ETHANOL EXTRACT OF *ORTHOSIPHON STAMINEUS* BENTH. EXHIBITS ANTIOBESITY EFFECT BY INHIBITING ANGIOGENESIS AND PANCREATIC LIPASE

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by

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This thesis is dedicated to.....

My parents

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In the name of Allah the Most Gracious and the Most Merciful

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LIST OF ABBREVIATIONS

°C	Degree Celsius
AAS	Atomic absorbance spectroscopy
ALT	Alanine transaminase
As	Arsenic
AST	Aspartate aminotransferase
ATR	Attenuated total reflectance
AV	Average
BMI	Body mass index
Cd	Cadmium
cfu	Colony forming unit
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal-regulated kinases
ET	Ethanol extract
EUP	Eupatorin
EW	50% ethanol extract
FFA	Free fatty acid
FTIR	Fourier Transforms Infrared
g	Gram
GAE	Gallic acid equivalent
h	Hour
H_2SO_4	Sulphuric acid
HDL	High-density lipoprotein
HFD	High fat diet
Hg	Mercury
HIFBS	Heat inactivated human bovine serum
HNO_3	Nitric acid
HPTLC	High performance thin layer chromatography
HUVEC	Human umbilical vein endothelial cells
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Half maximal inhibitory concentration

LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
mg/kg	Milligram per kilogram
MLT	Microbial limit test
MMP	Matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
NFKB	Nuclear factor-kappaB
ng	Nanogram
nm	Nanometer
NPEG	Natural products-polyethylene glycol
OD	Optical density
Pb	Lead
PBS	Phosphate buffer saline
PCA	Principal component analysis
p-NPB	*p*-nitrophenyl butyrate
ppm	Parts per million
QE	Quercetin equivalent
RA	Rosmarinic acid
R_f	Retention factor
SD	Standard deviation
SEM	Standard error of mean
SIN	Sinensitin
TC	Triglyceride
TG	Total cholesterol
TMF	5-hydroxy-6,7,3',4'-tetramethoxyflavone
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
W	Water extract
WAT	White adipose tissue
WHO	World Health Organization
µg/mL	Microgram per millilitre

µL	Microlitre
µm	Micrometer

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EKSTRAK ETANOL *ORTHOSIPHON STAMINEUS* BENTH. MEMPAMERKAN KESAN ANTIOBESITI DENGAN MENGHALANG ANGIOGENESIS DAN LIPASE PANKREAS

ABSTRAK

Dalam kajian ini, kesan antiobesiti *Orthosiphon stamineus* (OS) disiasat menggunakan ekstrak etanol (ET), ekstrak 50% air-etanol (EW) dan ekstrak air (W) daripada tumbuhan ini. Ekstrak yang mempunyai aktiviti antiangiogenik paling tinggi, iaitu ET telah menunjukkan kesan antiobesiti secara *in vivo*. Walau bagaimanapun, aktiviti ke atas enzim lipase pankreas adalah sederhana dengan ekstrak ET menunjukkan aktiviti yang paling tinggi diikuti oleh ekstrak EW dan W. Kesan menghalang percambahan sel preadipocyte (3T3-L1) turut diperhatikan dan ekstrak ET menunjukkan aktiviti paling kuat diikuti oleh ekstrak EW dan W. Aktiviti antiangiogenik dan profil kimia OS juga dikaji yang melibatkan sampel daripada lima lokasi berbeza diseluruh Semenanjung Malaysia iaitu Batu Kurau (BK), Kepala Batas (KB), Sik Kedah (S), Changkat Jering (CJ) dan Sungai Buloh (SB). Sampel dari ladang BK menunjukkan aktiviti antiangiogenik paling kuat diikuti oleh SB, CJ, KB dan S. Profil kimia menggunakan Principal Component Analysis (PCA) dan High Performance Thin Layer Chromatography (HPTLC) mendedahkan bahawa ekstrak W mempunyai kandungan fitokimia terendah (fenolik dan flavonoid) diikuti ekstrak EW dan ekstrak ET. Kesan ujikaji ekstrak ET ke atas tikus menunjukkan penurunan berat badan yang ketara yang berkadar langsung dengan peningkatan dos. Kesan penurunan berat badan (P<0.01) dinilai secara peratusan pengurangan berat tisu adipos berbanding berat badan. Apabila ekstrak etanol OS diberikan pada tikus secara oral pada dos 250mg/kg (0.86±0.2%), didapati peratusan berat tisu adipos

lebih rendah berbanding dengan kumpulan HFD (2.19±0.2%). Berat tikus pada akhir rawatan menunjukkan bahawa ekstrak etanol mencegah peningkatan berat badan pada dos 500mg/kg dengan peningkatan berat sebanyak 7% berbanding kumpulan HFD yang menunjukkan peningkatan berat sebanyak 31% daripada berat badan awal. Ekstrak etanol juga menghalang steatosis hati yang disebabkan HFD sepanjang tempoh kajian. Merujuk kepada profil lipid, kami mendapati bahawa ekstrak etanol menyebabkan penurunan paras kolesterol (TC) dan trigliserida (TG) yang ketara dan berkadar langsung dengan penigkatan dos. Pada dos 500mg/kg, didapati paras TG dan TC masing-masing adalah 2.02±0.1mmol/L dan 0.29±0.08mmol/L berbanding kumpulan HFD (TG-3.03±0.1mmol/L; TC-0.64±0.14mmol/L). Profil HDL dan LDL bagi semua kumpulan yang dirawat dengan ekstrak OS menunjukkan bahawa ia berjaya menurunkan paras LDL dalam darah dan mengurangkan risiko penyakit jantung. Ini disokong dengan keputusan indeks plasma atherogenik (AIP) yang digunakan untuk meramal risiko penyakit kardiovaskular. Rawatan HFD+OS menunjukkan risiko mendapat penyakit kardiovaskular semakin berkurangan dengan peningkatan dos ekstrak (OS-125 AIP = -0.51, OS-250 AIP = -0.53 dan OS-500mg /kg AIP = -0.73) berbanding kumpulan HFD (-0.31). Selain itu, ekstrak etanol OS secara jelas menurunkan paras AST dan ALT dalam darah dan ini membuktikan bahawa ekstrak OS mempamerkan kesan perlindungan hati. Ini adalah selari dengan keputusan histologi hati yang mempamerkan kesan perlindungan terhadap hati berlemak kesan pengambilan HFD. Secara keseluruhan, keputusan menunjukkan bahawa ekstrak etanol OS menunjukkan aktiviti antiangiogenik yang kuat telah mempamerkan kesan antiobesiti yang berkemungkinan sesuai untuk mengurangkan berat badan serta risiko penyakit kardiovaskular. Hasil kajian juga menunjukkan

bahawa aktiviti ini adalah amat bergantung kepada kawasan geografi dan juga cara penanaman sesuatu spesis tumbuhan.

