GARCINIA ATROVIRIDIS FRUIT EXTRACT MODULATES LIPID ACCUMULATION IN 3T3-L1 ADIPOCYTE CELLS

BY

HANISUHANA HAMIDON

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International Islamic University Malaysia

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ABSTRACT

Obesity is the accumulation of excess body fat to the extent it may have an adverse effect on health. It is undeniably a complex disease with risk factors combination of genetics, lifestyle, nutrition, increasing age, and lack of physical activity. Alarming increase of obesity prevalence since past few years brings the concern to explore effective therapy against obesity. The hydroxycitric acid, HCA, is a potential compound in weight management that possesses a high market demand. It was commonly reported to be originated from *Garcinia cambogia*. There is little attempt to study the potential of our locally available underexplored *G. atroviridis* on its HCA content. This also has opened the gap to study the extract lipid alteration potential the on 3T3-L1 cell culture model. This present study aims to quantify the amount of HCA available in the fruit extract as well as to investigate its lipid modulation properties in 3T3-L1 cell culture model. The fresh fruit was macerated using ethanol and freeze-dried. Then, the HCA was screened and quantified using FTIR and HPLC analysis, respectively. The extract was further tested in vitro for its lipid modulation potential. Firstly, the cell viability assay was conducted to assess the possible extract's cytotoxic effect on the cell line. Then, the cell was induced to differentiate using differentiation medium in the presence of the extract. Differentiated adipocytes were analysed qualitatively and quantitatively using Oil Red O stain. The treatment's effect on the adipogenesis-related proteins, PPARγ and C/EBPα were also investigated by means of western blot. The lipid content in the differentiated adipocytes was also confirmed with leptin ELISA assay. Finally, the adipolysis assay was conducted to identify the extract's effect on the breakdown of lipid. From the FTIR spectroscopy, the presence of HCA in the extract was confirmed based on the significant functional groups of hydroxyl, carbonyl, and lactone. Later, the HPLC analysis quantified about 11.35 ± 0.55 % (w/w) of HCA in the extract with retention time at 0.9 minute, corresponding to the standard. The extract was no cytotoxic on the cell line since the treatment did not cause any 50 % reduction in the cell viability, hence concentrations of 10, 45, and 60 µg/mL were chosen for subsequent assays. In adipogenesis assay, it was observed that increased co-treatment concentration inhibited the formation of intracellular lipid. This was further confirmed with spectroscopic absorbance of Oil Red O stain and absorbance value measured shows reduction. Co-treatment at 60 µg/mL significantly ($p < 0.05$) inhibited the adipogenesis. From the western blot analysis, the treatment only affects the expression of C/EBPα protein significantly ($p < 0.05$). It was concluded that the treatment supressed adipogenesis by inhibiting the C/EBPα expression. Meanwhile, the leptin assay finding was also in agreement with adipogenesis analysis since the leptin released was significantly reduced ($p < 0.05$) at increased treatment concentration. Finally, the extract enhanced the adipocytes breakdown (adipolysis) significantly ($p < 0.05$) at 10 and 45 µg/mL of extract. Taken together, the findings indicated that the fruit extract of *G. atroviridis* plays a role in alteration of lipid accumulation with the intervention of protein associated with adipogenesis and enhancing the adipolysis. Understanding adipogenesis and its molecular mechanisms may provide clues for future strategies development in preventing and managing obesity. Hence, the *G. atroviridis* fruit can be proposed as one of weight management agents due to its lipid modulation properties.

