Selective tropical fruit waste component exhibiting adipocyte differentiation inhibition, a future anti-obesity therapeutic strategy

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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EXHIBITING ADIPOCYTE DIFFERENTIATION
INHIBITION, A FUTURE ANTI-OBESITY
THERAPEUTIC STRATEGY**

ASYIFAH MOHAMED RASHID

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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**SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES
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“My success can only come from Allah. In Him I trust, and unto Him I look” – Al-

Qur’an 11:88

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ABSTRACT

The prevalence of obesity globally today has become significantly prominent and this has posed serious challenges and problems for professionals in the health sector to combat. The rising side effects of existing synthetic drugs used to medically treat obesity have also been concerning and the need for alternative measures of treatment is greatly in demand. In this thesis, a range of studies being done to investigate the potential of natural components, particularly, fruit waste components, as an alternative source for obesity treatment will be discussed. The elucidation of bioactive compounds present in the respective active fruit waste components will be identified and its biomechanism were investigated. In addition, comparative study to investigate the difference in effectiveness between the waste component and flesh of a fruit as well ripening stages of a fruit were also investigated in two separate chapters of this thesis. Finally, for a holistic investigation of experimental extraction condition, an optimization study to explore the most optimal condition to yield the highest amount of bioactive compound(s) from a fruit component was also determined.

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ABBREVIATIONS

ACN	Acetonitrile
CCK-8	Cell-counting kit 8
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAT-Enhancer binding protein
DAD	Diode array detector
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
FAS	Fatty acid synthase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HPLC	High performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
IFD	Induced fit docking
LC-MS	Liquid chromatography coupled with mass spectrometry
LPL	Lipoprotein lipase
MD	Molecular dynamics
MeOH	Methanol
NOB	Nobiletin
NADPH	Nicotinamide adenine dinucleotide phosphate
NOB	Nobiletin
NMR	Nuclear magnetic resonance
NOB	Nobiletin

PMF	Polymethoxylated flavones
PPAR	Peroxisome proliferator activated receptor
PPRE	PPAR-response element
RCS	Relaxed complex scheme
RT-qPCR	Quantitative real-time reverse-transcription polymerase chain reaction
SEN	Sinensetin
SPE	Solid Phase Extraction
SREBP	Sterol regulatory element binding protein
TAN	Tangeretin
VMD	Visual Molecular Docking
WST-8	Water soluble tetrazolium salt

LIST OF PUBLICATIONS

Journal papers (published)

1. Asyifah MR, Lu K, Yip Y, Zhang D, Averrhoa carambola L. Peel Extract Suppresses Adipocyte Differentiation in 3T3-L1 Cells, Food and Function, 2016, Accepted Nov 2015 DOI: 10.1039/c5fo01208b
2. Asyifah MR, Lu K, Ting HL, Zhang D, Hidden potential of tropical fruit waste components as a useful source of remedy for obesity, Journal of Agricultural and Food Chemistry, 2014, 62(16), 3505-3516
3. Lu K, Asyifah MR, Shao F, Zhang D, Development of HIV-1 fusion inhibitors targeting gp41, Current Medicinal Chemistry, 2014, 21(17), 1976-1996

Journal papers (submitted or in progress)

4. Asyifah MR, Zhang D, The effects of different extraction conditions on polymethoxylated flavones of Citrus aurantifolia peel and its bioactivity, Submitted for Peer-review Publication Apr 2016
5. Asyifah MR, Chia J, Zhang D, Adipocyte differentiation suppression study of flesh, peel, leaf and seed extract of Citrus aurantifolia: A comparative study, Submitted for Peer-review Publication Dec 2015
6. Asyifah MR, Yip Y, Zhang D, Three polymethoxylated flavones found in lime peel as a potential natural source for obesity treatment, Submitted for Peer-Review Publication Dec 2015

Conference papers

7. Conference Poster Presentation by Asyifah M R entitled *Three bioactive compounds in Citrus aurantifolia peel which inhibits adipogenesis with different degree of potencies, A potential source for anti-obesity therapeutics* at San Francisco Food Chemistry and Technology (FCT-2015) Conference, 16-18th Nov 2015
8. Conference Presentation on the *Latest discovery of (-)-epicatechin in Starfruit peel as a potential source of obesity treatment* at Milan, Italy, EGEA Conference 2015, 3-5th June 2015

PART I

INTRODUCTION AND LITERATURE REVIEW

Chapter 1

1. Introduction

1.1 Obesity

Obesity is a condition when fats have been accumulated in adipose tissues excessively or abnormally, and is predominantly caused by several environmental, social and even genetic factors. The consequence of obesity has strongly been associated with many complications in the form of cardiovascular diseases, type II diabetes, several types of cancers and even stroke. (1) With reference to body mass index (BMI), obesity is typically defined as one who possesses a BMI greater than or equal to 30. The prevalence of obesity globally today has become significantly prominent and this has posed serious challenges and problems for professionals in the health sector to combat. (2, 3) As highlighted by the World Health Organization (WHO) in their latest report in 2014, it has been found that over 1.9 billion adults worldwide were overweight, and more than 600 million of them were reported to be obese. As for children worldwide, 42 million under the age of 5 were also reported overweight or obese. (4) The propelling surge in the number of obese patients today can be particularly attributed to environmental and societal changes associated to an increase physical inactivity as well as the lack of public awareness on the importance of maintaining a healthy lifestyle. In view of this, there is certainly an urgent need to combat this problem as soon as possible, be it through finding more effective means to treat existing obese patients and also raising awareness to promote a healthier lifestyle amongst population globally.

1.2 Rising problems of present treatment

Factoring in the rising prevalence of obesity globally today, many forms of treatment and strategies to combat this disorder has been established. While the fundamental portion of the treatment emphasizes the need to engage in regular healthy physical activity in a form of structured exercises and having a proper dietary management, in terms of monitoring the kind of food and one's calorie intake daily, this alone would not suffice to effectively treat an obese patient. (5)

Figure 1 Scheme representing 3 synergistic strategies required for an effective obesity treatment

As represented in the above scheme in Figure 1, additional intervention via the means of prescribing effective weight-loss medications is also needed to further supplement the overall treatment so as to achieve effective results in obesity treatment. Among some of the synthetic weight-loss medications that have been approved by the US Food and Drug Administration (FDA) till date, include these three synthetic drugs known as Phentermine-topiramate (Qsymia), Lorcaserin (Belviq) and Orlistat (Xenical).

(6, 7) However, despite being made commercially available and approved by FDA, it has been found that the consumption of each of these respective drugs, may also cause severe side effects to patient's quality of life, affecting therapy compliance. (8, 9)

Figure 2 Chemical structure of Lorcaserin and a brief summary of how it works in treating obesity

The administration of Lorcaserin that serves to control food intake by binding itself onto a specific serotonin neurotransmitter receptor known as 5-HT_{2C} has a considerably poor selectivity as summarized in Figure 2.

The ability for Lorcaserin to not bind selectively to 5-HT_{2C} receptor has been reported to cause patients to suffer from serotonin syndrome, among which includes hallucinations and valvulopathy effects. (10, 11)

Phentermine-topiramate (Figure 3) results in weight loss via similar neurotransmitter-mediated suppression of appetite as that Lorcaserin and in addition, promotes an additional effect, by increasing the metabolism of the patient. (12, 13)

However, like the other synthetic drugs, the administration of Phentermine-topiramate also induces adverse side effects including cognitive and cardiovascular dysfunction. (13)

Phentermine-Topiramate

Figure 3 Chemical structure of Phentermine-Topiramate

Figure 4 Chemical structure of Orlistat and a brief summary of how Orlistat inhibits gastric and pancreatic lipases in the lumen of the gastrointestinal tract. Illustration adapted from Heck and others. (14)

The administration of Orlistat on the other hand, which is also aimed to control the food intake of patients, works in a slightly different mode of action. Instead of targeting the 5HT neurotransmitter receptor, Orlistat inhibits gastric and pancreatic lipases in the lumen of the gastrointestinal tract as illustrated in Figure 4, thereby preventing fats from being digested and entering into the bloodstream. (14) As effective as it may be, Orlistat has also been found to result in an increase in undigested stool triglycerides consequently resulting in adverse gastrointestinal effects. (15)

In view of the rising problems caused by the existing prescriptions made available to complement the overall treatment of obesity, there is an urgent need for more effective alternatives to be developed. In this regard, there are two approaches that we could consider: (i) Finding an alternative method of treatment, by developing new drugs or supplements that targets a different mode of action from existing drugs, such as Orlistat and Lorcaserin; (ii) Using an alternative source of therapy for obesity treatment. Instead of using synthetic drugs and focusing on the typical lead optimization, looking out for an alternative source, such as the use of phytochemicals extracted from natural sources such as herbs and fruit components, could serve as a promising alternative solution. (16, 17)

1.3 An alternative method of treatment: Targeting adipogenesis

As we have gathered from the respective mechanisms of existing drugs such as targeting serotonin neurotransmitter and inhibiting pancreatic as well as gastric lipases, these methods continue to raise concerns, as it is only able to produce a short-term effect in suppressing appetite of patients while risking the possibility of detrimental side effects in the long run. An urgent need for a less toxic drug administration alternative and possibly a new mechanistic approach to target treatment of obesity is therefore paramount.

Herein, the proposal of understanding the process of adipocyte differentiation (also known as adipogenesis) and obesity were then being explored as a possible alternative. Over the last decade, many studies have been done to understand the relation of adipocyte differentiation and its association with obesity. (18-21) Uncontrolled regulation of adipocyte differentiation has been found to be a major cause of obesity. This fundamentally occurs when homeostasis that controls glucose and lipid dynamics can no longer be maintained due to excessive energy present *in vivo* thereby causing lipids and triglycerides to accumulate excessively as a result of uncontrolled adipocyte differentiation. (22, 23) In view of this, it is therefore suggested that having an external ‘intervention’ in order to control and promote tight regulation of adipocyte differentiation, thereby maintaining the overall energy homeostasis could therefore be an alternative strategy in order to treat obesity. Similarly, in a review done by Spiegelman and others, they have addressed how disequilibrium in energy homeostasis caused by ‘obesity genes’ may ultimately result in changes in fat cell number and lipid content. (19) Among some of these ‘obesity genes’ involved in adipocyte differentiation

regulation are several adipogenic transcription factors such as the sterol regulatory element binding protein (SREBP) family, the CCAAT-enhancer binding protein (C/EBP) family and peroxisome proliferator activated receptors (PPARs). (22, 24, 25) Of these transcription factors, PPARs play vital roles in fatty acid metabolism and therefore, serve as key drug targets to treat obesity. (26)

Fundamentally, this suggests that should a drug or a phytochemical extract be able to alter these key genes effectively, it could therefore be a useful source to potentially treat obesity. To further investigate the potential effect of a drug or phytochemical extract in inhibiting adipocyte differentiation and its mechanistic role in treating obesity, a widely known 3T3-L1 preadipocyte cells would be the best model for the bioactivity assay experiment. (18, 20)

1.3.1 In vitro 3T3-L1 cell line model

In the last 3 decades, *in vitro* systems have been widely used in order to study the processes of adipocyte differentiation, which has essentially made the study of cellular events taking place during the transition from undifferentiated fibroblast-like preadipocytes into mature round fat cells possible. (Figure 5) Preadipose cell lines (preadipocytes) are cells that already committed solely to the adipocyte lineage. (27)

Figure 5 Cartoon figure of a typical adipocyte differentiation process where preadipocytes are induced to differentiate into mature round fat cells, commonly known as mature adipocyte cells

3T3-L1 cell lines, which are clonally isolated from Swiss 3T3 cells derived from disaggregated 17- to 19- day mouse embryos, are one of the most well-established and frequently employed cell lines for this study. (28-30) This cell line faithfully recapitulates the step in adipocyte differentiation over a period of 8 days when maintained in culture with fetal bovine serum (FBS), accelerated by inducing agents dexamethasone and 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. (19, 27, 31)

1.3.2 Oil Red O staining

When induced, preadipocyte cells will then undergo differentiation to form mature adipocyte where it stores fats in the form of triacylglycerol (Figure 4). Oil Red O ($C_{26}H_{24}N_4O$) is a (fat-soluble dye) diazo dye that can be used to stain these lipids (triacylglycerols). (32) The intensity of this dye can then be measured quantitatively using spectrophotometer, as this dye has the appearance of a red powder with maximum absorption at 519 nm. Fundamentally, the intensity of the dye reflects how well the adipocyte differentiation has taken place during the 8 days *in vitro* treatment.

Figure 6 Direct comparisons of cell morphology between cells induced for adipocyte differentiation (positive control) versus on that is not (negative control). X10 Magnification under an Olympus CKX41 phase contrast microscope

Figure 6 is a direct histological comparison between a typical undifferentiated 3T3-L1 cells (known as negative control) and differentiated adipocyte 3T3-L1 cells (positive control) when stained with oil red O dye at the end of an 8 days treatment. The undifferentiated cells i.e. negative control, unlike the positive, is not induced with

agents required to initiate the differentiation process of the cells. It is therefore expected that when treated with oil red O dye, low amount of stain is observed and this is very well seen in the figure above. Conversely, in a typical differentiated adipocyte cells, without treatment of any antagonists drugs as seen in the positive control, most sugars are allowed to be converted and stored as fats within the adipose tissue, and this is reflected with increasing amount of red stains observed when stained as shown in the figure. Understanding this difference in morphology and the representation of the stains is a concept widely used as an effective methodological tool to measure the effectiveness of a drug or phytochemical extract in inhibiting adipocyte differentiation. This concept on how it was used for biological screening in this research project will be further elaborated in section 1.5.1.

1.4 The need for alternative source of treatment

Next, as we have identified a promising alternative mode of action for obesity treatment, it is also crucial to address the need for a shift in focus from synthetic drugs to sourcing out for bioactive phytochemicals originating from natural sources. Taking the increasing detrimental effects and risks that synthetic drugs have on obese patients into account, a new host of alternative treatment originating from natural sources, such as herbs, plants and fruits could serve as a potentially prospective source for obesity treatment. (16) Tropical fruits for instance, are rich in a diverse array of bioactive phytochemicals. (33, 34)

In the last decade as highlighted by Devalaraja and others, several studies have looked into the potentials of bioactive phytochemicals originating from fruit flesh in altering diabetes and obesity pathophysiology. (35) However, as addressed in our

review, we further highlighted the missing gap in most studies where they mainly focused on phytochemicals present in the flesh component of a fruit while neglecting the other components including its seeds, rinds, leaves and peels. (17, 36) In recent comparative studies analyzing the antioxidants and anti-inflammatory capabilities of the different fruit components including its flesh, rinds and seeds, results have shown promising bioactivities in the peels, even more so than its flesh component. (36-40) Phytochemical composition analysis were reflective that they were abundantly present in these fruit waste components of which it also suggests that these components may have potential to exhibit therapeutic properties for obesity.

1.4.1 Targeting tropical fruit waste components

In order to further explore the possibility of using natural sources to treat obesity, we have narrowed the scope of our project focusing mainly on the fruit waste components. The idea of utilizing these waste components as potential therapeutics will not only help to supplement more alternatives for antiobesity treatment but also potentially reduce environmental concern as this simultaneously offers a solution to reduce landfill waste problems which have been highlighted by Venkat to exceed 55 million metric tonnes per year, nearly 29% of annual production. (41,80) Phytochemicals extracted from selected tropical fruit components, originating from varied sources: the flesh, seeds, leaves and peels; were found to effectively result in a promising anti-obesity effect. (41) The phytochemicals were found to work in several ways. First, as fatty acid synthase (FAS) inhibitors, where typically the formation and accumulation of fatty acids in body were prevented (42) and second, via the inhibition

of a process known as ‘adipocyte differentiation’, via several mechanisms. (22, 37, 43)

Acknowledging that previous studies have found significantly higher polyphenol contents in peel components of a fruit than its flesh and since it has been predicted to be the potential bioactive class of compounds responsible to treat metabolic disorders such as type II diabetes and antioxidant effects, we hypothesize that the possibility for these fruit waste components to exhibit a more potent effect on anti-obesity therapy than its flesh component is just as possible. (38, 40, 44)

1.5 Identification of bioactive phytochemicals in fruit waste components

While information of anti-obesity potential of fruits and its components is useful, knowledge of chemical composition, particularly in identifying the bioactive phytochemicals responsible for the component's bioactivity is also very important. This will provide information on specific function(s) that the fruit component extract has on anti-obesity properties and will then provide more opportunities for in-depth research to be done in order to maximize the yield of the bioactive compounds present in the extract, thereby increasing the medicinal value of the fruit component.(45, 46) In order to accomplish this, several experiments have to be done. First, preliminary data to investigate the potential of selected crude extract to inhibit adipocyte differentiation will have to be done. In this case, our primary biological screening investigation to test the extract's bioactivity potential is to use the 3T3-L1 preadipocyte cells, as mentioned previously. For an extract to be considered biologically active, it has satisfy two conditions:

- 1) Reduce lipid accumulation (Subject to Biological screening)
- 2) Extract is not toxic (Subject to Cell viability assay)

Following this, further isolation and purification can then be done to possibly elucidate the specific bioactive compound(s) responsible for the bioactivity.

1.5.1 Biological Screening and cell viability assay

As mentioned in section 1.3.1, 3T3-L1 cell is an established *in vitro* model used typically to monitor and study the mechanistic pathway of adipocyte differentiation. In this section, the use of 3T3-L1 preadipocyte cells and its lipid quantification technique, via Oil Red O staining to measure the potential efficacy of an extract to inhibit adipocyte differentiation will be further elaborated. Adipocyte differentiation is process that converts and stores excess energy in a form of fats in mature adipocyte cells that forms up the adipose tissue. As discussed previously, 3T3-L1 cell line has the ability to faithfully recapitulate the step in adipocyte differentiation over a period of 8 days. As seen in Figure 3, cells remains morphologically different when undifferentiated (negative control) versus one that has been fully differentiated (positive control). Therefore understanding this concept, we can then use this model as a tool for our biological screening of our sample extracts, originating from different fruit source and components. (22, 47)

In order to assess if a sample extract is able to inhibit adipocyte differentiation effectively, the extract is first processed and prepared in varied concentrations from lowest to highest. Typically, we would test it in a range of 50 to 400 mg/mL of crude extract, induce the respective amounts onto cells and let it undergo the normal process of differentiation over the period of 8 days.

	Negative control	Treated with bioactive compound	Positive Control	
A				After 8 days treatment
B	$Abs_{Neg} = 0.102$	$Abs_{sample} = 0.443$	$Abs_{Pos} = 1.367$	Absorbance @519 nm
C	$\frac{Abs_{sample} - Abs_{Neg}}{Abs_{Pos} - Abs_{Neg}} = \text{Normalized Lipid Accumulation}$			

Figure 7 Experimental scheme representing (A) Morphology of respective cells at 10x magnification (B) The absorbance value measuring the red coloration at 519 nm (C) The formula used for the determination of the efficacy of a sample extract, after normalizing against positive control and factoring in environmental factors by subtracting negative control.

Figure 7 is an experimental scheme to illustrate how the efficacy of the sample extracts can be measured. An effective adipocyte differentiation is measured by how well lipids are formed at the end of the 8th day. Here, the number of lipids is directly proportional to the amount of Oil Red O stain emitted by the respective cells. For cells that have been treated with a biologically active sample extract, it is expected that the extracts play an essential role in inhibiting adipocyte differentiation from taking place effectively. Herein, this phenomenon can be observed by lack of lipids formed at the end of the adipogenesis treatment. Essentially, when there are lower amount of lipids formed, lesser amount of Oil Red O stain is retained on cells and therefore, the colour intensity is expected to decrease when measured under spectrophotometer. The efficacy of the respective extracts can be measured relative to the positive control using the formula as represented in Figure 6C.

Fundamentally, the lower the normalized lipid accumulation is, the lesser amount of lipids have been formed, implying the more effective the extract may be in inhibiting adipocyte differentiation. However, we need to consider the fact that an extract remains ineffective until cell viability test proves otherwise. The fall in lipid accumulation as seen during the biological screening stage is merely indicative that the extract may play role in inhibiting adipocyte differentiation but it could also likely be due to toxicity effect of the extracts too. Hence, in order to confirm the bioactivity capacity was due to adipogenesis inhibitory effect, the sample extract was then further subjected to cell viability assay test under the same concentration range. Sample extracts that are found to reduce lipid accumulation during adipogenesis and exhibits non-toxic effect on cells within the tested range are then considered to be potentially bioactive.

Figure 8 Illustration on the use of water-soluble tetrazolium salt (WST-8) to measure the number of living cells, cell viability detection mechanism. Illustration is adapted from Dojindo CCK-8 protocol manual (48)

For the purpose of cell viability assay, water-soluble tetrazolium salt (WST-8) was used. The use WST-8 as a measurement of the number of living cells is illustrated in Figure 8. Cells that are active and viable are expected to produce nicotinamide adenine dinucleotide phosphate (NADPH) through dehydrogenase activities. This in turn, will act as reducing agent that essentially reduces WST-8 into WST-8 formazan yellow dye. The amount of dye that is produced by the dehydrogenase activities in cells is proportional to the number of living cells.