ETHANOL EXTRACT OF *ORTHOSIPHON STAMINEUS* BENTH. EXHIBITS ANTIOBESITY EFFECT BY INHIBITING ANGIOGENESIS AND PANCREATIC LIPASE

ABSTRACT

In this study, the antiobesity effect of *Orthosiphon stamineus* (OS) was investigated using ethanol extract (ET), 50% ethanol-water extract (EW) and water extract (W) of this plant species. Ethanol extract with the most potent antiangiogenic activity showed a significant antiobesity property *in vivo*. However, the activity towards the pancreatic lipase enzyme was modest with ET being the most potent followed by EW and W in decreasing order of reactivity. Activity towards inhibition of preadipocyte cell proliferation (3T3-L1) was also observed with ET being the most potent followed by EW and W. The antiangiogenic activity and chemical profile of OS collected from five different locations throughout peninsular Malaysia was also studied namely Batu Kurau (BK), Kepala Batas (KB), Sik Kedah (S), Changkat Jering (CJ) and Sungai Buloh (SB). BK plantation showed the most potent antiangiogenic activity with percentage of microvessels inhibition of 96.6±2.0%, followed by SB, CJ, KB and S. Chemical profiling using Principal Component Analysis (PCA) and High Performance Thin Layer Chromatography (HPTLC) reveal that W had the lowest amount of phytochemicals (phenolic and flavonoid) followed by EW and ET in increasing order. Treatment with the ET extract revealed significant weight loss effect in dose-dependent manner. Significant weight loss effect (P<0.01) was observed as the percentage of adipose tissue weight relative to body weight is reduced with oral feeding of the ET extract at dose 250 mg/kg (0.86±0.2%) compared to the HFD control group (2.19±0.2%). Weight of the rats

measured at the end of the treatment showed ethanolic extract prevent weight gain at dose of 500 mg/kg with only 7% increase of body weight compared to HFD group that showed 31% increase of body weight relative to initial weight. The ET also prevents HFD induced liver steatosis throughout the treatment period. From the lipid profile results, we found that ET significantly decreased cholesterol (TC) and triglyceride (TG) level in dose-dependent manner. At dose of 500 mg/kg the TG and TC were found to be 2.02±0.1mmol/L and 0.29±0.08mmol/L, respectively compared to HFD obese animals (TG- 3.03±0.1mmol/L; TC-0.64±0.14mmol/L). The HDL and LDL profile of the OS treated groups also showed significantly higher HDL level and lower in LDL level, suggesting that the extract may help to reduce the risk of heart disease. This is supported with the result of the atherogenic index of plasma (AIP) for prediction of risk of the cardiovascular event. Surprisingly, treatment of HFD+ET reduce the cardiovascular risk in a dose-dependent manner (OS-125 AIP = -0.51, OS-250 AIP = -0.53 and OS-500mg/kg AIP = -0.73, respectively) compared to HFD (-0.31) control. From the AST and ALT result, ET showed markedly lower AST and ALT level compared with HFD control group, suggesting that the OS extract exhibits a liver protective effect. This is consistent with the liver histology that ethanolic extract of OS prevents fatty liver disease. Overall, the results suggest that ET with strong anti-angiogenic activity showed a significant antiobesity effect and potentially useful for weight reduction as well as lowering the risk of cardiovascular diseases. The finding also shows that this activity is strongly dependent on the geographical location, specifically Batu Kurau plantation where the planting condition e.g. amount of water and sunlight, fertilizer as well as soil type are properly controlled.

CHAPTER 1: INTRODUCTION

1.1 Obesity

Obesity refers to abnormal or excessive fat accumulation in the body that presents a risk to health. Increase in adipocytes numbers and size contributes towards obesity. Proliferation and differentiation of adipocytes as well as fat accumulation are direct causes of obesity (Rohana and Roji 2011). Undoubtedly, obesity results from an imbalance between energy intake and expenditure that develops risk to metabolic disorders, atherosclerosis and diabetes (type 2) which have been affecting over 50% of adult population (Wellen and Hotamisligil 2003). Previous studies have shown that obesity facilitates development of chronic diseases such as inflammation-based pathologies, stroke, osteoarthritis, cancers, hypertension, sleep apnoea, and arthritis (Hotamisligil 2003, Singla 2010, González-Castejón and Rodriguez-Casado 2011). Age, developmental stage, physical activities and dietary habit are contributing factors that lead to excessive fat deposition in adipose tissues and other vital organs (Singla 2010).

Body mass index (BMI), a measure of weight relative to height, is used as the key assessment tool in measuring the degree of overweight index. Correspondingly, according to the Global Health Observatory (GHO) data from WHO, the median BMI in equivalence to optimum health for an adult population is in the range of 21-23 kg/m^2, BMI of more than 30 kg/m^2 is considered severely obese, whereas BMI of more than 40 kg/m^2 increase the risk rate of mortality. According to a report of World Health Organisation (WHO) in 2008, 35% of adults aged more than 20 years, were overweight, with BMI $\geq$ 25 kg/m^2 (34% men and 35% of women) (WHO 2014). The statistic shows evidence of increased prevalence of obesity around the

world and Malaysia in 2010 has been ranked by WHO sixth as the country with highest obesity rate in Asia (Verma, Chua et al. 2013). Therefore, there is a need to combat obesity and promote a healthy lifestyle.

