ENHANCED DIFFERENTIATION OF 3T3-L1 ADIPOCYTES BY EXTRACTS OF *Pleurotus giganteus* (Berk) KARUNARATHNA & K.D. HYDE

PUVANESWARI A/P PARAVAMSIVAM

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

University of Malaya

ENHANCED DIFFERENTIATION OF 3T3-L1 ADIPOCYTES BY EXTRACTS OF *Pleurotus giganteus* (Berk) KARUNARATHNA & K.D. HYDE

PUVANESWARI A/P PARAVAMSIVAM

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

University of Malaya

ABSTRACT

Pleurotus giganteus (Berk.) Karunarathna & K. D. Hyde is a culinary mushroom that has been consumed by the indigenous communities in Peninsular Malaysia for hundreds of years. Although large scale domestication of this wild mushroom is currently in progress, the findings on its medicinal properties are very limited. This study was conducted to evaluate the insulin-sensitising capabilities of *P. giganteus* by using the 3T3-L1 cell line model. The basidiocarps of *P. giganteus* were purchased from Nas Agro Farm, Sepang and extracted using hot water and methanol. The methanol extract of *P. giganteus* was fractionated to obtain the hexane, butanol and ethyl acetate fractions. The effect of *P. giganteus* extracts and fractions on the proliferation of 3T3-L1 preadipocyte cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Highest 3T3-L1 preadipocytes proliferation was obtained using hot water extract and butanol fraction of *P. giganteus*. Methanol extract and hexane and ethyl acetate fractions caused moderate proliferation of preadipocytes respectively. Subsequently, Oil Red O assay was carried out to investigate the effect of *P. giganteus* extracts and fractions on lipogenesis during adipocyte differentiation of 3T3-L1 cells. Ethyl acetate fraction at 100 µg/ml stimulated the highest lipid accumulation at 175.30 ± 2.7%. The glucose uptake of differentiated 3T3-L1 cells was measured by fluorescence based glucose uptake assay. The highest glucose uptake was recorded at 136.20 ± 10.5% in ethyl acetate fraction at 100 µg/ml respectively. The lipolysis of differentiated 3T3-L1 adipocytes was measured using ZenBio's non-esterified fatty acid and glycerol quantification kit. The ethyl acetate fraction at 100 µg/ml showed the highest free fatty acid release from 3T3-L1 adipocytes by 3.4 fold. Collectively, the ethyl acetate fraction at 100 µg/ml moderately induced cell proliferation of 3T3-L1 preadipocyte cells and highly stimulated lipogenesis, glucose uptake and lipolysis in 3T3-L1 adipocytes. Therefore, ethyl acetate fraction was

University of Malaya

selected to study for its effect on 3T3-L1 adipocytes at a molecular level. The gene expression quantification was done using real time PCR analysis with TaqMan® primer. Ethyl acetate fraction of *P. giganteus* significantly enhanced adipogenic differentiation in 3T3-L1 adipocytes via the upregulation of SREBP, PPARγ and Pi3k genes. The glucose uptake was facilitated by the highly expressed glucose transporters, namely, glut 1 and glut 4. These results suggest that ethyl acetate fraction of *P. giganteus* has insulin sensitising effect on adipocytes and therefore has the potential to act as an adjuvant for the management of type 2 diabetes.

University of Malaya

ABSTRAK

Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde adalah merupakan cendawan masakan yang dimakan oleh masyarakat Orang Asli di Malaysia selama beratus-ratus tahun. Cendawan jenis liar ini telah didomestikasikan dalam skala yang besar tetapi ciri-ciri perubatannya tidak banyak dilaporkan. Kajian ini telah dijalankan untuk menilai kebolehan ekstrak dan pecahan *P. giganteus* untuk berfungsi sebagai agen pemekaan insulin dengan menggunakan model sel 3T3-L1. Basidiokarpa *P. giganteus* dibeli dari Ladang Agro Nas, Sepang dan diekstrak menggunakan air dan metanol. Ekstrak metanol *P. giganteus* telah dipecahkan untuk mendapatkan pecahan heksana, butanol dan etil asetat. Kesan ekstrak *P. giganteus* terhadap percambahan sel pra-adiposit sel 3T3-L1 dinilai dengan menggunakan teknik 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromida (MTT). Ekstrak kues dan pecahan butanol dari *P. giganteus* paling banyak meningkatkan percambahan pra-adiposit sel 3T3-L1. Ekstrak methanol dan pecahan heksana dan etil asetat menunjukkan percambahan pra-adiposit sel 3T3-L1 yang sederhana. Seterusnya, analisa 'Oil Red O' telah dijalankan untuk menilai kesan ekstrak dan pecahan *P. giganteus* terhadap lipogenesis sel 3T3-L1 yang telah berbeza. Ekstrak etil asetat dengan kepekatan 100µg/ml telah merangsang pengumpulan lemak paling banyak iaitu sebanyak 175.3 ± 2.7%. Pengambilan glukosa oleh sel 3T3-L1 yang telah berbeza ditentukan dengan analisa pengambilan glukosa berdasarkan sinar pendarfluor. Pengambilan glukosa yang paling tinggi iaitu sebanyak 136.2 ± 10.5% telah diperolehi untuk pecahan etil asetat. Lipolisis sel adiposit 3T3-L1 yang telah berbeza ditentukan melalui kit kuantifikasi asid lemak bebas yang bukan-berester dan gliserol ZenBio. Ekstrak etil asetat dengan kepekatan 100µg/ml menunjukkan pembebasan asid lemak yang paling tinggi iaitu sebanyak 3.4 kali ganda. Secara keseluruhan, ekstrak etil asetat dengan kepekatan 100µg/ml telah mengaruh percambahan sel pra-adiposit 3T3-L1 pada kadar yang sederhana tetapi telah

University of Malaya

merangsangkan lipogenesis, pengambilan glukosa dan lipolisis dalam sel 3T3-L1 adiposit pada kadar yang tinggi. Oleh itu, ekstrak etil asetat dipilih untuk mengkaji kesannya terhadap sel adiposit 3T3-L1 pada tahap molekular. Analisa ekspresi gen dilakukan dengan menggunakan primer TaqMan® dan PCR masa sebenar. Ekstrak etil asetat dari *P. giganteus* telah meningkatkan pembezaan adipogenik dalam sel adiposit 3T3-L1 melalui ekspresi gen SREBP, PPARγ dan Pi3k. Pengambilan glukosa telah dipermudahkan oleh ekspresi pengangkut glukosa seperti glut 1 and glut 4 yang tinggi. Secara keseluruhan, keputusan ini menunjukkan bahawa pecahan etil asetat dari *P. giganteus* memperolehi kesan pemekaan insulin terhadap sel adiposit dan mempunyai potensi untuk digunakan sebagai pambantu dalam pengurusan diabetes jenis 2.

University of Malaya

ACKNOWLEDGEMENTS

This research project would not have been possible without the support of many people. First and foremost I offer my sincerest gratitude to my supervisors, Prof Dr. Vikineswary Sabaratnam and Prof Dr. Umah Rani A/P Kuppusamy, who have offered invaluable assistance, support and guidance. Heart felt gratitude also to Datin Professor Dr. Sri Nurestri Abd Malek and Prof Dr. Chua Kek Heng for the research grants and also for allowing me to use their laboratory facilities.

I was lucky enough to have the support from the Late Dr. Gowri Kanagasabapathy, a post-doc in Faculty of Medicine, University of Malaya. I would like to sincerely thank her for all her helps, guidance and emotional supports in helping me to complete my research.

During my daily work in Mycology and Plant Pathology Lab (B402) and Biochemistry Lab, I have been blessed with a friendly and cheerful group of fellow students and researchers. Cheers to Kamalanathan Ramachandaran, Priscilla Ann, Asweni Baskaran, Audrey Chew, Lavania Nair, Sarmila Mutukrishnan, Shanti Velayutham, Neeranjini Nallathamby, Wei Lun, Jing Yang, Tan Wei Cheat, Farhat, Loo Poh Leong, Huda, Chan Pui Man, Bavani Arumugam, Saraswathy Subramaniam, Nooshin, Tan Xien Cher, Sharjahan, John Pinto, Vinoth Kumar, Mdm. May Hing and last but not least Dr. Jegadeesh for making B402 feel like home away from home.

I would like to forward my sincere gratefulness to University of Malaya for providing the scholarship and funds for my research. This study was funded by the following research grants, UM-MOHE UM. C/625/1/HIR/MOHE/SC/02 (HIR F00002-21001) and University Malaya Postgraduate grant PV007/2012A.

University of Malaya

Finally, I am forever indebted to my husband Kamalanathan Ramachandaran, my father Paravamsivam Arasayah, my mother Kamala Periasamy, my brothers Maaran Paravamsivam and Thayalan Paravamsivam and other members of my family for their understanding, spiritual support, encouragement and providing a loving environment for me. Without them I wouldn't have the strength and support to complete my research.