1.5.2 Separation, isolation and identification

There are many challenges for the identification of bioactive compounds in biological samples. Biological extracts originating from different fruit components usually have very complex chemical composition and matrices. Therefore, it is necessary for extracts that have been identified to be biologically active after the screening stage, be subjected to further separation. This is usually done via silica gel column chromatography. After which, the respective fractions originating from the crude extracts can then be further analyzed using the various methods such as liquid chromatography coupled with mass spectrometry (LC-MS), high performance liquid chromatography coupled with diode array detector (HPLC/DAD), nuclear magnetic resonance (NMR) and so forth. (49, 50)

The process involved in identifying the bioactive compound in crude extracts usually starts with a rigorous step of separation, a second stage biological screening of all the separated fractions to identify the bioactive fraction(s) and finally, further comprehensive chemical analysis of the identified bioactive fraction(s). By the final stage, the chemical analysis is expected to be less complex in comparisons to the crude

extract of which LC-MS can then be used to determine the molecular weight (mass to charge ratio) of the bioactive compounds that aids in the identification process. Should the fraction be available in sufficient amount, further purification of the bioactive fraction can then be done via silica gel column chromatography or preparative HPLC, before further characterization of the bioactive compounds can be done, using NMR analysis.

1.5.3 Analysis of bioactive compounds using HPLC/DAD, LC-MS and NMR

Reversed-phase HPLC fundamentally works by using a nonpolar adsorbent surface and a polar eluent to separate a large variety of organic compounds with varied polarity, from very polar to non-polar, by changing the polarity of polar phase throughout the running time. The column that was used to separate all the extracts studied in this project are C₁₈ column material while the mobile phase employed are typically methanol in combination with aqueous acidified polar solvent as reviewed by Merken and Beecher. In our case, formic acid was used. (51)

Ultraviolet-visible (UV-Vis), fluorescence, refractive index (RI) are among some of the traditional detectors that have been coupled to HPLC but one that has been profoundly used in research studies for monitoring phytochemicals, including phenolic compounds and flavonoids would be the DAD. (51) Its ability to provide UV-Vis spectra of target compounds may provide possible chemical structural information that is useful for its identification and characterization process. In addition, as DAD is able to produce chromatograms at wide range of wavelengths, an optimal wavelength that produces maximum absorbance for improved selectivity and sensitivity can then be determined effectively. It is due to these reasons that HPLC/DAD was used in the analytical process of the research project.

However, as UV-Vis spectra of flavonoids as well as phenolic compounds are relatively similar, the probability of misidentifying certain compounds may occur.⁽⁵⁰⁾ Hence, to eliminate this uncertainty, most analysis is coupled with mass detector using LC-MS and referenced with pure standards purchased commercially. For extracts that can be further purified, the bioactive compounds were also subjected to NMR analysis for characterization of its chemical structure. The combination of a HPLC/DAD, LC-MS and NMR are the routine technique for analysis of the bioactive compounds identified from the crude extracts for this project.

1.6 Understanding the biological mechanism of the bioactive compounds in inhibiting adipogenesis (Theory)

Having to identify bioactive phytochemicals responsible for the suppression of adipogenesis is only the first step towards finding an alternative treatment for obesity. As the process of adipogenesis is regulated by multiple transcription factors namely SREBP, PPARs and C/EBP family, it is essential to understand the underlying mechanism that accounts for the suppression effect caused by the bioactive compound(s). (22, 24, 25) PPAR is essentially a transcription factor that is activated by ligand binding served to regulate the expression of genes involved in cellular lipid uptake and oxidation. (58) The point of interest then, is to look at how the phytochemicals can act as a possible ligand to the transcription factors that would then consequently affect the expression of target genes. (19-21) In order to achieve this, selecting the right set of adipogenic genes to be studied and establishing the method to analyze the effect of bioactive compounds on its expression is crucial. For this case, quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) was used.

1.6.1 Selected adipogenic genes for gene expression studies

Over the last decade, thorough studies have been done to identify the set of transcription factors that can promote adipogenesis in a dominant fashion. (18, 19, 52, 53) As illustrated in Figure 9, is a condensed version of the key transcription factors leading to adipocyte differentiation have been summarized and its respective role will be further discussed in this section.

Figure 9 Condensed diagram highlight the role of key (selected) transcription factors relevant in initiating adipocyte differentiation

PPARs are often the key drug target to treat obesity and obesity-related diseases as they play vital roles in fatty acid metabolism. (24, 25, 54) PPAR δ , PPAR α as well as PPAR γ are members of the PPAR subfamily of nuclear hormone receptor. Out of the three, PPAR γ has been found to act as a direct regulator of many fat-specific genes and also a “master” regulator that can trigger the entire program of adipogenesis. (19, 21) Unlike PPAR γ , PPAR α and PPAR δ are not expressed preferentially in fat, but there have been studies that suggests that these receptors could play a direct or indirect role in adipogenesis. (52, 55) While the precise role of PPAR δ remains inconclusive, (56) PPAR α has been found to be a key modulator to the synthesis of fatty acids, its

oxidation and metabolism of lipids which can essentially reduce the storage of energy. (57, 58) It is therefore been highlighted by Fruchart et al. and Kersten et al. that the ability to activate PPAR α could thus, be a useful strategy for the treatment of obesity and its related diseases. (59, 60)

C/EBP α is another “master” transcriptional regulator that acts cooperatively with PPAR γ during adipocyte differentiation. (19, 61) Upon induction during adipocyte differentiation, ligand binding activation causes PPAR γ to bind to distant enhancer regions while C/EBP α tends to bind at sites clustered in proximal promoter regions thereby, acting cooperatively to initiate adipogenesis. (19, 62) Following this, PPAR-response element (PPRE) in promoters such as adipocyte protein 2 (aP2) as well as the lipoprotein lipase (LPL) in turn, gets regulated. (24, 52)

Hence, understanding the mechanistic pathways and the specific roles of selected adipogenic genes enable us to study the possible effects of bioactive compound(s) resulting to the overall suppression of adipogenesis. Whether the bioactive compound(s) bind itself onto the master transcriptional regulators (C/EBP α and PPAR γ) thereby turning off the master switch of adipocyte differentiation or causing an upregulation of PPAR α gene, initiating fatty acid oxidation are some of the possibilities that can be investigated. (22, 60, 63, 64) Having highlighted the key adipogenic genes of interest, the following primers are then selected for use, in order to facilitate the genetic expression study in this research project:

Table 1. ‘Genes of Interest’ primer sequence used for polymerase chain reaction

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
LPL	ATCGGAGAACTGCTCATGATGA	CGGATCCTCTCGATGACGAA	55
PPAR β	TCCATCGTCAACAAAGACGGG	ACTTGGGCTCAATGATGTCAC	55
C/EBP α	CGCAAGAGCCGAGATAAAGC	CACGGCTCAGCTGTTCCA	56
PPAR γ	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC	57
aP2	CATGGCCAAGCCCAACAT	CGCCCAGTTTGAAGGAAATC	54
PPAR α	AGGCTGTAAGGGCTTCTTTCG	GGCATTGTTCGCGTTCTTC	54
GAPDH	GGTGAAGGTCGGTGTCAACG	CAAAGTTGTCATGGATGACC	51

1.6.2 RNA Isolation for gene expression studies (Theoretical Concept)

As the process of adipocyte differentiation have been well studied over the last decade, selecting the relevant genes to investigate the underlying mechanisms of bioactive compounds leading to the overall suppression of adipocyte differentiation has been made easier. It is then essential to strategize a technique in order to analyze these effects effectively. Figure 10 is a summarized illustration of how this may be done experimentally.

Figure 10 Cartoon illustration of experimental procedure used to determine the effect of crude extracts in up regulating or down regulating the respective genes of interest

As represented, 3T3-L1 cells will also be used for the purpose of this study. Subjected to 8 days treatment with varied concentrations of the bioactive crude extract, the cells are left to undergo adipogenesis regularly. At the end of the 8th day, the RNA of the respective cells are extracted and isolated as elaborated in section 2.4.1. RNA is isolated instead of the deoxyribonucleic acid (DNA) of the cells because unlike DNA, which gives all the information available (both coding and non-coding sequence) within

the genome of the cells, ribonucleic acid (RNA) are more specific and provides details of specific genes that were turned on at that moment in time for the production of a functional gene product, usually a protein. (65) As the objective of the experiment is to study the effect of crude extract on genetic expression leading to the overall inhibition of adipocyte differentiation, isolating the RNA of the cells instead of the DNA is absolutely crucial. The next step then, is to reverse transcribe the respective isolated RNA into complementary DNA (cDNA) before subjecting it to qPCR using the respective series of ‘gene of interest’ primers, as highlighted in Table 1.

1.6.3 Using RT-qPCR to assess the effect of bioactive compound on genetic expression (Theory)

RT-qPCR is used to evaluate the upregulation or downregulation effect of crude extract on the respective genes of interest. Only then, can we conclude if the adipogenesis suppression caused by a crude extract was due to the up/downregulation of specific genes or simultaneous regulation of a collective of genes. In order to determine this, the respective cDNA samples of cells treated at various concentrations of bioactive crude extract were then used for the following analysis.

Real-time qPCR is typically used to monitor the gene expression of the ‘gene of interest’ in relative to the ‘housekeeping genes’ as reference. (66) Housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and pathophysiological conditions. (67) Housekeeping genes are essential for two purposes: first, to indicate how well RNA has been extracted and second, to determine the quality of samples and the PCR itself. In our experiments, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the housekeeping gene used.

Figure 11 the four characteristic phases of PCR, evaluated by real-time PCR fluorescence acquisition, adapted from Pfaffl and others. (68, 69)

Before subjecting the respective cDNA samples for qPCR analysis, a specific ‘gene of interest’ primer (as listed in Table 1) along with a specific dye (SYBR green) that intercalates with double-stranded DNA and fluoresces when bound, were introduced into each samples. (70, 71) The administration of SYBR green allows us to monitor the real-time exponential growth of our cDNA promoted by the respective gene of interest primers after every cycle. Figure 11 shows a typical exponential growth in DNA after 40 cycles of PCR.

Figure 12 Figure taken and adapted from Roche LightCycler 480 SYBR green I master protocol, of a serially diluted samples containing cDNA. (72)

In order to interpret whether a gene of interest has been up regulated or down regulated by the extract, we will then look at how fast the DNA in the respective samples grow exponentially. In principle, the cycle number at which the DNA starts to grow exponentially corresponds to the amount of gene of interest expressed in the particular cDNA extract, i.e. if more genes have been expressed in a particular cDNA extract, the faster the DNA starts to grow exponentially as compared to the other (Figure 12). With reference to Figure 8, as the exponential growth of DNA occurred at an earlier cycle number in sample A than in sample B, the expression of that particular gene of interest is higher in sample A than sample B. Theoretically, these difference observed can then be used to measure if the expression of the gene of interest have been up or downregulated under specific cellular conditions and treatment. The tool used for

this measurement is the $\Delta\Delta C_t$ method. (73) To further complement this, gel electrophoresis was also typically used to support the overall data.

1.7 Molecular Modeling

Upon the completion of the gene expression test, the key protein that is involved in the regulation of adipogenesis suppression step can then be identified. Now, with the target protein elucidated and the bioactive phytochemicals in crude extract that results in the overall adipogenesis suppression identified, the molecular binding relationship between the active compound and the target protein can then be investigated. This can be achieved by a combinatorial approach in molecular modeling using molecular docking molecular dynamics simulation and free energy calculations. (22, 74, 75)

1.8 Aims of Project

With the increasing prevalence of obesity globally and acknowledging the need to combat this medical condition urgently, we suggested two approaches, the first, addressing the need to use an alternative method of treatment, targeting a mechanism that is different from the existing synthetic drugs and second, finding an alternative source for therapy such as the use of naturally existing phytochemicals and less dependent on synthetic drugs. The approach was brought forth in order to address the rising problems of existing synthetic drugs as it was commonly found to result in detrimental side effects, affecting patient's quality of life. (9) Till date, only three synthetic drugs have been FDA approved whilst the other drugs had to be discontinued due to the various side effects that it may cause. (5)

As adipocyte differentiation dysfunction is a fundamental process that results in obesity and its mechanism has been very well studied, targeting this system to combat obesity remains promising. (53) In the last decade, there have been a series of studies that suggests the ability of several fruit sources, mainly its flesh component, to induce a tight regulation in adipocyte differentiation. However till date, very little studies were done to identify the bioactive compounds responsible for this effect.⁽¹⁷⁾ In addition, with reference to comparative studies that have found the “waste components” of a fruit such as its seeds, peels and leaves extract to exhibit higher antioxidant capacity than its flesh, we have decided to narrow down the scope of our project targeting the fruit-waste components of fruit. We hypothesize that since the waste components exhibit higher antioxidant capacity, it likely suggests that higher amounts of useful phytochemicals are present in those components than its flesh.

In view of these series of studies, the aim of our research project is not only to identify potential fruit waste components that exhibits anti-obesity potential via the suppression of adipocyte differentiation but also to fill in the missing gaps that have not been addressed in previous studies. Herein the project proceeds to fulfill four main objectives:

- (1) Identify promising tropical fruit waste components commonly consumed in Singapore
- (2) Understand the underlying mechanism involved in crude extract leading to overall inhibition of adipogenesis
- (3) Identification of bioactive phytochemicals responsible for the bioactivity
- (4) Investigate the effects of extraction condition to yield maximum amount of bioactive compounds in extract

1.8.1 Identify promising tropical fruit waste components

Targeting tropical fruit waste components commonly consumed in Singapore remain as our topic of interest. As discussed by González-Castejón, tropical fruits have been widely recognized to contain diverse array of bioactive phytochemicals that could effectively treat obesity. (33) As Singapore is situated in a geographically strategic location that is significantly blessed with vast variation of tropical fruits that are reasonably priced, it is practical to target these fruits for our study. In addition, discovering promising potential of commonly consumed fruits amongst Singaporeans would be an interesting find. Naturally, people would be more inclined to understand how these fruits could be transformed into something that is medically useful in treating medical disorders such as obesity. Highlighting and spreading the discovery of these tropical fruits as a promising therapeutic source to treat disorders then would be an interesting task. This could significantly raise awareness amongst Singaporean and inculcate the habit of not wasting these fruits and to possibly incorporate the use of these therapeutic components in their daily diet. Hence, in order to achieve this in our research project, a comprehensive study and proper biological screening of the fruits and its components (as previously discussed in section 1.5.1) have to be done in the first phase of this study.

1.8.2 Understand the underlying mechanism involved in crude extract leading to overall inhibition of adipogenesis

Adipogenesis has a complex series of mechanisms involved in its process. (18) Understanding the underlying mechanistic pathways of crude extracts leading to the reduction of lipid formation in bio-organisms via the inhibition of adipocyte differentiation is therefore crucial. This will not only broaden the perspective of its mechanistic role but also in the long run, may be beneficial for medicinal chemists should they like to look at ways to optimize the potency of a promising drug/phytochemicals, or find new strategies to treat obesity. In order to achieve this, comprehensive study on the effects of crude extracts on respective adipogenic genes is done as discussed in section 1.6.

1.8.3 Identification of the bioactive compounds responsible for the bioactivity

Following the preliminary elucidation of how the crude extract of an active fruit component inhibits adipogenesis, identifying the bioactive phytochemicals would then be the next crucial step. This area, particularly in identifying, isolating and purifying the bioactive phytochemicals, as previously highlighted have not been widely studied. This is understandable due to the complex nature and matrices of biological samples. The presence of bioactive phytochemicals in minute amounts and the complex nature to separate, isolate and purify these compounds are among some of the challenges faced by researchers accounting for the lack of studies done in this area. (76) The objective of our work is therefore to overcome this challenge by means of combining several separation techniques including silica column chromatography coupled with HPLC.

The identification of bioactive phytochemicals would also be an essential measure in the long run as it helps to provide a greater understanding as to why the exact same fruit (or components) originating from different cultivars and countries exhibit vastly different medicinal potential. This observation is similarly seen by Taing and others, where they discussed the significantly different anti-obesity bioactivity capacity observed between mangoes originating from three different cultivars Nam Doc Mai, Kensington Pride and Irwin. (37) Fundamentally, different agronomic factors, including both physical and environmental condition can affect the overall chemical composition the fruit components and its bioactivity capacity. (17, 77) This area of research investigating the bioactivity capacity of fruit components (of same species) containing varied concentrations of identified bioactive phytochemicals in its chemical composition was also explored in this project.

1.8.4 Investigate the effects of extraction condition of the active component in the overall bioactivity

As extraction condition is vital in determining the overall chemical composition of the respective components of the fruit extracts, this phase of the project aims to address this. (78) Similar to studies that have done previously by Akowuah and others, the objective to find an optimized extraction condition is to allow the bioactive extract to yield the right chemical composition in order to induce maximal bioactivity. (45) Among some of the areas of optimization that will be considered would be similar to that of Buelga and others (79) which includes:

1. Temperature of extraction
2. Type of solvents used for extraction
3. Duration of extraction
4. Sample-to-solvent ratio
5. Number of cycles of extraction

The effectiveness of the extract subjected to the different extraction conditions will be analyzed via two means: subjected to *in vitro* bioactivity test using 3T3-L1 cells, and HPLC/DAD analysis for quantification, with reference to calibration standards. It is hypothesized that *extract that contains the highest amount of bioactive compound(s) is expected to exhibit the greatest bioactivity capacity.*

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PART II

EXPERIMENTAL

Chapter 2

2. Materials and methods

2.1 Materials

Fruits and its components Fruits and its respective fruit components were purchased from local supermarkets in Singapore. The fruits consist of lime (*Citrus aurantifolia*) peel, pomelo (*Citrus grandis*) flavedo, albedo and flesh, mandarin orange (*Citrus reticulata*) peels and seeds, mangosteen (*Garcinia mangostana*) rind, bilimbi (*Averrhoa bilimbi*) peel, starfruit (*Averrhoa carambola* L.), ripe and unripe banana (genus *Musa*, locally known as *Pisang Berangan*) peel, watermelon (*Citrullus lanatus*) seeds, flavedo and albedo as well as apple (*Malus domestica*). Lime (*Citrus aurantifolia*) leaves, flesh, seeds and peels used for comparative analysis studies, discussed in Chapter 5 of this thesis, all originated from the same plant and were obtained from a fruit farm in Malacca, Malaysia.

Reagents and Chemicals Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, Cell Counting Kit-8 (CCK-8, 96992), Oil Red O, dichloromethane (DCM), formic acid, dimethyl sulfoxide (DMSO), acetonitrile (ACN), pure standards (-)-epicatechin and (+)-catechin were purchased from Sigma Aldrich (St. Louis, MO, USA). Pure standards Nobiletin, Sinensetin and Tangeretin were purchased from Indofine Chemical Company Inc (Hillsborough, NJ, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Penicillin/streptomycin, Purelink RNA mini kit

and TRIzol reagent were from Invitrogen (Carlsbad, CA, USA). IScript cDNA synthesis kit was from Bio-Rad (Hercules, CA, USA). GoTaq colorless master mix was from Promega (Madison, WI, USA). Primers were synthesized by 1st Base Pte Ltd (Singapore, Singapore). Analytical grade ethanol and methanol were purchased from Merck (Damstadt, Germany).

2.2 Biological screening to measure the bioactivity of crude extracts

2.2.1 Adipocyte differentiation of 3T3-L1 cells

For biological screening process to measure the effectiveness of the respective crude extract to inhibit adipocyte differentiation process, *in vitro* model was used using 3T3-L1 preadipocytes cells. On a 24-well plate, 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO₂ to confluence (2000 cells/cm²). Confluent cells were maintained in DMEM supplemented with 10% FBS for 24 hrs. Post-confluent cells (Day 0) were then induced to adipocyte differentiation with a hormonal cocktail containing DMEM supplemented with 10% FBS, 2.5 µg/mL insulin, 0.5 µM dexamethasone, and 100 µM IBMX, as well as the respective crude extracts or pure standards at desired concentrations. After 48 hrs (Day 2), the medium was then replaced with DMEM containing 10% FBS and 2.5 µg/mL insulin. After another 48 hrs (Day 4), medium was changed to fresh DMEM containing 10% FBS. Every 48 hrs thereafter (Day 6), medium was replaced with DMEM containing 10% FBS. On day 8, the cells were harvested for analysis. Crude extracts and the respective isolated pure standards were added into DMEM to achieve the respective concentrations for the entire adipogenic period.

2.2.2 Oil Red O staining

3T3-L1 cells differentiated for 8 days were then washed twice with PBS (pH 7.4) and then fixed with 3.7% formalin (2 hrs). The cells were washed twice with distilled water and stained with freshly diluted Oil Red O solution (six parts of 0.5% Oil Red O stock solution and four parts of H₂O) with gentle agitation (60 min). Excess stain was removed with 60% aqueous ethanol, and the cells were further washed with distilled water three times before observation under an Olympus CKX41 phase contrast microscope. For quantitative determinations of accumulated lipid in cells, Oil Red O staining was eluted with isopropanol. The amount of dye eluted was quantified by spectrophotometer at 513 nm.

