexa-1(6),3-diene-1,3-dicarboxylate                                                     | 91694406 | 1,3-Dipropyl 5-(methoxyimino)-4,6-dimethyl-2-oxocyclohexa-1(6),3-diene-1,3-dicarboxylate          | Methanol      |
| 222 | 3,6-Epoxy-2H,8H-pyrimido[6,1-b][1,3]oxazocine-8,10(9H)-dione, 3,4,5,6-tetrahydro-4,5-dihydroxy-, [3R-(3.alpha.,4.alpha.,5.alpha.,6.alpha.)]- | 91695162 | 3,6-Epoxy-2H,8H-pyrimido[6,1-b][1,3]oxazocine-8,10(9H)-dione, 3,4,5,6-tetrahydro-4,5-dihydroxy-   | Ethyl acetate |
| 223 | Z,Z,Z-5,8,19-Trihydroxyeicosa-5,8,11-trienoic acid, methyl ester, tris (trimethylsilyl) ether                                                | 91696879 | Z,Z,Z-5,8,19-Trihydroxyeicosa-5,8,11-trienoic acid, methyl ester, tris (trimethylsilyl) ether     | Isopropanol   |
| 224 | 1H-pyrazole-3,4-dicarboxylic acid, 5-formyl-1-phenyl-, dimethyl ester                                                                        | 91697456 | dimethyl 5-formyl-1-phenyl-1H-pyrazole-3,4-dicarboxylate                                          | DCM           |
| 225 | 1-H-pyrrole-2,3-dihydro-1-methyl-2-(propan-2'-onyl)-4-heptafluorobutyryl                                                                     | 91697613 | 1-H-pyrrole-2,3-dihydro-1-methyl-2-(propan-2'-onyl)-4-heptafluorobutyryl                          | Methanol      |
| 226 | Fumaric acid, 2,2,2-trichloroethyl tridecyl ester                                                                                            | 91698227 | Fumaric acid, 2,2,2-trichloroethyl tridecyl ester                                                 | Isopropanol   |
| 227 | Picolinyl 13-methylpentadecanoate                                                                                                            | 91700136 | Picolinyl 13-methylpentadecanoate                                                                 | DCM           |
| 228 | Picolinyl 7,8-octadecadienoate                                                                                                               | 91700253 | Picolinyl 7,8-octadecadienoate                                                                    | Diethyl ether |
| 229 | beta.-L-Altropyranoside, phenylmethyl 2,4-bis(acetylamino)-2,4,6-trideoxy-, 3-acetate                                                        | 91701057 | beta.-L-Altropyranoside, phenylmethyl 2,4-bis(acetylamino)-2,4,6-trideoxy-, 3-acetate             | Methanol      |
| 230 | D-Ornithine, N,N'-bis(dimethylaminomethylene)-, ethyl ester                                                                                  | 91701067 | D-Ornithine, N,N'-bis(dimethylaminomethylene)-, ethyl ester                                       | Methanol      |
| 231 | 1,3-Benzenediol, O-(2-methoxyethoxycarbonyl)-O'-(3-phenylpropionyl)-                                                                         | 91703802 | 1,3-Benzenediol, O-(2-methoxyethoxycarbonyl)-O'-(3-phenylpropionyl)-                              | Methanol      |
| 232 | Succinic acid, 3-methylphenyl pentadecyl ester                                                                                               | 91704248 | Succinic acid, 3-methylphenyl pentadecyl ester                                                    | DCM           |
| 233 | Diethylmalonic acid, ethyl 2-fluoro-3-trifluoromethylphenyl ester                                                                            | 91708079 | Diethylmalonic acid, ethyl 2-fluoro-3-trifluoromethylphenyl ester                                 | Methanol      |
| 234 | Fumaric acid, 4-chloro3-methylphenyl nonyl ester                                                                                             | 91717687 | Fumaric acid, 4-chloro3-methylphenyl nonyl ester                                                  | Isopropanol   |
| 235 | Phthalic acid, 4,4-dimethylpent-2-yl isobutyl ester                                                                                          | 91719687 | Phthalic acid, 4,4-dimethylpent-2-yl isobutyl ester                                               | Hexane        |
| 236 | 4-Bromobutyric acid, 8-chlorooctyl ester                                                                                                     | 91719835 | 4-Bromobutyric acid, 8-chlorooctyl ester                                                          | Diethyl ether |
| 237 | Phthalic acid, butyl 8-quinoliny ester                                                                                                       | 91720685 | Phthalic acid, butyl 8-quinoliny ester                                                            | Hexane        |

|     |                                                                               |           |                                                                               |               |
|-----|-------------------------------------------------------------------------------|-----------|-------------------------------------------------------------------------------|---------------|
| 238 | Phthalic acid, monoamide, N-methyl-N-phenyl-, butyl ester                     | 91720686  | Phthalic acid, monoamide, N-methyl-N-phenyl-, butyl ester                     | Hexane        |
| 239 | Phthalic acid, butyl 4-methylthiophenyl ester                                 | 91720702  | Phthalic acid, butyl 4-methylthiophenyl ester                                 | Hexane        |
| 240 | Silane, dimethyl(dimethylpentyloxysilyloxy)isobutoxy                          | 91721173  | Silane, dimethyl(dimethylpentyloxysilyloxy)isobutoxy                          | Methanol      |
| 241 | Terephthalic acid, 3,7-dimethylocta-2,6-dienyl butyl ester                    | 91733466  | Terephthalic acid, 3,7-dimethylocta-2,6-dienyl butyl ester                    | Hexane        |
| 242 | Sebacic acid, but-3-yn-2-yl propyl ester                                      | 91737407  | Sebacic acid, but-3-yn-2-yl propyl ester                                      | Diethyl ether |
| 243 | Silane, dimethyl(4-ethylphenoxy)heptyloxy                                     | 91741339  | Silane, dimethyl(4-ethylphenoxy)heptyloxy                                     | Methanol      |
| 244 | 2-(6,7-Difluoro-1,3-benzoxazol-2-yl)-4,6-dimethylthieno[2,3-b]pyridin-3-amine | 91742968  | 2-(6,7-Difluoro-1,3-benzoxazol-2-yl)-4,6-dimethylthieno[2,3-b]pyridin-3-amine | Isopropanol   |
| 245 | (2-Fluoro-5-nitrophenyl)methanol, dimethylpentafluorophenylsilyl ether        | 91744302  | (2-Fluoro-5-nitrophenyl)methanol, dimethylpentafluorophenylsilyl ether        | Isopropanol   |
| 246 | 4,2'-Dihydroxy-3-methoxychalcone                                              | 134258872 | 4,2'-Dihydroxy-3-methoxychalcone                                              | Isopropanol   |
| 247 | Furazano[3,4-b]pyrazine-5-acetic acid, 6-hydroxy-, ethyl ester                | 135789682 | Furazano[3,4-b]pyrazine-5-acetic acid, 6-hydroxy-, ethyl ester                | Isopropanol   |
| 248 | Ethanone, 2-azido-1-(4-methyl-3-furazanyl)-, oxime                            | 136647801 | Ethanone, 2-azido-1-(4-methyl-3-furazanyl)-, oxime                            | Isopropanol   |
| 249 | Tungsten, tricarbonylchloro(.eta.5-2,4-cyclopentadien-1-yl)-                  | 250111904 | Chlorotricarbonyl-.pi.-cyclopentadienyltungsten                               | Isopropanol   |