University of Malaya

TABLE OF CONTENTS

University of Malaya

University of Malaya

University of Malaya

University of Malaya

LIST OF FIGURES

University of Malaya

LIST OF TABLES

University of Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Mol	Mole
ANOVA	One-way analysis of variance
α	Alpha
β	Beta
DNA	deoxyribonucleic acid
cDNA	complementary DNA
°C	degree celcius
DM	diabetes mellitus (type 2 diabetes)
dNTP	deoxynucleotide triphosphate
DMRT	Duncan Multiple Range Test
DMEM	Dulbecco's Modified Eagle's Medium
FFA	free fatty acid
γ	Gamma
g	Gram
GLUT-1	glucose transporter – 1
GLUT-4	glucose transporter – 4
H_2O_2	hydrogen peroxide
IBMX	3-isobutyl -1-methylxanthine
IDDM	insulin dependent diabetes mellitus
Kg	Kilogram
L	Litre
Mm	*Mus musculus*
mg	Milligram
mg/l	Milligram per litre
mg/ml	Milligram per millilitre
ml	Millilitre
µg	Microgram
µg/ml	microgram per millilitre
µl	Microliter
µM	Micromolar
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NCBI	National Centre for Biotechnology Information
NCD	Non-communicable disease
2-NBDG	2- (N-(7-Nitrobenz -2 – oxa – 1,3 – diazol – 4 – yl) Amino) - 2 – Deoxyglucose
NIDDM	non-insulin dependent diabetes mellitus
O_2	Oxygen
PCR	polymerase chain reaction
%	Percentage
±	plus-minus
PBS	phosphate buffer saline
P. giganteus	*Pleurotus giganteus*
PPAR-γ	peroxisome proliferator activated receptor - γ
RNA	ribonucleic acid
RT	reverse transcription
SREBP-1c	sterol regulatory binding protein- 1c
TAG	Triacylglycerol

University of Malaya

LIST OF APPENDICES

University of Malaya

CHAPTER 1

INTRODUCTION

One of the growing major health threats that human face today is the non-communicable disease (NCD) which kills more than 36 million people every year. Non-communicable disease is a non-contagious disease which continues for extensive duration of time and generally progresses slowly (WHO, 2013). Nearly 80% of NCD deaths occur in low and middle income countries. In Malaysia, NCD contribute to an estimated 73% of total death with cardiovascular diseases being the biggest contributor. It has been reported that, at least 63% of adults aged 18 years and above had at least one NCD risk factor. Diabetes mellitus (DM) is enlisted as one of the four top diseases from the total risk factors (NHMS, 2015). Population studies indicated that approximately 171 million individuals aged 20 and above suffered from DM globally in year 2000 and it was estimated to escalate up to 366 million by the year 2030 (Wild *et al*., 2004). Being a middle income country, Malaysia is currently embracing a major shift in lifestyles in parallel with urbanisation which is the right element for the diabetic outburst nationwide (WHO, 2013). In the year 2000, this alarming epidemic has been forecast to be a pandemic situation around 2010 to 2015 (Zaini *et al*., 2000).

The adverse outcomes associated with DM are the macrovascular and microvascular complications. The common macrovascular complications are cardiovascular diseases, atherosclerosis, strokes and peripheral vascular disease while microvascular complications are retinopathy, nephropathy and neuropathy (Stolar and Chilton, 2003). It was reported that amongst diabetic patients, 4.3% had their lower limbs amputated, 3.4% had strokes and 1.6% were on dialysis or had kidney transplants due to DM (Letchuman *et al*., 2010).

University of Malaya

The three major types of DM are type 1, type 2 and Gestational DM. Type 1 (insulin-dependent diabetes mellitus) normally has an early onset during childhood and needs life-long treatment due to deficient insulin production. Type 2, (Non-insulin dependent diabetes mellitus) is a chronic disease, which generally develops in later stages of life due to insulin resistance or insufficient production by β-cells in the islets of Langerhans (Matthaei *et al*., 2000). Type 2 is the most common form of the disease, accounting for 90-95% of cases reported (Li *et al*., 2004). Gestational DM occurs in non-diabetic pregnant women but normally resolves after delivery of the baby. There are many reasons for the occurrence of DM and this may vary depending on the types of diabetes.

Generally, it is a complex integration between genetic, metabolic framework, physical activities, eating habits and socio-cultural factors (Narayan *et al*., 2000). Genes (heredity) is a root cause for DM and one is genetically susceptible if one has a family history of DM compared to others. Besides heredity factors, obesity is also an important risk factor for diabetes especially type 2 because excessive fat stored will interfere with the body's ability to utilize insulin effectively and thus lead to high blood glucose level (Kahn, 2000). The modern life-style with declining wholesome nutritious food has also resulted in increasing rates of type 2 diabetes (Vinicor, 1998).

The combination of insulin resistance and obesity increases the risk of cardiovascular diseases and other obesity related disorders. Insulin resistance causes pancreatic cells to secrete more insulin to normalize the glycemic level when adipocytes show decreased insulin-sensitivity (Wilcox, 2005). Adipocytes are specialized cells which synthesize triglyceride during copious amount of glucose uptake. Triglycerides will be mobilized via lipolysis when there is energy discrepancy (Fonseca-Alaniz *et al*., 2007). Insulin, by binding to its receptors in adipocytes, activates tyrosine kinase signaling pathway which

University of Malaya

locates the glucose transporters resulting in the increase of glucose uptake. Glucose is stored in the form of triglyceride and excessive amount of triglycerides accumulation causes obesity. Besides, adipocytes in type 2 diabetes patients are insulin-resistant and are not capable of accumulating lipids to their fullest capacity (Anand & Chada, 2000). This implies that, adipose tissue itself offer an attractive avenue to treat complications related to DM. It has been reported that not only an excess of lipid accumulation is associated with an increased risk of type 2 diabetes, but deposition of triglyceride and free fatty acids in tissues other than adipocytes such as liver, muscle and pancreas have negative effect on insulin sensitivity which eventually leads to type 2 diabetes (Okuno *et al*., 1998; Yang *et al*., 2004; Spiegelman & Flier, 2001).

Targeting the adipocytes seems challenging because of its many overlapping functions and its integration into complex networks that are necessary for a proper alteration of energy homeostasis. The model chosen to carry out the research was the 3T3-L1 cell line. This is a preadipocyte cell line derived from disaggregated Swiss 3T3 mouse embryo cells. It is one of the preferred cell line for the study of adipocytes because 3T3-L1 preadipocytes constitute a reliable model for analysing the proliferation stage, commitment of preadipocyte to adipocyte, lipid accumulation during adipocyte differentiation and lipid mobilization. Deregulation of these processes has a great influence in the occurrence of type 2 diabetes and obesity (Amer & Ostman, 1974). These roles of adipocytes, however, are less defined in terms of the essential mechanisms involved for the treatment of DM and obesity (Nawrocki & Scherer, 2005).

This implies that, the adipocytes can be targeted to treat diseases related to abnormal function of adipocytes. Most of the commercial therapeutics stimulates anorexic signals in the central nervous system to suppress appetite or they inhibit absorption of nutrient in the intestine (Shi & Burn, 2004). Only selected therapeutics in the market interferes

University of Malaya

with adipocytes directly. Thiazolidinediones (TZDs) which is a class of peroxisome proliferator activated receptor γ (PPARγ) agonist is the most prominent example that targets the adipocyte (Nawrocki & Scherer, 2005). Some of the commonly used medicines for type 2 diabetes including insulin and rosiglitazone are known to cause adverse side effect to the host such as weight gain and therefore interest has been shifted to explore natural substances (Maregasi *et al*., 2008). The search for natural substances exhibiting potential insulinimic or insulin sensitizing properties is ongoing in the field of nutritional research. Various mushrooms have been shown to be ideal food for the potential dietetic prevention of hyperlipidemia and hyperglycemia due to high content of fibers, proteins, microelements and low fat content (Mahajna *et al*., 2008).

Mushrooms are gaining popularity in terms of health and wellness over the past decades due to their high nutritional and pharmaceutical properties. Traditionally, mushrooms with unique flavour and texture have been used as a major component of the folk medicine especially in China to promote health and longevity. Many of the mushrooms that were claimed to be curative by folk medicine contained important source of biologically active compounds and have been validated by recent scientific research (Eik *et al*., 2012). Mushrooms are being reported to contain substances which may prevent or reduce the severity of life threatening diseases such as cancer, cerebral stroke and heart disease (Wasser & Weis, 1999). Mushrooms have been reported to contain natural compounds (β-glucans) that show anti-hyperglycemic, anti-hypertriglyceridemic, anti-hypercholestrolemic, anti-inflammation and anti-arteriosclerotic (Kim *et al*., 2005; Kanagasabapathy *et al*., 2012b).

Pleurotus species are widely cultivated and consumed in the world and they are generally called oyster mushrooms. The fruiting body of this genus has nice texture and a pleasant flavor which makes it a popular edible mushroom worldwide (Khan , 2010).