1.1.1 Causes of Obesity

The etiology of obesity is complex and it does not have a simple and straightforward cause. Adipocytes have been shown to influence the pathogenesis of obesity-related disease due to its function as endocrine organ. Adipocytes apart from storing cell for fat and calories as triglycerides, also function as an active endocrine organ that communicates with the brain and peripheral tissue to secrete a variety of protein factors including chemokines, cytokines and hormone-like factors such as leptin, adiponectin and resistin (Calabro and Yeh 2007, German 2010). As body fat mass increases in obesity, enlargement of adipocytes causes molecular and cellular alteration affecting systemic metabolism where the concentration of anti-inflammatory adipokines is reduced and pro-inflammatory adipokines is increased (**Figure 1.1**). In addition, release of free fatty acids (FFA) and glycerol from adipose tissues also increased affecting the function of insulin in tissue to become resistant. The FFA causes an increase in hepatic output of glucose and triglycerides in the form of very low density lipoprotein (VLDL) that cause insulin resistance in muscle or hyperglycaemia. Accumulation of FFA in muscle cells and excess insulin secreted from the pancreas in response to high hepatic glucose production causes hyperinsulinemia. Altogether, hyperglycaemia and hyperinsulinemia increase blood pressure contributing to hypertension (Jung and Choi 2014).

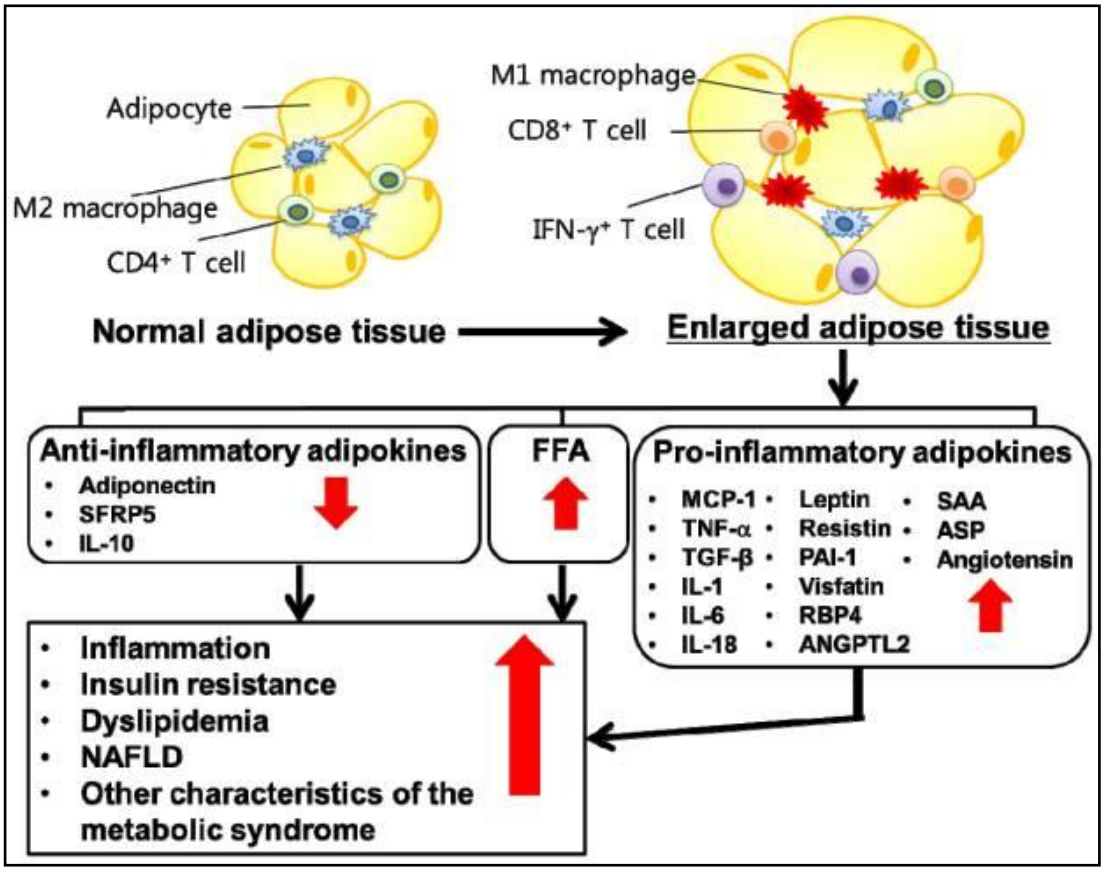

Figure 1.1 Endocrine function of adipose tissue and secretion of inflammatory adipokines in obese state **(Jung and Choi 2014)**

The endocrine function of adipose tissue undoubtedly has a strong association with obesity associated metabolic syndrome. There are more than 50 adipokines that have been identified with their functional roles to control metabolic functions, but most studies have been focusing more on the activities of leptin and adiponectin. The discoveries of leptin and adiponectin produced mainly by the adipose tissue have been shown to communicate with brain and peripheral organs to regulate energy homeostasis, glucose and lipid metabolism and cardiovascular function. Release of angiotensinogen, a potent vasoconstrictor with relation to excess body fat has increase hypertension. Inflammation of adipose tissues has induce higher expression of pro-inflammatory proteins such as TNF-α, interleukin-6 (IL-6), monocytes

chemotactic protein 1 (MCP-1), inducible nitric oxide synthase (iNOS), transforming growth factor β1 (TGF- β), procoagulant proteins such as plasminogen activator inhibitor type 1 (PAI-1), tissue factor and factor VII (Greenberg and Obin 2006, Jung and Choi 2014).

High amounts of adipose tissues are also associated with the increased number of macrophages where they function to scavenge moribund adipocytes. Adipocyte precursors also have potent phagocytic capacity and in response to specific stimuli, they can be transformed into macrophages-like cells that are present in obese compared to lean individual (Charrière, Cousin et al. 2003). This strongly supported by (Weisberg 2003) with evidence that macrophage infiltration of adipose tissue is characteristic of human obesity. This correlated with the report by (Jung and Choi 2014) that obesity induces switch of adipose tissue phenotypic from anti-inflammatory (M2) to pro-inflammatory (M1) macrophages.

In summary, the causes of obesity is mainly due to excessive number and enlargement of adipocytes that leads to dysregulated secretion of adipokines and increased release of free fatty acids (FFA) that modify the inflammatory responses, therefore contributing to development of metabolic disorder.

