COMPUTATIONAL SCREENING AND *IN VITRO* STUDY OF MALAYSIAN PLANTS FOR ANTI OBESITY PROPERTIES TARGETING PANCREATIC LIPASE

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
Å	Angstrom
°C	Degree celcius
µg/ml	Microgram per mililiter
µL	Microliter
3D	Three-dimensional
ASEAN	The Association of South East Asian Nations
BMI	Body Mass Index
BOG	octyl *β*–glucoside
EIU	Economist Intelligence Unit
FDA	Food and Drug Administration
FEB	Free energy of binding
FTIR	Fourier-transform infrared
g	Gram
GA	Genetic Algorithm
GHz	Gigahertz
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
kcal/mol	Kilo calories per mole
kg/m^2	Kilogram per square meter
L	Liter
MHz	Megahertz
mL	Mililiter
mM	milimolar

MUP	Methoxyundecyl phosphonate
NADI	Natural Product Discovery System
NIH	National Institute of Health
nm	Nanometer
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Bank
ppm	Part per million
PTL	Pancreatic triglyceride lipase
RMSD	Root-mean-square deviation
TAG	Triglyceride
THL	Tetrahydrolipstatin
TLC	Thin layer chromatography
v/v	Volume over volume
WHO	World Health Organization

PENYARINGAN PENGKOMPUTERAN DAN KAJIAN *IN VITRO* TUMBUHAN MALAYSIA UNTUK SIFAT-SIFAT ANTI OBESITI MENSASARKAN LIPASE PANKREAS

ABSTRAK

Lipase pankreas ialah enzim yang bertanggungjawab dalam pencernaan lemak diet kepada asid lemak bebas dan monoasilgliserol. Pengambilan lemak diet yang tinggi akan menyebabkan pengumpulan lemak berlebihan dalam badan dan seterusnya menyebabkan pertambahan berat badan dan obesiti. Obesiti telah menyebabkan masalah ekonomi dan kesihatan di Malaysia dan juga seluruh dunia. Untuk mencari alternatif bagi agen anti-obesiti, kaedah penyaringan *in siliko* dan *in vitro* digunakan dengan mensasarkan lipase pankreas untuk menghalang pencernaan lemak diet. Saringan *in siliko* dilakukan dengan menggunakan 3963 sebatian fitokimia dari pangkalan data NADI, di mana pendokan terhadap lipase pankreas (PDB ID: 1LPB) dilakukan. Daripada keputusan penyaringan, 13 tumbuhan dengan sebatian teratas telah dikenalpasti sebagai *Momordica charantia*, *Jatropha curcas*, *Endiandra kingiana*, *Calophyllum inophyllum*, *Garcinia mangostana*, *Chisocheton ceramicus*, *Aglaia argentea*, *Artocarpus champeden*, *Manilkara zapota*, *Dysoxylum hainanense*, *Capsicum annum*, *Oryza sativa* dan *Hopea hainanensis*. Ekstrak metanolik mentah tumbuhan terpilih ini telah diuji untuk kajian *in vitro* bagi menyiasat aktiviti perencatan terhadap lipase pankreas. Daripada penilaian *in vitro*, ekstrak metanolik *Manilkara zapota* dikenal pasti sebagai perencat yang berpotensi untuk lipase pankreas dengan 92.5 % perencatan, diikuti oleh *Oryza sativa* (77.7 %), *Garcinia mangostana* (60.0 %), *Endiandra kingiana* (58.0 %) dan *Momordica charantia* (13.3 %). Penilaian pendokan *Manilkara zapota* telah dinilai dan didapati

sebatioan yang diramalkan dengan sifat anti lipase adalah dari kumpulan terpene. Dengan menggunakan teknik bioassai terpandu, *Manilkara zapota* disekatkan kepada fraksi *n*-heksana, etil asetat dan air. Fraksi *n*-heksana menunjukkan 90.0 % perencatan terhadap lipase pankreas seterusnya dipecahkan menggunakan kromatografi turus. Selepas langkah pemecahan, daripada 14 pecahan yang terkumpul, 10 pecahan menunjukkan aktiviti perencatan melebihi 70.0 %. Pecahan ditandakan sebagai Fr1 yang memberi 90.8 % perencatan, diikuti oleh Fr2 (94.1 %), Fr3 (94.7 %), Fr4 (91.6 %), Fr5 (90.7 %), Fr8 (92.4 %), Fr10 (89.9 %), Fr14 (92.9 %), Fr15 (70.4 %) dan Fr16 (85.8 %). Pecahan-pecahan aktif yang diperolehi daripada *Manilkara zapota* adalah berpotensi dipencilkan sebatian aktifnya untuk diuji sebagai agen anti-lipase.

COMPUTATIONAL SCREENING AND *IN VITRO* STUDY OF MALAYSIAN PLANTS FOR ANTI OBESITY PROPERTIES TARGETING PANCREATIC LIPASE

ABSTRACT

Pancreatic lipase is the enzyme responsible for hydrolysis of dietary fats to free fatty acids and monoacylglycerol. High intake of dietary fats will cause excess accumulation of fat in the body and will further cause overweight and obesity. In order to find alternatives for anti-obesity agents, *in silico* screening and *in vitro* methods were employed, targeting pancreatic lipase to restrain hydrolysis of dietary fat. *In silico* screening was first done using 3963 phytochemical compounds from NADI database, which were docked onto pancreatic lipase (PDB ID: 1LPB). From the screening results, 13 plants with top hits compounds were identified namely, *Momordica charantia*, *Jatropha curcas*, *Endiandra kingiana*, *Calophyllum inophyllum*, *Garcinia mangostana*, *Chisocheton ceramicus*, *Aglaia argentea*, *Artocarpus champeden*, *Manilkara zapota*, *Dysoxylum hainanense*, *Capsicum annum*, *Oryza sativa* and *Hopea hainanensis*. The crude methanolic extracts of these selected plants were tested for *in vitro* study to investigate inhibition activity against pancreatic lipase. From *in vitro* assessment, the methanolic extract of *Manilkara zapota* was recognized as potential inhibitor for pancreatic lipase with 92.5 % inhibition, followed by *Oryza sativa* (77.7 %), *Garcinia mangostana* (60 %), *Endiandra kingiana* (58 %) and *Momordica charantia* (13.3 %). Docking assessment of *Manilkara zapota* was evaluated to discover the predicted compounds with anti lipase properties are from terpene group. Using bioassay guided technique, the extract of *Manilkara zapota* was partitioned into *n*-hexane, ethyl acetate and water

fractions. The *n*-hexane fraction which showed 90.0 % of inhibition against pancreatic lipase was further fractionated using column chromatography. After fractionation step, out of 14 fractions collected, 10 fractions showed inhibitory activity of more than 70.0 %. The fraction assigned as Fr1 exhibited 90.8 % of inhibition, followed by Fr2 (94.1 %), Fr3 (94.7 %), Fr4 (91.6 %), Fr5 (90.7 %), Fr8 (92.4 %), Fr10 (89.9 %), Fr14 (92.9 %), Fr15 (70.4 %) and Fr16 (85.8 %). The active fractions that were derived from *Manilkara zapota* could potentially be isolated for its active compounds to be tested as anti-lipase agents.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Obesity is an emerging global health burden, in developed and developing countries, despite their income levels (Oxfam, 2014). Obesity has been declared as global epidemic since 1997 by World Health Organization (WHO), affecting both adults and children (Oxfam, 2014). If this situation is not well control, the involved countries may have to deal with emerging challenges, for examples, increase in healthcare spending, increase in number of related diseases resulting from obesity and lost of productive years.