University of Malaya

Over the years, it has been reported that *Pleurotus* spp is an important genus of edible mushroom and it is a good source of bioactive compounds such as polysaccharides, fatty acids and statins with hypoglycemic as well as hypocholestrolemic properties, dietary fibers, minerals and vitamins (Li *et al*., 2011). *Pleurotus* spp is gaining recognition because they are well known for the high nutritional value and therapeutic properties. Some species of *Pleurotus* mushrooms have shown hypoglycemic activities in both experimental animals and human subjects. Figure 1.1 shows *Pleurotus giganteus* (Berk) Karunarathna & K.D. Hyde which is locally known as 'cendawan seri pagi' (morning glory mushroom). It is one of the largest edible mushrooms and has been treated as special food since ancient times (Udugama & Wickramaratna, 1991). In Malaysia *P. giganteus* is consumed by the indigenous people (Lee *et al*., 2009).

The objectives of this study were to:

1) Investigate the effect of *Pleurotus giganteus* (fruiting bodies) extracts on proliferation of 3T3-L1 cell line.
2) Investigate the effect of *Pleurotus giganteus* (fruiting bodies) extracts on insulin-like properties namely lipogenic and glucose uptake ability in 3T3-L1 cell line.
3) Investigate the effect of *Pleurotus giganteus* (fruiting bodies) extracts on lipid mobilisation in differentiated 3T3-L1 cell line.
4) Investigate the molecular mechanism (gene expression) of *Pleurotus giganteus* (fruiting bodies) extract's action on 3T3-L1 cell line.
5) Investigating the connection between adipogenesis and glucose metabolism using 3T3-L1 cell line.

University of Malaya

Figure 1.1: *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde (Jones *et al*., 2007)

University

CHAPTER 2

LITERATURE REVIEW

2.1 Traditional Complimentary Medicine

The occurrence of various types of challenges in medical world has extended the exploration of research to natural resources in search of cure. Practice of traditional complimentary medicine is common in Malaysia for minor ailment and overall well-being. The use of medicinal plants for treatment of DM dates back from the Ebers papyrus about 1550 BC (Vaidya, 2013). Before the discovery of insulin and other pharmaceutical preparations, traditional medicine mainly obtained from plants were used to treat DM. The use of herbal remedies and plant derivatives has been practiced for centuries in different systems of medicine like Ayurveda, Siddha, Unani, Naturopathy and others (Ravishankar & Shukla, 2007).

Traditional medicine comprises knowledge, skills, and practices based on theories, beliefs, and experiences indigenous to different cultures used in the maintenance of health. Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities. Traditional medicines are mainly used for therapeutic activity that refers to successful prevention, diagnosis and treatment of physical and mental illness, as well as beneficial alteration or regulation of the physical and mental status of the body. The knowledge of using the natural resources for medicinal purpose is in abundance due to multiplicity of ethnic groups with their own background of traditional healing practices and vast diversity of flora. Traditional complimentary medicine is still in practice even though the commercial drugs are readily available and widely accepted (WHO, 2013).

University of Malaya

A multitude of herbs and medicinal plants and some compounds purified from them have been studied for the treatment of DM as they may provide a basis of new synthetic antidiabetic medications with potent activity (Vats *et al*., 2002). Indeed, many of the traditional medicines that claimed to be curative have been validated by scientific research. For instance, the widely prescribed insulin-sensitizer metformin was derived from guanidine, a molecule isolated from *Galega officinalis L*. (French lilac) (Bailey & Day, 2004). Devising and implementing sound science is crucial in order to preserve the social value of research and to identify the level of evidence and the potential effects of traditional medicine practice on human health outcomes. International collaborative research on herbal medicine, discussing scientific validity, using appropriate outcome measures and determining appropriate study designs are some of the efforts can be executed to bring herbs and medicinal plant studies to a next level (Tilburt & Kaptchuk, 2008).

Mushrooms have been known as an extraordinary therapeutic agents in traditional folk medicines in addition to its culinary usage worldwide (Deepalakshmi & Mirunalini, 2011; Abdullah *et al*., 2012). Mushrooms had been used as therapeutic agent against many life threatening diseases especially in countries like China, India, Japan and Korea. The traditional knowledge about medicinal properties of mushrooms is most notable, where the application of mushrooms to maintain health was recorded as early as 100AD in China (Cimerman, 1999). Yet, it was only in 1960 that scientists investigated the basic active principles of mushrooms which are health promoting. The use of mushroom in health care began with treating simple and age old common diseases like skin disease to present day life threatening disease such as cancer (Jiskani, 2001).

University of Malaya

2.2 Medicinal mushrooms

Mushrooms have long been known by mankind for their flavour and texture. In this millenia, they have become increasingly attractive not only for their nutritional properties but for their medicinal properties too. About 900 species are known to possess potential therapeutic agents that may ensure wellness of humans (Mahajna *et al*., 2009). In Malaysia, wild mushrooms are collected from the forests, rubber and oil palm plantations as well as paddy fields by indigenous people for their culinary and medicinal properties. The commonly encountered wild edible species include *Schizophyllum commune* Fries (Figure 2.1A), *Lentinus squarrosulus* Mont and *Auricularia spp*. (Figure 2.1B) and *Termitomyces heimii* Natarajan (Figure 2.1C).

Mushrooms are recognised as an important source of biologically active compounds of both high and low molecular weight metabolites (Silva *et al*., 2012). Mushrooms have markedly been reported to contain substances which may prevent or heal life threatening diseases such as cancer, cerebral stroke and heart disease as well as reduced risk of blood cholesterol, improve hypolipidemic, hypoglycemic and hypotensive actions, antibacterial, antidiabetic, antioxidant activities and other disease conditions (Wasser & Weis, 1999). Nowadays mushrooms are widely being incorporated in daily diet including soup, health tonic, tea, pickles and herbal formulas (Cimerman, 1999). Furthermore, fresh mushrooms have been used as food and food – flavouring materials in soups and sauces for centuries.

The search for natural substances from mushroom exhibiting potential lipid mobilising and or insulin-like properties is ongoing in the field of nutritional research. Various mushrooms have been shown to be ideal food for the potential dietetic prevention of hyperlipidemia and hyperglycemia due to high content of fibers, proteins, microelements and low fat content. Mushrooms produce many different non-starch

University of Malaya

polysaccharide components, especially β glucans (Sobieralski *et al*., 2012). These polysaccharides are sometimes found to stimulate the immune system in humans which stop the proliferation of cancer cells. Mushrooms such as *Auricularia spp*, *Tremella fuciformis*, *Lentinula edodes* and *Grifola frondosa* have been used for thousands of years for the treatment of various diseases. These mushrooms have been widely used in China as culinary mushrooms and are also known for their pharmaceutical effects in herbal medicine (Byung *et al*., 2002; Kim *et al*., 2001). In addition, recently accumulated evidences have shown that culinary-medicinal mushrooms have integral role in the prevention of many age-related neurological dysfunctions as well, namely Alzheimer's and Parkinson's disease (Phan *et al*., 2015). Mushrooms have been reported to have hypoglycemic as well as hypolipidemic properties (Yuan *et al*., 1998). Apart from that, obesity has received significant attention due to its ability to promote cardiovascular disease and cancer. Several studies have reported the role of the mushrooms in reducing obesity.

For instance, effect of *Auricularia* spp on the serum and liver lipids as well as on the faecal excretion of bile acids and neutral steroids in rats was studied. In another study, it was found that mushroom has the ability to lower plasma total cholesterol levels in rats (Cheung, 1996). However, to date, very few detailed investigation with regards to the effect of the mushrooms on lipid metabolism in adipose tissue has been done.

University of Malaya

Figure 2.1: Selected edible mushrooms encountered in the wild (Jones *et al*., 2007)

A: *Schizophyllum commune* Fries.

B: *Auricularia spp*.

C: *Termitomyces heimii* Natarajan

2.3 *Pleurotus* spp

Origin

The oyster mushrooms are amongst the most popular edible mushrooms.

Genus: Pleurotus

Family: Pleurotaceae

Order: Agaricales

Oyster mushroom is one of the most widely cultivated and consumed edible mushroom in the world and they are generally called oyster mushroom. Oyster mushroom belonging to the genus *Pleurotus* (Quel) Fr., tribe *Lentineae Fayod*, family *Polyporaceae* (Fr.) Fr., is widely distributed throughout the Northern Hemisphere, such as Europe, North Africa, Asia and North America (OECD, 2006). To date, approximately 70 species of *Pleurotus* mushrooms have been recorded and new species are discovered more or less frequently although some of these are considered identical to previously recognised species. *Pleurotus* mushrooms commonly grow on broad-leaf hardwood in the spring and fall, especially cottonwoods, oaks, alders, maples, aspens, ash, beech, birch, elm, poplars and willows, being its favoured natural selective habitats (Stamets, 1993). *Pleurotus* spp grow within a temperature range of 15° to 30°C.