خلاصة البحث

البدانه هي عباره عن تجمع الدهون في الجسم الى درجة مضرة للصحه. تعتبر البدانة مرض معقد هنالك مزيج من العوامل المسببه للمرض منها العوامل الوراثيه و طريقة الحياة و التغذيه بالاضافة الى الزيادة بالعمر و انعدام اللياقة البدنيه. هنالك زيادات كبيره لموشرات البدانه في السنين القليله الماضيه هذه الزيادات تفرض الاهتمام لايجاد علاج فعال ضد البدانه. يعتبر الهايدروكسيستركاسد (ه س أ) مركب من المحتمل ان يساعد في تنظيم الوزن؛ يعتقد ان هذا المركب مستخلص من نبات الجارسينيا كامبوجيا. هنالك القليل من الدراسات حول وجود مركب ال (ه س أ) في هذا النبات. و هذا قد اعطى فرصه لدراسه قابلية هذه النبته لتغير الدهون في خلايا ال ٣ت٣-ل١ . هذه الدراسة الحاليه تهدف الى تحديد كمية ال ه س أ الناتج من مستخلص النبات بالاضافة الى معرفة مقدرته على تحوير الدهون في خلايا ال ٣ت٣-ل١ .تأخذ الفاكهه الطازجة من النبات ليتم استخلاصها ثم تجميدها بجفاف؛ بعد ذلك قمنا بفحص و تحديد ال ه س أ بواسطة اجهزة ال اف تي اي ا ر و ال ه بي ال سي. تم اختبار خاصيه تحوير الدهون للمستخلص في المختبر حيث بدأنا من اختبار بقاء الخلايا لمعرفة ما اذا كان المستخلص سام للخلايا ؛ بعد ذلك قمنا بتمييز الخلايا باستخدام وسط للتمايز.تم تحليل الخلايا الدهنيه المتمايزة نوعيا و رقميا باستخدام صبغة الدهن الاحمر و . تم ايضا اختبار المستخلص على البروتينات المرتبطه باللخلايا الدهنيه باستخدام اختبار لطخة وسترن. تم تأكيد محتوى الدهون في الخلايا الدهنيه المتمايزة من خلال فحص ال لبتين اليسا. اخيرا اجري فحص الخلايا الدهنيه لمعرفة تأثير المستخلص على تحطيم الدهون. اكدت نتيجة التحليل الضوئي اف تي اي ار وجود مركب ال ه س أ في مستخلص النبات بالاعتماد على مجاميع وظيفية كبيرة من الهيدروكسيل و الكاربونيل و اللكتون. تحليل ال ه بي ال سي اظهر كميه من ال ه س أ ما يقارب١١, ٣٥ ± ٠,٥٥% بوقت ٠,٩ دقائق. مستخلص النبات لم يؤثر على بقاء الخلايا الدهنيه في تركيزات المختاره ١٠و٤٥و٦٠ ملليلتر /مايكروغرام كانت التركيزات المختاره للمستخلص. في اختبار تكوين الشحوم كان من الملاحظ مع زيادة تركيز العلاج قلة نسبة الدهون داخل الخلايا ؛ قد تم تأكيد هذه النتائج با ختبارات امتصاص التحليل الضوئي لصبغة الدهن الحمراء وقياس القيمه الامتصاصيه التي اظهرت قيمة قليله من الدهون ؛ العلاج المصاحب بتركيز٦٠ مايكروغرام/ مل اضهر نتائج تثبيطيه بشكل ملحوظ (ب اقل من ٠,٥٠) . نتائج لطخة وسترن اظهرت ان العلاج اثر في س/ي ب ب أ بروتين فقط بشكل ملحوظ (ب اقل من ٠,٥٠). نتائج تحليل اللبتين كانت توافق نتائج تحليل تكوين الدهون. كل النتائج و الموجودات اظهرت ان مستخلص النبات من الفاكهه يعمل بدور كبير على تغيير تراكم الدهون مع تدخل البروتين المرتبط بتكوين الدهون و تحسين تحليل الدهون بشكل ملحوظ (ب اقل من ٠,٥٠). فهم طريقة تكوين الدهون و الاليه الجزيئيه يمكنها ان تقدم دلائل لاليات تطوير في منع و تنظيم البدانه في المستقبل. نبات ال ج. اتروفيريديس يعتبر كاحد النباتات المقترحه لتنظيم الوزن بسبب خصائص تحوير الدهون التي تحتوي عليها.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology).

.....
Muhammad Taher
Supervisor

.....
Deny Susanti
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology).

.....
Wan Mohd Azizi bin Wan Sulaiman
Internal Examiner

.....
Hasmah binti Abdullah
External Examiner

This thesis was submitted to the Department of Pharmaceutical Technology and is accepted as a fulfilment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology).

.....
Mohd Rushdi bin Abu Bakar
Head, Department of Pharmaceutical Technology

This thesis was submitted to the Kulliyyah of Pharmacy and is accepted as a fulfilment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology).

.....
Juliana binti Md Jaffri
Dean, Kulliyyah of Pharmacy

DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Hanisuhana Hamidon

Signature ..... Date 14/12/2017

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ap2	adipocyte protein2
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
BMI	Body mass index
BSA	Bovine serum albumin
C/EBPs	CCAAT-enhancer-binding proteins
C/EBPα	CCAAT/enhancer-binding protein α
CAMP	Cyclic adenosine monophosphate
CO_2	Carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CPT 1	Carnitine palmitoyltransferase I
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FRAP	Ferric reducing antioxidant power
FTC	Ferric thiocyanate
FTIR	Fourier transform infrared spectroscopy
GAE	*G. atroviridis* extract
GLUT4	Glucose transporter 4
HCA	Hydroxycitric acid
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBMX	3-isobutyl-1-methyl-xantine
ICRACU	Integrated Centre for Animal Research, Care and Use
IFN-γ	Interferon-γ
IL	Interleukin
IUPAC	International Union of Pure and Applied Chemistry