1.2 Statement of problem

Obesity prevalence is increasing because of various factors such as urbanization, economic quality, lifestyles and genetic factors (Hruby and Hu, 2015). Since the last three decades, rate of obesity in Asia has increased by more than doubled. From 1980 to 2013, the percentage of adults having Body Mass Index (BMI) at or more than 25 kg/m^2 had increased by 8.1 % in men and 8.2 % in women (The Economist, 2017). Table 1.1 shows the increment percentage of obese adults from 2010 to 2014 (The Economist, 2017). In four years, the number of obese people has increased up to more than 30 % in ASEAN countries. These numbers are increasing at alarming rate and have become a major concern in Malaysia. A research by Oxfam International, released on January 2014, revealed that Malaysian has become the fattest population in Southeast Asia. The research carried out by the United Kingdom Poverty And Disaster Relief Group has ranked Malaysia in 44^{th} place in a list of 125 countries (Oxfam, 2014). In 2015, another survey by World

Obesity Federation (http://www.worldobesity.org/) conducted in Malaysia reported that 15% of men age 18 and above were obese. While, for women, 20.6% age 18 and above were obese. It is also estimated that there will be 2.16 billion overweight people and 1.12 billion obese people with concurrent to other diseases (Segheto *et. al*., 2015).

Table 1.1: Percentage of obese adults and their increment on selected countries. Data is taken from The Economist report (2017).

	Percentage obese adults		
Country	2010	2014	Increase in number of obese people (%), 2010-2014
ASEAN country sample			
Indonesia	4.3%	5.7%	33%
Malaysia	10.5%	13.3%	27%
Philippines	4.1%	5.1%	24%
Singapore	5%	6.2%	24%
Thailand	6.7%	8.5%	27%
Vietnam	2.6%	3.6%	38%
Comparator countries			
Japan	2.9%	3.3%	14%
South Korea	4.2%	5.8%	38%
United Kingdom	25.5%	28.1%	10%
United States	31.2%	33.7%	8%

Current treatments given to obese patients are medication, controlled diet and exercise (Mahgerefteh *et. al*., 2013). Orlistat (**1**) and sibutramine (**2**) are examples of authorized drugs for obesity treatment (Figure 1.2). However, these drugs give negative side effects upon administration. Patients will experience nausea, drowsiness, severe pain in lower back and upper stomach and increased number of bowel movement after taking orlistat (**1**) (Dourish *et. al*., 2008). Sibutramine (**2**) cause patients to experience dry mouth, increase blood pressure, constipation and tachycardia (Dourish *et. al*., 2008). Controlled diet and physical activity to increase energy expenditure approaches, were only able to give promising effect in a short period of time. Obese patients appeared to gain weight after sometimes upon taking

these clinical treatments (de la Garza *et. al*., 2011). Hence, scientist is looking for different approaches and initiatives to treat obesity by targeting natural products.

Besides increasing prevalence of obesity and side effects of current treatments, Malaysia and related countries have to allocate a lot of money for healthcare expenses and clinical researches with the risk that the researches being halted due to negative outcomes (The Economist, 2017). Current treatments and improvement measures taken to improve health had cost Malaysia more than RM4 billion according to a survey by The Economist newspaper. Some studies conducted in a few countries showed that an obese person acquired higher cost for healthcare by more than 25% compared to a healthy person. Considering these factors, it is vital to find other treatments or new drugs by exploiting natural sources to find potential inhibitor to treat obesity (The Economist, 2017).

1.3 Objectives of the study

The objectives of the study are:

1. To investigate potential anti-obesity agents targeted pancreatic lipase from native plants in Malaysia using computational approach.
2. To understand the binding interaction of bioactive compounds with pancreatic lipase.
3. To apply bioassay guided approach in order to identify the active extracts, fractions and sub-fractions.
4. To perform *in vitro* pancreatic lipase assay and investigate the inhibitory activity of plant's extracts, fractions and sub-fractions.

CHAPTER 2

LITERATURE REVIEW

2.1 Obesity

2.1.1 Overview about obesity

Obesity has become a major health problem not only in Malaysia, but also worldwide affecting mostly adolescents and adults. Obesity is defined as the excess accumulation of fat in the body which exceeding the required amount needed by the body that may impair health. This is caused by imbalance between energy intake and energy expenditure (Roh and Jung, 2012). A report from Royal College of Physicians of London, stated obesity as body mass index (BMI) exceeding 30 kg/m^2, whereas overweight is stated as BMI between 25 kg/m^2 and 29.9 kg/m^2 (Nguyen and El-Serag, 2010). Epidemiology studies stated that increasing degrees of overweight and obesity is a crucial prognostic of decreased longevity (Anti-Obesity Drugs Report, 2003).

A campaign by National Institute of Health (NIH) was done to raise awareness about obesity because it is often considered as cosmetic problem by the society, rather than health problem (Puhl and Heuer, 2010). Lack of understanding about obesity and associated healthcare problems had caused this issue to be seldom ignored. Obesity is associated to many diseases such as sleep apnea, coronary heart disease, endocrine and metabolic disturbances, cancer, diabetes mellitus, hypertension as well as psychological problems (Kazemipoor *et al*., 2012). It is also associated to multidimensional impairments in health-related quality of life and psychosocial well-being (Arterburn *et. al*., 2005). In previous years, researchers have spent a lot of money for the treatment and also for the loss of the productivity. There

are few ways of treating obesity that involve combination of medication, behavioral and dietary modification, pharmacotherapy, exercise and gastric bypass surgery in utmost cases (Maria and Evagelia, 2009). Despite all the efforts and remedies provided by diet industry, they failed to give long term effect of weight loss for obese or overweight people. Individuals who are changing their diet to lose weight were estimated to return to their original weight within two to five years (Nelson-Dooley *et. al*., 2005).