The commonly cultivated *Pleurotus* species include *P. sajor caju, P. cystidiosus, P. eryngii and P. tuberregium*. The fruiting body of this genus has a nice texture and a pleasant flavour that makes them available all over the world especially in Asia. Some of the commonly available and locally grown *Pleurotus* spp in Malaysia include *P. florida* (Figure 2.2A), *P. sajor-caju* (Fr) Singer, (Figure 2.2B), *P. citinopileatus* (Singer) (Figure 2.2C), *P. flabellatus* (Berk & Broome)Sacc. (Figure 2.2D) and *P.cystidiosus*

University of Malaya

(Figure 2.2E). *Pleurotus* spp has notable place in nutraceutical science as they are rich nutritionally and possess possible medicinal uses, especially as anticancer (Martin *et al*., 2010), anti-cardiovascular disease (Alam *et al*., 2009), anti-diabetes (Hu *et al*., 2006a), antioxidant (Khan *et al*., 2010) and hepatoprotective (Wong *et al*., 2012). Moreover, these mushrooms may provide significant support against malnutrition as well due to their high nutritional values. Therefore, regular consumption of *Pleurotus spp.* can play integral role in health and disease prevention. However, more clinical researches are suggested for the evaluation and validation of medicinal benefits of *Pleurotus* mushrooms.

Previous study showed that the oral administration of *Pleurotus ostreatus* in experimental rats had blood glucose-lowering effect in both insulin-dependent and insulin-independent diabetic condition (Chorváthová *et al*., 1992; Krishna *et al*., 2009). In another study, Hu *et al*. (2006a) reported that blood glucose lowering effect was observed in rats with streptozotocin-induced diabetes which were treated with water soluble polysaccharides extracted from *P. citrinopileatus*. Meanwhile, *P. eryngii* improved insulin sensitivity and enhanced antihyperglycemic and antihyperlipidemic effects in db/db mice fed by 5% extract (Kim *et al*., 2010). According to Badole *et al*. (2008) the water extract of *Pleurotus pulmonarius* in combination with glyburide synergistically showed antihyperglycemic effect in alloxan-induced diabetic mice. In genetically diabetic mice, oral administration of 20% whole mushroom maitake powder and its chemically derived fractions prevented an increase in blood glucose levels by increasing insulin sensitivity (Mayell, 2001). Furthermore, a clinical study with a total of 120 patients randomly divided into three groups, with 40 patients in mushroom-fed group and the remaining groups with other treatments showed that feeding of *P. sajor-caju* mushroom significantly reduced fasting blood glucose level as well as blood cholesterol and HbA1c (glycosylated haemoglobin) in type 2 diabetic patients (Agrawal

University of Malaya

et al., 2010). Figure 2.2 shows the commonly available *Pleurotus* spp. mushrooms in Malaysia (Jones *et al*., 2007).

The oyster mushroom has many advantages as a cultivated mushroom:

- rapid mycelial growth
- high ability for saprophytic colonisation
- simple and inexpensive cultivation techniques
- some species are available for cultivation under different climatic conditions

Owing to these attributes, in terms of medicinal properties and flexibility in cultivation, the production and consumption of this mushroom has increased remarkably.

2.4 The fruiting bodies of *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde

Pleurotus giganteus (Berk) Karunarathna & K.D. Hyde which was originally described as *Lentinus giganteus,* is a synonym with *Panus giganteus* (Berk) Corner (Lee *et al*., 2009). It is known as 'uru paha' in Sri Lanka and 'zhudugu' in China (Karunarathna *et al*., 2011). It was one of the largest edible mushrooms which have been treated as special food since ancient times (Udugama & Wickramaratna, 1991). Confusion in the nomenclature of the mushroom was eventually solved by transferring *Lentinus giganteus* to *Pleurotus giganteus* based on the molecular evidence (Karunarathna *et al*., 2011). In Malaysia, *P. giganteus* is consumed by the indigenous people (Lee *et al*., 2009). It is locally known as 'Cendawan Seri Pagi' (morning glory mushroom). Currently, this mushroom a variety from China is commercially cultivated. Cultivation of this mushroom though new to Malaysia, has been very popular in Northern Thailand (Karunarathna *et al*., 2011) and China (Wong *et al*., 2012).

University of Malaya

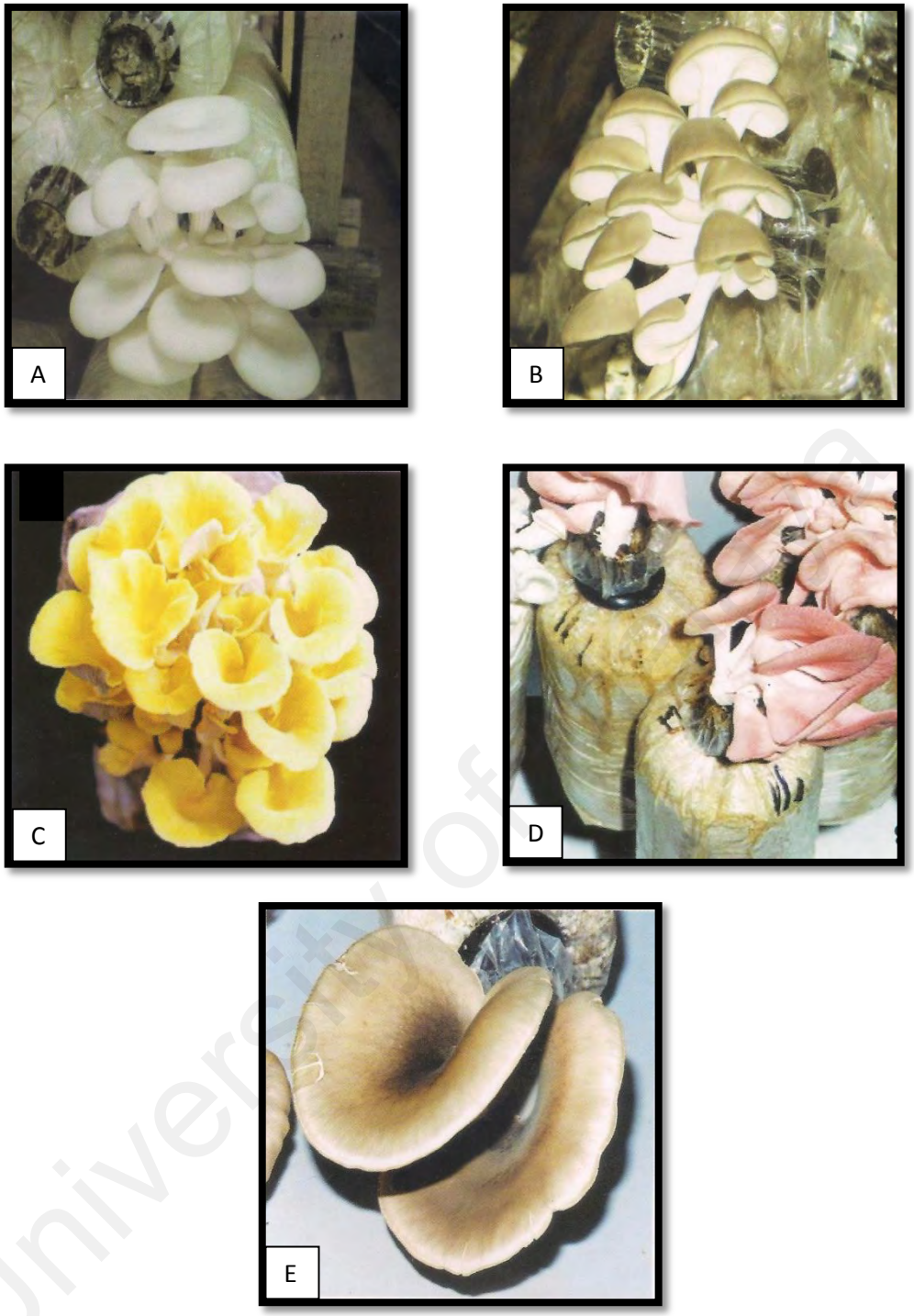


Figure 2.2: Commonly available *Pleurotus* spp. mushrooms in Malaysia. *P. florida* (A), *P. sajor-caju* (Fr) Singer (B), *P. citinopileatus* (Singer) (C), *P. flabellatus* (Berk & Broome) Sacc (D) *and P. cystidiosus* (E) (Jones *et al*., 2007)

University of Malaya

Various investigations have been carried out lately to evaluate the medicinal properties of *P. giganteus* both *in vivo* and *in vitro*. Phan *et al*. (2012) have reported that *P. giganteus* contained carbohydrate, dietary fiber, potassium and phenolic compounds. Aqueous extract of *P. giganteus* had significantly ($p<0.05$) stimulated neurite outgrowth activity (Phan *et al*., 2012) and hepatoprotective effect on rats (Wong *et al*., 2012). Aqueous and ethanol extracts of *P. giganteus* have shown antioxidant and genoprotection properties (unpublished data). In the present study, hot water and methanol extracts as well as hexane, ethyl acetate and butanol fractions of the fruiting bodies of *P. giganteus* were investigated for insulin-like properties for possible use as an alternative for treatment of type 2 diabetes.