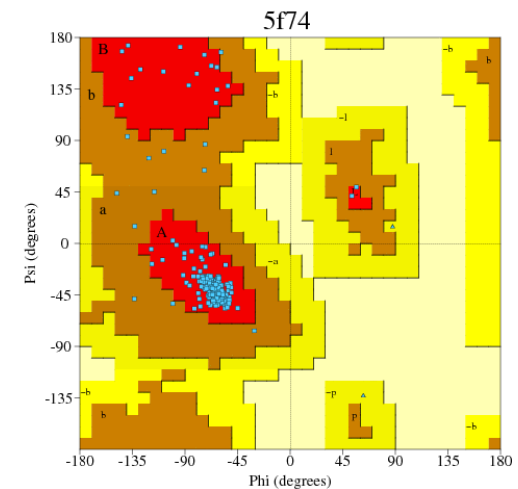
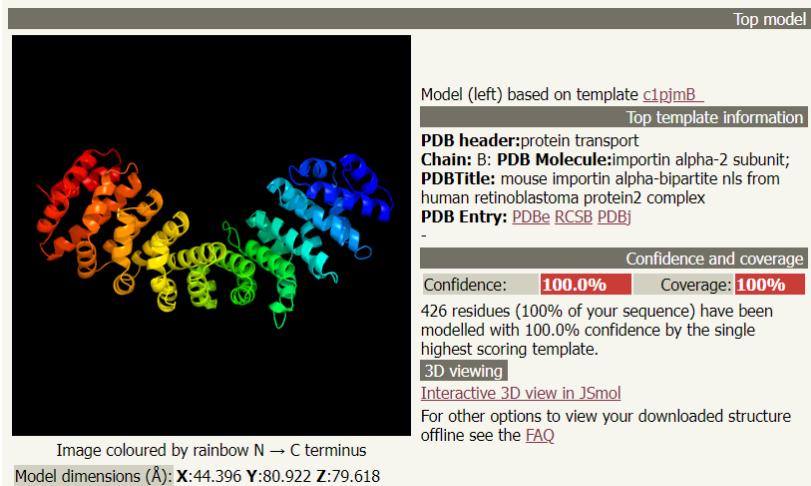
**Table S4:** Predicted protein structure with their Ramachandran plot

| Protein<br>(PDB<br>id) | Predicted 3D structure with validation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Ramachandran plot                                                                               |
|------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| ACC<br>(3FF6)          | <p>Top model</p>  <p>Model (left) based on template <a href="#">c3ff6D</a></p> <p>Top template information</p> <p><b>PDB header:</b> ligase<br/> <b>Chain:</b> D: <b>PDB Molecule:</b> acetyl-coa carboxylase 2;<br/> <b>PDBTitle:</b> human acc2 ct domain with cp-640186<br/> <b>PDB Entry:</b> <a href="#">PDB</a> <a href="#">RCSB</a> <a href="#">PDBj</a><br/> -</p> <p>Confidence and coverage</p> <p>Confidence: <b>100.0%</b> Coverage: <b>96%</b></p> <p>732 residues ( 96% of your sequence) have been modelled with 100.0% confidence by the single highest scoring template.</p> <p><a href="#">3D viewing</a><br/> <a href="#">Interactive 3D view in JSmol</a><br/> For other options to view your downloaded structure offline see the <a href="#">FAQ</a></p> <p>Image coloured by rainbow N → C terminus<br/> Model dimensions (Å): <b>X:</b>105.330 <b>Y:</b>108.517 <b>Z:</b>109.621</p> | <p>3ff6</p>  |

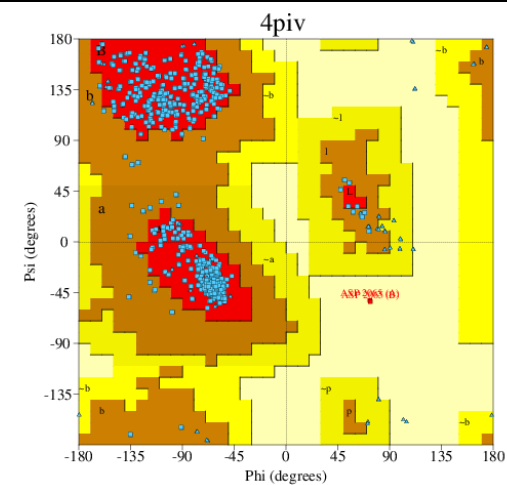
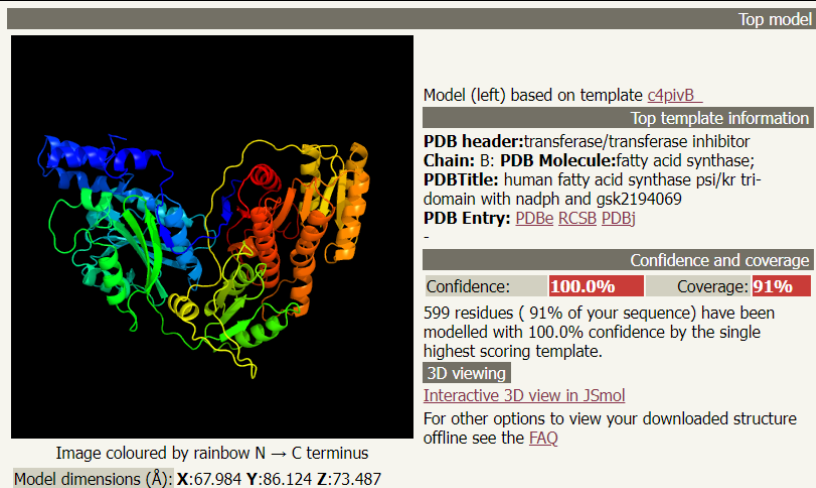
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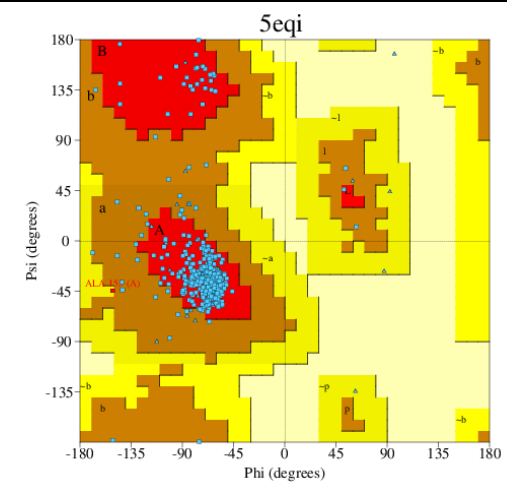
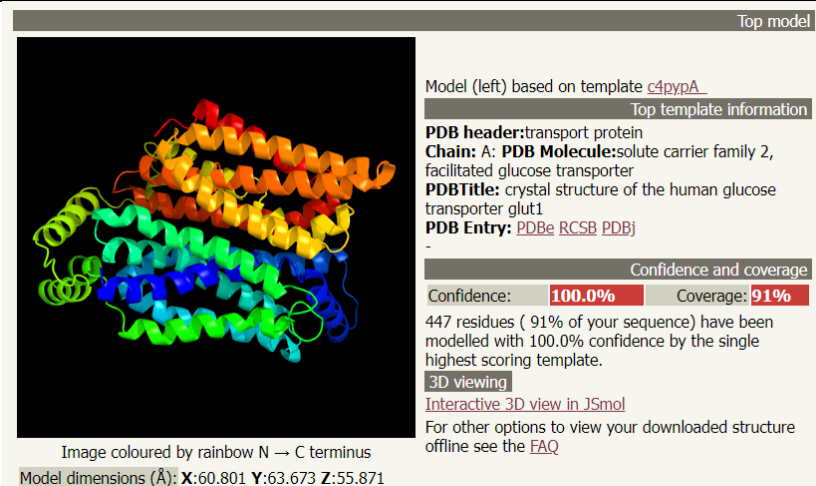
## ChREB P (5F74)