2.1.2 Obesity in Malaysia

Economist Intelligence Unit (EIU) from The Economist newspaper has conducted a survey and research in 2017 to study the prevalence of obesity in ASEAN countries, which covered Malaysia, Singapore, Indonesia, Thailand, Philippine and Vietnam (The Economist, 2017). The results were compiled in a report entitled "Tackling Obesity in ASEAN". Among ASEAN countries, Malaysia has the highest obesity prevalence in Southeast Asia with 13.3 % of obese people while overweight people were recorded at 38.5 %. Besides the highest prevalence of obesity, the condition had cost Malaysia between RM4.26 billion and RM8.53 billion for healthcare spending from the previous years. This is equivalent to 9.57 % to 19.36 % of Malaysia's healthcare spending (Figure 2.1). Malaysia had the largest number of productive years lost among females between seven and twelve years and second highest among males in between six and eleven years in ASEAN (The Economist, 2017).The reduction in productive years is the impact from affliction related to obesity. Increment in obesity statistics at alarming rate appeared significantly by lack of exercise and Malaysian eating habit. The report showed that only a third of Malaysian adults had ever exercised while only 14 % exercise

adequately. Other than that, Malaysian has strong culture of entertaining guests with food and often consumes unhealthy diet such as fast foods. Apart from that, Malaysian view obesity as cosmetic issue rather than a health issue.

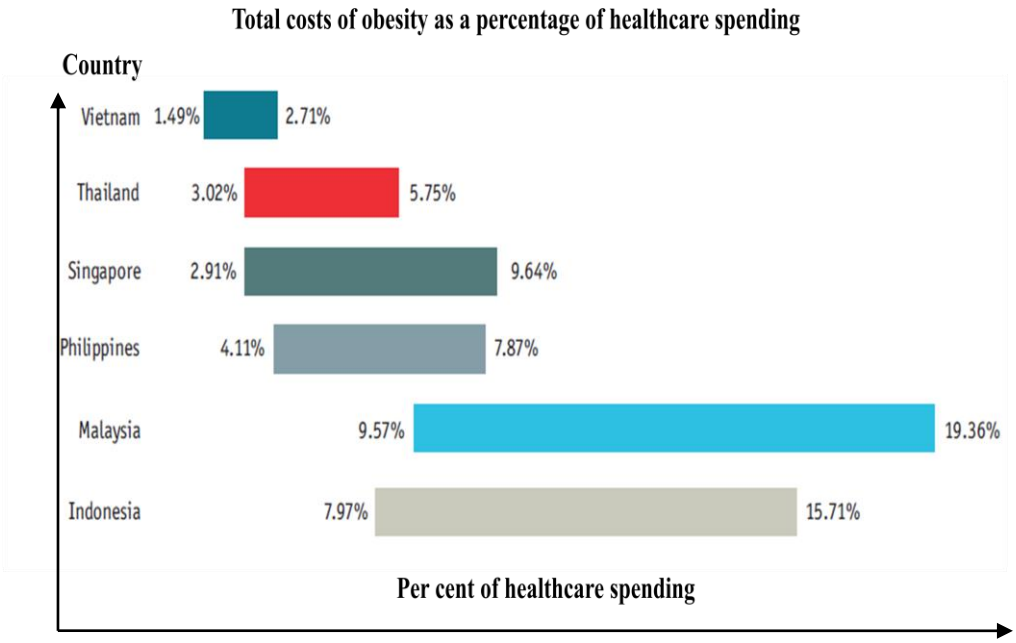


Figure 2.1: Percentage of total cost in healthcare spending according to ASEAN countries (The Economist, 2017).

2.1.3 Strategies and treatment of obesity

There are six strategies to treat obesity that involve different approaches summarized by Yun (2010). The available treatments of obesity that have been studied are lipase inhibitory, suppression of food intake, stimulating energy expenditure, inhibition of adipocyte differentiation, regulation of lipid metabolism and also combine effect of the treatments (Yun, 2010).

Lipase inhibitory effect is one of the promising treatments for obesity because there is no alteration of central mechanism (Birari and Bhutani, 2007). Pancreatic lipase hydrolyzes dietary fat before being absorbed by the intestine. Therefore, pancreatic lipase has been widely studied in order to determine its potential as anti-

obesity agents. Inhibition of pancreatic lipase restrained the dietary fat from being hydrolysed and absorbed into the intestine and then excreted as faeces (Godoy-Matos *et. al*., 2011). Phytochemicals that have inhibitory effect on pancreatic lipase are polyphenols, saponins, flavonoids and caffeines, which can be found in various type of tea leaves and other natural source (Kim and Kang, 2005; Han *et. al*., 2006 and Shimoda *et. al*., 2006).

Second strategy is suppression of food intake to regulate body weight which is related to hormonal and neurological systems (Yun, 2010). There are few receptors in the central nervous system that responsible in satiety regulation such as serotonin, dopamine, histamine and other associated receptors. Researches will target these receptors in order to treat obesity by reducing energy intake (Chantre and Lairon, 2002). Sibutramine is the new drug in the market that can treat obesity via appetite suppression. However, sibutramine have known side effects such as insomnia, dry mouth and constipation (Chaput *et. al*., 2007). *Garcinia cambogia*, Korean red ginseng (Kim *et. al*., 2005), *Camellia sinensis* (Kao *et. al*., 2000), sunflower oil (Ferrer-Lorente *et. al*., 2007) and *Citrus aurantium* (Klontz *et. al*., 2006) are few examples of plant that have appetite suppressing properties. These plants mainly contain of glycosides, saponins and flavonoids.

Third strategy of treating obesity is by stimulating energy expenditure (Yun, 2010). Evidence showed that many rodent models have become obese due to low energy expenditure. A mammalian will dissipate excess energy as heat to modulate energy expenditure and body weight. Hence, the key in this strategy is to increase energy expenditure by physical activity, obligatory energy expenditure or by

adaptive thermogenesis (Flatt, 2007). Extracts of *Pinellia ternate* (Kim *et. al*., 2006), *Panax ginseng* (Attele *et. al*., 2002) and *Ilex paraguariensis* (Pang *et. al*., 2008) are examples of plants that were proven to increase energy expenditure.

The fourth strategy is inhibition on adipocytes differentiation. The main role of adipocytes is storing triglycerides and release free fatty acids upon energy demand by the body which is important in maintaining energy balance and lipid homeostasis in the body (Yun, 2010). Hence, the processes of adipocyte proliferation and differentiation are targeted in anti-obesity material screening. However, a research by Lefterova and Lazar (2009) exposed that the inhibition of adipogenesis caused astherosclerosis and diabetes type 2. Bioactive compounds that are proven to inhibit adipocyte differentiation are sporamin from *Ipomoea batatas* that showed 84 % inhibition activity onto preadipocyte differentiation (Xiong *et. al*., 2009). Mycelia extract from *Cordyceps militaris* decreased lipid accumulation by 93.7 % (Shimada *et. al*., 2008) while ternatin from *Coriolus versicolor* decreased triglyceride accumulation by 87 % (Ito *et. al*., 2009).