Limited or no information is available concerning lipid mobilising and or insulin like properties of *P. giganteus* in adipose tissue. Further comprehensive chemical and pharmacological investigations are needed to clarify the exact mechanism of hypoglycemic effects before they can be developed for preventive and therapeutic purposes to improve the hyperglycemic status in type 2 diabetes. In the present study, extracts of the fruiting bodies of *P. giganteus* are used to investigate insulin – like properties as an alternative approach for treatment of type 2 diabetes.

2.5 Diabetes Mellitus

Diabetes mellitus (DM) is one of the world's oldest known ancient diseases and is described by the World Health Organization (WHO, 2013) as a metabolic disorder characterised by chronic hyperglycemia (excessive hepatic glycogenolysis and glyconeogenesis) due to reduced insulin secretion, insulin action or both. The prevalence of DM is rapidly increasing worldwide as well as in Malaysia (NHMS, 2015). One critical problem of DM is that it does not show any serious symptom and

University of Malaya

causes serious complications such as coronary heart disease (Silva *et al*., 2012). There are three major types of DM which include type 1, 2 and 3.

2.5.1 History of Diabetes Mellitus

The term diabetes is derived from the Greek word '**diabainein**' which means the extreme production of urine as the major symptom of DM. In 1675, Thomas Wills added the term Mellitus which means sweet taste in Latin. Matthew Dobson proved that the sweetness in urine was due to excessive output of sugar in the urine of people with DM (Dobson, 1776). The pathogenesis of DM was experimentally understood only in the 1900s. In 1910, it was discovered that the lack of a particular 'secretion' produced by the pancreas led to the development of DM and this 'secretion' was referred to as insulin (Patlak, 2002).

2.5.2 Type 1 Diabetes Mellitus

Type 1 diabetes which accounts for 5 – 10% of DM is an insulin-dependent diabetes mellitus (IDDM). It occurs due to the absolute deficiency of insulin, most often in children and young adults. This is an autoimmune disease in which the immune system targets the β-cells of the endocrine pancreas and results in the loss of insulin production. The disease disturbs glucose homeostasis and is reflected as 'hyperglycemia'. Type 1 is also known as childhood or juvenile DM as most people develop it at childhood. For treating type 1 diabetic patients, efforts are being made to transplant pancreas or insulin producing β-cells (NIDDK, 2013). To date, the most reliable cure has been daily injections of insulin for controlling blood glucose level. There are also rapid-acting insulin analogues in the market, which mimic the insulin secretion profile of the body, gradually replacing the conventional insulin (Garber, 2006).

University of Malaya

2.5.3 Type 2 Diabetes Mellitus

Type 2 diabetes is a non - insulin dependent diabetes mellitus (NIDDM), which comprises 90 - 95% of all DM cases (ADA, 2009). Type 2 diabetes is an important preventable disease, in which the body does not produce enough insulin or respond to insulin. In this type, insulin target tissues do not respond to insulin. This is described as 'insulin resistance'. The development of insulin resistance is a complex and progressive event. Previous studies have shown that various molecular mechanisms can lead to insulin resistance. Defects related to insulin receptor such as incomplete processing of insulin receptor, decrease in number of insulin receptor on the cell surface, auto-antibodies to the insulin receptor or decreased kinase activity of insulin receptor have been implicated in insulin resistance. Other events like increased insulin degradation or defective downstream signalling from insulin receptor also result in insulin resistance. Deficiency in translocation of glut 4 to the plasma membrane is another crucial factor of insulin resistance (James & Piper, 1994).

Insulin activates its signalling pathway by binding to its receptors in the target tissue which then suppress the glucose release from liver and kidney as well as the translocation of glucose transporters in muscle and adipose tissue which increase the glucose uptake and inhibit the release of free fatty acid (FFA) into the blood circulation. Insulin inhibits lipolysis caused by epinephrine and stimulates lipogenesis and glucose uptake in adipocytes (DeFronzo, 2013). During insulin resistance, pancreatic β cells respond to excess glucose in blood circulation by secreting more insulin to maintain normal glycemia and to overcome the decreased ability of some tissues to respond to insulin. However, interruption occurs between this pancreatic β cells and the peripheral tissue causing an increase in insulin secretion to compensate for the reduced insulin sensitivity (Akehi *et al*., 2008). This phenomena, however causes hyperinsulinemia

University of Malaya

when target tissues show decreased insulin sensitivity. In long term, the β-cells will slowly damage and insulin production will cease (ADA, 2009).

2.5.4 Gestational Diabetes Mellitus

Gestational diabetes mellitus develops in pregnant women. During pregnancy the need for insulin increases and gestational diabetes mellitus occurs at later stages of pregnancy. It is very similar to type 2 diabetes since there is defect in insulin secretion and responsiveness. Even though it is fully treatable, approximately 20 - 50% of affected women may develop type 2 diabetes later in their life (Akehi *et al*., 2008).

2.5.5 Current treatment for Diabetes Mellitus

Globally plenty of resources are spent for medicines, diets, physical training and so on since the complications of DM rising towards high death rate. Current DM therapies focus on controlling and lowering blood glucose to a normal level (Miller *et al*., 2014). The mechanisms of currently available antidiabetic agents include stimulation of β-cell of pancreatic islet to release insulin, resist the hormones which rise blood glucose, increase the number or rise the affinity and sensitivity of insulin receptor site to insulin, enhance the use of glucose in the tissue and organ, inactivate free radicals, resist lipid peroxidation and correct the metabolic disorder of lipid and protein as well as enhance the microcirculation in the body (Li *et al*., 2004). In Zhao *et al*'s study (as cited in Li, Zheng, Bukuru & Kimpe, 2004) based on these mechanisms, the drugs clinically used to treat DM are divided into insulin, insulin-secretagogues, insulin-sensitivity improvement factor, insulin-like growth factor, aldose reductase inhibitor, α-glucosidase inhibitor and protein glycation inhibitor respectively mentioned. Table 2.1 shows oral agents used to treat type 2 diabetes. These drugs are likely to cause adverse side effects to the host such as hypoglycemia, lactic acid intoxication, weight gain and gastrointestinal upset and hence interest has been diverted to natural substances

University of Malaya

(Maregasi *et al*., 2008). The search for natural substances with lipid mobilizing potential and or insulinemic properties is ongoing in the field of nutritional research.

2.6 Adipose tissue - a major player in metabolism

2.6.1 Adipose tissue structure

As shown in Figure 2.3, adipose tissue is constructed of different components including; adipocytes, connective tissue matrix, nerve tissue, stromovascular cells and immune cells. Adipose tissue is specialised and perfectly adapted for lipid metabolism which provides main storage site for excess energy in the form of triglycerides. Adipocytes synthesise triglyceride during abundant food supply and mobilise them via lipolysis when there is energy deficit (Fonseca-Alaniz *et al*., 2007). On average, there is about 15kg of adipose tissue in a man who weighs about 70kg (Shen *et al*., 2003). Adipocytes regulate the amount of stored fat in adipose tissue through the mechanisms of lipogenesis and lipolysis. Besides its storage function, adipose tissue is highly crucial for its mechanical functions as well, such as insulation and protection against mechanical forces. Moreover, it was discovered that adipose tissue also functions as an endocrine organ, with the discovery of leptin which is secreted from adipose tissue and have systemic signaling capacity (Zhang *et al*., 1994).

2.6.2 Lipogenesis

Lipogenesis is stimulated by insulin during the fed state in adipocytes (Fig 2.4). The uptake of glucose is increased via the recruitment of glucose transporters to the plasma membrane (Saltiel & Kahn, 2001). In the cytosol, glucose enters into glycolysis, an intermediate metabolite, dihydroxyacetone-phosphate is converted to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase (GPDH) which serves as the backbone of TAG.

University of Malaya

Table 2.1: Oral agents to treat type 2 diabetes

Primary action	Class	Agent	Side effects	Precautions
Increases insulin production in pancreas	Sulfonylureas (1st generation)	Tolbutamide (Ornase™)	Hypoglycaemia, weight gain, hyperinsulinemia, disulfiram reaction with alcohol	Chlorpropamide remains active for up to 60 hours. Use extreme caution with elderly patients or patients with hepatic or renal dysfunction
		Tolazamide (Tolinase™)		
		Chlorpropamide (Diabenese™)		
	Sulfonylureas (2nd generation)	Glyburide (Micronase™, Glynase™, Diabeta™)	Hypoglycaemia, weight gain, hyperinsulinemia	Clearance may be diminished in patients with hepatic or renal impairment
		Glipizide (Glucotrol, Glucotrol XL™)		
		Glimepiride (Amaryl™)		
Primarily decreases hepatic glucose production. Minor increase in muscle glucose uptake which may improve insulin resistance	Biguanide	Metformin (Fortamet™, Glumetza™, Glucophage™)	Nausea, diarrhoea, metallic taste, possible lactic acidosis	Due to increased risk of lactic acidosis, should not use if suspect frequent alcohol use, liver or kidney disease, or CHF
Decreases insulin resistance, increasing glucose uptake, fat redistribution; minor decrease in hepatic glucose output; preserves β-cell function; decreases vascular inflammation	Thiazolidinedione	Rosiglitazone (Avandia™)	Minor weight increase of 3-6 lbs., oedema	Should not use in patients with CHF or hepatic disease. Can cause mild to moderate oedema
		Pioglitazone (Actos™)	Minor weight increase of 3-6 lbs., oedema	
Slows absorption of complex carbohydrate from GI tract.	Alpha-glucosidase inhibitor	Acarbose (Precose™) Miglitol (Glyset™)	Gas and bloating, sometimes diarrhoea for both drugs.	Should not be used if GI disorders are concurrent.