2.2.3 Processing the data

As briefly discussed in Chapter 1, section 1.5.1, the absorbance value of the respective cells have already been quantified using spectrophotometer. In order to measure the effectiveness of the inhibition of adipocyte differentiation, formula 1 is being used.

$$\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{Neg}}}{\text{Abs}_{\text{Pos}} - \text{Abs}_{\text{Neg}}} = \text{Normalized Lipid Accumulation}$$

Formula 1. Normalized Lipid Accumulation

Subtracting the respective sample values against the negative control will account for the environmental factors during the experiment. Normalizing the sample values by dividing against the positive control will provide a simple calibration to measure the effectiveness of suppression in lipid accumulation during adipocyte differentiation. Normalized Lipid Accumulation ≥ 1 would suggest that no suppression occurs, while

normalized lipid accumulation < 1 may suggest a possible suppression in adipocyte differentiation. Cell viability assay and gene expression studies are required to further confirm its inhibitory effect of the respective sample extracts tested.

2.3 Cell viability assay

Cell viability was performed using Cell Counting Kit-8 (CCK-8, Sigma Aldrich) according to the manufacturer's protocol with slight modification as previously discussed by Choi et al. Briefly, 5000 cells were seeded into each well of the 24-well plate and cultured in 400 μ L DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO₂ for 24 hrs. The post-confluence cells were induced to adipocyte differentiation in the same manner as described above. On Day 6, Cell Counting Kit-8 (CCK- 8) purchased from Sigma Aldrich was used to determine cell viability. The cells were washed with PBS and 300 μ L of diluted CCK-8 (10%) was then added into each well and the plate was incubated in dark for another 2 hrs. The medium was then transferred to respective eppendorf tubes, mixed, and 100 μ L was transferred into each well of a 96-well plate before the absorbance was measured at 450 nm with a TECAN Infinite M200 reader.

2.4 Genetic expression analysis

2.4.1 RNA extraction and Reverse transcription-polymerase chain reaction

Total RNA was extracted from 3T3-L1 cells using TRIzol reagent and Purelink RNA mini Kit on day 8. Reverse transcription was performed with Iscript cDNA synthesis kit, according to the manufacturer's instructions. PCR reactions were performed using the GoTaq colourless master mix and Takara TP600 thermal cycler. The thermal cycle conditions were: after heating at 95°C for 5 min, PCR amplification was done with 40 cycles of 95°C for 30 sec, respective annealing temperature for 30 sec, 72°C for 30 sec, followed by a terminal extension at 72°C for 7 min. Primers used for PCR were described previously and shown in Table 1. The PCR products were subjected to electrophoresis on 2% agarose gels stained with ethidium bromide. The bands were scanned by G:Box and analysed via SynGene software; GAPDH was employed as the internal control.

2.5 LC-MS analysis for bioactive compounds

ThermoFinnigan LCQ Fleet MS which was equipped with Thermo Accela LC was used for LC-MS data collection. 0.1% of formic acid in water as solvent A. 0.1% of formic acid in ACN as solvent B. The column was flushed with 5% of B for one minute, and then increases the percentage of B from 5% to 95% over 5 minutes. Keep 95% for another 2 minutes and decrease to 5% over 1 minute. Flow rate 0.25mL/min. The full scan mass spectra from m/z 50-2000 were acquired with both negative mode and positive mode. Injection amount is 0.5mg/mL *10 µL in water.

2.6 Statistical analysis

Data are expressed as means $\pm$ SEM. Differences were considered statistically significant when the *p* value was less than 0.05, assessed using an unpaired Student's *t*-test.

2.7 Anti-obesity analysis of selected fruit waste component

2.7.1 Anti-obesity analysis of *Citrus aurantifolia* extract

2.7.1.1 Extraction of Lime peel

2kg of *C. aurantifolia* (lime), imported from Malaysia, were purchased from National Trades Union Congress (NTUC) Fair price in Singapore. The peels were separated and air-dried at room temperature for 24 hrs and brought to dryness by lyophilizing the peels further for another 48 hrs using Labconco freeze dryer #7670031. The dried peels were then grinded with Toyomi blender model BL2026 into powder form. The extract was then extracted with methanol twice at 30 °C for 45 minutes. For every 1 g of powder to extract, 10 mL of analytical grade methanol was used. The extract was filtered with Whatman filter paper no. 1 via Buchner filtration and the filtrate was then left under the rotavap to remove the remaining solvents. The extract was then further lyophilized to remove any moist present to yield 10% of crude *C. aurantifolia* peel extract.

The percentage yield of crude extract obtained was calculated using formula 2.

$$\text{Percentage yield} = \frac{\text{Weigh of final crude extract (g)}}{\text{Dried extract in powder form (g)}} \times 100 \%$$

Formula 2. Percentage yield calculation formula

2.7.1.2 Separation and isolation via column chromatography

Flash chromatography was carried out using silica gel 60 (Merck). Thin layer chromatography (TLC) was carried out on silica gel 60 F254, 0.2 mm layer (Merck). The first round of crude extract isolation was done using silica gel column chromatography under gradient elution. Solvent system started off with 100% chloroform to 40% methanol with 0.1% formic acid over time. 80 fractions were collected and analyzed to yield 12 separate fractions. The subsequent column chromatography for the combined fractions were done in 5 different rounds using hexane, hexane: ethyl acetate, ethyl acetate: methanol solvent system.

2.7.1.3 HPLC analysis

Waters 2695 separation mode equipped Waters 2996 photodiode array detector covering 210-400 nm range were used for data analysis. The separation was performed on an Agilent Zorbax SB-C18 column 5 μ M, 4.6 x 150 mm) with 0.1% of formic acid in water (A) and methanol (B) as the mobile phase. The column temperature was room temperature, and the injection volume was 100 μ L. The gradient elutions were as follows: The gradient elutions were as follows: 0 % of B to 100 % of B over 100 minutes, followed by 100% of B for 15 minutes and then decrease to 0% over 20 minutes to wash and condition the column. The flow rate was 1.0 mL/min. The data were processed with Origin software version 9.0.

2.7.1.4 Nuclear magnetic resonance for characterization of isolated compounds

^1H NMR and ^{13}C NMR was recorded at room temperature using Bruker Avance 500 MHz and 100 MHz spectrometers respectively. Chemical shifts for ^1H NMR spectra are reported as δ in units of parts per million (ppm) and referenced to the solvent signal of the deuterated solvent. Multiplicities were given as: s (singlet); d (doublet); dd (doublets of doublet).

2.7.1.5 Molecular docking – Relaxed Complex Scheme

In the relaxed complex scheme (RCS), a long molecular dynamics (MD) simulation of the *apo* protein will first be conducted to extensively sample the protein's conformations. The second phase involves the docking of ligands to a large ensemble of the *apo* protein's MD snapshots. (1, 2) In the case of PPAR α , there are no available *apo* structures in the Protein Data Bank (PDB). While it was possible to obtain a *holo* structure from the PDB to dock the ligands of interest, we noticed that there was a significant difference in the active site volume between the *apo* state and the ligand-bound activated state. Therefore, we apply the RCS to PPAR α in order to increase the accuracy when docking the PMFs.

Structure Preparation The PPAR α structure was obtained from the Protein Data Bank (PDB ID: 2REW) at a resolution of 2.35 Å. (3) The bound ligand in the X-ray crystal structure was removed to obtain the *apo* state. The protonation state of the ionizable residues were determined at physiological pH condition. The structure was then subjected to the LEaP module (4, 5) in AMBER suite where missing

hydrogen atoms were added and the topology files for molecular dynamics simulation generated.

Simulation Protocol The MD simulation was conducted using the SANDER module in AMBER 10.0. (4, 5) Any covalent bonds associated with hydrogen atoms are restrained using the SHAKE algorithm. (6) The reference pressure was 1.0 bar and the Berendsen barostat was used with the pressure-coupling constant of 2.0 ps. The temperature was maintained at 300 K, and the Berendsen thermostat was used with the coupling constant of 2.0 ps. The particle mesh Ewald method (7) with cubic spline approximation was used to treat the long-ranged Coulombic interactions, and the direct sum tolerance was set to 0.00001.

The protein was solvated with the TIP3P water molecules (8) in a octahedral box of $\sim 2.52 \times 10^5 \text{ \AA}^3$ in volume. We first minimize the solvent with 5000 steps of steepest descent method, followed by 5000 steps of conjugate gradient method. After which, the whole solvated protein system is minimized till the energy gradient converges at $0.01 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$. After minimization, the system is heated from 0 K to 300 K in 100 ps before subjecting to NPT simulation for 2 ns. After the MD simulation, a snapshot was extracted every 100 ps, resulting in a total of 20 snapshots used for the docking.

Docking Protocol Gridding was done with the centroid of the co-crystallized ligand in the structure, generating a box size of $12 \text{ \AA} \times 12 \text{ \AA} \times 12 \text{ \AA}$. The structures of nobiletin, sinensetin and tangeretin for induced-fit docking were generated at pH 7.0. The induced-fit docking was first carried out by Glide (9-11), then residues within $5.0 \text{ \AA}$ of the ligands were further refined with Prime.(12, 13) Docked poses that are within $30.0 \text{ kcal mol}^{-1}$ of the best docked pose are re-docked with a higher precision. (9)

2.7.2 Comparative study of four different components of *Citrus aurantifolia* from one plant source

2.7.2.1 Extraction of lime peel, seed, flesh and leaf component

290 g of *Citrus aurantifolia* fruit, and 102 g of its leaves were obtained from the same plant in Malaysia. Both the fruit and leaf were rinsed with tap water and gently brushed to wash off debris on surface. The peel, flesh, and seeds of the fruit, as well as the leaf were separated and placed in different containers, respectively. The samples were lyophilized for 72 hours before they were grounded into powder using a Toyomi blender (model BL 2926). A total of 35 g, 11 g, 9 g, and 30 g of powder from the lime peel, flesh, seeds, and leaf were added into four 500-mL round bottom flasks (RBF), respectively. Based on a solvent-to-sample ratio of 10:1, 75% aqueous ethanol was added to each of the RBF. The powder was extracted twice at 2 hours interval. All extractions were performed in a water bath at 25°C with the aid of a temperature controller. Buchner filtration was used to separate the extract from the powder, and the filtrate was dried using a rotary evaporator and the extract was further lyophilized for 48 hours to remove any moisture present to yield 20 %, 40 %, 12%, and 22 % of crude peel, flesh, seed and leaf extract, respectively. The extracts were stored in -80 °C freezer until use. The percentage yield of crude extract obtained was calculated using Formula 2.

2.7.2.2 HPLC analysis and quantification of polymethoxylated flavones (PMFs) in samples

Each 1 mg/mL of pure NOB, SEN and TAN as well as 3mg/mL of lime leaf, peel, flesh, and seed extracts were dissolved in water, in triplicates. Waters 2695 separation mode equipped with Waters 2996 photodiode array detector covering 210-400 nm range were used for data analysis. The separation was performed on an Agilent Zorbax SB-C18 column 5 μ m, 4.6 x 150 mm) with 0.1% of formic acid in water (A) and methanol (B) as the mobile phase. The column was run at room temperature and the injection volume was 100 μ L. The gradient elution was as follow: 0 % of B to 100 % of B over 100 minutes, followed by 100% of B for 15 minutes and then decrease to 0% over 20 minutes to wash and condition the column. The flow rate was 1.0 mL/min. The peaks were detected at 260 nm and identified by standard substances. The following reference compounds were used as markers: SEN, NOB and TAN. The external standard method was used for the HPLC quantification and the results are reported as percent of dry sample weight.

2.7.3 Extraction optimization of selected PMFs in *Citrus aurantifolia* peel

2.7.3.1 Sample preparation and extraction of lime peel

3.5 kg of *C. aurantifolia* (lime), imported from Malaysia, were purchased from National Trades Union Congress (NTUC) Fair price in Singapore. The peels were separated and air-dried and brought to dryness at room temperature for 72 hrs. The dried peels were then grinded with Toyomi blender model BL2026 to yield 208 g of lime peel powder and stored in – 80 °C until use. Three grams of lime peel powder were extracted with 30 mL of the following solvents: water, methanol, 50% aqueous methanol, ethanol, 50% aqueous ethanol, chloroform, at varying temperature conditions (20 - 60 °C) and extraction time (1 – 4 hrs). During the extraction process, the round-bottomed flasks were covered with plastic wrap to prevent the evaporation of solvent. All extractions were done in two cycles and three independent replicates of extractions were done for each sample set under the various extraction condition. After the respective extraction, the extracts were filtered with Whatman filter paper no. 1 via Buchner filtration and the filtrate then left under the rotavap to remove the remaining solvents. Extracts were further lyophilized to its powder form and labeled accordingly before storing in - 80 °C freezer until further used for analysis.

2.7.3.2 Extraction optimization experimental design

Single factor experiment was used to determine the optimum extraction condition for lime peel similar to other extraction optimization studies that have been done previously (14, 15). The type of solvent system was determined by subjecting the extracts on 5 different systems: water, methanol, 50% aqueous methanol, ethanol, 50% aqueous ethanol, chloroform. Ethanol was found to exhibit the highest yield of PMFs and therefore used for further optimization. Different ethanol concentrations (0, 20, 50, 80, 100%, v/v) were first applied with other variables remained constant at 20 °C for 60 min. Then different extraction times of 60, 120, 180, 240 and 300 min at 20 °C were applied using the best ethanol concentration determined. Finally, the extraction was carried out based on different extraction temperatures (20, 30, 40, 50 and 60 °C) applying the best ethanol concentration and extraction time. The best ethanol concentration, extraction temperature and extraction time were determined based on the highest concentration of all three PMFs (SEN, NOB and TAN) yielded based on HPLC quantification analysis. Following this, bioactivity assay test on 3T3-L1 preadipocyte cells were done on selected 3 sample extracts labeled **1.1**, **1.2** and **1.3** respectively. The three sample extracts were selected based on the significance difference in PMFs (SEN, NOB and TAN) concentrations present in the respective extracts, with sample **1.1** having the least amount of quantified PMFs in its crude peel extract and **1.2** having the highest. The bioactivity of the respective sample extracts were then measured by treating 3T3-L1 cells with varied concentrations of the extracts (50, 100 and 250 mg/L) over a period of 8-days. The differentiated cells were then stained with a specialized fat-soluble diazo dye known as Oil Red O stain, which is able to selectively bind itself to triglycerides and lipids resulting in bright red color observed, of which the amount of accumulated lipids can then be quantified (16). The

lesser the accumulated lipids formed in cells the more effective the extract is in inhibiting adipocyte differentiation. Cell viability assay is also done to ensure that toxicity effects do not interfere in its bioactivity result.

2.7.3.3 Quantification of PMFs via HPLC analysis

The extracts were subjected to qualitative and quantitative HPLC analysis. HPLC analysis was performed using a Waters 2695 separation mode equipped with an automatic injector and a Waters 2996 photodiode array detector covering 210-400 nm range. Agilent Zorbax SB-C18 column (5 μ M, 4.6 x 150 mm) was used. The temperature was maintained at 25 °C, with injection volume of 100 μ l and flow rate of 1 mL/min. All the markers (1mg/mL) and samples (5mg/mL) were separated using gradient elution with 0.1% of formic acid in water (A) and methanol (B) as the mobile phase. The gradient elution were as follows: 0 % of B to 100 % of B over 100 minutes, followed by 100% of B for 15 minutes and then decrease to 0% over 20 minutes to wash and condition the column. The peaks were detected at 260 nm and identified by standard substances. The following reference compounds were used as markers: SEN, NOB and TAN. The external standard method was used for HPLC quantification and results are reported as percentage of dry peel weight. The data were processed with Origin software version 9.0.

2.7.4 Analysis of *Averrhoa carambola* peel

2.7.4.1 Extraction of Averrhoa carambola peel

20 pieces of unripe (green) star fruit were purchased from a local wet market. The fruits were washed and peeled. The peels were further cut into smaller pieces and stored in a $-80\text{ }^{\circ}\text{C}$ freezer (72 h). The frozen samples were then lyophilized (72 h, $-80\text{ }^{\circ}\text{C}$). Dried peels were ground into fine powder using a blender. 53.0 g of crude powder was extracted with 50% aqueous ethanol (1500 mL) in a water bath ($30\text{ }^{\circ}\text{C}$) for 24 h. The mixture was filtered through Whatman no.4 filter paper, and the filtrate condensed by rotary evaporation. The condensed filtrate was further lyophilized (72 h, $-80\text{ }^{\circ}\text{C}$) to obtain brown solids (21.2 g) and stored in a $-80\text{ }^{\circ}\text{C}$ freezer until use. The crude extraction yield is exceptionally high with an average percentage of 40% however this has been further validated experimentally in triplicates.

2.7.4.2 Separation via silica gel-chromatography

5.0 g of the crude extract (brown solid) was re-suspended in methanol for further extraction. The clear solution of the methanol phase was separated by filtration. The methanol insoluble part was collected as a single fraction (Fraction 15). The homogeneous methanol extract was concentrated under vacuum before subjecting it to further separation via silica gel chromatography with a sequential gradient elution of DCM : MeOH:formic acid from 100:0:2 to 0:100:2, to yield 14 fractions labelled as Fractions 1–14 respectively. The fractions were then condensed and lyophilized into powder form and stored in a $-80\text{ }^{\circ}\text{C}$ freezer until they were used for analysis.

2.7.4.3 HPLC analysis of crude peel extract

Waters 2695 separation mode equipped Waters 2996 photo-diode array detector covering the 210–400 nm range was used for data analysis. The separation was performed on an Agilent Zorbax SB-C18 column 5 micrometer, 4.6 × 150 mm) with 0.1% of formic acid (A) and methanol (B) as the mobile phase. The column temperature was room temperature, and the injection volume was 80 µL. The gradient elutions were as follows: 0% B/100% A was increased linearly to 100% B/0% A over 100 minutes, held isocratic for 15 minutes, decreased to initial conditions over 15 minutes and held isocratic for 5 minutes. The flow rate was 1.0 mL min⁻¹.

2.7.4.3 Molecular Docking

The induced-fit docking (IFD) protocol in Maestro suite was carried out to predict the binding of PPARα (PDB ID: 2REW) (3) with (–)-epicatechin. The overall procedure has four stages: Briefly, during Stage 1 initial softened-potential Glide docking is performed on a vdW scaled-down rigid receptor; (9-11) a scaling of 0.5/0.5 was set for receptor/ligand vdW radii, respectively. The top 20 poses for each test ligand was retained. In Stage 2, receptor sampling and refinement was performed on residues within 5.0 Å of each ligand for each of the generated ligand:protein complexes. The side-chains, as well as the backbone and ligand, underwent subsequent energy minimizations. Stage 3 involved re-docking the test ligands into their respective 20 structures that are within 30.0 kcal/mol of their lowest energy structure. Finally, the ligand poses were scored in Stage 4 using a combination of Prime and GlideScore scoring functions in which the top ranked pose for each ligand was chosen as the final result. The SP scoring function was used in all docking stages.

Visualization of the protein-ligand complexes was done with the Visual Molecular Dynamics (VMD) software. (17)

2.7.5 Comparative analysis of *Berangan* banana peel at different ripening stages

2.7.5.1 Extraction of *Berangan* banana peel

Berangan banana were purchased from local wet market in Singapore. Half of the bunch was processed and the unripe peels were separated and freeze dried for 48 hrs. The remaining banana were wrapped in a plastic bag and left to ripe at room temperature for 6 days before peels were separated and freeze dried. The dried peels of the respective unripe and ripe bananas were grinded with Toyomi blender model BL2026 into powder form. 64.7g of unripe banana peel (green) and 27.7 g of ripe banana peel (yellow) were then extracted with 100% methanol twice at 25 °C for 60 minutes as optimized by González-Montelongo and others (18). For every 0.3 g of powder to extract, 2 mL of analytical grade methanol was used. The extract was filtered with Whatman filter paper no. 1 via Buchner filtration and the filtrate was then left under the rotavap to remove the remaining solvents. The extract was then further lyophilized to remove any moist present to yield 8 % and 19 % of crude unripe and ripe berangan peel extract respectively. The percentage yield of crude extract obtained was calculated using Formula 2.

2.7.5.2 HPLC analysis of crude peel extract

Waters 2695 separation mode equipped Waters 2996 photodiode array detector covering 210-400 nm range were used for data analysis. The separation was performed on an Agilent Zorbax SB-C18 column 5 μ M, 4.6 x 150 mm) with 0.1% of formic acid in water (A) and methanol (B) as the mobile phase. The column temperature was room temperature, and the injection volume was 100 μ L. The gradient elutions were as follows: The gradient elutions were as follows: 0 % of B to 10 % of B over 30 minutes, followed by 10 % of B to 80 % for 20 minutes and then decrease to 0% over 20 minutes to wash and condition the column. The flow rate was 1.0 mL/min. The data were processed with Origin software version 9.0.

2.7.5.3 Separation of crude unripe banana peel extracts using Solid Phase Extraction (SPE)

Method was followed closely as discussed by Shui and others (19). 40mg of unripe banana peel extract was dissolved in 4 mL of ultrapure water and was then subjected to solid-phase extraction (SPE) using a 3mL end-capped C₁₈ column (Isolute), which were initially activated and conditioned with methanol and ultrapure water respectively. 2 mL of sample were introduced into column and 3 mL of ultrapure water was used to elute most of the organic acid and sugar yielding Fraction 1 and further 2.5 mL of methanol to elute the remaining less polar compounds, yielding Fraction 2.