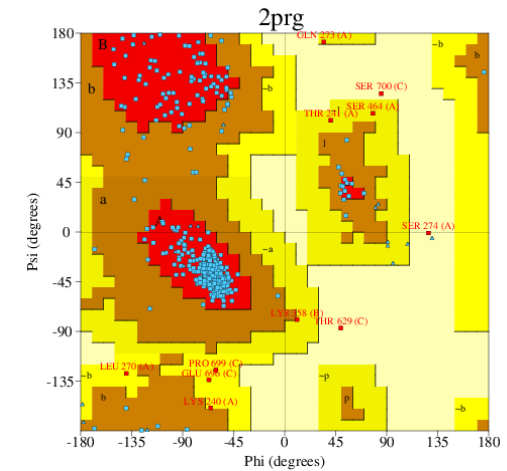
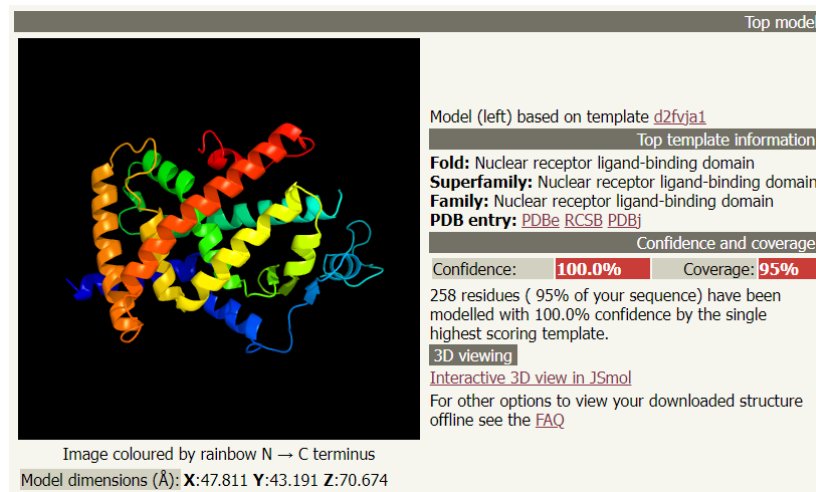
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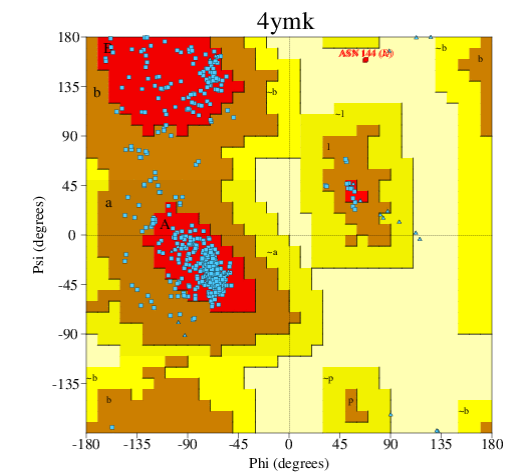
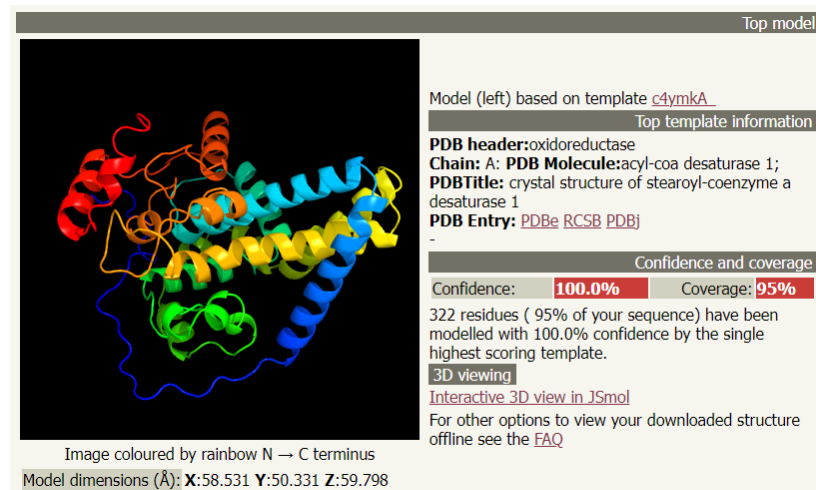
## GLUT1 (5EQI)