1.1.2 Current Treatment for Obesity

Modern lifestyle and unhealthy eating habits result in increased number of health related problems including obesity. According to (WHO 2015), out of 1.9 billion of adults population in 2014, 18 years and older were overweight. Out of this population, over 600 million were obese. Current available treatments for obesity include appetite suppressants or anorexics that control food intake by modulating the

central nervous system. Sibutramine (serotonin and noradrenalin re-uptake inhibitor) and rimonabant (cannabinoid receptor antagonist) are preeminent examples. These treatments require new options such as sibutramine which can cause constipation and headache with minimum weight loss (Luque and Rey 1999). The use of rimonabant was terminated due to serious psychiatric side-effects (Leite, Mocelin et al. 2009). Other treatment involves drugs that inhibit the absorption of specific nutrients in food such as orlistat by inhibiting pancreatic lipase and therefore reduce systemic absorption of dietary fat (Korner and Aronne 2004, Seo, Choe et al. 2011). However, orlistat was reported to cause steatorrhea and increase risk of developing deficiencies in lipid soluble vitamins and essential fatty acids (Heck, Yanovski et al. 2000).

Gastric bypass surgery or bariatric surgery are alternative options recommended for severely obese individuals who suffer from serious health problem related to obesity that is hard to be treated with diet and physical exercise alone. Bariatric surgery is an approach to help lose weight by surgery on the stomach and/or intestines that subsequently restrict food intake and promote weight loss as well as reducing risk of type 2 diabetes. However, the best results can be achieved when the patients follow healthy eating habit and regular exercise. Undeniably, the cost for the surgery is quite high and some side effects have been identified, include bleeding, infection, leaks at the site where intestines were sewn together, diarrhoea and blood clots that may affect lungs and heart. Moreover, deficiency in nutrient absorption also has been reported (NIH 2011). Hence, a better option of obesity treatment should be explored to overcome the side effect of taking synthetic drugs and surgery.

1.1.3 Angiogenesis and Obesity

Angiogenesis is a process of new capillary blood vessels from existing blood vessel and it is an important natural process that occurs for healing and reproduction process (Adair and Montani 2010). The process is controlled by the body through a balanced production of growth and inhibitory factors in healthy tissues. Disturbances in the body production of specific growth and inhibitory factors may lead to an excessive and insufficient growth of blood vessel that directly affects the health of an individual. The excessive blood vessel proliferation may cause tumor growth and spread, it may also cause rheumatoid arthritis, diabetic blindness, psoriasis, obesity and others. In this scenario, the new blood vessel nourishes the diseased tissues and some of the new blood vessels are in fact abnormal and leaky which may damage normal tissues. On the contrary, insufficient blood vessel formation may cause infertility, heart disease, stroke, ulcers and scleroderma. The insufficient blood vessels formation causes improper restoration of blood circulation and increases the risk of tissue death (Koch 2003, Adair and Montani 2010, Kumar, Kavimani et al. 2012).

The angiogenesis in relation to obesity is not fully established. However, the increase in proliferation and differentiation of adipocytes has been previously reported by many other researchers who have shown strong association between these two pathology (Bråkenhielm, Cao et al. 2004, Voros, Maquoi et al. 2005, Cao 2007, Nishimura, Manabe et al. 2007, Lijnen 2008, Christiaens and Lijnen 2010). The adipose tissue undergoes expansion and regression throughout adult life and this requires parallel growth of the capillary network to support the survival of the tissues. Thus, adipose tissues are among the tissues that have highest angiogenic capacities (Lemoine, Ledoux et al. 2013).

Ledoux, Queguiner et al. (2008) have outlined the role of angiogenesis in development of fat cells and their study has found that the adipose tissue from severely obese adults grafted on chick chorioallantoic membrane (CAM) was able to recruit its own endothelial cells to induce angiogenesis (Ledoux, Queguiner et al. 2008). Apart from that, adipose tissue previously reported to promote wound healing and revascularize ischemic tissues of myocardium, suggested that adipose tissue produces angiogenic molecules. Their finding has shown the link of adipogenesis and angiogenesis apart from other factors that promote adipose tissue angiogenesis such as hyperplasia, hypoxia and inflammation (Hausman and Richardson 2004, Cao 2007, Nishimura, Manabe et al. 2007).

Adipocytes apart from fat storing organ, form as an important endocrine organ that secretes a variety of protein factors including chemokines, cytokines and hormone-like factors such as leptin, adiponectin and resistin (German 2010). Hausman and Richardson (2004) in their review indicate that adipogenesis is regulated by factors that drive angiogenesis. Vascular endothelial growth factor (VEGF) is the most critical growth factor in initiating the formation of immature blood vessels. In addition, the expression of VEGF is influenced by hypoxia, insulin, growth factors and several cytokines which are directly connected with the endocrine function of adipocytes (Hausman and Richardson 2004). This is supported by Nishimura et al. (2007) in their live-cell imaging study of unfixed living adipose tissue labelled with a combination of lectin (red), BODIPY (blue), acetylated LDL (blue) and Hoechst 33342 (green) in *db/db* mice (Nishimura, Manabe et al. 2007). The study revealed that adipogenesis of the adipose tissue takes place within adipogenic/angiogenic cell clusters that contain various stromal cell and blood vessels. This also further determined with administration of anti-VEGF antibodies to

the mice and it inhibited not only angiogenesis but also the formation of adipogenic/angiogenic cell clusters. This clearly indicates that coupling of adipogenesis and angiogenesis is essential for the differentiation of adipocytes in obesity and VEGF is the mediator of the process.