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PART III

RESULTS AND DISCUSSION

Chapter 3

3. Biological screening of Tropical Fruit Waste components

3.1 Introduction

The prevalence of obesity and the plummeting reliance on existing synthetic drugs have raised the urgent need for medicinal scientists to find an alternative method of treatment to combat this disorder. (1) As discussed in the introduction section of this thesis, it is the aim of the project to shift the focus from synthetically designing potent drugs to sourcing out for alternatives from natural sources particularly, the fruit waste components of tropical fruits. Addressed in our review, which highlighted the very limited amount of studies done in the last decade focusing on this area, there is an urgent need to start off the project with biological screening of selected fruits and its components before subjecting it to further analysis. (2)

Figure 13 Schematic representing the pathways for the first stage of biological screening

In the above scheme as represented in Figure 13, is the general layout of which the biological screening of tropical fruit waste components were facilitated in this project. The first phase of the project started off having to identify potentially

promising tropical fruits that have been studied previously, typically via thorough literature reviews. Among some of the things that were looked out for were the fruit's antioxidant capacity, concentrations of phytochemicals in its crude extract as well as its use in traditional medicine for treatment of different kinds of ailments. Next, before subjecting the selected fruits and its components to the biological screening stage using 3T3-L1 cells (as explained in the first chapter of this thesis), the respective components were first subjected to extraction conditions as recommended by previous literatures. Different concentrations of the respective extracts were prepared accordingly and treated on 3T3-L1 cells for 8 days before its bioactivity capacity were measured and quantified using Oil Red O stain, in three individual replicates. (3-6) The bioactivity capacity of the respective extracts was then classified into three categories: Low, High or No bioactivity.

Following this, only extracts that exhibit high bioactivity and seemingly a dose-dependent trend were then further subjected to cell viability assay analysis using CCK-8. (7, 8) The purpose of this stage of the project is to ensure that the suppression of lipid accumulation that was observed during adipocyte differentiation in 3T3-L1 cells were primarily due the possible presence of potent bioactive phytochemicals in its extract and not due to it being toxic. (9) Only extracts that exhibit promising high bioactivity effect in its biological screening test and remain non toxic within the tested concentration range were considered to be a viable source to be subjected for further analysis.

3.2 Biological screening results of selected extracts

Selecting and identifying relevant tropical fruit waste components is essential in order to further narrow down the scope and achieving the objectives set for this project. In view of this, thorough investigations to examine existing studies done previously, listing promising tropical fruits were gathered. Kim and others have highlighted the promising potential of *Citrus grandis* Osbeck, commonly known as pomelo, extract in exhibiting high antioxidant and antidiabetic activity. (10) In addition, citrus fruits have been used traditionally over centuries long to relieve symptoms of common cold, indigestion as well fever. A fair amount of studies have been done *in vivo* and *in vitro* to identify bioactive phytochemicals present in its components, particularly in its potential role as a promising antioxidant source. (11-13)

Figure 14 Summary of all the 18 fruit components subjected to biological screening before extraction

Considering this, three fruit species and its respective components of the *Citrus* family were particularly selected for the biological screening in this project: *Citrus grandis* (pomelo), *Citrus aurantifolia* (lime) and *Citrus reticulate blanco* (mandarin orange). Among other fruits were the peels of *Averrhoa carambola* (starfruit), *Averrhoa bilimbi* (cucumber tree), *Malus domestica* (Apple), *Garcinia mangostana* (Mangosteen), *Citrullus lantus* (Watermelon) and the local *Musa* genus (Berangan banana), as summarized in Figure 14. (14-16) Table 2, represents the biological screening results of the respective fruit extracts compiled in the first phase of this project.

Table 2. Summary of selected fruit components biologically screened against 3T3-L1 cells, along with its bioactivity and toxicity results for the relevant extracts.

	Fruit & Component	Bioactivity	Toxicity
1	<i>Citrus grandis</i> (Tambun Pomelo) Flavedo	Low*	-
2	<i>Citrus grandis</i> (Tambun Pomelo) Albedo	No Bioactivity	-
3	<i>Citrus grandis</i> (Tambun Pomelo) Flesh	No Bioactivity	
4	<i>Averrhoa Bilimbi</i> (Bilimbi) Peel	No Bioactivity	-
5	<i>Citrus aurantifolia</i> (Lime) Peel	High	Not Toxic
6	<i>Citrus aurantifolia</i> (Lime) Seed	No Bioactivity	-
7	<i>Citrus aurantifolia</i> (Lime) Flesh	No Bioactivity	-
8	<i>Citrus aurantifolia</i> (Lime) Leaf	High	Toxic
9	<i>Averrhoa carambola</i> (Star fruit) Peel	High	Not Toxic
10	<i>Citrus reticulata</i> (Mandarin Orange) Peel	High	Toxic
11	<i>Citrus reticulata</i> (Mandarin Orange) Seed	No Bioactivity	-
12	<i>Citrullus lantus</i> (Watermelon) Flavedo	No Bioactivity	-
13	<i>Citrullus lantus</i> (Watermelon) Albedo	No Bioactivity	-
14	<i>Citrullus lantus</i> (Watermelon) Seed	No Bioactivity	-
15	Unripe <i>M. genus</i> Berangan Banana Peel	High	Not toxic
16	Ripe <i>Musa genus</i> Berangan Banana Peel	High	Not toxic
17	<i>Malus domestica</i> (Apple) Peel	No Bioactivity	-
18	<i>Garcinia mangostana</i> (Mangosteen) Peel	No Bioactivity	-

The effect of respective extracts at various concentrations on adipocyte differentiation is monitored *in vitro* and quantified after 8 days of treatment; by measuring the Oil Red O stain using spectrophotometer. Each respective extracts were prepared in varied concentrations of 50, 100, 200 and 400 mg/L respectively and were subjected to 3T3-L1 cells treatment, in triplicates as well as on three separate plates to ensure repeatability. The bioactivity capacity of the extracts was measured by how well the extracts were able to suppress lipid accumulation at the end of the 8th day of cell treatment by proportionately measuring the amount of oil red O stained in cells as previously elaborated in section 1.3.2 of this thesis. In principle, the more stains there is, more lipids were accumulated and vice versa.

The respective bioactivity of the extracts were quantified accordingly as discussed in the method section of this thesis, section 2.1.3, and were further classified into three main categories: High, Low and No bioactivity as represented in Table 2. The following scheme in Figure 15 further elaborates how the effects of the respective extracts were further classified.

Figure 15 (i) Normalized lipid accumulation data and (ii) Cytotoxicity results of the respective extracts (A) *Citrus aurantifolia* peel extract (B) *Citrus reticulata* peel extract (C) *Citrus grandis* flavedo extract and (D) *Citrullus lantus* albedo extract

Figure 15A(i) represents the bioactivity of lime peel extract, which shows an obvious dose-dependent trend in the normalized lipid accumulation. Treatment with 50 mg/L of crude lime peel extract only induces 25% of lipid accumulation suppression while the administration of 400 mg/L of extract suppresses over 80% of lipid accumulation. As the lime peel extract exhibits a significant degree of lipid suppression during treatment over the range of crude concentration, it is therefore categorized as one that exhibits “High” bioactivity. Following the scheme represented in Figure 13, extracts that were definitively categorized to exhibit high bioactivity were further subjected to a cell viability assay using CCK-8. Lime peel extract were subjected to cell viability test and as represented in the diagram 11A(ii) exhibits no significant effect on 3T3-L1 cell viability within the concentration range of interest. This therefore, classifies lime peel extract as one of the promising fruit component that remains viable for further analysis in this project.

Figure 15B(i) on the other hand, represents the normalized lipid accumulation of mandarin orange peel extract that shows rather promising suppression effect as the lime peel extract. However, further subjecting to CCK-8 represented in Figure 15B(ii), it was found that the mandarin orange peel does affect the 3T3-L1 cell viability at the tested concentration, deeming it a non viable fruit component to be brought forward for further analysis. The cell viability test further suggests that the initial suppression of lipid accumulation observed in biological screening stage was likely due to the peel extract’s toxic effect rather than being attributed to any presence of potentially active phytochemicals in the extract. The flavedo (green peel) component of *Citrus grandis* was classified as an extract that primarily exhibits low

bioactivity. As seen in Figure 15C(i), only slight suppression of lipid accumulation were seen on 3T3-L1 cells administered with 400 mg/L of extract while not much activity was seen at lower administration between 50-200 mg/L. This data were repeatable on the three replicates and remains inconclusive if the flavedo extract was essentially an active extract. Therefore, under this circumstance, the flavedo extract was defined as exhibiting low bioactivity, which essentially leaves it as a less promising component to be brought forward for further analysis.

Extracts were primarily classified as exhibiting no bioactivity when it shows very little or no significant suppression of lipid accumulation on 3T3-L1 cells during the 8-days treatment. As represented in Figure 15D(i), watermelon peel extract were among the many extracts identified to exhibit no bioactivity during adipocyte differentiation. Administration of watermelon peel extract at 50-400 mg/L does not affect the adipocyte differentiation process in any way of which, optimal amount of lipids were able to form effectively throughout. Upon this biological screening, it is therefore classified as among one the fruit extracts that were deemed to be a no longer viable source to treat obesity due to its inability to induce any sort of suppression in lipid accumulation during the adipocyte differentiation stage. Similarly, this is the case for the other fruit component extracts such as apple peel, mangosteen peel, lime flesh and cucumber tree peel to name a few, as represented in Table 2.

Out of the eighteen fruit components screened in the first phase of the project, only three extracts exhibit promising potential to treat obesity via the suppression of lipid accumulation during adipocyte differentiation. The three fruit extracts as highlighted in Table 2 are, *Averrhoa carambola* (Starfruit) peel, *Citrus aurantifolia*

(Lime) peel and last but not least, the peels of both ripe and unripe *Musa genus* (Berangan banana).

3.3 Approach for each targeted fruit components

Having identified the three viable fruit extracts to work on, specific directions were set for the respective fruit extract, in order to achieve the collective goals set for this research project.

3.3.1 *Citrus aurantifolia* (Lime) Peel

Citrus aurantifolia (lime) peel extract have been found in the biological screening stage to exhibit bioactivity in a dose-dependent manner. This phenomenon is observed due to the presence of biologically active phytochemicals in the crude extract. Besides having to identify the specific phytochemicals responsible for the inhibition of adipocyte differentiation, understanding the underlying mechanism leading to the overall suppression is also essential. The comprehensive study, as will be presented in Chapter 4 of this thesis, from identifying to understanding the biological mechanism and also, elucidating the chemical binding relationship between the identified phytochemical(s) and specific adipogenic protein serves to provide a thorough study accounting for the potential of lime peel extract as a promising alternative source to supplement obesity treatment. This set of data, along with further *in vivo* studies in the future, could essentially promote the shift from over-reliance on synthetic drugs to the use of natural extracts for obesity treatment in the long run.

The following scheme in Figure 16 summarizes the overall direction for the study of lime peel extract in this project. Besides conducting a thorough research to account for the bioactivity potential of lime peel extract as a promising alternative source for obesity treatment, a comparative study to justify the importance of focusing on the waste components instead of depending on the flesh component is also needed.

Figure 16 Schematic of research project direction in three main stages for *Citrus aurantifolia* extract

As represented in stage 2 of the scheme, the next study then is served to analyze the fundamental difference in chemical composition between the different fruit components originating from the same plant. Herein, we are looking at the possible

comparisons of the seed, flesh, peel and leaf component of lime, similar to a study conducted by Taing and others in assessing the antioxidant capacity of the flesh and peel component of Mango originating from the same source.(17) In Table 2, we have observed how the four components of lime, despite originating from the same plant exhibit bioactivity differently. In the initial biological screening stage, it has been found that the lime peel extract exhibits high bioactivity while the seed and flesh extract remained inactive. The lime leaf extract on the other hand, was seemingly toxic thus making it a less viable source. Chapter 5 of this thesis presents the findings for this subject matter.

Finally, having identified the bioactive phytochemicals and assessing the key difference in chemical compositions between the four extracts, experimental extraction conditions to yield maximum concentrations of the bioactive phytochemicals should then be explored. (18) This study value-adds the significance of the research as it starts to explore the best conditions required should large-scale production for lime peel extracts, as an alternative source for obesity treatment, be considered. Chapter 6 of this thesis discusses on this aspect of the research work.

3.3.2 *Averrhoa carambola* (Starfruit) Peel

Similar to stage 1 of lime peel extract, the research work for starfruit extract peel is directed to provide the same comprehensive study to account for its potential as anti-obesity therapeutic in the long run. The identification of the phytochemical accounting for its bioactivity and studies to understand the underlying mechanism of the extract as well as the chemical binding relationship with key adipogenic protein were also done. Chapter 7 of this thesis presents the full research findings of starfruit peel extract.

3.3.3 *Musa genus* (Berangan banana) Peel

The biological screening between the different ripening stages of the *Berangan* peel (ripe versus unripe) exhibits a seemingly interesting observation. Figure 17 represents the respective normalized lipid accumulation of the respective extracts.

Figure 17 Normalized lipid accumulation data of ripe and unripe *Berangan* banana peel extract respectively

Between the ripe (yellow) and the unripe (green) *Berangan* banana peel extracts, it appears that while both extracts does not affect the cell viability of 3T3-L1 cells, they exhibit significantly different potencies in the suppression of adipocyte differentiation. Elaborate studies to investigate the chemical composition between the two extracts to account for this initial observation would provide a significant clue in identifying the like bioactive phytochemical in banana peel extract responsible for the suppression bioactivity. The oxidation process that takes place during ripening stage may play an essential role in altering the phytochemical composition of the banana peel extract at different ripening stage. (19, 20) This will be further discussed in Chapter 8 of the thesis.

3.4 Chapter Summary

At the end of the biological screening stage of this project, we have managed to successfully narrow down the scope of the research work, focusing mainly on three tropical fruits namely, *Citrus aurantifolia*, *Averrhoa carambola* and *Musa* genus, particularly the local berangan banana. Having focus mainly on *Citrus aurantifolia*, the comprehensive study is designed to investigate and provide answers to the following fundamental questions:

1. *How are tropical fruit waste component useful in treating obesity?*
2. *What are the relevant bioactive phytochemicals responsible for the overall suppression of adipocyte differentiation in the respective extracts?*
3. *Why should we focus on the waste component of a fruit instead of its flesh?*

The study of *Averrhoa carambola* and *Musa* genus is also done to partly answer the same questions. The exploratory methods as well as the results and discussion of the respective fruit extract identified in this biological screening stage will be further elaborated in the coming chapters. Fundamentally, with the completion of the first phase, we have consequently identified potential fruit components that may exhibit anti-obesity properties via the inhibition of adipocyte differentiation process. In the following chapters, the bioactive compounds present in these respective extracts and the underlying mechanism it plays resulting in the overall bioactivity will be further explored and discussed.

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Chapter 4

4. Citrus aurantifolia peel as a source for obesity treatment

4.1 Introduction

Citrus aurantifolia, commonly known as lime or *limau nipis*, has been widely used over centuries long for several purposes such as a common food source and key ingredient in local dishes as well as in traditional medicines, to treat different kinds of illnesses such as colds, sore throats, coughs, stomach ailments, digestive issues and tonic problems in Asia. ⁽¹⁾ Despite its popularity in the region, very little studies have been done in the past to investigate the medicinal value of this fruit, particularly in its peel component. Thorough chemical composition analysis of the *citrus* family, mainly in its flesh component though, has been made widely available highlighting the presence of useful phenolics such as naringenin, hesperetin, diosmin and naringin in its extract. ⁽²⁻⁴⁾ However, there has been an apparent lack of discussion in the potential use of these citrus fruits, particularly its peel component, as a potential source for obesity treatment. In light of this, as discussed in the biological screening stage of our research work, as the peel component of the lime extract was among one of the promising fruits identified to exhibit a considerably significant effect in the suppression of lipid accumulation during adipocyte differentiation, it is of our interest to therefore focus on lime peel and to understand the underlying mechanism involved in its extract, leading to the overall positive suppression of adipocyte differentiation. In order to accomplish this, this chapter discusses a wide range of studies done on lime peel from discussing the bioactivity of the crude extract to the isolation and purification processes involved to identify the bioactive compounds present in the

extract. Following this, the underlying molecular mechanism that results in the overall suppression of adipocyte differentiation and the possible chemical binding relationship between the individual isolated bioactive compound(s) identified and the key adipogenic protein receptor, PPAR α , were also elucidated.

Name	Fruit	Part Used	Extract in MeOH	Crude Extract	Yield (%)
<i>Citrus Aurantifolia</i>					10 %

Figure 18 Summary of extraction for crude Lime peel.

4.2 Effects of crude lime peel extract on adipocyte differentiation

Lime peel was first processed and extracted under the experimental conditions discussed in section 2.8.1.1 to yield 10% of methanolic lime peel extract, as summarized in Figure 18. The effects of the crude lime peel extract on the suppression of adipocyte differentiation were then evaluated after 8 days of treatment using varied concentrations of the extract on 3T3-L1 adipocyte cells. As summarized in Figure 19, the effects of lime peel extract on the overall suppression of adipocyte differentiation by measuring the amount of lipids accumulated using Oil Red O stain, relative to the positive control was evaluated. Figure 19A is a histological summary of stained 3T3-L1 cells on day 8 of the adipogenic period, after treatment with 50-400 mg/L of crude lime peel extract respectively. Based on the results obtained, crude lime peel extract have been found to induce suppression in adipocyte differentiation in a dose-dependent manner. As evident in the histological data in Figure 19A, the number and size of lipid droplets were seemingly decreasing when treated with an increasing dosage of lime peel extract accordingly.

Figure 19 Effect of lime peel extract on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with treatment of 400 mg/L, 200 mg/L, 100 mg/L and 50 mg/L crude LPE respectively throughout the whole adipogenic period. Positive control is a group of cells that were induced to undergo adipocyte differentiation without any additional drug treatment. On day 8, cells were stained with Oil Red O. (A) Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2), 4X objective (lane 3) and photographed with camera (lane 4) (B) Quantification of lipid accumulation determined by Oil Red O against normalized positive control (C) Cytotoxicity of crude LPE measured by cell viability assays. * $p < 0.05$ compared with the positive control. ** $p < 0.01$ compared with the positive control.

Cross-referencing with the normalized lipid accumulation data as represented in the chart in Figure 19B, this similar trend was further confirmed. Comparing between cells when treated with relatively lower concentrations of 50 mg/L versus one at high concentrations of 400 mg/L of crude extract respectively, it has been found that the treatment with higher dosage of lime peel was able to significantly induce over 90 % inhibition in adipocyte differentiation while only 20% inhibition was observed, when the cells were subjected to a lower concentration of lime peel extract. In order to verify the dose-dependent effect of crude lime peel extract leading to the overall suppression of adipocyte differentiation, as validated in both the histological analysis and normalized lipid quantification, cell cytotoxicity assay was also conducted at varied concentrations ranging from 50-1000 mg/L. As represented in Figure 19C, the crude lime peel extract exhibits no significant effect on the cell's viability in comparisons to that of the untreated cells. This is a good indication suggesting that the suppression of lipid accumulation in a dose-dependent manner was solely due to inhibitory effect of phytochemicals present in crude LPE and not due to its toxicity effect.

4.3 Identification of bioactive compounds in crude lime peel extract

Having seen the positive bioactivity effect of crude lime peel extract in inducing significant suppression in adipocyte differentiation in a dose-dependent manner, the next step involves the identification of precise phytochemical(s) responsible for this bio-mechanism. In order to facilitate this investigation effectively, the crude extract was subjected to a series of separation steps using silica gel chromatography, with dichloromethane and methanol as the mobile phase, to yield 12 separate fractions labeled Fractions 1 to 12 respectively. The LC elution profile of the 12 fractions can be found in Appendix I. Following this, each individual fraction were then prepared in two concentrations of 100 mg/L and 200 mg/L respectively and further subjected to adipocyte differentiation assay on 3T3-L1 preadipocyte cells, in triplicates. Summarized in Figure 20, is the quantification and histological data of selected fractions with varying bioactive capacity. Out of the 12 fractions, only one fraction (Fraction 2) showed a significant inhibition in TG accumulation. This is a significant indication of the possible presence of bioactive phytochemical(s) concentrated in Fraction 2, likely responsible for the suppression of adipocyte differentiation initially observed in crude LPE.

Figure 20 Effect of respective fractions of LPE at 200 mg/L concentration. (A) Observation of cells after Oil Red O staining under phase contrast microscopy at 10 X objective (lane 1) 40X objective (lane 2) and under naked (lane 3) (B) Quantification of lipid accumulation determined by Oil Red O absorbance at 513 nm normalized against positive control. Fraction 2 exhibited the only potency in inhibition of lipid accumulation during adipocyte differentiation

The chemical composition analysis of Fraction 2 then was further facilitated, upon freeze-drying, via reversed phase HPLC coupled with LC-MS. As observed in Figure 21A, three major peaks were identified and labeled as Compound **1**, **2** and **3** with retention time of 16.3 min, 18.7 min and 21.7 min and m/z ratio of $[M+H]^+ = 373.13$, $[M+H]^+ = 403.14$ and $[M+H]^+ = 373.13$ respectively. Further isolation and purification of bioactive Fraction 2, in order to identify and characterize these three compounds, yields 1.7 mg of compound **1** with over 95% purity, 14.1 mg of compound **2** with over 98% purity and 4.3 mg of compound **3** with over 95% purity (Figure 21B).