LDL	Low density lipoprotein
LPS	Lipopolysaccharides
MDI	Mixture of dexamethasone, IBMX, and insulin
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NF-κB	Nuclear factor-kappa B
PBS	Phosphate buffered saline
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor-γ
ppm	Parts per million
PVDF	Polyvinylidene fluoride
rpm	Rotation per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBA	Thiobarbituric acid
TBS	Tris-buffered saline
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor α
TPC	Total phenolic content
TXB_2	Thromboxane-B2
UNTX	Untreated

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Obesity is a global public health problem. Malaysia has been rated as the highest among Asian countries for obesity, thus "winning" the title of "The Fattest Country in Southeast Asia". In Malaysia, according to National Health and Morbidity Survey in 2011, 2.5 million of Malaysian adults were obese. The prevalence has increased dramatically to 15.1 % in 2011 from 4.4 % in 1996 (Ministry of Health Malaysia, 2014). Meanwhile, the worldwide obesity prevalence has been increasing since past few years and according to World Health Organisation (WHO) (2015), in 2014 more than 1.9 billion adults worldwide were overweight and among the figure, 600 million were obese. Such alarming figure brings major concern on the need to explore effective therapy against obesity since many studies have been reported that obesity is a major risk factor for diabetes, cardiovascular, several types of cancer (such as endometrial, breast, colon and prostate), pulmonary, osteoarticular, and metabolic diseases (Reaven, 2011; Huang et al., 2014).

Up till today, natural product continues to play essential roles in healthcare. In 1985, the WHO reported that approximately 65 % of the world population depends on the plant-derived traditional medicine for their primary health care, meanwhile among the remaining population of developed countries, plant products still play indirect role in the healthcare system (Cragg & Newman, 2013). Herbal remedies are also being accepted in many developed countries. It is believed that herbal remedies promote healthier living and viewed as holistic and balanced approach of healing (Ekor, 2014).

Due to this, natural product contributes an important role in finding of the new chemical entities for drug discovery. Natural source-derived drugs served as leads that suitable for optimisation by synthetic means. It was estimated that about one quarter of all drugs in current pharmacopeias were derived from plants (Efferth & Koch, 2011). The natural product becomes the interest in drug development due to its structural diversity as well as their ability to possess highly selective and specific biological activity based on mechanism of action (Cragg & Newman, 2013).

Traditional herbal medicines may have some potential in managing obesity. The fruit rind of *Garcinia atroviridis*, also known as Asam Gelugor was reported as one of unique sources of hydroxycitric acid (HCA). The Asam Gelugor exhibits a distinct sour taste and has been safely used for centuries for cooking flavourings. HCA is a compound that have the potential as a weight management agent and possesses high demand. However, the exact mechanism of the compound in targeting obesity is not well elucidated and this opened the gap to understand the HCA content in the fruit of *G. atroviridis* as well as its lipid modulation potential in vitro.

1.2 RESEARCH PROBLEM

Obesity is the accumulation of excess body fat to the extent it may have an adverse effect on health. It is undeniably a complex disease with risk factors combination of genetics, lifestyle, nutrition, increasing age, and lack of physical activity. Alarming increase of obesity prevalence since past few years brings the concern to explore effective therapy against obesity. In terms of medication approach for obesity, the approach only provides short-term solution and other problems such as its effectiveness and safety. The HCA has been proven beneficial in the treatment of obesity-related complications such as inflammation, oxidative stress, and insulin

resistance. It was commonly reported to be originated from *G. cambogia*. There was little attempt to study the potential of our locally available underexplored *G. atroviridis* on its HCA content as well as to understand the HCA effect on 3T3-L1 adipocyte cell line, especially its ability to exhibit lipid modulation properties.

1.3 OBJECTIVES OF THE RESEARCH

The general objective of this research was to assess the lipid modulation potential of *G. atroviridis* fruit extract in vitro using 3T3-L1 adipocyte cells. Meanwhile the specific objectives of this research were:

1. To extract and quantify the HCA content in the fruit extract of *G. atroviridis*.
2. To determine the effect of the extract on the lipid accumulation and break down in 3T3-L1 adipocyte cells.
3. To quantify the expression of protein involved in adipogenesis (PPARγ and C/EBPα) in 3T3-L1 adipocyte cells.