The fifth strategy is regulation on lipid metabolism by prompting triglyceride hydrolysis to decrease fat store in the body (Yun, 2010). Various flavonoids identified in the extract of *Nelumbo nucifera* were proven to suppress body weight gain in mice (Ohkoshi *et. al*., 2007).

The sixth strategy is the combination effect of strategies mentioned above (Yun, 2009). Catechins from green tea have showed anti-obesity effect by increasing

energy expenditure and lipolytic activity, lowering adipocyte differentiation and lipogenic activity and suppress appetite (Boschmann and Thielecke, 2007).

2.1.4 Commercial drugs and their side effects

There are many authorized drugs for the treatment of obesity available in the market. Intake of prescribed medicine should be combined with changes in eating and physical activity which can help people lose weight by less than 10% of their body weight. However, some drugs for obesity treatment may be linked to serious health problem.

There are several synthetic drugs that have been used for obesity treatment which are orlistat (**1**), sibutramine (**2**), lorcaserin (**3**), rimonabant (**4**), phentermine (**5**) and topiramate (**6**) (Figure 2.2), naltrexone/bupropion, and liraglutide,. However, sibutramine and rimonabant were withdrawn from market because of their harmful side effects (Gadde *et. al*., 2018).

Orlistat (**1**), or also known as Xenical® has been available since 1999. The substance tetrahydrolipstatin (THL) derived from lipstatin produced by *Streptomyces toxytricini,* appears to be a general inhibitor for mammalian lipases (Lookene *et. al*., 1994). Orlistat is approved for adolescent age 12 years and above (Rogovik and Goldman, 2011). This drug acts by blocking the digestion of about 30% of dietary fat ingested within the lumen of gastrointestinal tract (Dourish *et. al*., 2008). Orlistat does not get absorbed by the body and it only acted inside of the intestine (Moreno *et. al*., 2003). From medical records, individual can lose about 5 to 7 pounds after maximum two years of consumption. However, orlistat can give side effects such as

diarrhea, gas, stomach pain and rare cases of severe liver injury (Mohamed *et. al*., 2014).

Lorcaserin (**3**), sold as Belviq® is an appetite suppressant drug. It acts on serotonin receptors in the brain which causes reduction of energy intake. Consumer will feel full after taking small amounts of food. The effectiveness of the drug is proven by loss of 5% of initial body weight in one year. However, there are known side effects of lorcaserin such as constipation, dry mouth, cough, tiredness, nausea dizziness and headaches (Kim *et. al*., 2014).

Phentermine-topiramate or its commercial name, Qsymia®, is also an appetite suppressant drug. Phentermine (**5**) will suppress appetite, while topiramate, (**6**) an approved drug use to treat epilepsy, have distinct weight loss side effect. The side effects due to these combination drugs are constipation, dry mouth, insomnia, dizziness, taste alterations and tingling of hands and feet. Phentermine, benzphetamine, diethylpropion and phendimetrazine are drugs that sold under many names are also appetite suppressant drug (Kim *et. al*., 2014 and Hendricks, 2017).

Naltrexone/bupropion or its commercial name, Contrave, acts by suppressing food intake. Effects from consuming this drug are dizziness, headache, insomnia, vomiting, dry mouth and diarrhea. Liraglutide also suppress food intake by decrease appetite and increases the feeling of fullness over effects on central nervous system (Gadde *et. al*., 2018).

Sibutramine (**2**) sold as Meridia® inhibits re-uptake of serotonin and norephinephrine to cause reduction in food intake. Obese people whom consume this drug will commonly experience constipation, dry mouth, headache and insomnia. There are also possibilities for stroke and heart attack when taking this drug in long term period (Mohamed *et. al*., 2014). Rimonabant (**4**) or Acomplia® also acted by reducing food intake by blocking the cannabinoid-1 receptor. Common side effects are dizziness, diarrhea and nausea (Lunagariya *et. al*., 2014). Both sibutramine and rimonabant have been withdrawn from the market because of their serious side effects. Sibutramine can lead to heart attack and stroke in high risk cardiac patients. Rimonabant can cause probable serious psychiatric disorder (Ado *et. al*., 2013).

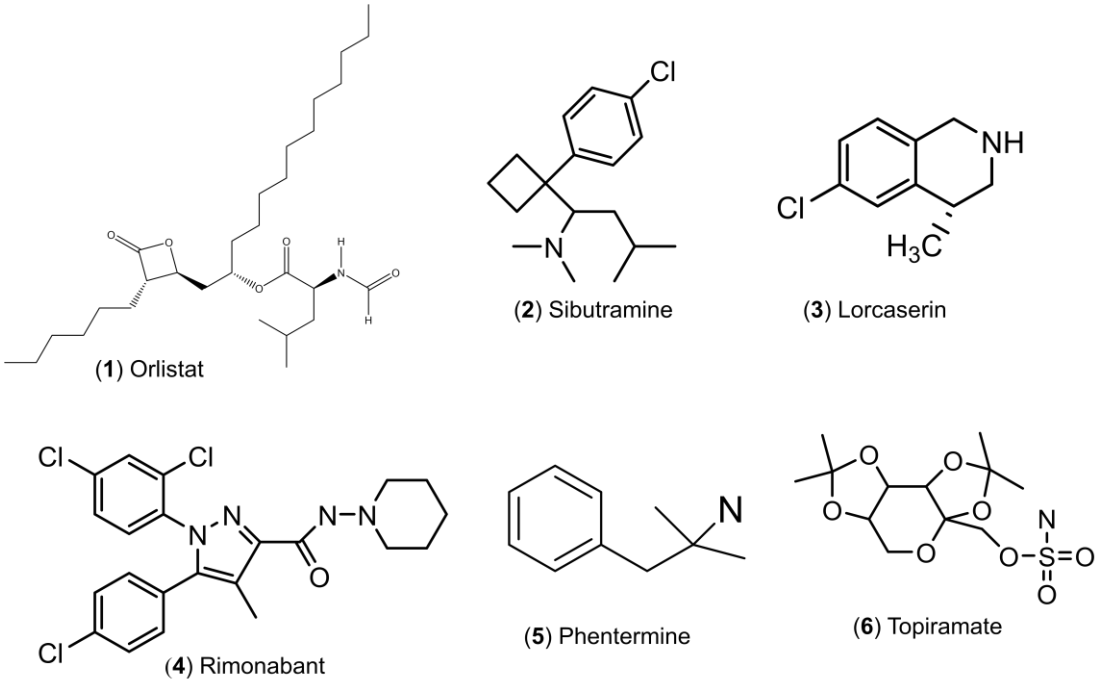


Figure 2.2: Chemical structure of orlistat (**1**), sibutramine (**2**), lorcaserin (**3**), rimonabant (**4**), phentermine (**5**) and topiramate (**6**).