Figure 21 (A) HPLC chromatogram of crude fraction 2 exhibiting 3 prominent peaks labeled 1, 2 and 3 respectively (B) HPLC chromatogram of purified peaks 1, 2 and 3 along with the chemical structures of Sinensetin, Nobiletin and Tangeretin respectively

The isolated compounds **1**, **2** and **3** were subjected to elaborate characterization analysis using ^{13}C NMR, ^1H NMR and HPLC coupled with HR-MS as well as subjecting it to HPLC co-injection with pure compounds purchased commercially. These series of analysis have allowed us to successfully identify the three compounds **1**, **2** and **3**, present in bioactive fraction 2 to be a group of polymethoxylated flavones (PMFs); sinensetin, nobiletin and tangeretin respectively. While the actual spectral data is attached in the appendix of this thesis, here are the summarized characterized data. *Compound 1*: Yellow powder; m/z 373 $[\text{M}+\text{H}]^+$; ^1H NMR (500MHz, CD_3OD) δ : 7.64 (1H, dd, $J = 2.0, 8.5$ Hz, H-6'), 7.53 (1H, d, $J = 2.0$ Hz, H-2'), 7.16 (1H, s, H-3), 7.11 (1H, d, $J = 8.5$ Hz, H-5'), 6.68 (1H, s, H-8), 3.86-4.02 (15H, $5 \times \text{OMe}$).

Compound **1** was identified as 5,6,7,3',4'-pentamethoxyflavone (Sinensetin) from the spectral data along with co-injection and comparisons of retention time of pure standards via HPLC.⁽⁵⁾ *Compound 2*: White powder; m/z 403 $[\text{M}+\text{H}]^+$; ^1H NMR (500 MHz, CDCl_3) δ : 7.56 (1H, dd, $J = 2.0, 8.5$ Hz, H-6'), 7.41 (1H, d, $J = 2.0$ Hz, H-2'), 6.99 (1H, d, $J = 8.5$ Hz, H-5'), 6.62 (1H, s, H-3), 3.95-4.10 (18H, s, $6 \times \text{OMe}$); ^{13}C NMR (125 MHz, CDCl_3) δ : 177.45 (C-4), 161.12 (C-2), 151.95 (C-4'), 151.47 (C-7), 149.30 (C-3'), 148.42 (C-9), 147.74 (C-8), 144.10 (C-5), 123.99 (C-6), 119.66 (C-1'), 114.83 (C-6'), 111.22 (C-10), 108.55 (C-5'), 106.86 (C-3,C-2'), 55.99, 56.11, 61.71, 61.87, 62.00, 62.30 ($6 \times \text{OMe}$). Compound 2 was identified as 5,6,7,8,3',4'-hexamethoxyflavone (Nobiletin) from these spectral data along with references against standard via HPLC.⁽⁶⁾ *Compound 3*: Yellow powder; m/z 373 $[\text{M}+\text{H}]^+$; ^1H NMR (500 MHz, CDCl_3) δ : 7.87 (2H, d, $J = 2.0$ Hz, H-2', H-6'), 7.02 (2H, d, $J = 9.0$ Hz, H-3', H-5'), 6.61 (1H, s, H-3), 3.89-4.10 (15H, s, $5 \times \text{OMe}$). Compound **3** was

identified as 5,6,7,8,4'-pentamethoxyflavone (Tangeretin) from the spectral data along with co-injection and comparisons of retention time of pure standards via HPLC. (7)

4.4 Potency analysis of the three bioactive compounds

As sinensetin, nobiletin and tangeretin have been identified as the primary phytochemicals present in crude lime peel extract responsible for the overall bioactivity of the extract, it is then crucial to understand the potencies of the different compounds in suppressing adipocyte differentiation. Hypothetically, as the three PMFs are structurally similar, it is expected that they exhibit similar potencies when it comes to the suppression of adipocyte differentiation. However, in our study, this was not the case. Following the isolation stage, the respective bioactive compounds, sinensetin, nobiletin and tangeretin were subjected to further bioactivity assay analysis, under same experimental conditions, as discussed in section 2.2, at varied concentrations ranging from 25 μ M to 200 μ M in triplicates and on three separate plates.

Figure 22 (A) Lipid accumulation quantification of the respective isolated bioactive compounds (i) Sinensetin, (ii) Nobiletin and (iii) Tangeretin respectively along with its (B) cytotoxicity results measured by CCK-8 assay, highlighting Nobiletin as the most potent compound.

Figure 22A summarizes the normalized lipid accumulation data focusing on four specific concentration ranges of which the respective compounds were found to induce the most significant inhibitory trend. For this case, the range of interest for sinensetin was between 75 – 150 μM , nobiletin between 25 - 100 μM and tangeretin between 100 - 200 μM respectively. Generally, from the observed chart as represented in Figure 22A(i), (ii) and (iii), all three PMFs suppresses adipocyte differentiation in a dose-dependent manner. This set of data is conversely different from that revealed by

Kang and others of which in their paper, they reported the effect of sinensetin in particular, to enhance adipocyte differentiation instead.⁽⁸⁾

Addressing the hypothesis that PMFs having similar chemical structures, as seen in Figure 23, to exhibit similar potencies in suppression during adipocyte differentiation, our set of results have proved otherwise. The differences in potency effects were significantly apparent for all the three compounds. As observed in the charts presented Figure 22A (i) to (iii), nobiletin, which contains an extra methoxy group at the benzopyranyl or phenyl moiety, in comparisons to both tangeretin and sinensetin respectively, exhibits the greatest potency in adipocyte differentiation. This is consistent to studies done previously reporting the ability of nobiletin to suppress adipocyte differentiation in 3T3-L1 cells however no comparisons between the other two PMFs were made in any of these studies.⁽⁹⁻¹¹⁾

Figure 23 Chemical structures of Sinensetin, Nobiletin and Tangeretin

Based on our set of data, nobiletin was able to induce approximately 20 % inhibition at a concentration of as low as 25 μ M relative to the positive control, versus sinensetin and tangeretin, which were only capable of inducing similar effect of inhibition at a higher concentration of 75 μ M and 100 μ M respectively (Figure 22A). Similar trends were observed when the dosage of the respective compounds were increased accordingly indicating a repetitive potency trend of which, nobiletin being the most potent compound followed by sinensetin and lastly tangeretin, being the least potent of the three. The negative value of normalized lipid accumulation of nobiletin at 100 μ M as shown in Figure 22A-ii can be accounted due to the highly potent nobiletin to suppress inherent or natural extent of adipocyte self-differentiation that takes place in negative control under natural environment. Thus, when the value of lipid accumulation was subtracted from that of negative control for analysis, negative normalized value was obtained.

Likewise, cell viability assay of 3T3-L1 cells treated with relevant concentration ranges of the respective compounds showed no significant effect of the respective drugs on the cell viability (Figure 22B). This analysis further confirms the sole inhibitory effect of all three PMFs (sinensetin, nobiletin and tangeretin) against adipocyte differentiation without interference of any toxicity effect that might have occurred within the dosage range tested.

4.5 Underlying mechanism of crude lime peel extract on adipogenesis

While the relevant bioactive compounds and its individual potencies have been successfully identified and addressed, the underlying mechanism of crude lime peel extract and the summative role of compounds **1**, **2** and **3** (sinensetin, nobiletin and tangeretin), leading to the overall suppression of adipocyte differentiation has yet to be determined. As discussed in the earlier section of this thesis, the effects of the crude extract on the genetic expression of key adipogenic genes involved were then further studied in order to achieve this.

As shown in Figure 24, the RT-PCR analysis reveals the ability of the lime peel extract to simultaneously downregulate C/EBP α and PPAR γ , which are essentially the key transcription factors, in a dose-dependent manner. This set of data is consistent with a research conducted by Choi and others that discussed the effects of nobiletin on the expression of C/EBP α and PPAR γ , which are essentially the key protein receptors involved in regulating the expression of many adipogenic proteins.⁽¹⁰⁾

Also, treatment with crude lime peel extract was also found to induce a further downregulation in the expression of tissue specific gene namely LPL and aP2 that are known to be typically involved in fatty acid metabolism and transport processes. While no significant effect were observed on PPAR δ expression, an apparent upregulation of PPAR α gene in a dose-dependent manner was particularly interesting and this phenomenon will be further elaborated in the next section.

Figure 24 Effect of crude LPE on the expression of adipogenic genes. In biological replicates, adipocyte differentiation was performed with or without treatment of 400 mg/L, 200 mg/L, 100 mg/L and 50 mg/L of crude LPE throughout the whole adipogenic period. On day 8, cells were harvested and mRNA expressions were evaluated by RT-PCR. The bands were analyzed using G:Box-SynGene software. Neg: negative control; Pos: positive control (without treatment of crude LPE). Three biological replicates were used to represent the error bars. * $p < 0.05$ compared with the positive control. ** $p < 0.005$ compared with the positive control.

Overall, the underlying mechanism involved in crude lime peel extract while suppressing adipocyte differentiation can be better understood. It is believed that based on this genetic expression studies, crude lime peel extract does so via a simultaneous downregulation of key adipogenic genes particularly C/EBP α and PPAR γ that acts as a ‘master switch’ thereby leading to a further downregulation of its respective target genes such as those observed in aP2 and LPL. In addition, with the upregulation of PPAR α , it is therefore believed that all this collectively leads to the overall effective suppression of adipogenesis by the extract, as accounted in the bioactivity phases of this study.

4.6 Effect of isolated bioactive compounds on PPAR α upregulation

PPAR α is essentially a transcription factor that is activated by ligand binding served to regulate the expression of genes involved in cellular lipid uptake and oxidation. (12) In a number of studies, it has been shown that the upregulation of PPAR α may have a role in the pathology of obesity and obesity-related diseases since its pharmacological activation helps reduce the risk of cardiovascular disease. Furthermore, as many PPAR α agonists have shown beneficial effects in the treatment of various metabolic diseases, (13-15) it is therefore the capacity of the bioactive compounds to upregulate the PPAR α gene that becomes our primary interest despite the fact that we still acknowledge that the overall reduction of adipocyte differentiation, deduced from our genetic expression analysis of crude LPE, was due to the simultaneous downregulation of master adipogenic gene PPAR γ and C/EBP α as well as upregulation of PPAR α .

Figure 25 Effect of 100 μ M of Nobiletin, Sinensetin and Tangeretin on the upregulation of PPAR α in 3T3-L1 preadipocyte cells at the end of 8 days treatment of adipocyte differentiation. The bands were analyzed using G:Box-SynGene software. Neg: negative control; Pos: positive control (without treatment of crude LPE). Three biological replicates were used to represent the error bars. * $p < 0.05$ compared with the positive control. ** $p < 0.01$ compared with the positive control.

From our analysis of the effect of the respective bioactive compounds (100 μ M) on PPAR α gene expression during adipocyte differentiation, it revealed that all three PMFs caused a significant upregulation in the expression of PPAR α gene, which might be caused by direct agonism of another transcription factor or signal that affects PPAR α expression. Upon treatment of induced 3T3-L1 cells with 100 μ M of the respective PMFs during adipocyte differentiation, Nobiletin have been found to induce the highest magnitude of upregulation in the PPAR α expression at mRNA level by a scale factor of x2.13, followed by Sinensetin (x1.74) and Tangeretin (x1.32) relative to the negative control (Figure 25). This set of data correlates with the potency trend similarly observed in the bioactivity test addressed in previous section.

Nobiletin, which was deduced to be the most potent compound of the three PMFs has also been found to upregulate the expression of PPAR α gene most effectively. This observation, along with similar studies done on known PPAR α agonist, may support the fact that the ability of these bioactive compounds to inhibit adipocyte differentiation could be accounted due to the PPAR α agonist properties of these PMFs. Essentially, the more effective a compound is able to bind and interact with PPAR α , the more likely it is to regulate its gene expression thereby, leading to an increased likelihood to effectively suppress adipocyte differentiation and total lipid accumulation in cells. However, a conclusive statement can be made on the effect of suppression of lipid accumulation via upregulation PPAR α , further studies demonstrating the knockdown of PPAR α totally rescuing the impairment in lipid accumulation is still required.

4.7 Chemical binding relationship accounting for the different potencies

In order to further investigate the reasons why these three PMFs, despite structurally similar, exhibit different degree of upregulation towards PPAR α gene, thereby resulting in different potencies to suppress adipocyte differentiation, we then explored the binding mode of Nobiletin, Tangeretin and Sinensetin within the active site of PPAR α . As proposed in previous study, interaction with the AF-2 helix is crucial to PPAR α upregulation. Specifically, ligands that are able to form an interaction with TYR 464, are able to regulate PPAR α . (13) From Figure 26, the TYR 464 of the AF-2 helix was able to form bifurcated hydrogen bonds with the two oxygen atoms of the methoxy groups in Nobiletin. Comparing it to Sinensetin, TYR 464 was only able to form one hydrogen bond with one of the methoxy groups, despite that both Nobiletin and Sinensetin have two methoxy groups on their phenyl moiety. This could be due to the lack of methoxy group at the benzopyranyl moiety in Sinensetin that may serve to further optimize the orientation of the PMF, allowing it to form more interactions as well as bifurcated hydrogen bonds within the active site, as observed in Nobiletin. As for Tangeretin on the other hand, it was only able to establish an indirect interaction with TYR 464 via a bridging water molecule because it lacks an additional methoxy group in its phenyl moiety to form hydrogen bonds with. Even though Tangeretin has the additional methoxy group on its benzopyranyl moiety, it docks at a position further away from TYR 464 because compared to the phenyl moiety, the benzopyranyl moiety is able to form more interactions in the active site only if it moves away from TYR 464.

Figure 26 Crystal structure of PPAR α (left) and predicted PPAR α -PMFs binding structure (right). Hydrogen bonds formed by the PMFs and the surroundings are indicated as green dotted lines.

While the phenyl methoxy groups are required to form favorable hydrogen bonds with TYR 464, the benzopyranyl methoxy group is also required at the same time to help orientate the PMF so that it is able to form the most number of hydrogen bonds with TYR 464 as observed in Nobiletin. Therefore, our docking has proposed a relationship between the strength of interaction with TYR 464 and the PMFs' PPAR α upregulating activity, thereby accounting for the different potencies in suppressing adipocyte differentiation observed experimentally.

4.8 Chapter summary

The ability of crude lime peel extract to significantly inhibit adipocyte differentiation in 3T3-L1 cells were accounted due to the presence of three bioactive compounds: nobiletin, sinensetin and tangeretin. Although the chemical composition of lime has been well studied and the presence of these three PMFs are already known, its specific role to inhibit adipocyte differentiation have never been reported except for nobiletin.(9-11) Despite being classified under the same category of compounds (i.e. PMFs), and tangeretin as well as sinensetin, being structural isomers of one another, it has been found that the three compounds exhibit very different potencies in inhibiting adipocyte differentiation. Bioactivity analysis showed nobiletin to exhibit the highest potency followed by sinensetin and tangeretin respectively. Through gene expression analysis, we deduced that it is the ability of these bioactive compounds in crude LPE to simultaneously downregulate master adipogenic genes, PPAR γ and C/EBP α as well as upregulate the expression of PPAR α gene that accounts for the overall suppression of adipocyte differentiation. However, it is the ability of these bioactive compounds to act as a PPAR α agonist that remains as our primary interest in investigating the cause for the different potencies exhibited by the three PMFs in suppressing adipocyte differentiation. Further gene expression analysis of the three PMFs against PPAR α gene revealed a correlation between the ability of the respective PMFs to upregulate the expression of PPAR α gene with the degree of potency that each PMF exhibits; nobiletin of which exhibits the highest degree of suppression, was also observed to induce the highest magnitude of upregulation in PPAR α gene expression during adipocyte differentiation. Computational docking that was done to investigate the possible difference in binding mode between the three

PMFs, likely to account for the trend observed experimentally, further sets thing into perspective. Our study suggested that it is the fundamental ability of nobiletin to form the most number of favorable hydrogen bonds with TYR 464 that accounts for its greatest potency. This is particularly aided by the presence of additional methoxy group in both its phenyl and benzopyranyl moiety that serve to enhance its orientation within the PPAR α active site, making it more superior than its other 2 PMFs, tangeretin and sinensetin, which were lacking of a methoxy group in either moieties.

In conclusion, this comprehensive set of data accounts for the potential of crude LPE as a plausible supplement that can be used for the treatment of obesity and obesity-related disorders particularly due to the three bioactive compounds that is present namely nobiletin, tangeretin and sinensetin. However, further experimental studies is still required to provide a conclusive data, particularly on the direct interaction between the bioactive compounds elucidated and PPAR α and this can be done via luciferase assay. (16) Also, better understanding on the effectiveness of the crude extract *in vivo* and the determination of an acceptable dosage range for consumption of LPE is still required before further developmental studies be done to further explore the most economical way of extracting and processing these lime peel without having to compromise its bioactivity as a possible alternative supplement source for anti-obesity therapeutic.

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Chapter 5

5. Comparative study of adipogenesis suppression capacity of the different components of *Citrus aurantifolia*

5.1 Introduction

In the previous chapter of this thesis, we have addressed the potential of the peel component of *Citrus aurantifolia* (lime) to suppress adipocyte differentiation in a dose-dependent manner. In our work, we have identified three PMFs: nobiletin, sinensetin and tangeretin as the primary compounds responsible for the suppression of lipid accumulation during adipocyte differentiation. (Chapter 4) In a our review paper as well as in Chapter 1 of this thesis, we have also highlighted how most research studies have solely focused on the functionality of the flesh components as a potential source of therapy for varied diseases without having to assess its waste components that were sometimes found to contain higher bioactive phytochemicals than its flesh itself. (1-4)

Therefore, as part of a thorough study to explore the potential of lime as a source for obesity treatment, this study aims to do a comparative study of the different components of lime namely the extracts of peel, flesh, seed, and leaf, originating from the same plant source, particularly on its role in inhibiting adipocyte differentiation. In addition, having identified the three PMFs as the primary compounds responsible for the observed anti-adipogenic activity (Chapter 4), the study also aims to investigate the chemical composition of the respective components, to account for the

significant difference in bioactivity observed and deduce if there is a relationship between the abundance of PMFs present in the respective components and its bioactivity. Cell viability assay was also conducted to ensure that any observed bioactivity was solely due to the inhibitory property of the bioactive phytochemicals present in the extracts and not due to toxicity effect.

5.2 Crude lime peel extract exhibits the highest degree of suppression

The effects of the four separate crude components of *Citrus aurantifolia* on adipocyte differentiation were investigated at various concentrations on 3T3-L1 cells. The overall effect was evaluated based on its ability to suppress lipid accumulation effectively during the 8-days treatment when the cells were induced to undergo adipogenesis. The lipids accumulated were then quantified using Oil red O stain, a fat-soluble diazo-dye that stains triglycerides and lipids resulting in bright red color observed. Figure 27B summarizes the effects of the respective crude extracts from all four components on adipocyte differentiation, representing the quantified analysis of the total amount of accumulated lipids formed, relative to the positive control.

Based on the analysis, both the crude lime flesh and seed remain seemingly ineffective in inducing any sort of suppression during adipogenesis even at its highest concentration of 400 mg/L. As for the crude lime peel, it was found that adipocyte differentiation was suppressed in a dose-dependent manner and this is also evident in the decrease in the size and number of lipid droplets with increasing concentration, as shown in Figure 27A.

Figure 27 (A) Observation of cells after Oil Red O staining under phase contrast microscope at 10X objective when treated with various concentrations of respective lime extract components. (B) Summarized quantification of lipid accumulation effect of all four lime extract components on 3T3-L1 adipocyte cells determined by Oil Red O stain relative to normalized positive control (untreated cells). * $p < 0.05$ compared with the positive control. ** $p < 0.01$ compared with the positive control.

In comparisons to the cells treated with 50 mg/L of crude lime peel resulting in approximately 15 % inhibition of adipocyte differentiation, cells treated with 400 mg/L of crude lime peel was shown to induce significant inhibition in adipocyte differentiation of about 68 % relative to the positive control. Interestingly, 3T3-L1 cells treated with lime leaf extract also reflected some degree of suppression in lipid accumulation between 50-100 mg/L particularly, however when treated at higher concentrations between 200-400 mg/L, the lime leaf extract induces cell death, thereby also resulting in a normalized lipid accumulation values at the negative range as represented in Figure 27B. This result is also evident in the histological study as reflected in Figure 27A whereby floating cells were seen at 10x magnification when cells were treated with 200-400 mg/L of leaf extract. The toxicity effects of the respective components were then being conducted to further confirm this observation.

5.3 Lime leaf extract is toxic when administered at 200-400mg/L

Cell Counting Kit-8 (CCK-8) assay was employed for this analysis and it showed that the treatment of 3T3-L1 cells for the three crude lime components particularly, the flesh, seed and peel, at concentrations ranging from 50-400 mg/L, placed no significant effect on 3T3-L1 cell viability compared to that of untreated cells (Figure 28). This is a good indication suggesting that the suppression of lipid accumulation initially observed on cells, when treated with crude lime peel at varied concentrations, was solely due to inhibitory effect of phytochemical(s) present in the crude extract and not due to its toxicity effect.

Figure 28 (In clockwise) Cytotoxicity of crude lime leaf, peel, seed and flesh extract respectively, measured by cell viability assay test kit, CCK-8.

The lime leaf extract however, appears to induce toxicity effects on the cells especially at higher concentrations thereby resulting in a significant reduction in cell count of about 78 % and 97 % when treated with 200 mg/L and 400 mg/L of the extract respectively. This cell viability test further confirms the toxicity effect that lime leaf extract exhibits thereby making it a less favorable component to be used as a potential source for obesity treatment.