1.4 HYPOTHESIS OF THE RESEARCH

The HCA rich extract from *G. atroviridis* fruit has the ability to modulate lipid accumulation in differentiated 3T3-L1 cell line without any toxic effect as well as affecting the protein expression of the adipogenesis-related proteins, PPARγ and C/EBPα.

1.5 SIGNIFICANCE OF THE STUDY

Since Malaysia is the fattest country in Southeast Asia, this study is relevant to country in terms of managing obesity using natural resources. Obesity is implicated in development many chronic diseases, and the government's spending on health care

services is increasing in order to meet the rising demand for curative care for chronic disease patients. Thus, the finding is hoped to suggest novel source of treatment and management for obesity, which later leads to increment of quality of life among Malaysians and eventually decreases the burden of health care services together with reducing the government expenditure.

1.6 EXPERIMENTAL DESIGN

In this study, the investigation was divided into two parts. The first part was the HCA screening and quantification. Meanwhile, the second part involved the cell culture investigation using 3T3-L1 cell line. Figure 1.1 shows the flowchart of the experimental design of this research.

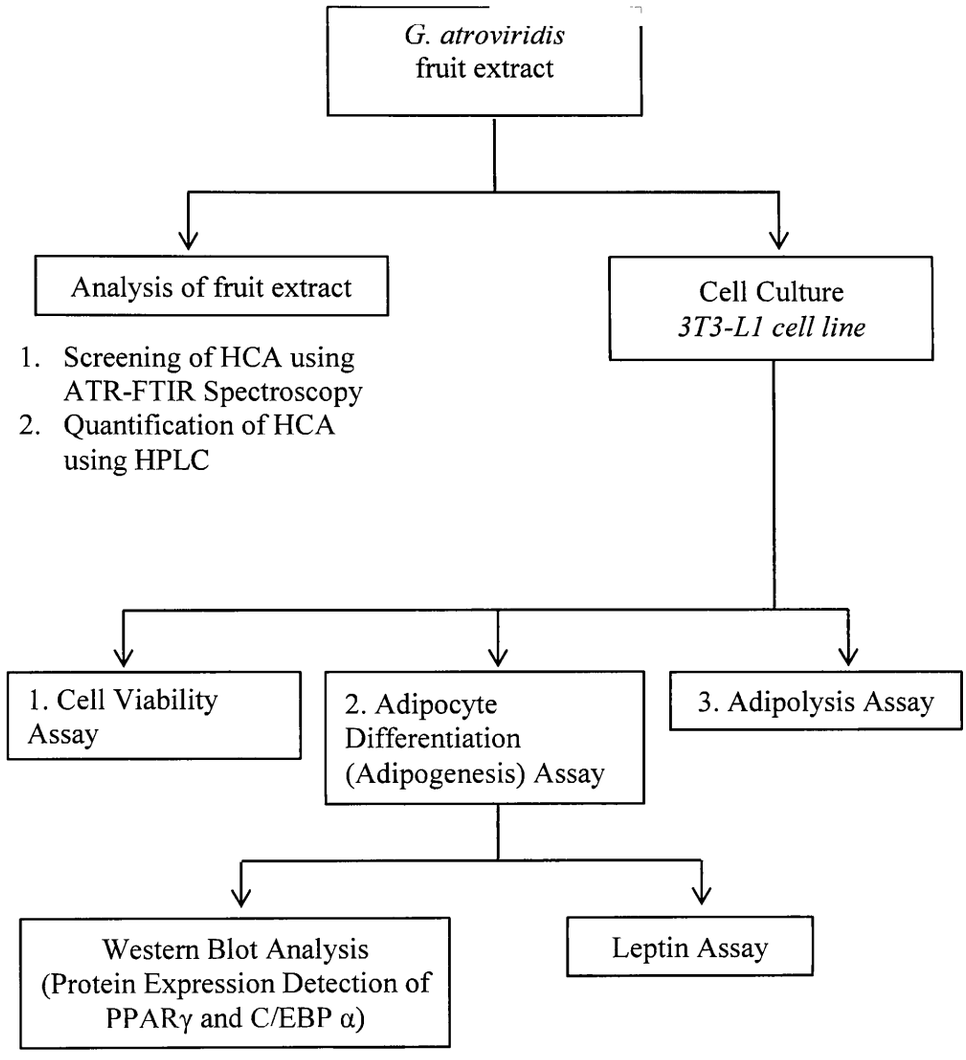

Figure 1.1 Flowchart of experimental design

CHAPTER TWO

LITERATURE REVIEW

2.1 GENERAL OVERVIEW

Obesity is the accumulation of excess body fat to the extent it may have an adverse effect on health, resulting in reduced life expectancy, and/or increased health problems (Mohamed, Ibrahim, Salah, & Dine, 2014). Alarming increase in obesity prevalence brings major concern on the need to explore effective therapy against obesity since many studies have been reported that obesity is a major risk factor for diabetes, cardiovascular, several types of cancer (such as endometrial, breast, colon and prostate), pulmonary, osteoarticular, and metabolic diseases (Reaven, 2011; Huang et al, 2014). Current treatment for obesity that available in market involves two mechanisms of action which are reducing intestinal fat absorption through inhibition of pancreatic lipase and appetite suppressant (Yun, 2010).