University of Malaya

Table 2.1: (continued)

Primary action	Class	Agent	Side effects	Precautions
Decreases hepatic glucose production and increases insulin secretion	Sulfonylureas and Biguanide	Glucovance™ (Glyburide and Metformin)	Hypoglycemia, weight gain, lactic acidosis.	Should not be used if suspect frequent alcohol use, liver or kidney use or CHF
		Metaglip™ (Glipizide and Metformin)		
Decreases hepatic glucose production, increases glucose uptake, decreases insulin resistance, and preserves β-cell function	Thiazolidinedione and Biguanide	Avandamet™ (Rosiglitazone and Metformin)	Edema, possible lactic acidosis	Should not be used if suspect frequent alcohol use, liver or kidney use or CHF
Decreases hepatic glucose production, increases glucose uptake, decreases insulin		Actoplus Met ™ (Pioglitazone and Metformin)	Same caveats as individual components	Same caveats as individual components
Decreases insulin resistance and increases insulin secretion	Thiazolidinedione and Sulfonylureas	Avandaryl ™ (Rosiglitazone and Glimepiride)		

(Centers for Disease Control and Prevention, 2007)

University of Malaya

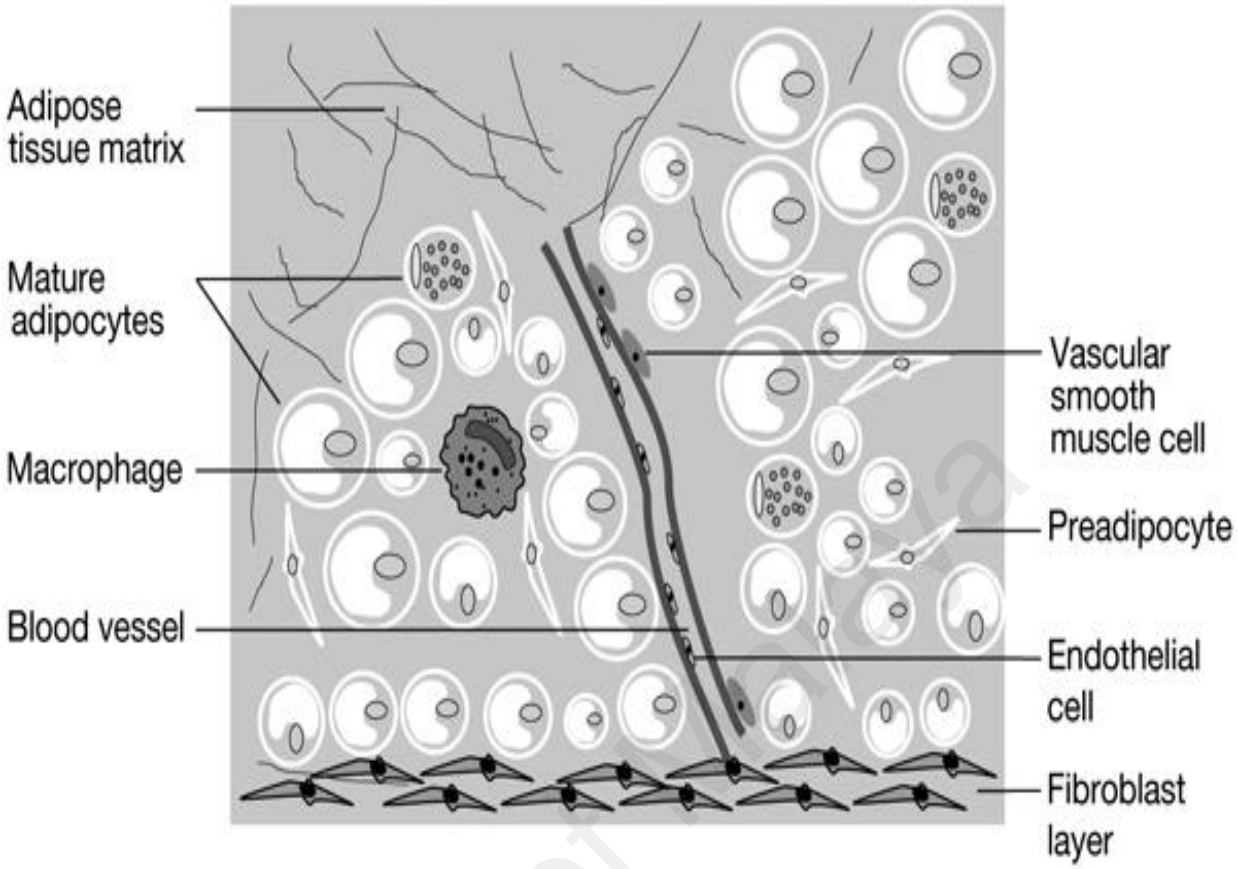


Figure 2.3: Cellular compartments of the adipose tissue (Schäffler *et al*., 2005)

In addition, acetyl-coA is used by adipocytes for *de-novo* fatty acid synthesis via acetyl-coA carboxylase (ACC) and the multifunctional enzyme fatty acid synthase (FAS) (Stoops *et al*., 1975). TAG is incorporated into lipid droplets, which form at the ER and are covered with PAT family proteins (perilipin, adipose differentiation and tail-interacting protein) (Martin & Parton, 2006; Bickel *et al*., 2009).

As mentioned earlier, besides uptake of glucose, adipose tissue stores and manages fatty acids circulating in the blood. Several membrane proteins are also associated in their uptake such as fatty acid transport protein 1 (FATP1) and CD36, especially for long-chain fatty acids (Wilsie *et al*., 2005). Intracellularly, fatty acids are transported by fatty acid binding proteins (FABPs). After entering the cells, fatty acids are esterified by acyl-coA-synthetase and used for triacylglyceride (TAG) synthesis on the endoplasmic reticulum (Coleman & Lee, 2004). These anabolic pathways are activated by insulin

University of Malaya

which in turn induces the major transcription factor for fatty acid and triglyceride synthesis, sterol regulatory element binding protein 1c (SREBP-1c). Figure 2.4 shows the regulation of lipogenesis in adipocytes.

2.6.3 Lipolysis

Stored triglyceride is hydrolysed to yield non-esterified fatty acids (NEFAs) and glycerol during lipolysis. This process is activated in adipose tissues during fasting and regulated by the hormones noradrenaline and insulin. As shown in Figure 2.5, the binding of noradrenaline to β-adrenergic receptors, coupled to adenylate cyclase via the stimulatory Gs-protein, leads to the production of cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA). PKA subsequently phosphorylates hormone sensitive lipase (HSL) and perilipins, the target proteins. The active HSL migrates to lipid droplet surface and cleaves the first fatty acid from TG yielding diglyceride (DG). Diglyceride lipase then cleaves the second fatty acid from DG to yield monoglyceride and cleaves monoglyceride lipase (MG), yielding fatty acid and glycerol (Yin *et al*., 2003). The non-esterified fatty acid and glycerol are exported to muscle and liver respectively. Insulin antagonises lipolysis by reducing PKA activity via reduction in cAMP levels, mediated via activation of phophodiesterase 3B (PDE3B) **(**Duncan *et al*., 2007). Figure 2.5 shows hormonal control of adipocyte lipolysis.

2.6.4 Glucose uptake

Glucose is a universal energy source in most organisms. Glucose metabolism is a process which converts glucose into energy in the form of adenosine triphosphate (ATP) and it is the primary source of energy and biomaterials for the maintenance of cell homeostasis. In humans, glucose is absorbed by the intestines and then into the blood.