Figure 29 HPLC chromatograms of 3mg/mL crude seed, flesh, peel and leaf extract against pure standard NOB when injected at 100 μ L for each run under same separation conditions

5.4 Chemical composition analysis of the four components

In a study we conducted earlier, three bioactive compounds: tangeretin, sinensetin and nobiletin, were elucidated to be responsible for the inhibition of adipocyte differentiation caused by crude lime peel extract. (Chapter 4) Therefore, it has been hypothesized that the presence or absence of these compounds in the respective components could account for the initial bioactivity observed. Figure 29 shows the extensive chemical composition of the respective methanolic extract components of lime separated via reverse-phase HPLC, which are vastly different from one another. This accounts for the different bioactivity observed when the cells treated with the four different components. As expected, when comparing closely against the retention time of the three pure standards, both lime flesh and seed appears to exhibit no distinct peaks within the retention time of the bioactive compounds. This allows us to conclude that the probable absence or rather, the presence of bioactive compounds only in minute amounts in the lime flesh as well as the seed component accounts for the ineffectiveness of the respective extracts to induce a significant degree of inhibition during adipogenesis.

Table 3. Summarized quantification of respective PMFs present in crude lime leaf and peel extract. The external standard method was used for the HPLC quantification. The results are reported as percent of dry sample weight.

Lime Leaf (3 mg/mL)	Rep 1	Rep 2	Average	Stdev	Quantification (µg/mL)	% Quantification
Sinensetin	7.32	8.54	7.93	0.86	1.96	0.07%
Nobiletin	70.74	69.58	70.16	0.82	42.29	1.41%
Tangeretin	18.56	17.69	18.13	0.61	9.93	0.33%

Lime Peel (3 mg/mL)	Rep 1	Rep 2	Average	Stdev	Quantification (µg/mL)	% Quantification
Sinensetin	4.91	4.90	4.90	0.00	1.26	0.04%
Nobiletin	31.53	31.18	31.35	0.24	20.43	0.68%
Tangeretin	9.76	7.95	8.85	1.28	5.25	0.18%

On the contrary, further analyzing the chemical composition of lime peel against the leaf, it has been found that the presence of the bioactive compounds within the extract alone is insufficient in determining the component as a potential source for therapy. This is because we realize that both the lime leaf as well as the peel component does contain a significant amount of bioactive compounds, particularly sinensetin, nobiletin and tangeretin. The respective components have also been subjected against co-injection tests with the pure standards to confirm the identity of the observed peaks. Further quantification of the amount of respective bioactive compounds have also been done and it has in fact indicated that the leaf extract to contain higher amounts of all sinensetin, nobiletin and tangeretin at 0.07 %, 1.41 % and 0.33% as compared to the peel component that contains only 0.04 %, 0.68 % and 0.18% respectively (Table 3). The toxicity effect exhibited by the leaf extract however remains a fascinating issue and this effect outweighs the presence of high

bioactive compounds present in its chemical composition thereby still making it a less favorable source.

5.5 Discussion

Four components of the *Citrus aurantifolia* flesh, peel, seed and peel were obtained from the same plant source in a farmhouse in Malaysia for this study. It was important this was ensured in order to account for the differing environmental as well as agricultural factors that can potentially affect the overall chemical composition of the respective components should it originate from difference plant source. (5) The ability of crude lime peel extract to significantly inhibit adipocyte differentiation in 3T3-L1 cells in a dose-dependent manner in comparisons to its other components including the flesh, leaf and seed, further justifies that the waste-component of a fruit may not necessarily be a less valuable source. In this case, we have also seen that in addition to existing research finding that suggests that the flesh of citrus family (*Citrus aurantium*) being a potential source for obesity treatment (6), it appears that during adipogenesis of 3T3-L1 cells, treatment with methanolic extract of flesh does not cause any significant suppression in lipid accumulation and so does the seed extract of the fruit. This could be due to the absence or presence of the bioactive compounds particularly sinensetin, nobiletin and tangeretin only in minute amounts within its chemical composition thereby making it an ineffective source for suppression of lipid accumulation. This is particularly evident in the HPLC analysis which reflected insignificant concentration observed in both flesh and seed extract at the retention time range of the active compounds initially elucidated from the lime peel extract (Figure 29).

Further analysis of the chemical composition comparing between the leaf and peel extract of lime, we have found that both the lime and leaf possess a considerably significant amount of the three bioactive PMFs. It was initially expected that the leaf to exhibit higher amount of bioactivity in comparisons to peel due to the presence of higher amount of PMFs in leaf as compared to the peel extract, as summarized in Figure 30, however this was not the case. From the initial comparisons in lipid accumulation quantification of cells after treatment with the respective extract, both lime peel and leaf exhibited similar inhibitory trend at the beginning when induced with 50 mg/L of extract. Following that, it appears that at 100 mg/L, cells treated with leaf extract exhibit higher degree of suppression in lipid accumulation of about 27 % as compared to the peels, which exhibits inhibition of roughly only 19 %.

Figure 30 Calibration standard curve of the respective standards sinensetin, nobiletin and tangeretin used to quantify the amount of respective PMFs present in the respective crude lime extracts. Bottom right is an overlapped HPLC chromatogram of lime peel and leaf against the pure standards sinensetin, nobiletin and tangeretin.

This could be due to the effects of PMFs which are present in larger amount in leaf extract as compared to the peel however, this hypothesis remains inconclusive as this set of result in leaf could essentially be affected also due to the toxicity effect, which was more significantly observed in cells when treated with leaf extract at higher concentrations (Figure 28). The toxicity effect exhibited in leaves could possibly be due to a similar defensive mechanism of a protein crosslinker that naturally takes place in leaves as discussed by Konno and others (7) however, further experimental analysis is still required to fully confirm and understand this phenomenon. At present, the toxicity effect observed in the leaf extract renders it as a less favorable source for obesity treatment when compared against the peel extract.

5.6 Chapter Summary

This series of comparative study of the four different component of *Citrus aurantifolia* from the same plant source, have certainly broadened our perspective especially in support of the investigation of fruit waste component as a potential source of medicine instead of just solely focusing on the flesh alone, as we have initially discussed in a review paper. (4) As observed in the chemical composition between the four components, we have found that the waste components, leaf and peel in particular, to contain a significant amount of bioactive compounds (PMFs) in comparisons to the flesh component that barely shows any prominent peaks of PMFs in its composition, that essentially attributes to the lack of bioactivity observed in the flesh component. However, we have also found that the higher concentrations of PMFs present does not necessarily correlate to a higher degree of inhibitory activity in adipocyte differentiation as toxicity effects of the particular also have to be factored in, as seen in the lime leaf extract. Out of the four components, lime peel appears to be the most promising component as it exhibits the highest degree of inhibition in adipocyte differentiation, without affecting the viability of cells. However, further exploration is still necessary for its effective use in both modern and traditional system of medicines, especially in its role as an alternative supplement for obesity treatment in the near future.

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Chapter 6

6. Effect of different extract conditions on PMFs in *Citrus aurantifolia* peel and its bioactivity

6.1 Introduction

In Chapter 4, we have addressed the potential of the peel component of *Citrus aurantifolia* to suppress adipocyte differentiation in a dose-dependent manner while in Chapter 5, we have identified the peel component as the most promising component for the suppression of adipocyte differentiation. These therapeutic effects of lime extract have been ascribed mainly to its PMFs, particularly nobiletin (NOB), sinensetin (SEN) and tangeretin (TAN), the most dominant constituent in the peel, which have been reported to be effective in the inhibition of lipid accumulation during adipocyte differentiation in biological systems (1-5). It is essentially the ability of lime peel extract to induce a tight regulation during adipogenesis, thereby enhancing the maintenance of the energy homeostasis, that is indicative of its potential for obesity treatment in the long run (6, 7).

The stability of different lime peel extracts from the same plant material however, may vary widely with respect to their PMFs concentration and consequently, affect its overall bioactivity capacity. One of the possible factors that could account for this would be the extraction condition of the different extracts (8). Several extraction parameters such as the type of solvent, solvent concentration,

extraction temperatures and extraction time are the major consideration for enhancement of extraction efficiency to obtain optimal amount of bioactive compounds (in this case, PMFs) in the lime peel extract (9-11).

To the best of our knowledge, studies on the effect of different extraction conditions on anti-obesity bioactivity of fruit peel are still scarce. Therefore, the aim of this study was to identify the effect of different extraction parameters on the yield of three main PMFs in lime peel: SEN, NOB and TAN as well as the inhibition effects of these extracts on 3T3-L1 cells during adipogenesis. The parameters studied are 1) type of solvents of varying polarities, 2) extraction time and 3) temperature. The study also aimed to determine the correlation between the concentrations of PMFs in these extracts and their overall bioactivity to inhibit adipocyte differentiation in 3T3-L1 cells.

Figure 31 Chemical structures of markers

6.2 HPLC analysis

SEN, NOB and TAN were previously reported to be present in the crude extracts of lime peel via a series of isolation and purification technique and have been found to be responsible in suppressing lipid accumulation during adipocyte differentiation (5). The best-optimized extraction condition to yield the highest percentage of these compounds of interest from lime peel is therefore favored. In order to further investigate this, all the markers (Figure 31) were firstly determined in a single run of HPLC via a gradient elution of 0.1 % formic acid in water: 0.1 % formic acid in methanol under the conditions discussed in the experimental section, of which they were separated within a total time of 75 min. The respective peaks of SEN, NOB and TAN were confirmed by comparison of their retention times with reference standards. The standards were resolved and eluted at 62.2, 67.0 and 71.2 min with respect to SEN, NOB and TAN (Figure 32). The markers were shown to exhibit good linearity in the range from 5.0 to 1000.0 ng in the calibration curves that were obtained by HPLC analysis.

Figure 32 HPLC chromatogram of *Citrus aurantifolia* (lime) peel extracts subjected under different extraction parameters: (a) reference markers (b) 100% water, 1h (c) 100% Ethanol, 4h (d) 100% methanol, 1h

Precision and accuracy of the method were done similar to Akowuah et al. where the standard solutions of SEN, NOB and TAN were determined seven times per run on three consecutive days and accuracy of the method were evaluated through recovery studies (8). The results showed very good precision, ranging from 0.5 to 100.0 $\mu\text{g/mL}$. The recovery experiment was performed at three concentrations of the

standard added to sample solutions of which the marker content had been determined. The results of the recoveries of SEN, NOB and TAN were in the range of 95-105 %.

The HPLC procedure was applied to the determination of the concentration of the PMF markers in the respective lime peel samples subjected to various extraction conditions. The quantitative analysis was performed under the same gradient elution conditions using external standard technique. The conditions set were sufficient to give well-separated peaks for identification of the respective compounds of interest.

6.3 Method of quantification

As the three PMFs have been identified to be the primary compounds that is responsible for the significant bioactivity exhibited by lime peel extract, it is therefore essential to establish a standardized method in order to effectively determine the concentrations of SEN, NOB and TAN in the respective sample crude extract, after subjecting to various extraction conditions.

Pure standards of sinensetin, nobiletin and tangeretin with at >99% purity were purchased commercially for this purpose. Runs in multiple replicates were done. Calibration standard curves were determined after running seven times at each respective concentration of standards, on three consecutive days and accuracy of the method were evaluated through recovery studies. Figure 33 are the processed calibration curves of the respective standard compounds, used for the purpose of concentration quantification of PMFs in extract for this project.

Having established the standard curves, it is important that the peak areas of the respective PMFs identified in the crude extract lies within the calibration standard range. In order to identify the best concentration of extracts to be prepared for effective HPLC quantification analysis, an additional optimization method was done in this phase of the project. Lime peel crude extract were prepared at various concentration between 1 – 5 mg/L and were left to run under the pre-determined optimized HPLC condition for analysis in triplicates. Figure 33 shows the respective results of the respective extracts.

Figure 33 HPLC graph (overlapped) of one sample crude lime peel extract prepared at concentrations 1-5 mg/L respectively at the different zoomed time scale.

With thorough comparisons of nobiletin, sinensetin and tangeretin peak areas in the respective crude extract against the respective calibration standard curves, it has been standardized that 5 mg/L of crude extract was the best concentration to be used for future analysis of all the crude extract samples after subjecting to various extraction condition. This is because based on the preliminary analysis as shown in figure 33, it has been deduced that 1-4 mg/L concentration of crude lime peel extract when subjected to rather mild extraction conditions may yield a significantly low amount of NOB, SEN and TAN that may not be effectively detectable during analysis or may have a considerably low peak area that falls out of range of the calibration

curve set. Similarly, preparing a higher concentration of extract of >5 mg/L may also produce significantly higher peak area values that could fall out of the calibration range. In addition, overly concentrated crude sample subjected to HPLC analysis may also cause clogging of columns as well. Hence, considering all these factors, 5mg/L of sample extract was the standardized concentration used in order to effectively detect and quantify the respective concentrations of SEN, NOB and TAN in our sample extracts.

6.4 Effect of different extraction condition on PMF concentrations yielded

As shown in Table 4, all the different solvent extraction systems yielded significantly different concentrations of the PMF markers. The amount of NOB, the primary PMF responsible for the suppression of adipocyte differentiation in lime peel (1, 2, 5) was higher in both 100% methanol and ethanol extract in comparisons to the 50% aqueous methanol or ethanol extract. The order of increasing amount of NOB, in different solvent systems were as follows water < 50 % ethanol < 50 % methanol < 100 % methanol < chloroform < 100 % ethanol.

Table 4 Percent concentrations of marker PMFs in *Citrus aurantifolia* peel extracts at 1 h, 20 °C of extraction

Marker	Water	Methanol	Aq. Methanol (50%)	Ethanol	Aq. Ethanol (50%)	Chloroform
SEN	0.023c	0.047c	0.025c	0.089c	0.026c	0.039c
NOB	0.311b	0.710b	0.436b	1.213a	0.426b	0.746b
TAN	0.057c	0.212b	0.118b	0.375b	0.117b	0.244b

Different letters in row for each extract indicate significantly different values ($P < 0.05$).

This is expected due the relatively non-polar composition of the compound as observed in its chemical structure, where the presence of the *o*-methyl groups renders them lipophilic in its properties (Fig 31). However, it remains interesting that despite chloroform being less polar than ethanol, a significant amount of NOB were yielded in its ethanol extract than the chloroform extract ($P < 0.05$) similarly, also for its other two PMFs, SEN and TAN. This observation is vastly different from what that has been observed previously, where it was mentioned that CHCl_3 was found to yield the greatest amount of SEN from its crude. From our results, we have found a better

solvent that can give a higher amount of all the three PMFs and ethanol was therefore the chosen solvent used for further optimization in the subsequent experiments.

Table 5 Summarized average percent concentrations of marker PMFs in *Citrus aurantifolia* peel extracts at various extraction conditions. All samples were done independently in triplicates.

Run	% of Ethanol	Temperature (°C)	Time (hr)	Percentage conc. of PMFs % (w/w)		
				SEN	NOB	TAN
1	0	20	1	0.023c	0.311b	0.057c
2	20	20	1	0.029c	0.383b	0.086c
3	50	20	1	0.026c	0.426b	0.117b
4	80	20	1	0.033c	0.515b	0.145b
5	100	20	1	0.089c	1.213a	0.375b
6	100	20	2	0.078c	1.412a	0.419b
7	100	20	3	0.081c	1.448a	0.464b
8*	100	20	4	0.111b	1.603a	0.553b
9	100	20	5	0.102b	1.630a	0.554b
10	100	30	4	0.079c	1.466a	0.487b
11	100	40	4	0.070c	1.251a	0.405b
12	100	50	4	0.082c	1.259a	0.352b
13	100	60	4	0.094c	1.118a	0.313b

Different letters in row for each extract indicate significantly different values ($P < 0.05$).

Summarized in Table 5, are the effects of SEN, NOB and TAN concentrations when extracted under the respective extraction conditions. As single factor experiment was used to determine the optimum extraction condition for lime peel, several parameters were explored in this regard. Observed in experimental run 1 to 5, the effects of polarity (ethanol concentration) in the solvent system were further

investigated. Similarly from the initial solvent system test, the presence of water in the respective solvent extract is expected to yield lesser amount of PMFs due to its relatively lipophilic nature in its chemical structure. This effect is observed accordingly as when the percentage of ethanol extract increases gradually from 0 to 100 %, the percentage of all the three PMFs in lime peel extract also increases significantly ($P < 0.05$) from 0.023 to 0.089 % for SEN, 0.311 to 1.213 % for NOB and 0.057 to 0.375 % for TAN respectively.

By considering the highest amount of SEN, NOB and TAN yielded, 100 % ethanol was chosen as the best extraction solvent for further extraction steps applying different extraction durations. Our result then is particularly different from some of the other studies that have been previously done, which favors the use of binary solvent system particularly aqueous alcohol as a more effective system in comparisons to mono-solvent system in extracting bioactive compounds (9, 12, 13). However, we may argue that as the primary bioactive compounds that may be of interest by Choi and others as well as Ghafoor and others were antioxidants, it is expected that the compounds to contain -hydroxy groups in its chemical structure. Therefore, it remains invalid for our study as we claim to particularly focus on the yield of PMFs from the lime peel extract rather than polyphenols.

As shown further in Table 5, particularly between experimental runs 5 – 9, the percentage of PMFs yielded from the crude lime peel extract were significantly different among the different extraction times. For all three PMFs, the results showed that extraction time plays a role in yielding a higher percentage over time. However, the rate of percentage increment starts to plateau, when left to extract for a period

longer than 4 hrs and this could be explained by the Fick's second law of diffusion, where final equilibrium among the solute concentrations in the solid matrix and in solvent was estimated to be achieved after the optimal extraction duration is reached (14). After considering the cost of extraction time and its practicality based on the percentage of all the PMFs yielded, the best extraction time was determined to be 4 hrs.

Applying both 100 % ethanol and 4 hrs extraction time, the uses of different extraction temperatures were studied. From the results, it was observed that, 20 °C was the best extraction temperature as it yielded the highest amount of the respective PMFs as compared to the other extraction temperatures used ($P < 0.05$). It has been found that the yield of PMFs, particularly the primary compound of interest NOB, decreases with increasing temperature from 20-60 °C. This phenomenon could be explained as high extraction temperature may cause some loss of organic solvent through evaporation, and also degradation of bioactive compounds (9, 11). Taking all the three parameters into account, we have seen how solvent system, extraction time and the temperature plays a significant role in yielding a vastly different amount of PMFs. Our series of experiments have thereby suggests that the most optimized extraction condition, to yield the highest amount of PMFs in lime peel extract is under the following condition: 100 % ethanol, 4 hrs extraction time at 20 °C. This method of determination however does have its setback as we continue to assume that the extraction parameters are independent of one another. In order to further enhance and assess both the individual and interaction effect among process variables on the responses, three-dimensional (3D) response surface plots from developed models may also be used in parallel with our existing experimental method (15).

6.5 Adipogenesis suppression analysis of crude lime peel extracts containing different PMF concentrations

As SEN, NOB and TAN isolated from *Citrus aurantifolia* peel have shown promising potential in the inhibition of adipocyte differentiation in 3T3-L1 cells, the effect of the different concentrations of PMFs present in crude lime peel extract, as a result of being subjected to different extraction conditions, on its overall inhibitory potential remains in question. In order to further investigate the effect of the different amount of PMFs yielded from the respective lime peel extract on the overall crude extract's ability to suppress lipid accumulation during adipocyte differentiation, we have selected three samples as summarized in Table 6 for analysis. Experiments **1.1**, **1.2** and **1.3** have been extracted under three separate conditions to yield a significantly different amount of SEN, NOB and TAN respectively.

Table 6 Selected three sample extracts for bioactivity assay representing its summarized percent concentrations of the respective marker PMFs in *Citrus aurantifolia* peel extracts and its extraction conditions

Expt	Solvent sys	Temperature (°C)	Time (hr)	Percentage conc. of PMFs % (w/w)		
				SEN	NOB	TAN
1.1	100 % Water	20	1	0.023c	0.311b	0.057c
1.2	100 % Ethanol	20	4	0.111b	1.603a	0.553b
1.3	100 % Methanol	20	1	0.047c	0.710b	0.212b

Different letters in row for each extract indicate significantly different values ($P < 0.05$).

As seen from Figure 33, the peak area of the respective markers correlates with the concentration of the respective PMF in the extract. As quantified, the order of the increasing amount of PMFs in the respective extracts are as follows: **1.2** > **1.3** > **1.1**, where extract **1.1** contains the least amount of PMFs (SEN=0.023 %, NOB=0.311 %, TAN=0.057 %) and **1.2** possesses the highest amount of PMFs (SEN=0.111 %, NOB=1.603 %, TAN=0.553 %) by an average of slightly over 6 fold, as it has been extracted under the optimized condition.

The effects of the three separate crude lime peel extracts **1.1**, **1.2** and **1.3** on adipocyte differentiation were investigated at various concentrations on 3T3-L1 preadipocyte cells, a well-established mammalian model assay used to investigate the effectiveness of a potential component or drug to treat obesity (6, 16). The overall effect was evaluated based on its ability to suppress lipid accumulation effectively during the 8-days treatment when the cells were induced to undergo adipocyte differentiation. The lipids accumulated were then quantified using Oil Red O stain. The ability of the extract to inhibit adipocyte differentiation is defined by how well it is able to prevent lipid from accumulating over the span of 8-days treatment relative to its positive control. Figure 34B (next page) summarizes the effects of the respective crude extracts on adipocyte differentiation, representing the quantified analysis of the normalized total amount of accumulated lipids formed, relative to the positive control.