There are a few numbers of drugs available in the market to ameliorate or prevent obesity, but other consideration must be taken such as the costs, efficacy, and side effects. In finding the effective therapy against obesity, the adipose tissue has become an important target and understanding the process of adipogenesis has become important. Adipogenesis is an important driver of the expansion of adipose tissue mass during the state of excess calories intake that later links to obesity. Antiobesity agent suppresses lipid accumulation during adipogenesis in 3T3-L1 preadipocytes, associated with a decrease in peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα) and fatty acid synthase (FAS) protein expression (Cheong et al., 2015).

Phytocompounds dietary supplements usually contain a complex mixture of phytochemicals which have additive or synergistic interactions. Garcinia extract has been used in the traditional Ayurvedic medicine (Lim, 2012). Garcinia is a plant under the family of Clusiaceae that is commonly used as a flavouring agent. Different types of phytochemicals including flavonoids and organic acids have been identified from this plant. Among all types of organic acids, hydroxycitric acid has been identified as a potential supplement for weight reducing and management agent. The HCA originated from *G. cambogia* able to reduce lipid accumulation in 3T3-L1 adipocyte (Kim, Kim, Kwon, & Park, 2004). HCA could be a promising antiobesity agent since it has the ability to inhibit adipocyte differentiation, reduce fatty acid synthesis, and epididymal fat accumulation through reducing ATP-citrate lyase activity, and suppress the appetite (Mohamed et al., 2014). In vivo studies meanwhile have contributed to the understanding of the antiobesity effects of HCA via regulation of serotonin level and glucose uptake (Chuah, Ho, & Beh, 2013). In term of safety, there is no significant adverse effects of HCA have been reported and it demonstrates the safety, bioavailability, and efficacy of HCA in weight management (Preuss et al., 2004).

Previous hypothesis stated that the abundant amount of fat leads to health risk. Hence, the idea of targeting the process of lipid formation to treat obesity has emerged. Targeting adipogenesis would be a direct approach to reduce the amount of fat present (Nawroki & Scherer, 2005). The process of adipogenesis involves series of transcriptional processs. Molecular interactions of CCAAT-enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPAR) families were suggested to be involved in the process of differentiation (Nicholson et al., 2007; Niemela, Miettinen, Sarkanen, & Ashammakhi, 2008; Moreno-Navarrete & Fernández-Real, 2012). The peroxisome proliferator-activated receptor- γ (PPARγ) is

a transcription factor that expressed mainly in adipose tissue (Cornelius, MacDougald, & Lane, 1994). It is the master regulator of adipogenesis and absolutely necessary and sufficient for adipogenesis. There are two major isoforms of PPARγ which are PPARγ1 and PPARγ2 (Rosen, Eguchi, & Xu, 2009). Recent studies showed that PPARγ2 affects adipogenesis mainly since it was observed in adipocytes of morbidly obese individuals (Sarjeant & Stephens, 2012). Other than that, PPARγ2 is also required for adipocyte differentiation and lipid metabolism (Rosen et al., 2009). During adipocyte differentiation, PPARγ2 is involved in the sequential mRNA expression of adipocyte phenotypic markers, such as adipocyte protein2 (ap2) and fatty acid synthase (FAS) (Tontonoz, Hu, & Spiegelman, 1994).

Based on the above mentioned facts, this study is anticipated since there is no study has been documented on the effect of HCA from *G. atroviridis* on adipocytes cell. Findings from this study could help in understanding the mechanism of action of HCA as a natural source for lipid formation inhibitor as well as providing new insights towards the discovery of new natural and safe antiobesity agent.

2.2 OBESITY

Overweight and obesity are closely associated with each other, with the definition of the abnormal or excessive fat accumulation that may impair health. Obesity is characterised with the increase in number or size of adipocytes, where adipocytes play a vital role in monitoring lipid and glucose metabolism (Guilherme, Virbasius, Puri, & Czech, 2008).

According to WHO (2015), the body mass index (BMI) value of greater or equal to 25 is described as overweight. Meanwhile, the BMI value of greater than or equal to 30 is the indication for obesity. Recently, obesity has become a serious public