## PPAR $\gamma$ (2PRG)



## SCD (4YMK)



# SREBP (6K9M)

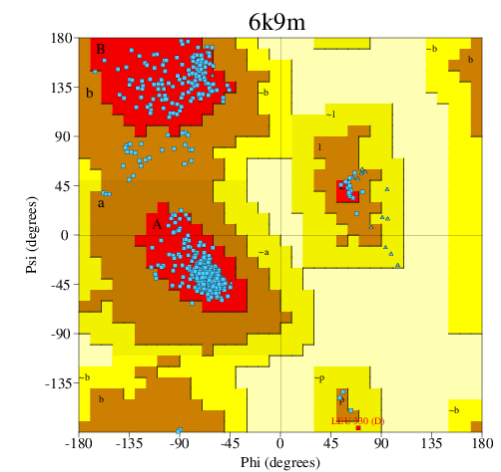
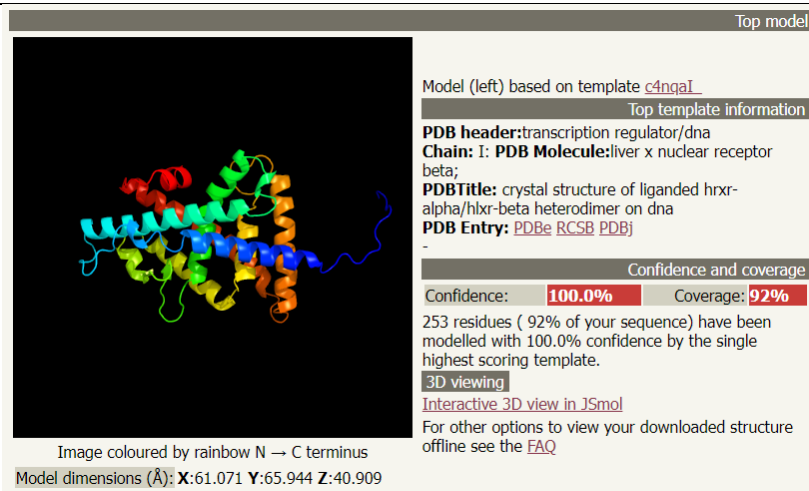
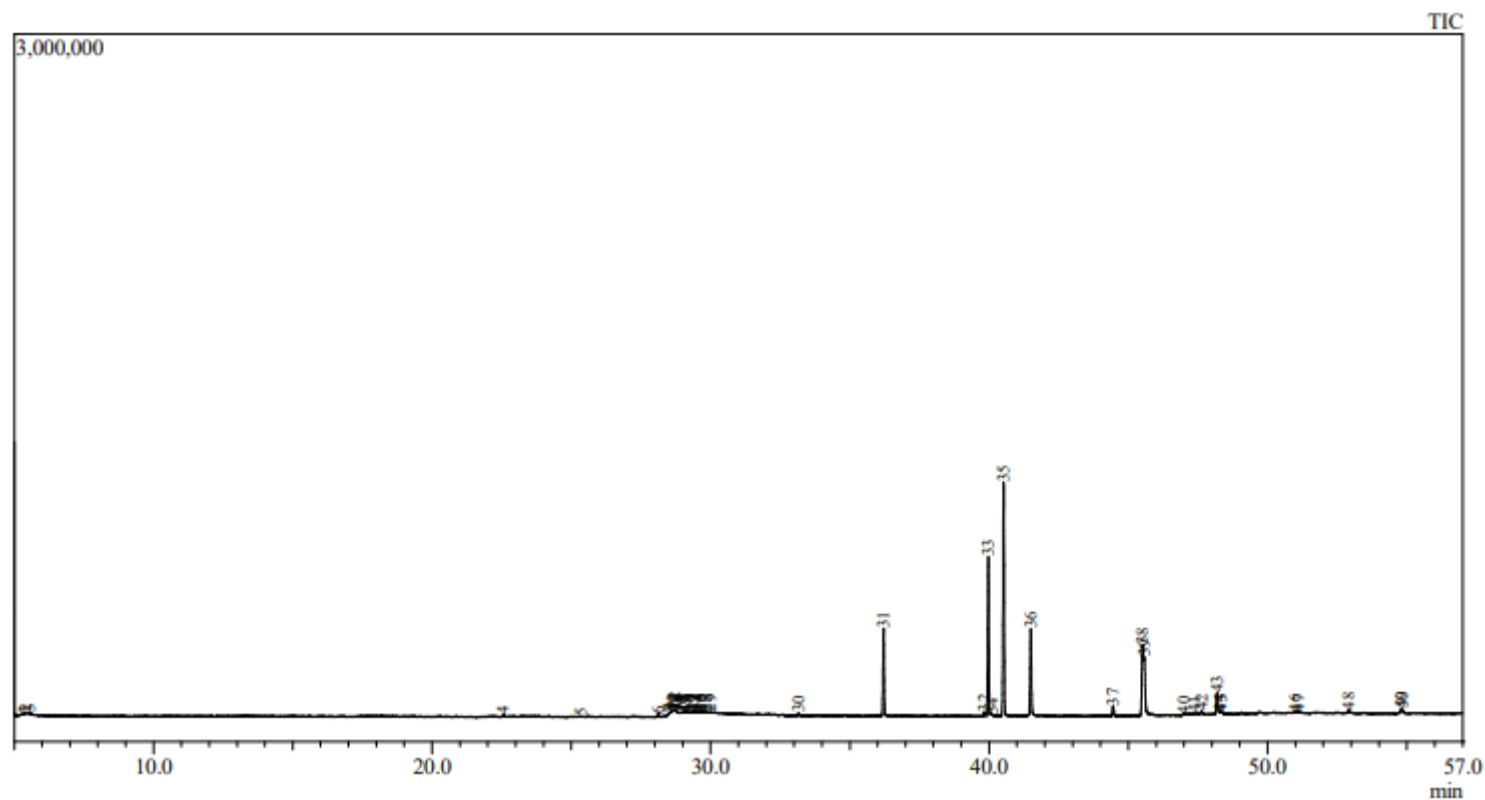