2.2 Lipid digestion and absorption

Obesity is a multifactorial and heterogeneous syndrome affected from environmental factors and genetic susceptibility. The environmental factors are mainly due to daily diet and lifestyle (Brettfeld *et al*., 2015 and Nirmala *et. al*., 2008). Iqbal and Hussain (2009), reported that triglyceride (TAG) is the most abundant type of lipid complexes found in food that give about 90-95 % of total energy attained from dietary fats. Digestion of TAG begins in the mouth and stomach by the action of lipase and continued in the small intestine. Less than 30 % of dietary lipids are digested in the mouth and stomach. Major hydrolysis of TAG happened in small intestine by the action of pancreatic lipase. Lipid digestion in small intestines occurs in systemic manner involving bile salts and pancreatic lipase (Iqbal and Hussain, 2009). Lipids will be delivered to the intestinal lumen as crude lipid emulsion particle. Most dietary fats exist as emulsion to permit transportation throughout the water-rich environment in the body because lipids are hydrophobic in nature. In the presences of lipid emulsion, bile salts are released into lumen to help the formation of micelles. Meanwhile, pancreatic lipase coheres to the lipid emulsion and hydrolyzed the ester bond of the TAG to produce free fatty acid and monoacylglycerol. After the hydrolysis process, micelles with phospholipids in vesicles are formed. Micelles are water soluble lipid materials that contain bile salts. This allows lipid substances to be transported in aqueous environment and absorbed into enterocyte (Lunagariya *et. al*., 2014). Inside enterocytes, the hydrolysis product of TAG will undergo biosynthesis process of TAG. Lipids would be then stored in the body in the form of TAG (Kerr *et. al*., 2015). The process of lipid digestion and absorption is shown in Figure 2.3.

Fat-rich diet will cause excess TAG in the body. Digested dietary triglyceride which hydrolyzed to fatty acid and glycerol are absorbed by small intestine. Excess triglyceride in the body will accumulate in the adipose tissue causing obesity (Schwartz and Wolins, 2007 and Ahmadian *et al*., 2007). As the result from the lipase absences or the lipase inhibition, dietary fats are not hydrolyzed and being excreted through stools. This causes reduced weight or slow growth, loss of fluids and decrease of fat-soluble vitamins (Lowe, 1997).

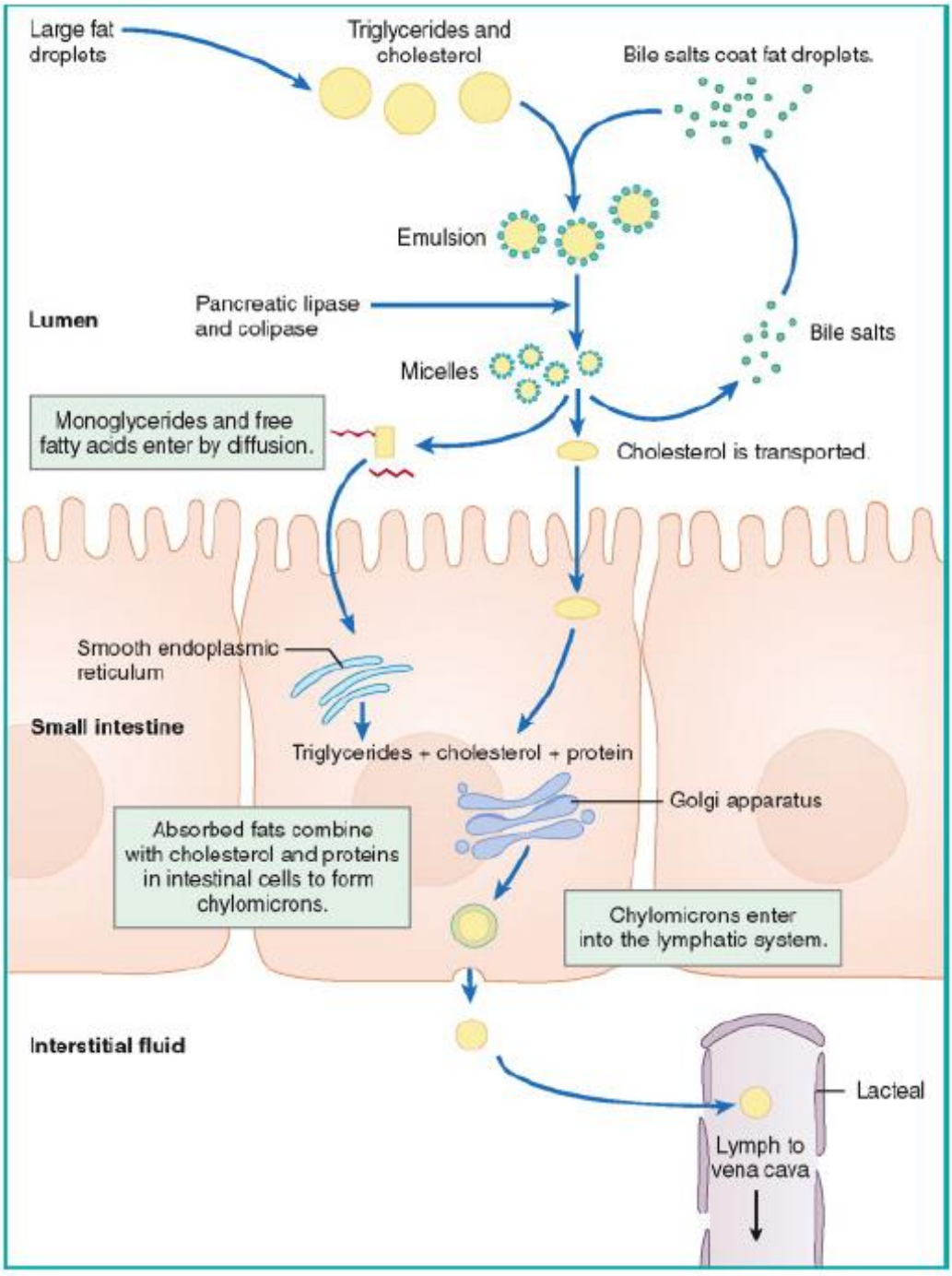


Figure 2.3: Mechanism of dietary lipid digestion and absorption. A mixture of fat droplets binds with cholesterols and bile salts to form micelle. Hydrolysis by lipase results into free fatty acids and will be absorbed into enterocyte and monoacylglycerides are re-synthesis into triacylglyceride (Carroll, 2007).