Figure 34 Effect of respective sample lime peel extract **1.1**, **1.2** and **1.3** on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with treatment of 50 mg/L, 100 mg/L and 250 mg/L of crude sample extract respectively throughout the whole adipogenic period. Positive control is a group of cells that were induced to undergo adipocyte differentiation without any additional drug treatment. On day 8, cells were stained with Oil Red O. (A) Observation of respective cells after Oil Red O staining under phase contrast microscopy at 10X objective (B) Quantification of lipid accumulation determined by Oil Red O against normalized positive control. * $p < 0.05$ compared with the positive control. ** $p < 0.01$ compared with the positive control.

In general, as all the sample extracts contain the three bioactive compounds in its crude, although in differing concentrations, it was expected that differentiation to be suppressed in a dose-dependent manner when induced at 50, 100 and 250 mg/mL concentration respectively. This is evident in the decrease in size and number of lipid

droplets (red stains) observed with increasing concentration, as shown in the histological diagram in Figure 34A and the constant decrease in the normalized lipid accumulation as observed in the chart represented in Figure 34B for all three sample extracts. Looking further when comparing the cells treated with 100 mg/L of the respective sample crude extract, it has been found that sample **1.1**, that possesses the least amount of PMFs in its extract, induces the lowest rate of suppression in lipid accumulation at approximately only 18 % while sample **1.2**, which contains a higher concentrations of all the three PMFs of over 6 fold, to induce a more significant suppression of about 74 % (Figure 34B). Similarly, this trend is subsequently observed when cells were treated at 50 mg/L and 250 mg/mL by the respective sample extracts.

The negative value of normalized lipid accumulation of cells when treated with 250 mg/L of sample **1.2** and **1.3** respectively can be accounted due to the highly potent PMFs present in the extract causing it to suppress inherent or natural extent of adipocyte self-differentiation that takes place even in the negative control under natural environment. Thus, this value of lipid accumulation when subtracted from that of negative control for analysis causes a negative normalized value to be obtained. However, this essentially also means that samples **1.2** and **1.3** contain a significant amount of PMFs present in its extract that thereby allows it to induce a 100 % suppression of lipid accumulation during adipocyte differentiation without incurring any toxicity effect as evident in the morphological data shown in Figure 34A along with the cell viability test that follows it.

6.6 Correlations between concentrations of PMF and bioactivity trend

Sample extract **1.1**, **1.2** and **1.3** contain varied concentration of PMFs as a result of treatment under different extraction conditions. As summarized in the previous section, data showed that all the three samples were able to suppress adipocyte differentiation in a dose-dependent manner, which is indicative of the potential of lime peel extract as a potential alternative source for anti-obesity treatment (16). The degree of suppression exhibited by the respective samples however, have been found to be significantly different, where sample extract **1.2** exhibits the highest potency in suppression followed by extract **1.3** and **1.1** respectively at all concentrations (Figure 34B and Figure 35).

Figure 35 Quantified normalized lipid accumulation of sample extract **1.1**, **1.2** and **1.3** overlapped with one another, exhibiting sample **1.2** inducing the highest degree of adipogenesis suppression followed by sample **1.3** and **1.1** respectively.

This observation further confirms the correlation between the concentration of PMFs present in a lime peel extract and its ability to suppress adipocyte differentiation effectively. Herein, it has been observed that sample **1.2** that have been quantified to contain the highest concentration of SEN, NOB and TAN in its crude extract to also exhibits the highest degree of suppression. Subsequently, sample **1.3** which contains a lesser amount of the respective PMFs was seen less active in comparisons followed by sample **1.1** that was found to be the least active of the 3 as it contains the lowest concentrations of the respective PMFs. We could therefore confirm that the extraction condition plays a significantly role in yielding the amount of effective bioactive compounds that can essentially determine if the crude lime peel extract could potentially be used as a form of alternative supplementary source of obesity treatment in the long run.

6.7 Chapter Summary

Different extraction conditions affected the amount of PMFs yielded in the crude lime peel extract and essentially its bioactivity potential. We have seen from the series of experimental results that lime peel extracted using the most optimized condition (100 % ethanol, 4hrs, 20 °C) yields the highest concentrations of PMFs, particularly SEN, NOB and TAN, that subsequently result in the extract (sample extract **1.2**) being the most potent. The solvent extraction systems and the HPLC method developed in this study could be applied to further analyze PMFs and the rest of the chemical compositions in lime (*Citrus aurantifolia*) peel. The ability of SEN, NOB and TAN in crude lime peel to collectively act together to suppress lipid accumulation during adipocyte differentiation, presents it as a highly prospective alternative source for obesity treatment in the near future.

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Chapter 7

7. *Averrhoa carambola* L. peel as a potential source for obesity treatment

7.1 Introduction

Averrhoa carambola L. also known as star fruit, have been used as traditional medicine, over centuries long, to treat various illnesses such as headaches, fever, skin disorders, nausea, diabetes and diarrhea.⁽¹⁻³⁾ A tropical fruit, widely cultivated within the Southeast Asian region, star fruits have been discovered to contain a significantly high amount of phytochemicals, particularly, polyphenols which makes it a great source of natural antioxidants that could effectively scavenge free radicals.⁽⁴⁾ While the flesh components of star fruit have been widely studied for various medicinal purposes, the peel component of the fruit however, were often neglected by researchers for its medicinal value. Matsusaka and others conducted a comparative study of star fruit, which further revealed the necessity to re-evaluate the potential of not just the flesh component of star fruit but also the peel. In their comparative assessment, the study revealed that the ethanolic peel extract contain significantly higher amount of polyphenols than its flesh and juice component, by almost 16-fold.

(5)

Factoring in this recent discovery revealed by Matsusaka and others, we then decided to further investigate the potential of star fruit peel to effectively suppress adipocyte differentiation on 3T3-L1 cells. As mentioned in Chapter 1 of this thesis,

the ability for an extract to effectively inhibit the process of adipocyte differentiation, would essentially suppress the ability for excess energy to be converted and stored as lipids (and triglycerides) in mature adipocytes. In this study, we not only investigate the potential of the crude star fruit peel extract in suppressing adipocyte differentiation but also identified one of the primary bioactive compounds responsible for the bioactivity. Subsequently, the biomechanism and mode of action of this active compound along with its binding mode onto target proteins were further analyzed in order to achieve a comprehensive analysis to account for the potential ability of star fruit peel as a promising natural source that may be considered for supplementing obesity treatment in the near future.

7.2 Crude star fruit peel extract inhibits adipocyte differentiation in a dose-dependent manner

Name	Fruit	Part Used	Extract	Extract in H ₂ O	Yield(%)
<i>Averrhoa Carambola</i>					44

Figure 36 Different stages in the star fruit peel extraction process. Star fruit peel was first freeze-dried (72 hrs, -86°C) and ground into fine powder, followed by extraction with 50% aqueous ethanol (53 g in 1500 mL, 24 hrs, 30°C). The mixture was then filtered through Whatman no. 4 filter paper, condensed and freeze-dried (72 hrs, -86°C) to obtain brown solids (21.2 g).

The ability of star fruit extract (Figure 36) to inhibit adipocyte differentiation were evaluated by measuring the amount of lipids accumulated at the end of 8 days treatment of adipogenesis on 3T3-L1 cells after subjecting to various concentration of star fruit peel extract at 10, 100, 500 and 1000 mg/L respectively. The experimental

procedure is explained in the biological screening experimental section of this thesis, section 2.2. As evident in the gradual decrease in the number of lipid droplets observed and shrinkage in cellular size with increasing dosage of star fruit peel extract respectively, shown in Figure 37A and the decreasing trend of the normalized lipid accumulation in cells treated with the respective crude extract at various concentrations relative to the positive control as represented in Figure 37B, it is sufficient to suggest the star fruit peel suppresses adipocyte differentiation in a dose-dependent manner.

Treatment with 10 mg/L of star fruit peel extract suppresses about 8 % of lipid accumulation, which then gradually increases its effect when treated at higher concentration, inducing over 24% at 100 mg/L, 83% at 500 mg/L and almost completely at highest concentration of 1000 mg/L. In order to ensure that the decrease in lipid accumulation observed in the biological screening stage was not due to toxic effect of the peel extract, cell viability of star fruit extract on 3T3-L1 cells were done using CCK-8. 3T3-L1 cells subjected to peel extract treatment at a wide range between 200 and 1000 mg/L concentration placed no observable toxic effect compared to that of untreated cells (Figure 37C). This analysis verifies that the initial suppression effect of star fruit peel extract in a dose-dependent manner observed during biological screening was solely due to the potential inhibitory effect of bioactive phytochemicals present in the peel extract and not due to toxicity.

Figure 37 Effect of star fruit peel extract on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with or without treatment of 1000 mg/L, 500 mg/L, 100 mg/L, 10 mg/L star fruit peel extract for the whole adipogenic period. On day 8, cells were stained with Oil Red O. (A) Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2), 4X objective (lane 3) or photographed with camera (lane 4). (B) Quantitation of lipid accumulation determined by Oil Red O. (C) Cytotoxicity of star fruit peel extract measured by cell viability assays. * $p < 0.05$ compared with positive control. ** $p < 0.01$ compared with positive control.

7.3 Effect of star fruit peel on adipocyte-specific gene expression

The process of adipocyte differentiation involves a complex series of collective effects of several adipogenic gene expressions, as discussed in Chapter 1.6 of this thesis. (6-10) Hence, in order to determine if the inhibition of adipocyte differentiation observed during biological screening was a result of the mediation of the peel extract thereby, leading to an alteration in the differentiation program, the effect of star fruit peel extract on the mRNA expression of a number of adipocyte-specific genes were analyzed via RT-PCR. Similar to that of the lime peel extract that was previously discussed in Chapter 4, star fruit peel extract revealed a similar trend in effects to the related adipogenic transcription factors. As summarized in Figure 38, RT-PCR analysis indicate that star fruit peel extract down regulates the expression of two essential master adipogenic transcription factors namely, C/EBP α and PPAR γ in a dose-dependent manner. This then is deduced to cause a further down regulation of its target genes such as those of aP2 and LPL. While PPAR δ gene remains unaffected with treatment of the peel extract, a significant up-regulatory effect was observed in the expression of PPAR α , in a dose-dependent manner. The apparent alterations in mRNA levels of adipocyte-specific genes suggests that star fruit peel extract suppresses adipocyte differentiation via simultaneous upregulation of PPAR α and downregulation of master adipogenic genes such as C/EBP α as well as PPAR γ .

Figure 38 Effect of star fruit peel extract on the expression of adipogenic genes. Adipocyte differentiation was performed with or without treatment of 1000 mg/L, 500 mg/L, 100 mg/L, 10 mg/L star fruit peel extract for the whole adipogenic period. On day 8, cells were harvested and mRNA expressions were evaluated by RT-PCR. The bands were analysed using ImageQuant TL (version 7.0). Negative: negative control; Positive: positive control (without treatment of star fruit peel extract). Three replicates were used to represent the error bars. * $p < 0.05$ compared with positive control, ** $p < 0.005$ compared with positive control.

7.4 Identification of Bioactive Fractions and Relevant Molecules of Star Fruit Peel Extract

Fruit Peel Extract

Having addressed the potential of crude star fruit peel extract to induce effective suppression of adipocyte differentiation in a dose-dependent manner, it is then crucial in the following step to identify the active compounds responsible for this bioactivity. In order to do this, similar elaborate separation and isolation technique was done on star fruit peel extract as it was done for lime peel extract. However, as the chemical composition of star fruit peel extract is vastly different to that of lime peel, 15 fractions were yielded upon subjecting to separation using silica column chromatography, labeled F1 – F15 respectively (Figure 39A).

- (A) The homogeneous methanol extract was subjected to further separation via silica gel chromatography with a sequential gradient elution of DCM:MeOH:formic acid from 100:0:2 to 0:100:2, to yield 14 fractions

Figure 39 (A) TLC spots of fractions 6-12 after separation via silica column chromatography. (B) Effect of star fruit peel extract fractions on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with or without treatment of 100 mg/L each fraction for the whole adipogenic period. On day 8, cells were stained with Oil Red O. Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2) or photographed with camera (lane 3) objective. F1 (1.5 mg) and F3 (1.2 mg) were excluded from analysis as the amount is too small.

These 15 fractions, F1-F15 were freeze-dried into powder form and further prepared to two concentrations at 100 mg/L and 200 mg/L respectively, before subjecting it to adipogenesis treatment on 3T3-L1 cells in triplicates and on three independent analysis. The objective of this experiment is to consequently narrow down and identify only the active fractions that is likely to contain significant amounts of bioactive phytochemicals present and eliminate those that exhibits no observable bioactivity. As summarized in Figure 39B which highlighted selected histological images of cells when treated with the respective fractions, it was essentially found that only one fraction, F12 to exhibit significant inhibitory effect with the lowest amount of lipid accumulated at the end of 8-days treatment. This is also indicative that extract of F12 may contain the most concentrated amount of bioactive compound(s) that is likely responsible for star fruit peel extract's suppression capacity in the first place. Cell viability assay on F12 extract was also done to further verify and confirm that the suppression effect observed on 3T3-L1 cells was not due to the toxic effect but due to presence of bioactive compounds in the extract.

In order to effectively identify the active compound(s) present in bioactive F12, the F12 extract was subjected to thorough chemical analysis using reversed phase HPLC coupled with LC-MS. As represented in Figure 40A, two major peaks with the respective retention time and m/z ratio of 23.6 min, $[M+H]^+ = 291.14$ (Peak 1) and 32.8 min, $[M+H]^+ = 827.25$ (Peak 2) were identified. As molecular weight of Peak 2 is considerably high, exceeding that of 500, it has been deduced that the compound was not likely be able to effectively fit itself within the binding pocket of any target receptors and hence less likely to be the bioactive compound that was responsible for

the suppression of adipocyte differentiation. This leaves us with peak 1 and it has been hypothesized that the likely bioactive compound in star fruit peel extract lies within peak 1, possessing a retention time of 23.6 min under the standardized HPLC parameters set, and a m/z ratio of 291.14. However, due to minute amounts of F12 left in the experiment, further purification and isolation to effectively separate the two major peaks could not be achieved. In order to identify the active compound that likely lies in Peak 1, an alternative method of compound identification and confirmation was sought.

Herein, the co-injection HPLC technique was used for this purpose. In a study conducted by Shui and others, they revealed (+)-catechin and (-)-epicatechin as the primary polyphenols abundantly present in star fruit extract. (10) Considering this, we purchased pure compounds of (+)-catechin and (-)-epicatechin with over 98% purity to facilitate the identification of such compounds in bioactive fraction, F12. As pure (+)-catechin has a retention time that falls out of the range of the two major peaks identified in F12 as represented in Figure 40D, it is clear that (+)-catechin is not the compound of interest in this matter. On the other hand, (-)-epicatechin, when subjected to reversed phase HPLC under the standardized parameters set for analysis appears to elute out at a retention time aligned with that of peak 1 in F12 (Figure 36D versus Figure 40A). In order to further confirm the identity of peak 1, a co-injection analysis, subjecting to the addition of 20 % (Figure 40B) and 50 % volumes of pure (-)-epicatechin relative to F12 (Figure 40C) indicated a proportional change in the respective peak area of peak 1, as verified in the overlapping chromatogram summarized in Figure 36E. This series of analysis further validates the identification of peak 1 in F12 to be likely (-)-epicatechin.

Figure 40 HPLC analysis of Fraction 12 (F12). (A) HPLC trace of SFP extract fraction 12. (B) HPLC chromatogram of SFP fraction 12 with 20% co-injection of (-)-epicatechin. (C) HPLC chromatogram of SFP fraction 12 with 50% co-injection of (-)-epicatechin. (D) HPLC chromatogram of pure (+)-catechin and (-)-epicatechin overlapped with one another (E) Overlapped HPLC chromatogram of SFP fraction 2 extract, highlighting the difference in peak area of peak 1, when co-injected at different volumes, 20% and 50% respectively.

In addition, to further confirm and account the role of (-)-epicatechin as the likely bioactive compound present in star fruit peel extract responsible for its bioactivity, the adipogenic suppression effect of the pure compound was investigated on 3T3-L1 cells and this, will be further elaborated in the following section.

7.5 (-)-Epicatechin suppresses lipid accumulation in 3T3-L1 cells

(-)-epicatechin was identified as the likely active compound present in bioactive fraction F12, that is also essentially present in the main crude star fruit peel extract, responsible for the positive bioactivity of the crude peel extract. The next step then, is to confirm and determine the potency of (-)-epicatechin, in its pure form, to suppress adipocyte differentiation effectively. Here, various concentration of (-)-epicatechin were prepared at 10, 50, 100 and 200 μM respectively, and subjected to adipogenesis assay on 3T3-L1 cells. Similarly, cell viability assay using CCK-8 was also done to account for its toxicity effect. Based on the diagram presented in Figure 41C, (-)-epicatechin remains non-toxic within the tested range between 0 to 500 μM concentration. Based on the normalized lipid accumulation analysis, inducing 10 μM of (-)-epicatechin does not seem to exhibit any significant effect on adipocyte differentiation however, with increasing dosage from 50 to 200 μM , the rate of adipogenesis suppression gradually increases from slight 10% to a significant 90% when treated with 200 μM of (-)-epicatechin (Figure 41B). The histological diagram, as represented in Figure 41A further validates the dose-dependent suppression effect of (-)-epicatechin on 3T3-L1 cells with the observable decrease in size and number of lipids accumulated with increasing concentrations of (-)-epicatechin treatment from 0 – 200 μM respectively.

Figure 41. Effect of (-)-epicatechin on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with or without treatment of 200 μM , 100 μM , 50 μM , 10 μM (-)-epicatechin for the whole adipogenic period. On day 8, cells were stained with Oil Red O. (A) Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2), 4X objective (lane 3) or photographed with camera (lane 4). (B) Quantitation of lipid accumulation determined by Oil Red O. (C) Cytotoxicity of (-)-epicatechin measured by cell viability assays. * $p < 0.05$ compared with positive control. ** $p < 0.01$ compared with positive control.

Therefore, it is with the collective set of data, combining both the adipogenic assay of pure (-)-epicatechin and factoring in F12 as the only bioactive fraction identified to induce significant suppression of lipid accumulation that we conclude the ability of star fruit peel extract to suppress adipocyte differentiation as partly due to the presence of active polyphenols such as those of (-)-epicatechin.

7.6 Molecular docking of (-)-epicatechin binding to PPAR α

Having identified (-)-epicatechin as one of the primary bioactive compounds in star fruit peel responsible for the suppression activity of adipocyte differentiation and the key target protein receptors that were mainly affected during the suppression, it is then interesting to further explore the chemical binding relationship between the two. In this case, the upregulation of PPAR α was of particular interest, as factoring in the experimental studies that we did with lime peel extract, as discussed in Chapter 4 of this thesis along with the set of literature reviews, particularly highlighting the role of upregulation PPAR α in adipogenesis suppression Chapter 1.6, we believe that it is partly due the binding of (-)-epicatechin to this target protein (PPAR α) that consequently results in the overall suppression of adipogenesis.

Figure 42 shows the binding mode of (-)-epicatechin towards PPAR α . In the predicted binding complex, (-)-epicatechin occupies the U-shaped ligand-binding pocket in a similar way as that of synthetic fibers BMS-631707 and GW735 (Figure 42B). Of which, BMS-631707 (12, 13) acts as a PPAR α / γ dual agonist while GW735 is a high-affinity ligand for PPAR α . (11) Comparing and assessing the binding modes of these two synthetic fibers provides greater insight with the regards to the binding properties of (-)-epicatechin towards PPAR α .

Figure 42 Predicted PPAR α -(−)-epicatechin binding structures. (A) The final snapshot of the 3-ns MD simulation of PPAR α -(−)-epicatechin complex. The PPAR α backbone is represented by the yellow ribbon, and (−)-epicatechin is represented with vdw and is color coded as follows: carbon, cyan and oxygen, red. (B) Superposition of the structures of GW735 (green) and BMS-631707 (mauve) bound to PPAR α . (C) Chemical structures of the compounds described in (B). (D) Hydrogen bonds formed by the (−)-epicatechin and the surroundings are indicated as green dotted lines.

Figure 42B shows the overlay of bound BMS-631707, GW735 and (-)-epicatechin in their respective configurations. The dihydroxyl phenol group of (-)-epicatechin is seemingly situated at a similar orientation and position as the carboxylate group of BMS-631707 and GW735 respectively. While both the hydroxyls on the benzene ring were oriented towards the AF-E helix and held in place through a network of hydrogen bonds with Y464 (Figure 42D). The same network of hydrogen bonds with the AF-2 helix occurs in the binding of ligands to PPAR α and rosiglitazone in the ternary complex with PPAR γ . (14) It is essentially the conservation of such network of hydrogen bonds is regarded impetus for ligand-mediated activation of these receptors. Based on this analysis, it is deduced that upon binding, (-)-epicatechin stabilizes the AF-2 helix through establishing the aforementioned network of hydrogen bonds, thereby causing a shift in the equilibrium favoring towards the active configuration of PPAR α receptor. Furthermore, the presence of chromene group in (-)-epicatechin enables it to project deeper in the cavity formed by helices H3 and H10, forming hydrogen bonds with the backbones of A454 and V270, that essentially allows it to firmly bind itself onto the PPAR α receptor (Figure 42D).