**Figure S1:** GCMS spectra of Hexane extracts



Peak 19: 3-Ethoxy-4-hydroxyphenylacetonitrile

Peak 31: Methyl palmitate

Peak 33: Methyl elaidate

Peak 35: Methyl stearate

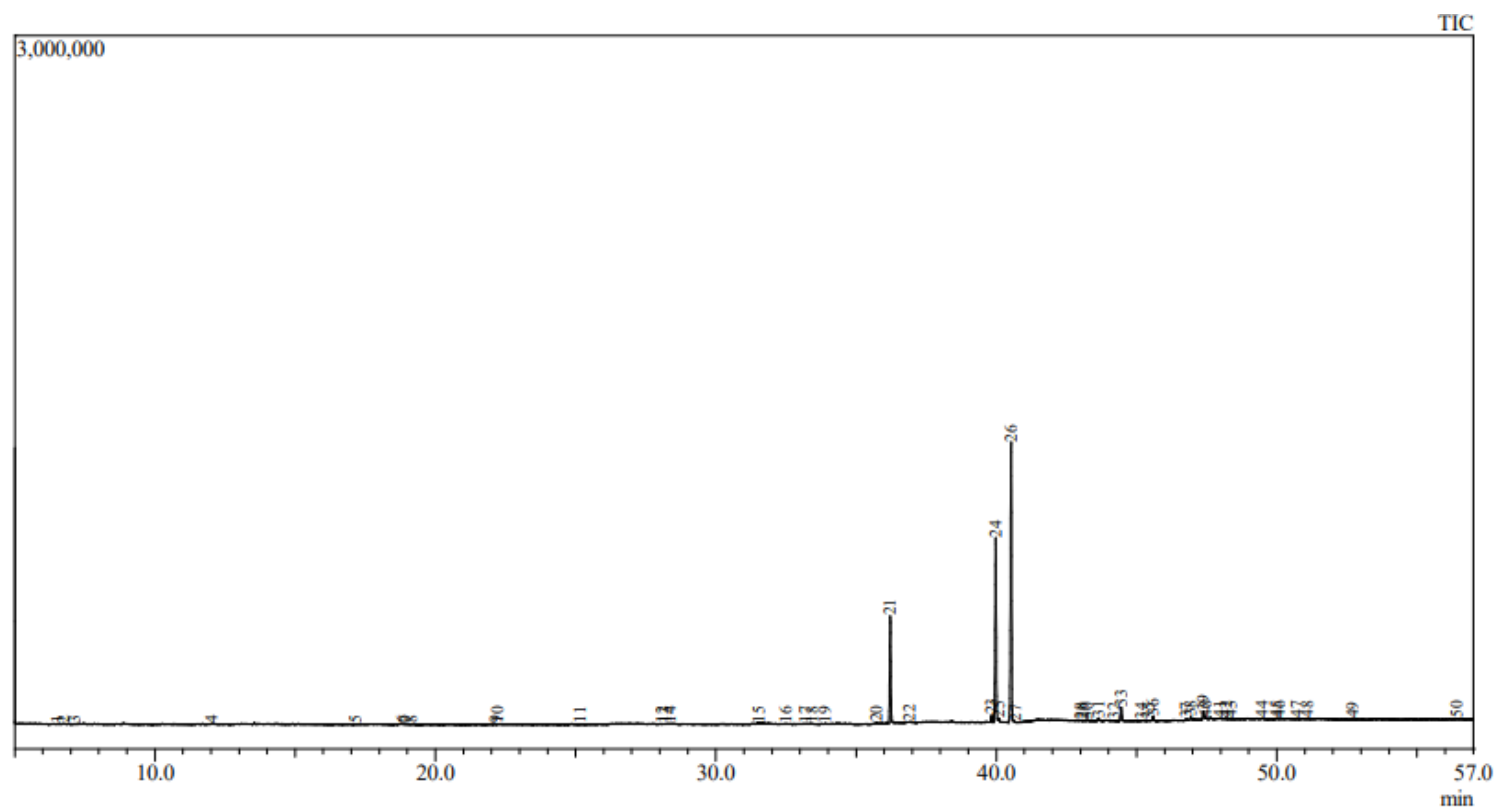
Peak 36: Hexacosene

Peak 37: Methyl arachidate

Peak 38: Bis (2-ethylhexyl) adipate

Peak 43: Bis (2-ethylhexyl) phthalate

**Figure S2:** GCMS spectra of Dichloromethane extracts



Peak 21: Methyl palmitate

Peak 23: Methyl linoleate

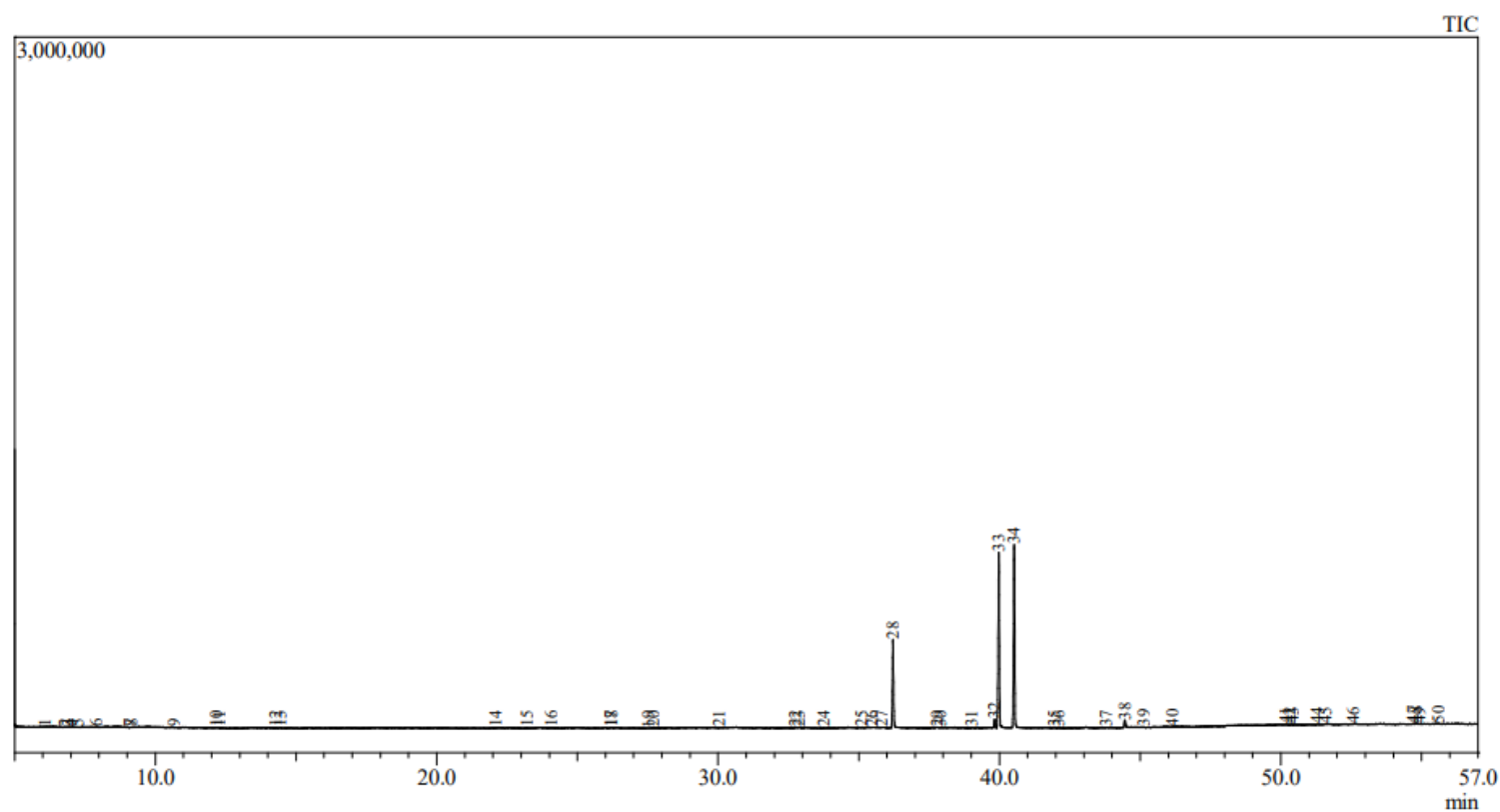
Peak 24: Methyl elaidate

Peak 26: Methyl stearate

Peak 33: Methyl arachidate

Peak 39: Oxiran-2-ylmethyl tetradecanoate

**Figure S3:** GCMS spectra of Methanol extracts



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Peak 28: Methyl palmitate

Peak 38: Methyl arachidate

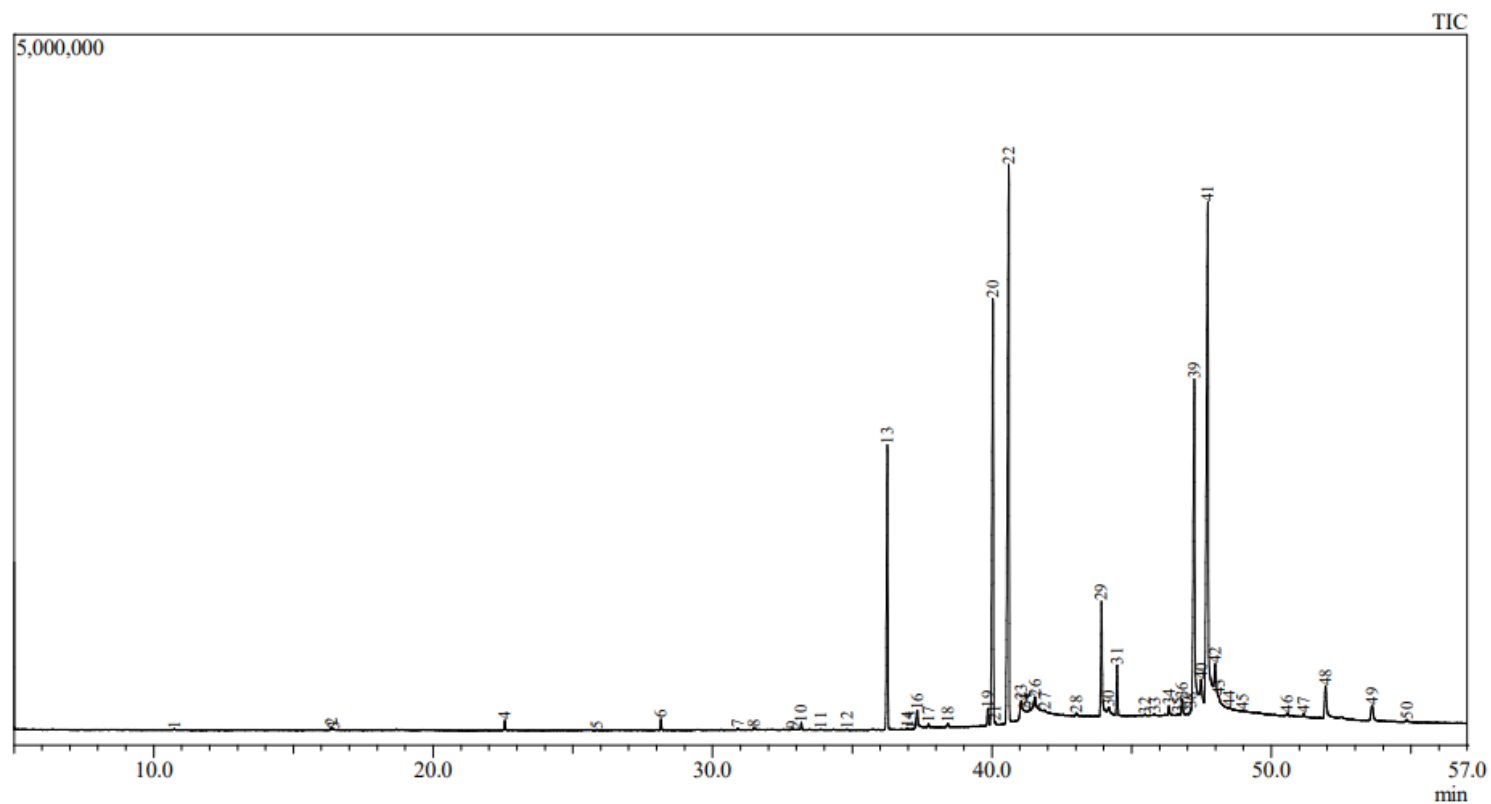
Peak 32: Methyl linoleate

Peak 33: Methyl elaidate

Peak 34: Methyl stearate

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**Figure S4:** GCMS spectra of Acetone extracts