2.3 Natural products as source of lipase inhibitor

Phytochemicals from natural sources affect on various pathways which gives the anti-obesity effects, such as inhibition of lipase enzyme, stimulation of energy expenditure, suppress appetite, inhibition of adipocyte differentiation and regulation of lipid metabolism. There are many studies done in search for new anti-obesity drugs by exploiting natural products. Vast types of dietary phytochemicals such as flavonoids, polyphenols, saponins, tannins, alkaloids and terpenes (Table 2.1) that have lipase inhibitory properties are potential alternatives for obesity treatment (Ado *et. al*., 2013 and Mohamed *et al*., 2014). .

Table 2.1: Types of phytochemicals and classes that have anti-obesity properties (de la Garza *et. al*., 2011).

Phytochemicals	Examples of phytochemicals class
Polyphenols	Flavonoids, isoflavones, chalcones, phenolic acids
Terpenoids	Carotenoids, sesquiterpenes
Alkaloids	Pyridine, xanthine
Organosulfur	
Phytosterols	

Various plants were widely screened for their lipase inhibitory activity in order to find active anti obesity agents. Natural resources can be exploited to find alternative for obesity treatments with a possibility of less or no side effects. Alias *et. al*. (2017) screened 24 crude plant extracts for lipase inhibitory activity with porcine pancreatic lipase *in vitro* assay. Four extracts were found to have inhibition more than 70 % which was considered as high activity. The most effective extract as pancreatic lipase inhibitor was *Phyllanthus niruri* with IC_{50} value of 27.7 µg/ml and followed by *Orthosiphon stamineus* (34.7 µg/ml), *Murraya paniculata* (41.5 µg/ml)

and *Averrhoa bilimbi* (55.2 µg/ml). The plant parts used were leaves, and whole plant for *Phyllanthus niruri*. However, these extracts were less potent when compared to orlistat which have IC_{50} value of 1.7 µg/ml.

Ado *et. al*. (2013) tested 98 plants from Malaysia on pancreatic lipase to determine their inhibitory activity. Methanolic extracts of all plants were subjected to *in vitro* assay and 19.4 % of the extracts showed inhibition by more than 80.0 %. Four plants namely *Aleurites moluccana* L. Willd (leaves), *Archidendron jiringa* (Jack) I.C Nielsen (flowers), *Averrhoa carambola* L. (fruits) and *Cynometra cauliflora* L. (leaves) showed 100 % inhibition against pancreatic lipase. An isolated compound from *Cynometra cauliflora* known as kaempferol 3-*O*-rhamnoside was also determined as an active lipase inhibitor.

In another study, Lee *et. al.* (2010) have successfully isolated new C-glycosylated flavones known as luteolin 6-*C*-β-D-boivinopyranoside from *Eremochloa ophiuroides* which was predicted to be potential lipase inhibitor.

Shin *et. al*., (2003) tested 200 herbal medicines for anti lipase activity and discovered that *Alpinia officinarum* is an effective lipase inhibitor with IC_{50} value of 9.6 mg/ml. Water extract of *Alpinia officinarum* was tested on *in vitro* assay of pancreatic lipase and *in vivo* test on hyperlipidemic mice. 3-methylethergalangin was successfully isolated and identified as potential lipase inhibitor with IC_{50} of 1.3 mg/ml. The effectiveness of this compound as lipase inhibitor was considered good when compared to orlistat with IC_{50} value of 0.8 mg/ml.

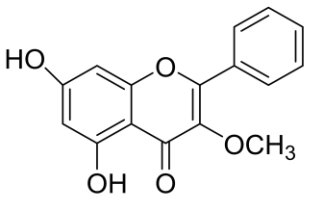
Kim *et. al*., (2010) studied on inhibitory effects of ethanolic extract of *Morus bombycis* against pancreatic lipase. The root of *Morus bombycis* showed a good inhibitory effect with IC_{50} value of 2.07 µg/ml, even though it is less effective when compared to orlistat, with IC_{50} value of 0.154 µM.

Green tea beverages that are widely consumed have shown the ability to inhibit pancreatic lipase by 66.5 % because of the presence of catechins in the green tea (Koo and Noh, 2007 and Juhel *et al*., 2000). Martins *et. al*., (2009) studied on inhibitory activity of mate tea, the sources of beverages that being consumed in Brazil. Mate tea is known to contain saponins and caffeine which have a good inhibition against pancreatic lipase.

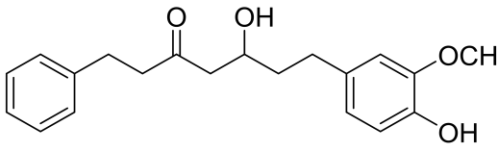
Kumar *et. al*. (2012) screened 33 Indian medicinal plants for their anti lipase activity. *Cassia siamea* roots extract is the most effective lipase inhibitor with 74.3 % inhibition. *Cassia siamea* is an edible plant and its leaves are used in making curry. Other plants with high inhibition activity are the leaves of *Chukrasia tabularis* (67.6 %), roots of *Vigna radiata* (64.6 %), fruits of *Lagerstroemia indica* (70.1 %), whole plants of *Justicia gendarussa* (61.1 %) and resin of *Ferrula asaefoetida* (72.5 %).

Crude extracts and isolated compounds derived from natural products have been documented for its ability to reduce body weight such as, 3-Methylethergalangin (**7**) and 5-Hydroxy-7-(4'-hydroxy-3'methoxyphenyl)-1-phenyl-3-heptanone (**8**) were isolated from *Alpinia officinarum* (Shin *et. al*., 2003 and Shin *et. al*., 2004), new isolated compound that identified as pancreatic lipase inhibitor, 3-O-trans-p-coumaroyl actinidic acid (**9**) from *Actinidia arguta* (Jang *et. al*., 2008),

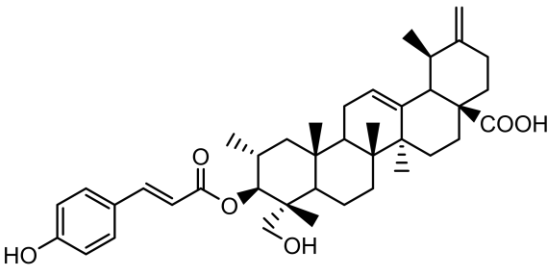
luteolin 6-*C*-β-D boivinopyranoside (**10**) from the leaves of *Eremochloa ophiuroides* (Lee *et. al*., 2010), cassiamin A (**11**) was isolated from *Cassia siamea* (Kumar *et. al*., 2012), carpesterol (**12**) was isolated from fruits of *Solanum stramonifolium* (Chanmee *et. al*., 2013), kaempferol-3-*O*-rhamnoside (**13**) from *Cynometra cauliflora* (Ado *et. al*., 2013) (Figure 2.4). From previous studies, natural products are highly potential as an alternative for anti-obesity drugs with less unpleasant effects.