A review by Daquinag et al. (2011) on vascularization of adipose tissue as an anti-obesity approach also indicates the roles of angiogenesis in an expansion of white adipose tissue (WAT). Development of obesity as a result of adipocytes hypertrophy (increase in cell size) as well as hyperplasia (increase in cell number) associated with the expansion of WAT that requires angiogenesis to feed the process. Depletion of nutrient and oxygen through inhibition of WAT neovascularization can prevent an onset of obesity in both genetic and diet-induced obesity models and this has been previously reported with the use of an anti-angiogenic drug to inhibit WAT expansion (Daquinag, Zhang et al. 2011). Rupnick et al. (2002) in their study has shown that the genetically obese leptin-deficient mice from different obesity model treated with anti-angiogenic agent (TNP-470, angiostatin (kringle 1-4 domains of plasminogen), endostatin (a C-terminal fragment of collagen XVIII), Bay-129566 (a matrix metalloproteinase inhibitor) and thalidomide resulted weight reduction and adipose tissue loss in dose-dependent manner. The treated mice showed decreased in endothelial proliferation and increase in apoptosis when compared with the control group, evidence that adipose tissue mass can be regulated by its vasculature (Rupnick, Panigrahy et al. 2002). This is strongly supported by Bråkenhielm et al. (2004) in their study using systemic administration of angiogenesis inhibitor, TN-470 (AGM-1470) tested on high caloric diet-fed *wt* mice as well as in genetically leptin-deficient *ob/ob* mice. They have shown reduction in adipose tissue vascularization that selectively affects the growth of adipose tissue with a decreased in insulin and

serum level of low density lipoprotein cholesterol (LDL-C) indicating that expansion of adipose tissue can be controlled via angiogenesis inhibitor (Bråkenhielm, Cao et al. 2004).

Another study by (Voros, Maquoi et al. 2005) on angiogenesis modulation during development of adipose tissue tested in murine models of obesity showed that fat pad growth in both nutritionally induced or genetic obesity in mice is accompanied by increased vascularization. They suggested the potential role of pro- and antiangiogenic factors in obesity-related angiogenesis similar as reported by Hausman and Richardson (2004) on VEGF signalling modulation that affects the development of adipose tissue in obesity (Hausman and Richardson 2004). Cutchins et al. (2012) in their study correlate VEGF expression in angiogenesis with the function of the inhibitor of differentiation-3 (Id3) released during expansion of adipocytes. The expanding adipose tissue results in hypoxia that induces VEGF expression by adipocytes. However, in the Id3-deficient mice model shows that the expression of VEGFA was attenuated, causing a decrease in microvascular blood volume in the adipose tissue (Cutchins, Harmon et al. 2012). This further supports the concept that inhibition of angiogenesis in adipose tissue may prevent obesity.

The natural compounds with antiangiogenic properties also have been reported to have a positive effect on the treatment of obesity. (Kim 2010) in his patent report discovered that *Psoraleae semen* extract, *Sieges beckie* herbal extract and *Corni fructus* extract which exhibit antiangiogenesis effect also demonstrated antiobesity activity. Furthermore, Mojzis et al. (2008) in their review summarize the potential of bioactive plant compounds specifically flavonoid and chalcones in angiogenesis modulation by regulating the expression of VEGF, matrix metalloproteinases (MMPs), EGFR and inhibit NF_KB, PI3-K/Akt, ERK1/2 signalling

pathways, causing strong antiangiogenic effects (Mojzis, Varinska et al. 2008). In addition, various studies on plant rich flavonoid has shown significant antiobesity effect such as green tea extract (Chantre and Lairon 2002, Lin, Della-Fera et al. 2005), Lotus leaf extract (Du, You et al. 2010), *Morus bombycis* root extract (Kim, Lee et al. 2010), *Nelumbo nucifera* leaves extract (Ono, Hattori et al. 2006) etc. which support that natural antiangiogenic agent also is a promising therapeutic agent for the treatment of obesity.

Overall, recent evidence on angiogenesis modulation as the target to prevent the growth of adipose tissue may offer a better option in the treatment of obesity and other related metabolic disorder. Anti-angiogenic therapy from natural sources with promising therapeutic efficacy should be further explored to combat obesity with lower risk of side effects.

1.2 Potential of Herbal Extract as Antiobesity Agent

Herbal medicines have received great attention as alternative medicines due to increase awareness on the high therapeutic values of natural products. The demand promotes more scientific study to ensure safety, quality and efficacy of the herbal medicine. Herbal medicine also called botanical medicine or phytomedicine is basically the application of the plant material such as root, leaves, bark, flowers, seeds and berries used in the maintenance of health, prevention, diagnosis and improvement or treatment of the illness. Herbal medicine has been in existence since ancient times and is the oldest treatment system in the world. The naturally occurring chemicals and compounds found in plant that is effective in healing various illnesses is a key for herbal medicine practice worldwide. The value of herbal medicine in

preventing and treatment of illness has increased due to improvement in analysis and quality control as well as advances in scientific research.

Extensive studies have been carried out by researchers to identify the value of plants and its constituents in lowering the risk of obesity and related metabolic syndrome. Several plant extracts such as *Sorbus commixta* (Japanese rowan), *Viscum album* (European mistletoe), *Rosmarinus officinalis* (rosmary), *Morus bombycis* (mulberry), and *Ginkgo biloba* (ginkgo) have been reported for their potent lipase inhibitory effect that may help in reducing dietary fat absorption with IC_{50} value less than 20 µg/mL (Kim, Lee et al. 2010, Yasser Bustanji 2010, Bustanji, Al-Masri et al. 2011, Lee, Kim et al. 2012). In other studies, green tea extract standardized to contain 25% catechin had direct inhibition on gastric and pancreatic lipases as well as stimulation of thermogenesis in its action of reducing fat accumulation in a body (Chantre and Lairon 2002). Potent secondary metabolites mainly phenolic and flavonoid from plant extract have been reported to contribute to this activity. Catechin, epicatechin and epigallocatechin gallate (EGCG) from *Camellia sinensis* (tea), *Morinda citrifolia* (noni), *Centella asiatica* (pegaga) and *Momordica charantia* (bitter gourd) were found to inhibit proliferation and differentiation of preadipocytes as well as potent pancreatic lipase, suggested the potential of these plant extracts as antiobesity agents (Lin, Della-Fera et al. 2005, Sahib, Hamid et al. 2011, Sergent, Vanderstraeten et al. 2012).

The approach on using plant and herbal extracts has great potential in combating diseases including obesity. Current interest in finding scientific evidence on safety and efficacy of herbal medicine becoming an alternative for development of new antiobesity agents as they were previously reported to contain high antioxidant properties which are the key to lowering risk of many diseases (Zhang,

Gan et al. 2015). This study aims to evaluate anti-obesity activity of *Orthosiphon stamineus* (Misai Kucing) extracts *in vitro* and *in vivo*.