Peak 13: Methyl palmitate

Peak 20: Methyl elaidate

Peak 22: Methyl stearate

Peak 31: Methyl arachidate

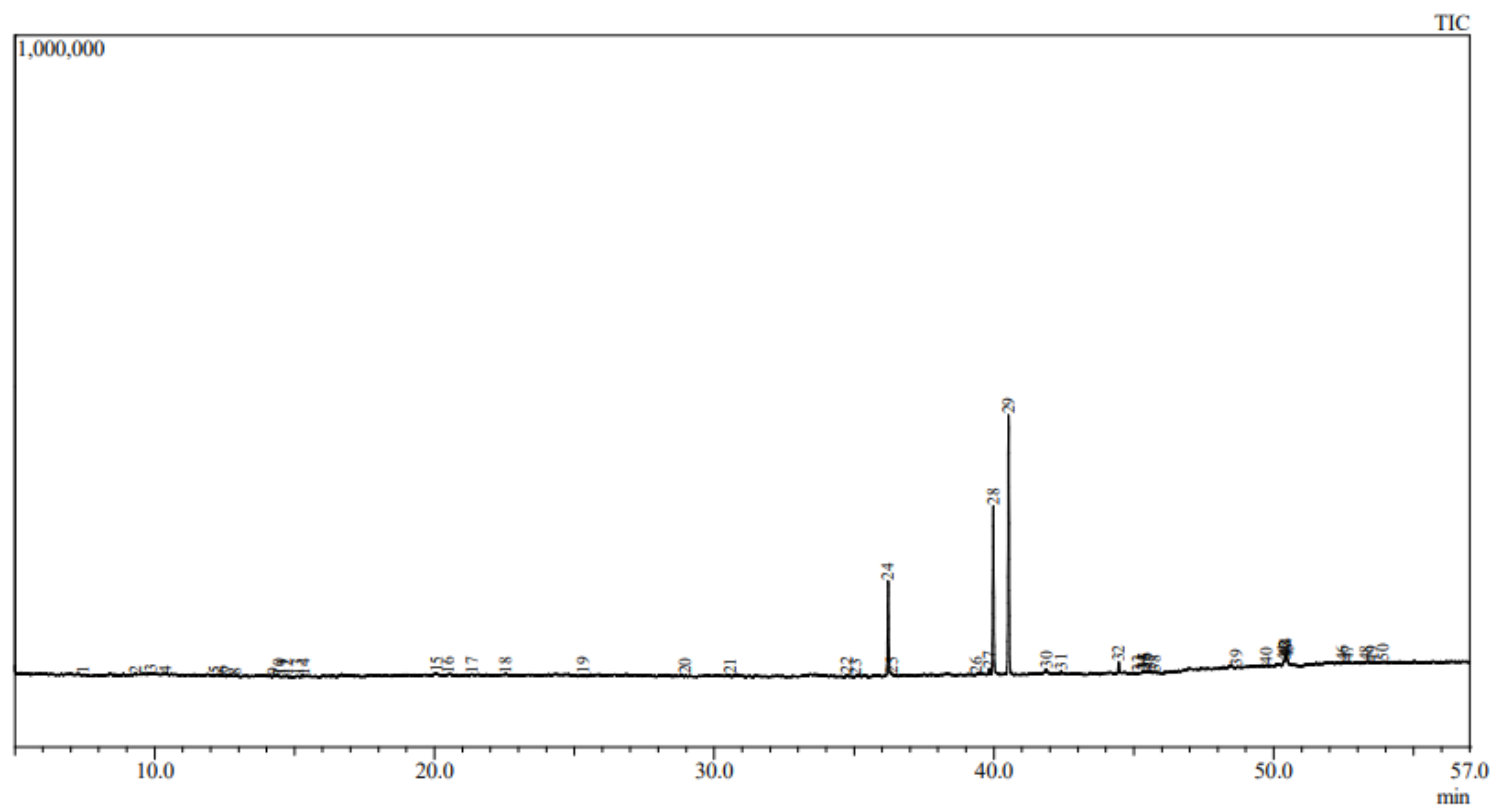
Peak 39: Glycidyl oleate

Peak 41: Glycidyl palmitate

Peak 42: Glycidyl stearate

Peak 43: Methyl behenate

**Figure S5:** GCMS spectra of Isopropanol extracts



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Peak 24: Methyl palmitate

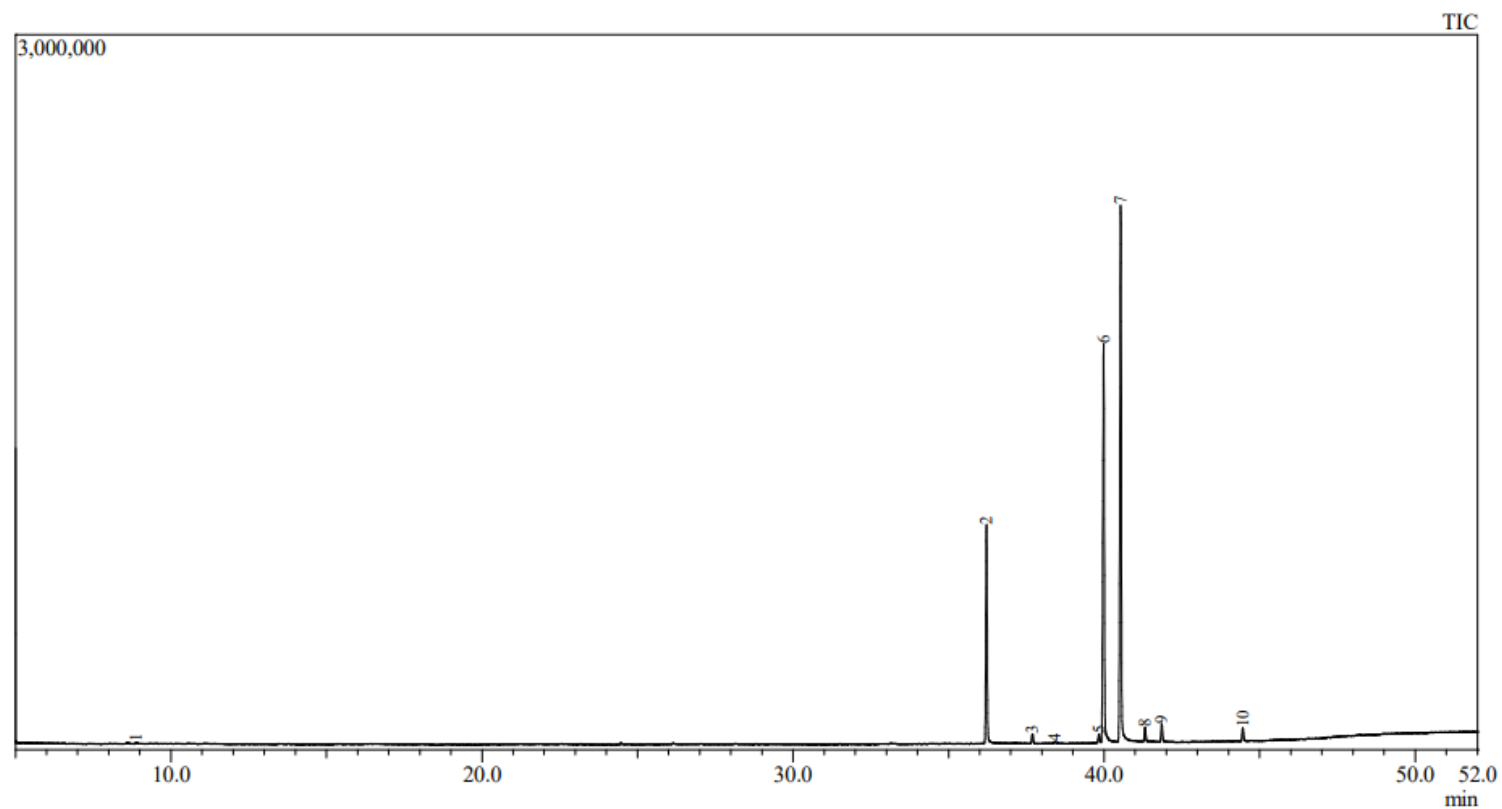
Peak 28: Methyl elaidate

Peak 29: Methyl stearate

Peak 32: Methyl arachidate

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**Figure S6:** GCMS spectra of Ethanol extracts