University of Malaya

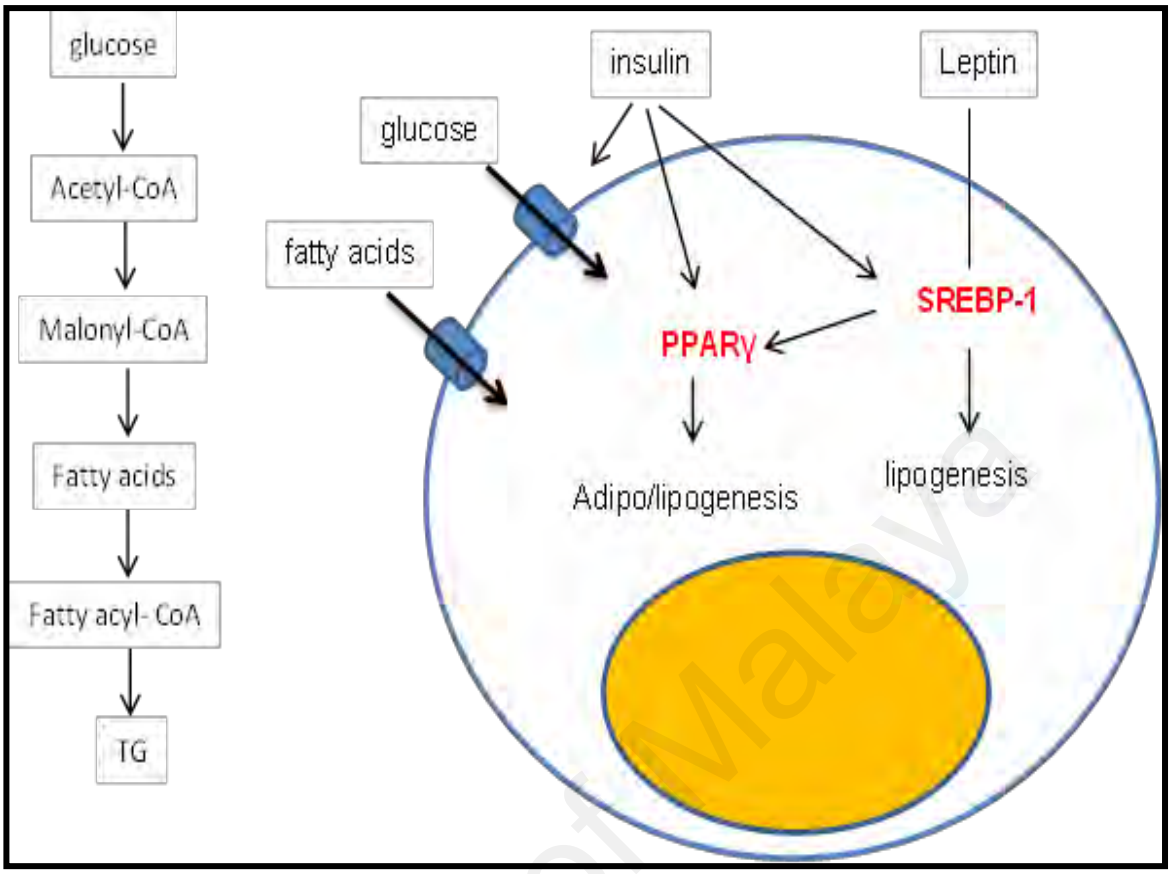


Figure 2.4: Regulation of lipogenesis in adipocytes (Kersten., 2001).

PPARγ: peroxisome proliferator-activated receptor gamma
SREBP-1: sterol regulatory element binding protein-1
TG: triglyceride

Extra glucose is stored in the muscles and liver as glycogen and triglyceride in adipocytes which is hydrolysed to glucose and released into the blood when needed. Blood glucose levels must be maintained within homeostatic levels to ensure optimal supply of glucose for normal cell function and survival. Glucose uptake in cells is achieved by the action of glucose transporters, which facilitate glucose movement down a concentration gradient, in contrast to energy-dependent uptake of glucose in the gut or kidney (Leney & Tavare, 2009).

University of Malaya

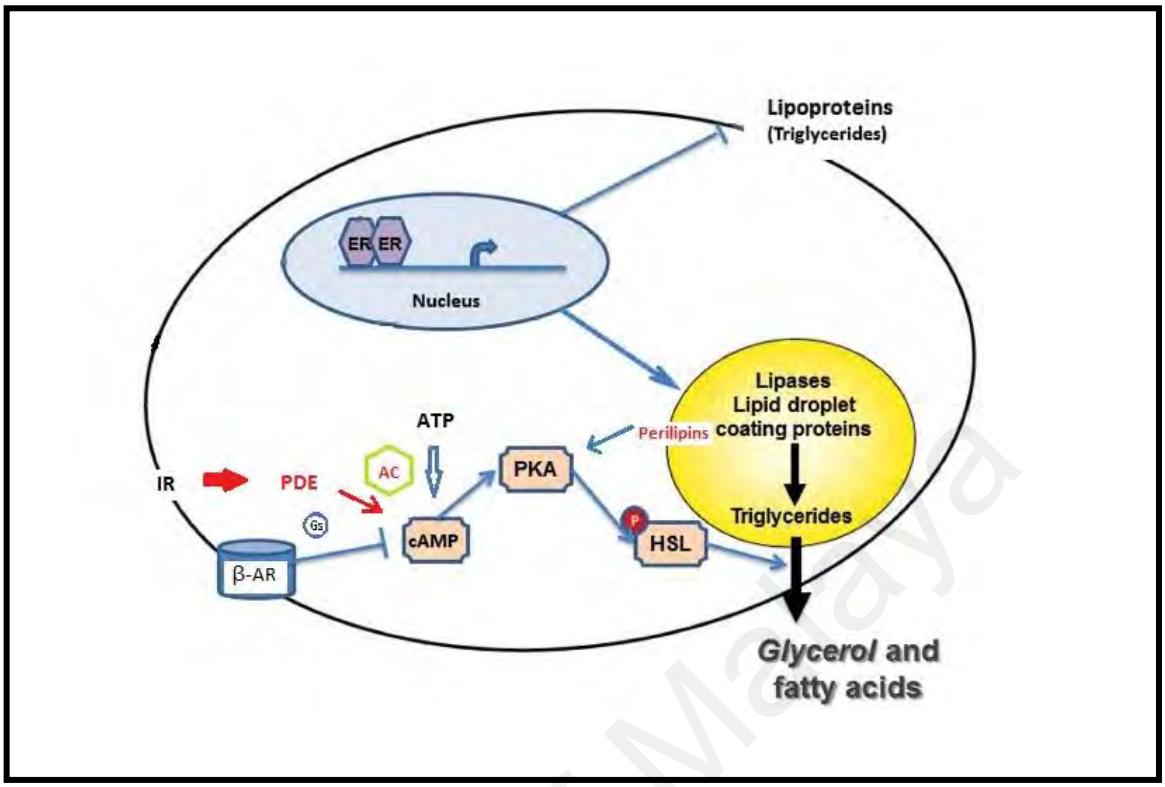


Figure 2.5: Hormonal control of adipocyte lipolysis (Morimoto *et al*., 2000)

ER : endoplasmic reticulum
AC : adenylate cyclase
PKA : protein kinase A
HSL : hormone sensitive lipase
β-AR : beta adrenergic receptors
cAMP: cyclic adenosine monophosphate
ATP : adenosine triphosphate
Gs : G protein coupled receptor
IR : Insulin receptor
PDE : phosphodiesterase

2.6.5 Adipogenic genes

Adipocyte differentiation which is known as adipogenesis or lipogenesis, is the process of fat cell development, accompanied by coordinated changes in cell physiology, hormone sensitivity and expression of adipogenic genes (Moreno-Navarrete & Fernández-Real, 2012). Confluent 3T3-L1 preadipocytes can be induced to differentiate into adipocytes when exposed to the appropriate differentiation stimulating cocktail including insulin at a non-physiologically high concentration, dexamethasone and 3-isobutyl-1-methylxanthine. Upon induction, the process of differentiation of

University of Malaya

preadipocytes is controlled by adipogenic genes expressions. Adipogenic transcription factors, including peroxisome proliferator-activated receptor (PPARγ) and sterol regulatory element binding protein 1c (SREBP-1c) play integral role in the complex transcriptional cascade of adipogenesis. The PPARγ is part of a superfamily of nuclear receptors. It is highly expressed in adipose tissues and stimulates transcription of many specific adipocyte genes that is critical in initial steps of adipogenesis. Generally, the antidiabetic agents, thiazolidinediones (TZD) compounds activate PPARγ. PPARγ is an important ligand-dependent nuclear receptor transcription factor that plays an important role in converting adipofibroblasts, fibroblasts, or preadipocytes into differentiated adipocytes. PPARγ plays integral role in many biological processes such as cellular conversion/differentiation, insulin sensitivity, type-2 diabetes, atherosclerosis, and cancer (Rosen & Spiegelman, 2001). Besides that, several other genes that are responsible for insulin signalling cascade and glucose and lipid metabolism, are also regulated as a result of PPARγ activation in mature adipocytes.

The SREBP protein is a transcription factor with important roles in adipogenesis, insulin sensitivity and fatty acid homeostasis. It also increases the transcriptional activity of PPARγ, raising the number of cells in the process of differentiation. SREBP is a transcription factor which is capable of mediating some of the effects of the hormone on insulin target genes. Abundance of SREBP-1c is found in the adipose tissue and liver, both of which are insulin sensitive. Besides that, SREBP-2, another SREBP isoform that is encoded by a separate gene is more selective in activating genes involved in cholesterol homeostasis where as SREBP-1c is involved in lipid synthesis and glucose metabolism (Le Lay, 2002).