1.3 *Orthosiphon stamineus* Benth.

Orthosiphon stamineus Benth., commonly known as Misai Kucing or Cat's whiskers is herbaceous shrub from the family of Lamiaceae. It has wispy stamens of its flower shaped look like cat's whiskers (**Figure 1.2**). Leaves of this plant are used commonly in Southeast Asia and European countries for herbal tea, widely known as Java tea. The old folk's practice of this plant reported on the use to treat menstrual disorder, gallstone, influenza, hepatitis and jaundice (Ahamed Basheer and Abdul Majid 2010). Moreover, this plant has been used traditionally as a diuretic agent and to treat kidney and bladder inflammation, gout, diabetes, rheumatism, abdominal pain, allergy, inflammation and hypertension (Ahamed Basheer and Abdul Majid 2010, Ismail, Hanapi et al. 2010).

1.3.1 Classification and Description

Table 1.1 Taxonomy classifications of *O. stamineus*

Family	**:**	**Lamiaceae**
Genus	:	*Orthosiphon*
Species	:	*stamineus*
Scientific name	:	*Orthosiphon stamineus* (Benth)
Vernacular name	:	Misai Kucing (Malaysia); Cat's whiskers (English); Kumis Kucing (Indonesia); Mao Xuhua (China)

Figure 1.2 Photographs of *Orthosiphon stamineus* flower and leaves

1.3.2 Chemical Constituents and Pharmacological Properties of *O. stamineus*

Various studies on *O. stamineus* revealed a range of chemical compounds. According to an Assessment Report by a European Medicinal Agency (EMA) released in 2010, the compound identified from this plant include mineral (potassium 3%), diterpenes (orthosiphols A-E 0.2%), triterpenes, essential oil (0.02-0.06%), sesquiterpenes, lipophilic flavones like sinensitin (0.1-0.19%), isosenensitin and euphatorin flavonol glycosides; rosmarinic acid (0.1-0.5%), and other caffeic acid derivatives like mono and dicafeyl tartaric acid as well as lithospermic acid, phytosterols (β-sitosterol) and essential oil (0.7%), isositol, pimarane, isopimarene and staminane diterpenes, triterpenes (ursolic acid, oleanolic acid, betulinic acid, hydroxybetulinic acid, maslinic acid, α-amyrin, β-amyrin), and chromenes (Hossain, Salehuddin et al. 2006, HMPC 2010, Hossain and Ismail 2013).

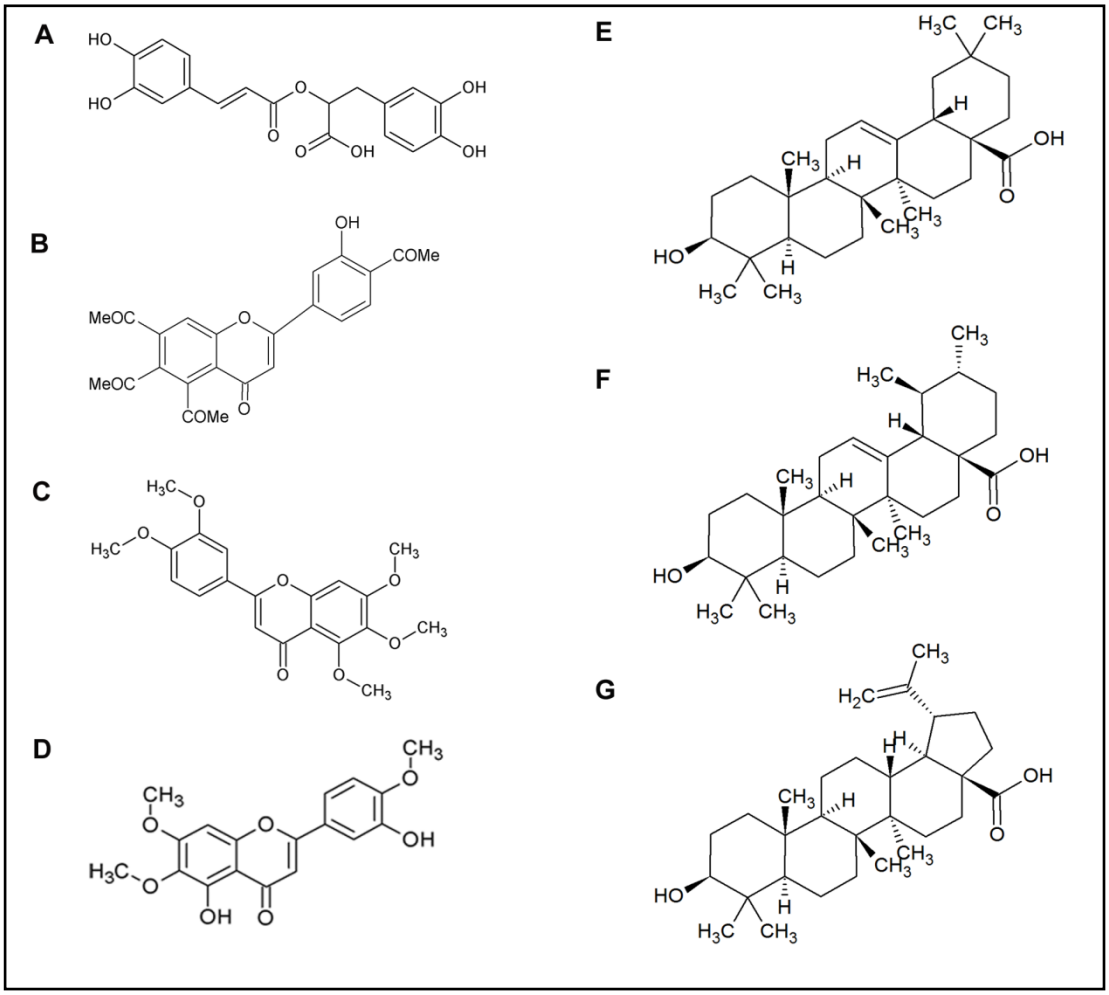

Figure 1.3 The structure of the chemical constituents (A- Rosmarinic acid, B- 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, C- Sinensitin, D- Eupatorin, E- Oleanolic acid, F- Ursolic acid and G- Betulinic acid) commonly found in *O. stamineus* extracts.