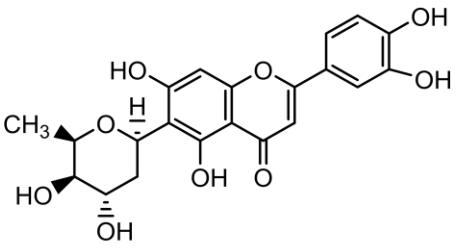
(**7**) 3-Methylethergalangin



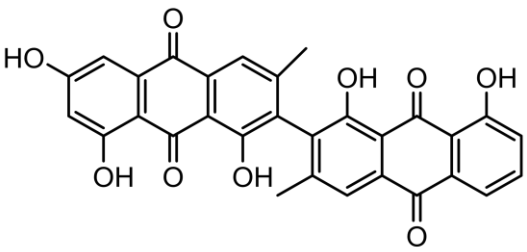
(**8**) 5-Hydroxy-7-(4'-hydroxy-3'methoxyphenyl)-1-phenyl-3-heptanone



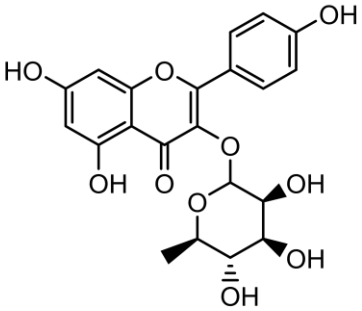
(**9**) 3-O-trans-p-coumaroyl actinidic acid



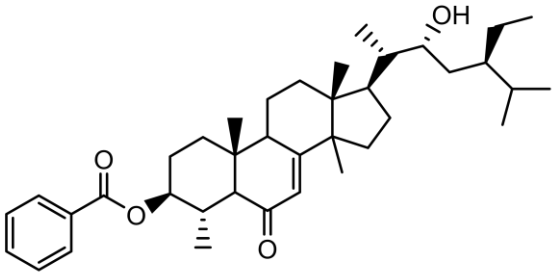
(**10**) Luteolin 6-*C*-β-D boivinopyranoside



(**11**) Cassiamin A



(**12**) Carpesterol



(**13**) Kaempferol-3-*O*-rhamnoside

Figure 2.4: Chemical structure of 3-methylethergalangin (**7**) 5-hydroxy-7-(4'-hydroxy-3'methoxyphenyl)-1-phenyl-3-heptanone (**8**), 3-O-trans-p-coumaroyl actinidic acid (**9**), luteolin 6-*C*-β-D boivinopyranoside (**10**), cassiamin A (**11**), carpesterol (**12**) and kaempferol-3-*O*-rhamnoside (**13**).

In conclusion, previous studies have showed that natural resources, especially those being consumed as herbal medicines, can provide an alternatives remedy in an attempt to control alarming statistics of obesity that continue to increase.

2.4 Strategies and target protein in this study

In this study, strategy of treatment to treat obesity was focused on one of the most potential and promising strategy which is inhibition of pancreatic lipase, an enzyme responsible for hydrolysis of dietary fat, in attempt to reduce energy intake through gastrointestinal mechanism, without modifying the central mechanism (Yun, 2010). Inhibition of pancreatic lipase does not alter the central mechanism, but prevents any interference on central mechanism of human body. Utilization of natural remedies for weight loss purpose is widely used because of its reliability, cost and safety compared to available treatment (Kazemipoor *et. al*., 2012). *In silico* screening and *in vitro* assay approaches are employed to find potential lead for lipase inhibitor.

2.4.1 Pancreatic lipase

Lipase is a water soluble enzyme secreted by pancreatic acinar cells, which is responsible for hydrolyzing long chain substrates such as triglycerides to glycerol and free fatty acids as shown in Figure 2.5 (Eydoux *et. al*., 2008). In most of lipases, the active binding pocket for substrate is restrained by the so-called lid, formed by a surface loop. In the presence of lipase inhibitors, the lid was found to undergo conformational changes which lead to accessible of solvent in the active site (Eydoux *et. al*., 2008).

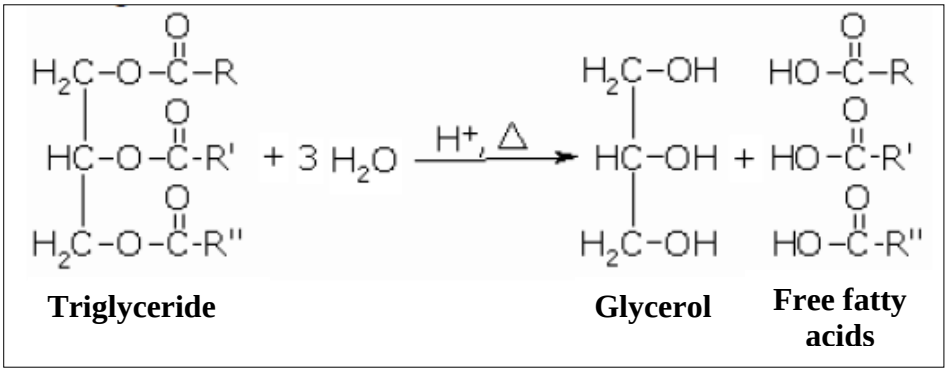


Figure 2.5: Hydrolysis of triglyceride to produce glycerol and free fatty acids (Hermansyah *et. al*., 2007).

Pancreatic lipase or also called triglyceride lipase is an important enzyme that needed for all aspects of fat metabolism. Of the known pancreatic lipases, pancreatic triglyceride lipase (PTL), the archetype of the lipase family, is clearly essential for the efficient digestion of dietary triglycerides. It fulfils the uptake of fats into various tissues, and the mobilization of fats inside cells. The correlative benefaction of pancreatic lipase to the substantial lipid hydrolysis is approximately 70-90 %. Triglyeride lipase became a key function in dietary fat absorption by hydrolyzing triglycerides into diglycerides and then into monoglycerides and free fatty acids (Winkler *et. al*., 1990). The hydrolysis of dietary triglycerides is critical for their utilization because triglycerides are not absorbed by intestinal absorptive cell. Dietary triglycerides must be cleaved into free fatty acids and glycerol before they are absorbed. In humans, triglyceride lipases are found in the gastrointestinal tract, bound to epithelial surfaces, and inside fat storage cells. Although some lipases will degrade a broad range of ester compounds, they all hydrolyze the ester bonds in acylglycerols, including the triglycerides that comprise greater than 95% of the dietary fats in the diet. Thus, inhibition of pancreatic lipase prevents the hydrolysis of triglyceride. The hydrolysis of dietary triglyceride is crucial for absorption and

utilization of the free fatty acids. Inhibition of pancreatic lipase resulted in excretion of dietary triglycerides in the stools (Winkler *et. al*., 1990).