7.7 Discussion

In our series of work done on star fruit peel extract, we have revealed that more importantly, peel component of the fruit, which were often neglected in many previous studies have been proven to exhibit high potential to suppress lipid accumulation via the inhibition of adipocyte differentiation. The biological screening revealed that the crude peel extract was able to induce this suppression in a dose-dependent manner. Further separation and purification steps of the crude peel extract enabled us to identify (-)-epicatechin as one of the primary polyphenol compounds present in crude star fruit peel extract that is responsible for its bioactivity.

Genetic expression studies which reveal the direct effect of the crude peel extract on the expression of key adipogenic genes further elucidated the biomechanistic pathway that may take place, leading to the overall suppression effect of adipocyte differentiation and decrease in lipid accumulation, observed in 3T3-L1 cells. Our study revealed that similar to that of crude lime peel extract, star fruit peel extract also suppressed adipocyte differentiation via the simultaneous downregulation of master adipogenic genes like PPAR γ and C/EBP α as well as upregulation of PPAR α . This is not particularly a new phenomenon as in studies conducted by Fruchart and others as well as Kersten and others, it has been shown that similar effects of PPAR α upregulation resulting in the sequential regulation of gene expressions involved in cellular lipid oxidation and uptake, were observed when bound to polyphenols. (15, 16)

Here, in particular, we believe that when 3T3-L1 cells is treated with star fruit peel extract, some (-)-epicatechin that is present in the peel extract then binds itself onto the PPAR α receptor in a particular manner as revealed in the previous computational section of this thesis, activating the PPAR α receptor and with the simultaneous downregulation of PPAR γ and C/EBP α , collectively resulted in the overall suppression of adipocyte differentiation via elevation of fatty acid oxidation and lipolysis. (17)

7.8 Chapter summary

The study of star fruit peel as a potential source for obesity treatment, in our knowledge, has never been studied. Although the chemical composition analysis of star fruit flesh are widely available, very little studies were done to focus on the peel component. The identification of (-)-epicatechin, as the primary compound in star fruit peel responsible for the suppression of adipocyte differentiation, were never previously reported. As (-)-epicatechin is also commonly found in wide range of natural resources such as in tea, blackberry, apple, black grape and broad bean, we believe that this work, revealing (-)-epicatechin as a key compound that can suppress lipid accumulation could lead to the identification of more alternative natural sources that may be considered for obesity treatment. (18, 19) Our data have presented a comprehensive study that not only addresses star fruit peel as a potential fruit peel candidate that could be used to treat obesity, but also revealed a complementing set of data identifying (-)-epicatechin as one of the primary compound present in its crude extract responsible for the bioactivity and also the probable underlying mechanism involved that essentially results in the overall effective suppression caused by the peel extract.

Although more studies have to be done such as that of an *in vivo* as well as the physiological relevance of these results, the identification of the bioactive compound and the molecular relationship of the relevant active compounds to target protein receptors elucidated, are useful in highlighting the potential of star fruit peel extract as a prospective candidate for alternative source of treatment for obesity and its related diseases in the near future.

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Chapter 8

8. Comparative analysis of Banana *berangan* peel and its suppression effect on adipocyte differentiation

8.1 Introduction

Banana (*Musa* sp.) is a common tropical fruit that is widely available throughout the region and is heavily consumed for its known source of vitamin C (ascorbic acid) and potassium.(1) In addition, there have been several comprehensive studies done on the *Musa Cavendish* pulp to examine the antioxidant activity of the fruit and its composition analysis revealed the presence of several useful phytochemicals present such as gallocatechin, β -carotene and vitamin E. (2) Besides the pulp component of banana, its peel component, which is often underutilized, have also recently gained much attention in the medicinal field for research. (3, 4) In a study conducted by Someya and others, banana peel have been found to be a good source of antioxidants that may essentially be useful to battle against heart disease and even cancer. (2)

Taking this study into consideration, it would be worthwhile to investigate the potential of banana peels to supplement obesity treatment via its ability to suppress lipid accumulation effectively during adipocyte differentiation. As Emaga and others have also previously addressed the effect of chemical composition at varied stages of maturation in banana peel (5), the present study has been aimed to evaluate and compare the *in vitro* suppression effect of lipid accumulation when treated with banana peel at two varied maturation stage of the peel extract (ripe versus unripe), as

well as to analyze the general difference in chemical composition between the two crude extracts. The banana (commonly known as *Pisang Berangan*) used for this analysis was purchased from the Singapore wet market at Marsiling.

8.2 Ripe and unripe banana peel exhibits suppression of adipocyte differentiation in different potencies

Banana peel were first extracted at two different maturation stage, the first, in its early ripening stage, when the peel was green and the second was when the peel was left to ripen for 7 days and its skin turned yellow as shown in Figure 43. The peels were extract under the exact same experimental condition as discussed by González-Montelongo and others, and labeled unripe banana peel (UBP) and ripe banana peel (RBP) extract respectively.(6)

Figure 43 Unripe banana berangan (left) and left to ripen after 7 days (right) used for extraction of UBP and RBP respectively

Both UBP and RBP extract were subjected to *in vitro* biological screening at various concentrations of 25-100 mg/L and 50-400 mg/L, as described in section 2.2 of this thesis, respectively. As seen in the histological diagram as presented in Figure 44A and 45B, both treatments with UBP and RBP extract induce a shrinkage in cellular size and decrease the number of lipids accumulated in a dose-dependent manner. However, the results that are particularly of interest to us lies in the vastly different potencies that the two extracts, UBP and RBP, exhibit towards the suppression of lipid accumulation during adipocyte differentiation. First, cytotoxicity analysis confirms that 3T3-L1 cells remain viable when treated at various concentrations of the respective banana peel extracts (Figure 44D).

Thorough analysis of the normalized lipid accumulation, as summarized in Figure 44C, suggests that there is a significant drop in potency of the banana peel extract as it matures from unripe (green) stage to the ripen (yellow) stage. On individual treatments with 100 mg/mL of the respective extract yielded an approximately only 10% suppression of lipid accumulation with RBP extract while 100% suppression was observed when treated with UBP extract. RBP extract however still induces a dose-dependent suppression in lipid accumulation during adipocyte differentiation however a higher concentration of RBP extract is required ranging from 50 – 400 mg/L to yield the same effect exhibited by UBP which utilizes a far less, almost a quarter-fold concentration range of 25 -100 mg/L.

Figure 44 Effect of the respective banana peel extract on adipocyte differentiation in 3T3-L1 cells (A) Observation of respective cells treated with RBP extract at 50-400 mg/L after oil red O stain under phase contrast microscope at lane (1) 40X, lane (2) 10X, lane (3) 4X objectives respectively and lane (4) photographed with camera (B) Observation of respective cells treated with UBP extract at 25-100 mg/L after oil red O stain under phase contrast microscope at lane (1) 40X, lane (2) 10X, lane (3) 4X objectives respectively and lane (4) photographed with camera (C) Quantification of lipid accumulation on cells treated with respective peel extract against normalized positive control (D) Cytotoxicity of crude banana peel extract measured by cell viability assay. * $p < 0.05$ compared with positive control. ** $p < 0.01$ compared with positive control.

This interesting experimental observation not only reveals how banana peel extract could be a prominent source of supplement for obesity treatment in the long run, but it also further revealed how the different maturation stage of the peel plays an essential role in altering affecting the overall potency of the fruit. It is then hypothesized that there is a possible alteration in chemical composition that takes place during this ripening stage (or maturation stage) of the fruit that consequently result in a significant decrease in the concentration of the active compound relative to the overall chemical composition of the peel extract, hence, the observable drop in potency observed between UBP versus RBP extract.

8.3 Chemical analysis of ripe versus unripe banana peel extract

Following this hypothesis, the chemical composition of the respective crude UBP and RBP extract were then investigated. In order to account for the vast difference in the potencies observed between the two extracts, the respective HPLC chromatograms were then further analyzed and overlapped with one another as summarized in Figure 45.

Figure 45 HPLC analysis of Crude RBP and UBP extract respectively and the overlapping chromatograms of the two bananas peel extract

In general, during the ripening stage, it appears that there is an observable drop in the three main peaks labeled peak 1-3 respectively, with a retention time that lies within the first 8 minutes. In addition, from the unripe to ripen stage of the peel extract, there appears to be a deliberate fall in the number of peaks formed at retention time between 37.5 - 50 minutes. These findings observed in the HPLC analysis then could not conclude on the possible identification of the likely active compound responsible for the bioactivity observed in banana peel extract. The drop in potencies observed between the crude UBP and RBP extract could be due to either reduction in concentration of polar compounds that was reflected in peaks 1-3 or the less polar compounds (peaks 4-11) that was eluted out at the slightly later retention time between 37.5 – 50 minutes. In fact, it could also be due to a combinatorial fall in concentrations of all the peaks that account for the fall in potency observed between the UBP and RBP extract.

8.4 Separation of crude unripe banana peel extract

Further investigation is therefore required to effectively identify the bioactive compounds present in banana peel extract. In order to achieve this, we focused on the more potent peel extract, UBP as it is more likely to contain a higher concentration of active compounds than the RBP extract. Applying a similar concept as those used to identify the bioactive compound in lime peel extract, as discussed in Chapter 4 of this thesis, a less tedious technique of separation of crude extract was used for this purpose – solid phase extraction.(7)

Figure 46 HPLC chromatogram of (A) Crude UBP extract (B) Fraction 1 of UBP extract (C) Fraction 2 of UBP extract.

As presented in Figure 46A, UBP extract has two main regions of peaks: regions I and II respectively. In order to effectively identify the possible region at which the active compound likely lies in, solid phase extraction technique was used to separate the two main regions of peaks as seen in Figure 46B and 47C respectively. These samples, labeled as Fraction 1 and Fraction 2 was then lyophilized, processed and prepared at 2 concentrations of 10 and 20 mg/L, in triplicates and on 3 individual plates for biological screening.

Figure 47 Effect of UBP fraction 1 and 2 extract on adipocyte differentiation in 3T3-L1 cells (A) Observation of cells after Oil Red O stain under microscope at 4X and 10X objectives against positive and negative control (B) Quantification of lipid accumulation of the respective fraction extract against normalized positive control. * $p < 0.05$ and * $p < 0.01$ compared with positive control.

Figure 47 summarizes the respective effect of the UBP Fractions 1 and 2 extracts on the suppression of lipid accumulation during adipocyte differentiation in 3T3-L1 cells. As observed, Fraction 2, which contains mainly less polar compounds, as they were eluted out at later retention time between 37.5 - 50 mins (Figure 46C) induce a more significant lipid accumulation suppression of approximately 30% & 50% at 10 and 20 mg/L respectively than that of Fraction 1 extract, which induces only approximately 10% suppression when treated with 10 and 20 mg/L of the said fraction. Although more experiments is required to further validate this results, the experimental HPLC and biological screening that we have gathered so far suggests that the likely presence of bioactive compound in banana peel extract that is responsible for the bioactivity observed likely lies in region II of chromatogram.

8.5 Discussion

Based on the preliminary sets of data, our studies have shown that both banana peel extracts, unripe and ripe remain as a valuable source that could potentially be used to supplement obesity treatment in the long run. The two sample extracts that were processed and tested at two different maturation stage, exhibited significantly different potencies in suppressing lipid accumulation during adipocyte differentiation. The UBP extract was found to exhibit greater potency than RBP extract by almost four-fold. At a treatment concentration of 50 mg/L of UBP extract, it was able to induce an approximately 40% suppression in lipid accumulation relative to the positive control while it takes slightly over four times concentration of RBP extract, at 200 mg/L, to yield a similar effect (Figure 44C).

This difference in potency observed between the two extracts were further analyzed by examining the respective chemical composition of the crude via HPLC, under standardized pre-determined parameters set as described in section 2.8.5.2 of this thesis. The HPLC chromatogram showed a significant alteration in the overall chemical composition of the peel extract during the ripening stage. Peaks 1-3 that were initially present in high concentrations in UBP extract seem to be present in lesser concentrations in RBP extract judging from the respective peak areas from the respective chromatograms. In addition more peaks that were eluted out within the 35-50 mins of the analysis in UBP extract become less defined in RBP extract. (Figure 45)

The initial analysis by comparing the chromatograms of both peel extracts was still insufficient to hint us the likely chemical properties of the possible bioactive compounds in the banana peel – whether or not it is polar or non-polar. Hence, to continue forth, elaborate solid phase extraction of the more potent extract i.e. UBP was done to understand the property of the likely bioactive compound(s) further. UBP extract was selected for this purpose as it is deduced that more potent an extract is, the more likely that it contains a higher concentration of bioactive compound than the less potent RBP extract. Solid phase extraction yielded two main fractions of which, fraction 1 was made up of mainly three polar compounds labeled as peak 1-3 and fraction 2, the less polar set compounds, peaks 4-12 (Figure 46). Biological screening of the respective fractions suggests that the active compound is likely present in fraction 2 than in fraction 1, due to the lack of bioactivity observed when treated with fraction 1 extract. Although more studies and a greater range of concentration

treatment with the respective fraction is needed to validate this preliminary results, the consistent triplicates of the biological screening is enough to hint that the likely bioactive compound present in banana peel extract is eluted out within 37.5-50 mins and is inferred to be less polar compound.

8.6 Chapter Summary

In our existing experimental results, we have gathered significant results suggesting the potential of banana peel extract as a prospective fruit to be considered in supplementing obesity treatment. In addition, from the series of chemical analysis crude separation and biological screening assay on the respective extracts, we managed to identify the chemical property of the like bioactive compound(s) present in banana peel responsible for the inhibition of adipocyte differentiation process in 3T3-L1 cells. More studies however, are still required in order to further validate and confirm this set of results. First, elaborate separation and purification of ideally all the 9 peaks eluted between 37.5-50 mins of the chromatogram (peaks 4-12) and the individual *in vitro* biological screening assay of each individual fractions are still necessary to effectively identify the precise bioactive compounds. Following this, chemical characterization using methods like NMR or crystallization coupled with HR-MS would be some of the useful tools to confirm the identity of these compounds. Next, the biological mechanism of how the active compound interacts with the relevant adipogenic protein receptors are also required in order to effectively provide a comprehensive picture in supporting the potential of banana peel as a prospective candidate to be used to supplement obesity treatment in the near future.

In addition, a more elaborate series of samples that covers a wider range of ripening stages of the peel would also be useful in investigating and accounting for the likely chemical reaction that takes place during the ripening stage of the fruit. Such studies will then provide us greater understanding on the overall chemical processes that takes place during ripening and possibly allow scientist to think of ways that we can look into in order to preserve the bioactive compounds that is abundantly present in the unripe peels from being affected during the ripening stage.

Finally, like every other fruit waste components that have been identified as a prospective source to supplement obesity treatment, in this thesis, more elaborate studies investigating its effects *in vivo* would also be necessary following up the proceedings of this project. Only then can clinical trials be arranged and the full potential of natural sources as an alternative source for obesity treatment in the future, be fully realized.

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PART IV

CONCLUSION

Chapter 9

9. Conclusion

As summarized in Figure 48, the four main objectives that was set for this project has been achieved through the different experimental studies conducted and discussed in the respective Chapters 3-8 of this thesis.

Figure 48 Summary of project

In the biological screening stage of this project, where a series of tropical fruits and its waste components were screened for its bioactive ability to suppress adipocyte differentiation using our *in vitro* model of 3T3-L1 cells, three main fruits were picked for further studies. The three fruits and its components that indicated promising results from its crude extract were namely, *Citrus aurantifolia* (lime) peel, *Averrhoa carambola* (starfruit) peel as well as the peels of both ripe and unripe banana commonly known as pisang *Berangan* (Chapter 3).

9.1 Summary on *Citrus aurantifolia*

A series of comprehensive studies were done on the peels of *Citrus aurantifolia* in particular, to effectively identify three bioactive polymethoxylated flavones present in its crude extract, responsible for adipocyte differentiation suppression. The underlying mechanisms that accounts for the observed suppression was deduced to be due to a simultaneous effect that the crude lime peel extract has on downregulating key adipogenic genes such as PPAR γ and C/EBP α as well as upregulating PPAR α genes. The different potencies exhibited by the three respective bioactive polymethoxylated flavones: nobiletin, tangeretin and sinensetin, prompted further investigation to be done. Genetic expression studies revealed a significant correlation between the suppression potency and its ability to upregulate the expression of PPAR α . For instance, nobiletin, which exhibited the greatest potency in adipocyte differentiation suppression, was also found to induce the highest degree of PPAR α gene upregulation in comparisons to that of tangeretin and sinensetin, respectively. Computational modeling was also used to complement the experimental findings to elucidate the possible chemical interactions that may be involved between the respective pure compounds and PPAR α receptor to account for the different potencies exhibited by the aforementioned PMFs.

Moving forward, the lime peel project was further complemented with a comparative analysis to assess how potent the crude peel component is relative to the other components of the fruit. Herein, as discussed in Chapter 5, lime peel was found to be the most effective crude extract in suppressing adipocyte differentiation in comparisons to its leaf, seed and flesh extract of the fruit. Further qualitative and quantitative chemical analysis of the respective components revealed a direct

correlation between the respective concentrations of the three PMFs present in the extract and the suppression capacity of the respective crude extracts exhibit. In addition, to achieve the aim in investigating the effects of extraction conditions on the overall chemical composition of an extract and subsequently its overall bioactivity capacity, lime peel extract was used for this purpose as elaborated in Chapter 6 of this thesis.

Our studies revealed that different extraction conditions affected the amount of PMFs yielded and as predicted in the initial hypothesis, it does affect the overall capacity of the extract to suppress adipocyte differentiation as well. In the study, we found that subjecting the peel extract to an optimized extraction condition of 100% ethanol for 4 hrs, at 20 °C, with a sample to solvent ratio of 1:10 and 2 extraction cycle, yields the highest concentrations of PMFs relative to the other conditions set and subsequently exhibits the greatest potency in adipocyte differentiation as well.

9.2 Summary on *Averrhoa carambola* peel extract

In a parallel study on *Averrhoa carambola* peel extract as elaborated in Chapter 7, (-)-epicatechin was identified as one of the primary bioactive compounds present in extract that accounts for its suppression effect during adipocyte differentiation. The underlying mechanism revealed similar genetic expression effects as that observed in lime peel extract. Computational docking was also done to support the experimental findings to elucidate the possible chemical interaction that takes place between (-)-epicatechin and key PPAR α receptor accounting for its overall upregulation, thereby leading to the overall suppression in adipocyte differentiation and lipid accumulation in 3T3-L1 cells.

9.3 Summary on *Berangan* banana peel extract

Finally, preliminary data between the ripe and unripe *Berangan* banana peel revealed an interesting finding indicating how ripening stage could alter the chemical composition of fruit significantly thereby, affecting the overall potency of the respective fruit peel extracts. Further studies are still needed to identify the bioactive compounds present in banana peel that is responsible for this bioactivity. Only then, can we subsequently conduct relevant experiments to understand the ripening process that takes place and perhaps strategize agronomic ways to prevent such chemical modifications from happening.

9.4 Overall summary and future work

Overall, the work that has been done throughout the entire duration of this PhD project has broadened our understanding towards the potential of tropical fruit waste component as a potentially resourceful tool that could be used to treat obesity in the long run. The mechanism of which the fruit extracts play is different from those of commercially synthesized drugs such as Orlistat, Phentermine-topiramate and Lorcaserin as addressed in the first chapter of this thesis, however the mode of action is not entirely novel either. Several *in vivo* studies have been done to show the potential effects of obesity treatment via the suppression of adipocyte differentiation caused by a potent compound or drug. (1-5) However, like every other pharmaceutical drug, it is crucial that this initial finding be carefully assessed and studied thoroughly before subjecting it to further rounds of clinical trials, in order to materialize the use of these components to supplement obesity treatment in the long

run. Although typically potent extracts, coming from a natural food source is implicated to exhibit lesser toxic effects on human, it may not be used as a convenient excuse to conveniently skip the *in vivo* and clinical trials phase, for precautionary and safety reasons. (6, 7) As such, moving forward, despite the fact that there are still considerably significant amount of work that has to be done to follow up on this work, the series of studies revealed in the project have shown promising potential of the use of such components to treat obesity in the long run. While obesity remains a pressing problem till date, greater awareness to promote healthy practices such as regular exercises and monitoring a proper and balanced diet on a daily basis are of paramount importance when it comes to achieving a holistic approach, when treating obese patients. (8)

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APPENDIX I

LC elution profile of lime peel extract showing 12 fractions that were obtained through silica gel chromatography using gradient elution: 100% Chloroform to 40% Methanol. Fraction 2 was identified to be highly concentrated with the bioactive compounds.

(NMR data for isolated Peak 1, identified as Sinensetin)

p3_B2_A_3b 1H AV500 MeOD

¹H NMR data for isolated Peak 2, identified as Nobiletin

p10_B2_A_2b 1H AV500 CDCl₃

¹³C NMR data of isolated peak 2, identified as Nobiletin

P10_B2_A_2b_2 13C BBFO2400 CDCl₃

¹H NMR data of isolated peak 3, identified as tangeretin

p5_B2_A_2c 1H AV500 CDC13