In addition to the components mentioned, Awale et al. (2003) have reported that *O. stamineus* also consists of oxygenated sesquiterpenes (Awale, Tezuka et al. 2003). **Figure 1.3** showed the chemical structure of the compounds commonly found in *O. stamineus* extracts.

Scientific studies have shown that extracts of *O. stamineus* leaves exhibit range of pharmacological properties such as antibacterial, antioxidant, hepatoprotective effect against paracetamol-induced liver damage and thioacetamide-induced hepatotoxic model rats, chemopreventive activity against human hepatocellular carcinoma cell line, HepG2, nephroprotective activity in rats and others. Moreover, *O. stamineus* extract is widely reported to have strong antioxidant properties. A study conducted by Chin et al. (2008) recorded that *O. stamineus* has strong antioxidant activity *in vivo* as the extract of the herb enhance the activity of hepatic glutathione-s-transferase in rat livers and the effect seen was dose-dependent (Chin, Ismail et al. 2008). Various researchers have worked extensively on various parts of the plant with different solvent as well as supercritical CO_2 extracts and proved that *O. stamineus* has various polyphenols, flavonoid and other bioactive phytoconstituents which attribute to its antioxidant property (Akowuah, Zhari et al. 2004, Akowuah, Ismail et al. 2005, Yam, Basir et al. 2007, Ahamed Basheer and Abdul Majid 2010, Ho, Noryati et al. 2010, Abdelwahab, Mohan et al. 2011, Farhan, Razak et al. 2012, Yam, Lim et al. 2013). A report by (Sahib 2009) revealed that the presence of a high level of anti-oxidants and phenolic content compounds in *O. stamineus* extract exhibit a strong antiangiogenic properties. Moreover, flavones eupatorin standard in comparison with chloroform extract of *O. stamineus* which rich in flavones eupatorin was found to inhibit migration of human umbilical vein endothelial cells (HUVECs) and contribute to significant antiproliferative effect against various cancer cells (Doleckova, Rarova et al. 2012). Ahamed et al. (2012) also reported the ability of *O. stamineus* extract to inhibit migration as well as tube formation of HUVECs by suppressing VEGF-induced phosporylation of VEGF receptor-2 in HUVECs with the phytochemicals profile showed higher amount of

rosmarinic acid, protein and phenolic contents (Ahamed, Aisha et al. 2012). Strong antiangiogenic property of *O. stamineus* indicates that this plant has higher potential for the treatment of various diseases involving modulation of vasculature including obesity.

Apart from that, the extracts of *O. stamineus* was found to be safe with no sign of toxicity effect *in vitro* and *in vivo* which suggested that it could be the safe natural source for treatment of critical diseases (Maheswari, Maryammal et al. 2008, Ahamed Basheer and Abdul Majid 2010, Kannappan, Madhukar et al. 2010, Alshawsh, Abdulla et al. 2011, Salleh, Rajab et al. 2011).

1.4 Hypothesis

1. *O. stamineus* has anti-obesity properties due to its anti-angiogenic properties.
2. Extract that contain the highest amount of phenolic and flavonoid compounds gives the strongest anti-obesity property.
3. Influence of localities on phenolic and flavonoid constituents affects antiangiogenic and anti-obesity properties.

1.5 General objective

To study the antiobesity property of *Orthosiphon stamineus* extracts and to establish the chemical profile in relation to its antiobesity effect.

1.6 Research objectives

1. To evaluate anti-obesity effect of *O. stamineus* extract *in vivo* and *in vitro* via its anti-angiogenic property.
2. To determine the amount of phenolic and flavonoid content of various solvent extract of *O. stamineus* from various locations.
3. To develop the quality control parameter by establishing the chemical profiles and general quality assessment of *O. stamineus* leaves extracts as an antiobesity agent.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and Chemicals

10% Formalin	Sigma-Aldrich, Germany
3T3-L1 Cells	ATCC, Rockville, MD, USA
6-aminocaproic acid	Sigma-Aldrich, Germany
Aluminium chloride	Sigma-Aldrich, Germany
Amphotericin B (Fungizone)	Sigma-Aldrich, Germany
Aprotinin	Sigma-Aldrich, Germany
Dimethyl sulfoxide	Sigma-Aldrich, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco, Life Technology, UK
Earle's Salt (M199) medium	Gibco, Life Technology, UK
Ethanol	Riedel-de Haën, Germany
Ethyl acetate	Riedel-de Haën, Germany
Foetal bovine serum (FBS)	Gibco, Life Technology, UK
Fibrinogen	Calbiochem, USA
Folin-Ciocalteu reagent	Sigma-Aldrich, Germany
Formic acid	Fisher Scientific, USA
Gallic acid	Sigma-Aldrich, Germany
Gentamicin injection B.P 80 mg / 2 mL	Sigma-Aldrich, Germany
Hexane	QRëc™, Germany
Hydrochloric acid	QRëc™, Germany
L-glutamine	Sigma-Aldrich, Germany
Lipase (Type II; crude from porcine pancreas)	Sigma-Aldrich, Germany
Methanol	Riedel-de Haën, Germany

Orlistat	TOCRIS Bioscience, UK
Penicillin-streptomycin solution	Gibco, Life Technology, UK
Phosphate buffered saline (PBS)	Sigma-Aldrich, Germany
p-Nitrophenyl butyrate	Sigma Chemical Co. (LLC)
Potassium acetate	Sigma-Aldrich, Germany
Quercetin	Sigma-Aldrich, Germany
Sodium carbonate	Sigma-Aldrich, Germany
Sulphuric Acid	Sigma-Aldrich, Germany
Suramin	Sigma-Aldrich, Germany
Thrombin 1000 unit	Sigma-Aldrich, Germany
Tris-HCl buffer	Sigma Chemical Co. (LLC)
Trypsin	Gibco, Life Technology, UK