Peak 2: Methyl palmitate

Peak 6: Methyl elaidate

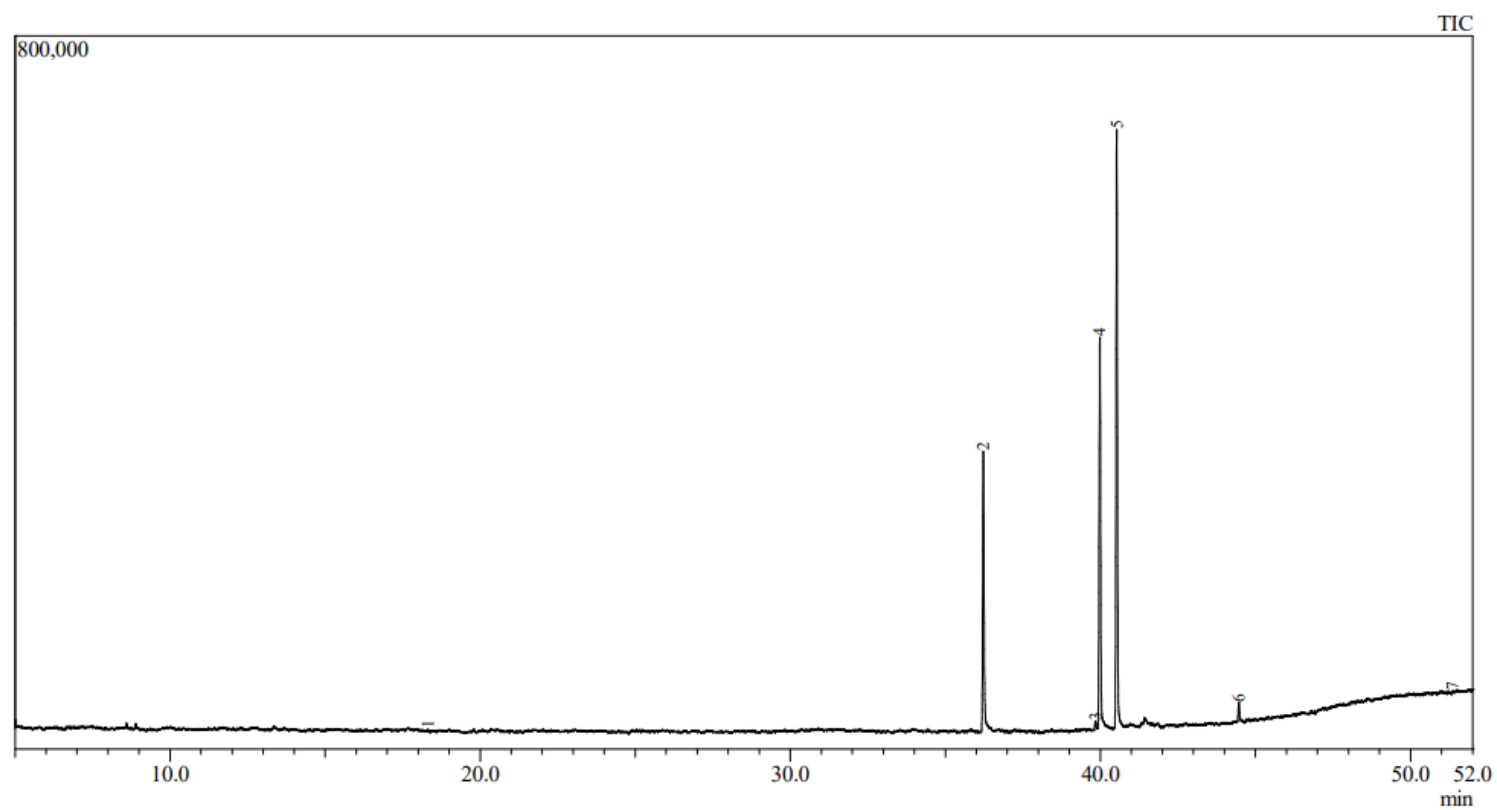
Peak 7: Methyl stearate

Peak 8: Ethyl oleate

Peak 9: Ethyl stearate

Peak 10: Methyl arachidate

**Figure S7:** GCMS spectra of Ethyl Acetate extracts



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Peak 2: Methyl palmitate

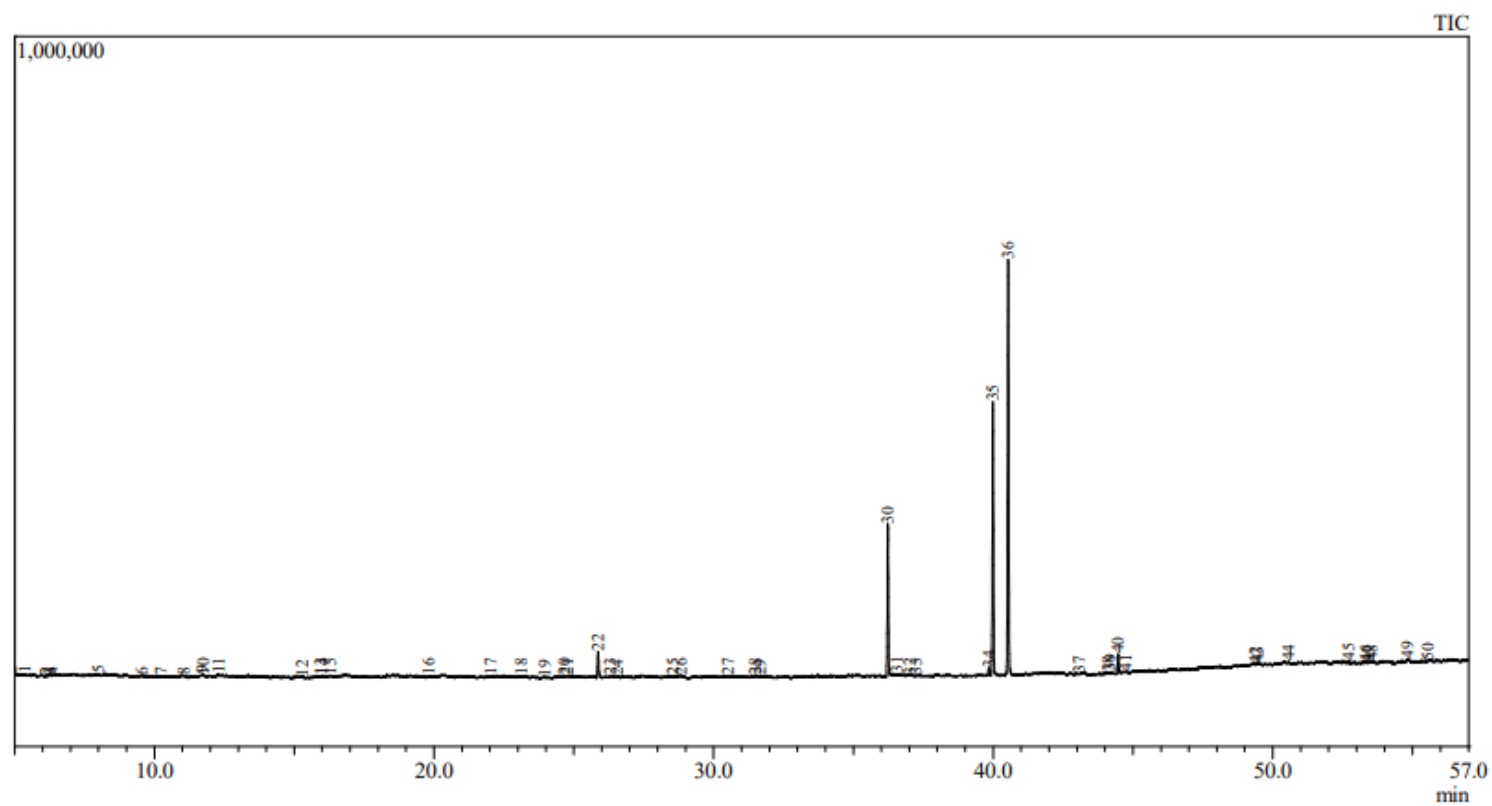
Peak 4: Methyl elaidate

Peak 5: Methyl stearate

Peak 6: Methyl arachidate

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**Figure S8:** GCMS spectra of Diethyl Ether extracts



Peak 22: Butylated hydroxytoluene

Peak 40: Methyl arachidate

Peak 30: Methyl palmitate