The three-dimensional structures of various triglyceride lipases classes that reported previously are belong to a class of serine esterase. Serine in triglyceride lipases made up the catalytic triad that essential in hydrolyzing triglyceride. The active serine possessed a nucleophilic characteristic (Egloff *et. al*., 1995b).

Lipase has a group of amino acid that act as surface loop to prevent serine reaching the solvent and the loop will rearranged in the presence of substrate to make an entrance. In order to investigate the rearrangement of surface loops to give access to catalytic triad, some researches had attempted to synthesize inhibitors that can mimic hydrophobicity of triglyceride. There are many small compounds synthesized as inhibitor for lipase. However, a phosphonate inhibitors with long alkyl chain have a greater resemblance with naturally occurring triglyceride (Egloff *et. al*., 1995a).

2.4.1(a) Crystal structure of pancreatic lipase

Crystallographic structure of pancreatic lipase that is used for computational simulation in this study was explained comprehensively by Egloff *et. al*., (1995a). A complex structure of pancreatic lipase was deposited in Protein Databank Website (www.rcsb.com), existed with covalently bounded phosphonate inhibitor and detergent molecules at 2.46 Å resolutions (Figure 2.6). The phosphonate inhibitor is methoxyundecyl phosphonate (MUP) and detergent molecules are octyl β-glucoside. Detergent molecules were needed for the crystallization of the protein. Visualization after model refinement showed that the MUP is covalently bonded to oxygen of Ser152 (Figure 2.7) which suggest the charactacteristic of MUP as potent inhibitor for pancreatic lipase. From the crystallographic structure, MUP is identified to exist as *R* and *S* enantiomers. These enantiomers were fitted into electron density map with refined partial occupancies for both enantiomers. Occupancies of *R* enantiomer have been refined to 0.65, while 0.40 for *S* enantiomer. These enantiomers existed in the active site of pancreatic lipase to mimic the alkyl chain of triglyceride. Five detergent molecules known as octyl β-glucoside also refined in lipase structure.

The active site of lipase consists of hydrophobic groove next to the active serine and opposite to catalytic triad are Ser152, Asp176, and His263 (Figure 1.7). Since lipase catalyzes lipid, hydrophobic interaction become the major contribution to the free energy of binding of inhibitor. Hydrophobic interaction is important to maintain the conformation and stabilizes the protein. Pancreatic lipase has a lid-like structure which to prevent an access of substrate to the active site. However, in the presence of hydrocarbons molecules, the lid-like structure will undergo conformational changes to give access to active site.

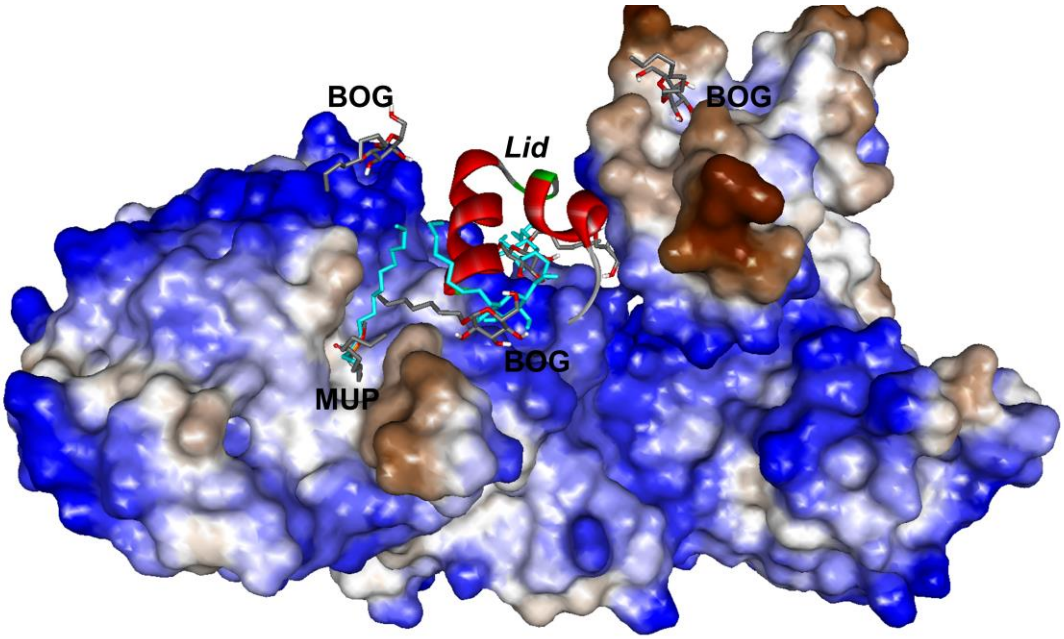


Figure 2.6: Surface representation of crystallographic structure of pancreatic lipase (PDB ID: 1LPB).

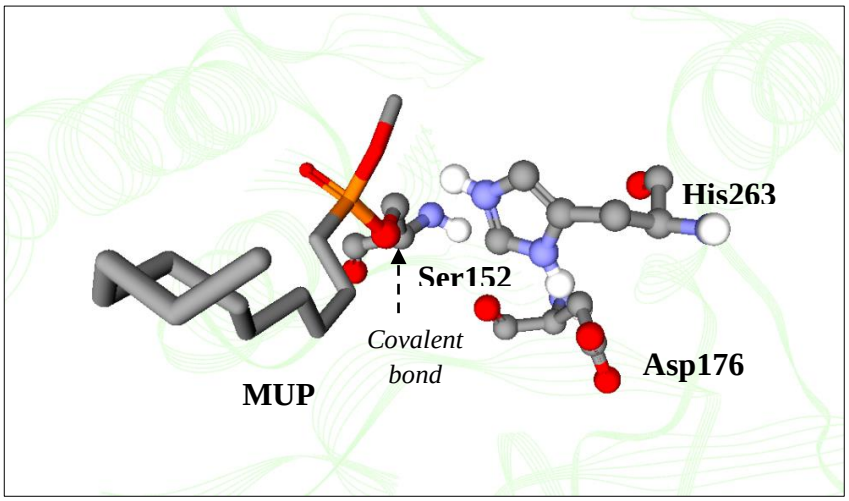


Figure 2.7: The catalytic triad of pancreatic lipase comprises of Ser152, Asp176 and His263.