2.2 Apparatus and Equipments

6-,12-, 24-, 48- and 96-well plates	Corning, USA
Autoclave	Hirayama, Japan
Biosafety cabinet	ESCO, USA
Cell culture flasks (25 and 75 cm^2)	BD Bioscience, USA
Disposable petri dishes	Sterilin, UK
Dissecting set	AVEAids, Malaysia
Erlenmeyer flasks	Schott Duran, Germany
Filter papers	Whatman, USA
Fluorescence microscope (EVOS)	AMG, USA
Freeze dryer	Labconco, USA
Fourier Transform Infrared Spectrometer (FTIR)	Perkin Elmer, USA
Fume hood	ESCO, USA
Grinder	Retsch GmbH, Germany
Haemocytometer	Marienfeld, Germany
High Performance Thin Layer Chromatography (HPTLC)	Camag, Switzerland
Muffle Furnace	Thermolyne, USA
Carbon dioxide incubator	Fisher Scientific, Germany
Incubator shaker	Sartorius, Germany
Inverted light microscope	Olympus, Japan
Microcentrifuge tubes	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
Microplate reader	Tecan, Switzerland

Multiwave3000 Microwave	Perkin Elmer, USA
Minisart filter 0.22 µm	Sartorius, USA
Needles	Becton Dickinson, USA
Oven	Memmert, Germany
pH meter	Oakton, USA
Refrigerator	Samsung, Korea
Rotary evaporator	Buchi, Switzerland
Round bottom flasks	Favorit®, Thailand
Screw cap centrifuge tubes	Axygen®, USA
Serological pipettes	BD Falcon, USA
Syringes	Becton Dickinson, USA
Thin layer chromatography plates	Merck, USA
Ultrasonic cleaner UC-10	Lab Companion™, China
Universal fit pipette tips	Corning Inc., USA
UV Spectrophotometer Lambda 45,	Perkin Elmer, USA
Volumetric flasks	Pyrex, USA
Vortex	VELP, Europe
Water bath	Protech-Electronic, Malaysia
Weighing Balance	Fisher Scientific, Germany

2.3 Sources of Plant Material and Authentication

Fresh leaves of *Orthosiphon stamineus* were collected from 5 different locations throughout peninsular Malaysia, mainly from Batu Kurau, Kepala Batas, Sik, Changkat Jering and Sungai Buloh. The plant sample was authenticated and deposited at the Herbarium of School of Biology, Universiti Sains Malaysia with voucher number of 11009. Localities of *Orthosiphon stamineus* used in this study are summarised in the **Table 2.1**.

Table 2.1 Location of *Orthosiphon stamineus* collected sample

No.	District	State	Code
1	Batu Kurau	Perak	**BK**
2	Kepala Batas	Pulau Pinang	**KB**
3	Sik	Kedah	**S**
4	Changkat Jering	Perak	**CJ**
5	Sungai Buloh	Selangor	**SB**

2.4 Gravimetric Analysis

All gravimetric tests were carried out following the standard method stated in Malaysia Herbal Monograph Volume 1.

2.4.1 Determination of Foreign Matter

Leaf samples of *Orthosiphon stamineus* of approximately 100g were spread in a thin layer form on a piece of white paper. Foreign matter was sorted with the naked eye and with the use of a magnifying glass. The foreign matter found was separated; the weight and percentage was then calculated per 100 g of sample (Committee, Ismail et al. 1999).

2.4.2 Determination of Total Ash Content

O. stamineus leaves samples of 5 g were weighed in tarred crucibles. Samples were then incinerated by increasing the temperature gradually, not exceeding 450°C until it is free from carbon. Then, the crucibles were cooled in a desiccator and the ash was weighed. Percentage of total ash content was calculated per gram of leaves sample (Committee, Ismail et al. 1999).

2.4.3 Determination of Loss on Drying

Plant materials of 2 g were weighed accurately in tarred flat-bottomed dishes. The samples were then dried in the oven at 100-105°C for 5 h until constant weight was achieved. Percentage of loss on drying with reference to the air dried sample was then calculated (Committee, Ismail et al. 1999).

2.4.4 Determination of Extractive Value

Five grams of powdered plant material was macerated separately for 24 h in 100mL of 95% ethanol and 50% ethanol with constant agitation for 7 h. The solution was filtered with Whatman No. 1 filter paper and 20 mL of the filtrate was dried at 105°C on watch glasses until constant mass. Then, a percentage of ethanol extractive value was measured against initial weight of the air dried powdered sample.

In addition, percentage yield for water soluble extract was carried under reflux. Five grams of plant materials was dissolved in 100 mL of distilled water and refluxed for 1 h. The remaining extract solution was filtered and the 20 mL of the filtrate was transferred into watch glasses which have been preheated to constant weight. The filtrate was then oven dried at 105°C for 3 h. The water soluble

extractive value was calculated against the weight of the air dried powdered sample according to Ismail et al.(Committee, Ismail et al. 1999).

2.5 Determination of Heavy Metal

The lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) content present in *Orthosiphon stamineus* leaves were determined using Atomic Absorption Spectrometer (AAS) as per standard method of British Pharmacopoeia 2008 (Commission 2008). Briefly, 0.5 g of dried powder of *O. stamineus* leaves was weighed and transferred into Teflon vessels. Ten millilitre of nitric acid was then added to the *O. stamineus* sample and heated to 200°C under 120 psi pressure using microwave (Perkin Elmer, USA). The samples in triplicate were then diluted with 50mL of distilled water and filtered with 2µm membrane syringe filter. The filtrates were then analysed using AAS against nitric acid as blank and standard reference of lead, cadmium, arsenic and mercury solution were prepared in 2% nitric acid. The plant samples were sent to KbioCorp Laboratory for analysis.

2.6 Microbial Limit Test

Powder dried leaves of *O. stamineus* were subjected to Microbial Limit Test (MLT) as per the British Pharmacopoeia 2008 method consisting of total viable aerobic count, total yeast and mould count, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.* and *Pseudomonas aeruginosa* count (Commission 2008). For total aerobic, yeast and mould count, 90 mL phosphate buffer at pH 7.2 was added to 10 g of dried powdered *O. stamineus* leaf. Then, 1 mL of the mixture was transferred into a petri dish prior containing 20 mL sterilized molten Soybean-Casein digest agar and