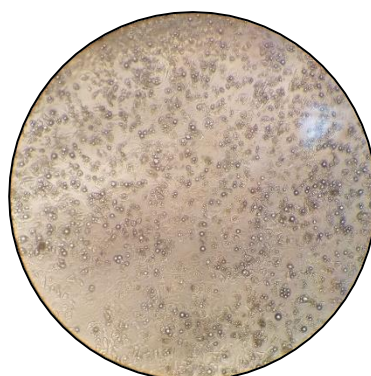
Peak 35: Methyl elaidate

Peak 36: Methyl stearate

**Figure S9:** Example of Oil Red O-stained 3T3-L1 cells



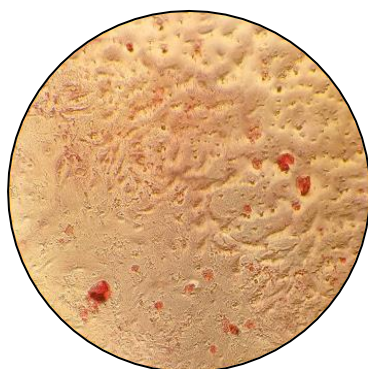
3T3-L1 cells before  
differentiation



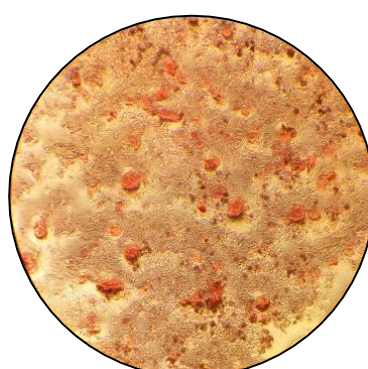
3T3-L1 cells after  
differentiation

**Figure S10:** Example of Oil Red O-stained 3T3-L1 cells

Methanol extracts treated cells  
after stained with Oil Red O stain.

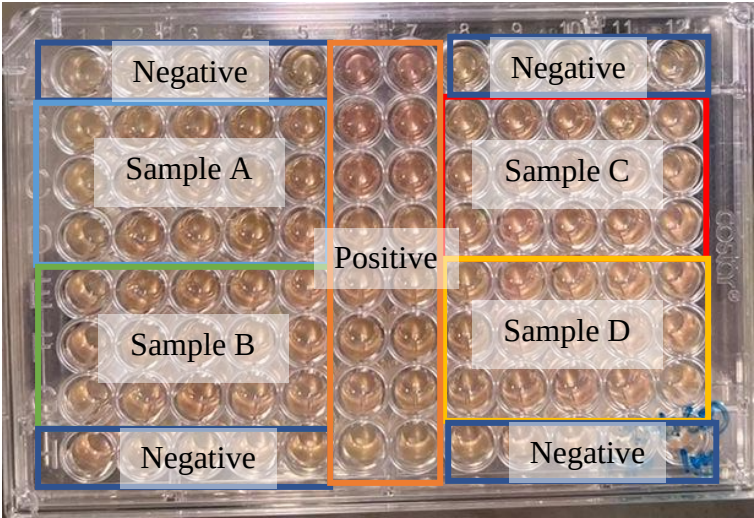


0.000025 mg/mL

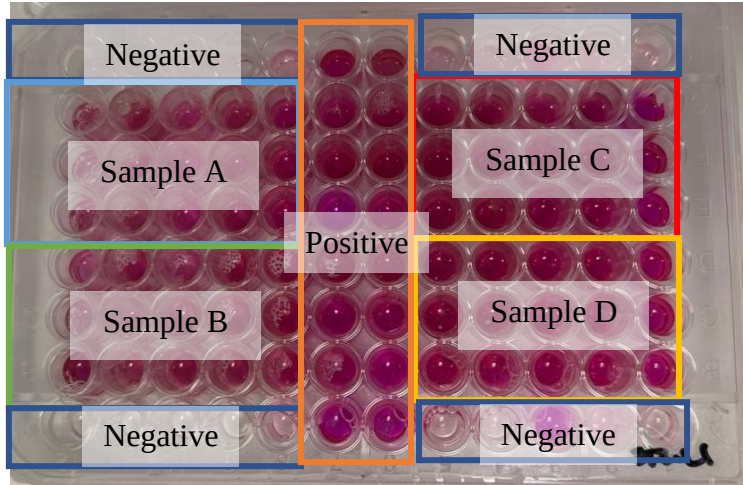


0.25 mg/mL

**Figure S11:** Documentary evidence of assay



MTT assay



Adipogenesis  
assay