The specific transporter proteins called the glut proteins (glut 1 and glut 4) are controlled by insulin. The attachment of ligand bound insulin on insulin receptor

University of Malaya

substrates of adipocytes leads to phosphorylation of insulin receptor substrates (IRS). The IR is a transmembrane receptor, composed of two α-subunits, and two β-subunits linked by disulphide bonds. The binding of insulin to the receptor causes conformational change in the β-subunits which promotes tyrosine autophosphorylation of the β-subunits of the IR (Lee *et al*., 2014). Mechanistically the insulin-dependent tyrosine phosphorylation of IRS proteins generates docking sites for the src homology 2 (SH2) - domain containing downstream effector phosphatidylinositol 3'-kinase (PI3K) (Fröjdö *et al*., 2009). PI3K pathway is the major pathway activated by insulin receptor (IR). It also induces glucose uptake, glycogen synthesis, cell growth and cell differentiation as well as anti-apoptotic effect. When docked, PI3K is positioned to phosphorylate the lipid phosphatidylinositol 4, 5-bisphosphate (PIP_2) a reaction that produces phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3) a second messenger that stimulates phosphoinositide-dependent protein kinase 1 (PDK). PIP_3 recruits Akt (also known as protein kinase B) to the cell membrane. This is followed by the phosphorylation of both Akt and Thr^{308} which is mediated by PDK 1 and GSK3α (Chang *et al*., 2004). Thus, the phosphorylated Akt facilitates glucose uptake in adipocytes by allowing the translocation of Glut 4 located in intracellular storage vesicles to the plasma membrane (Shepherd *et al*., 1998). Figure 2.6 shows PI3K pathway activation by active insulin receptor.

In parallel, insulin stimulates glucose transport in adipocytes via the regulated translocation of vesicles containing the glucose transporter to the plasma membrane. There are four Class 1 glucose transporters (1-4). Glut 1 is expressed in adipose and muscle tissue, which are the insulin responsive sites for glucose disposal. It was reported by Perrini *et al*. (2004) that in 3T3-L1 adipocytes, the pool of glut 1 is located in intracellular membranes and insulin stimulates its translocation to the plasma membrane. However, glut 1 is responsible for basal glucose uptake whereby its

University of Malaya

redistribution to the plasma membrane is modestly stimulated by insulin. Glut 1 is expressed in both preadipocytes and adipocytes and its expression level does not change during adipocyte differentiation.

In contrast, glut 4 is a high affinity glucose transporter that is expressed abundantly in adipose tissue and it is highly insulin-responsive. Glut 4 is only expressed in adipocytes and its expression is also regulated by PPARγ (Herman & Kahn, 2006; Fonseca-alaniz, 2007). The translocation of Glut 4 to the plasma membranes of adipocytes is an early metabolic event that happens after cell differentiation and after the activation of PPARγ. Glut 1 and Glut 4 in 3T3-L1 adipocytes are qualitatively similar in their response to insulin and in their subcellular distribution. Nevertheless, the fold increase in glut 4 (10-20 fold increase) at the cell surface in response to insulin is several time higher than that of glut 1 (2-4 fold increase) (Pessin *et al*., 1999).

2.6.6 3T3-L1 Cell Line

Many models and techniques are being used in order to evaluate and understand adipocyte biology (Poulos *et al*., 2010). The model chosen to perform the experiments of this study was the 3T3-L1 cell line. 3T3-L1 cell line is the most used model and constitute a reliable and suitable model for analysing the proliferation stage, commitment of preadipocyte to adipocyte and lipid accumulation during adipocyte differentiation (Kuppusamy *et al*., 1994). After hormonal treatment these cells can differentiate and acquire morphological and biochemical characteristics of adipocytes. 3T3-L1 is a pre-adipose cell line which was originated from clonal expansion of murine Swiss 3T3 cells and contains only a single cell type (Green & Kehinde, 1974).

University of Malaya

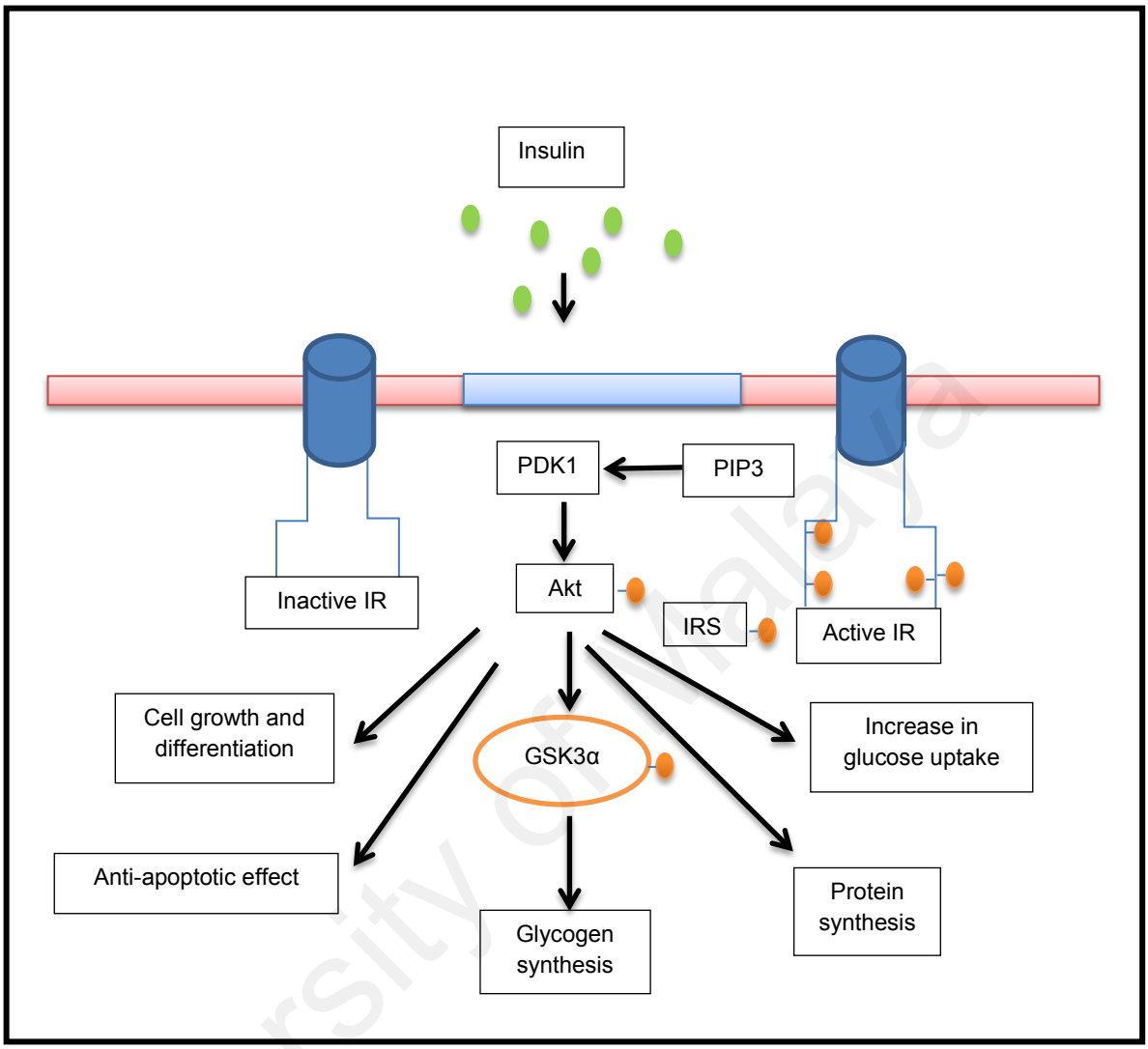


Figure 2.6: PI3K pathway, the major pathway activated by insulin receptor (IR) (Sussman *et al.*, 2011)

PDK = phosphoinositide-dependent protein kinase

Akt = protein kinase B

GSK3α = Glycogen synthase kinase 3 alpha

IRS = insulin receptor substrates

PIP3 = phosphatidylinositol 3, 4, 5-trisphosphate

University of Malaya

This cell line has been used widely in more than 5000 published articles on adipogenesis and the biochemistry of adipocytes for the last 30 years, because of its potential to differentiate from fibroblast to complete adipocytes. Several investigations have used 3T3-L1 cells because it helped in identifying key molecular markers including transcription factors and various pathways during pre-adipocyte differentiation (Poulos *et al*., 2010). Numerous protocols can be used to induce differentiation from preadipocytes to adipocytes, but the most commonly used agents are insulin, dexamethasone and isobutylmethylzanthine (IBMX) at concentrations of 1 ug/ml, 0.25 um and 0.5 um, respectively. Preadipocytes contain less lipid droplets accumulated, but four days after induction they start to accumulate lipids that grow in size and number over the differentiation time. Lipid metabolism in adipocytes is hugely regulated by hormonal agents such as insulin and epinephrine. Deregulation of lipid metabolism has a great influence in the occurrence of type 2 diabetes and obesity (Amer & Osman, 1974). Furthermore, the contribution of adipose tissue to lipogenesis in human is less well defined in pathological situations such as DM and obesity than in rodents. Therefore, understanding the mechanisms involved in the regulation of preadipocyte proliferation, differentiation, lipolysis as well as glucose uptake into adipocytes are essential for the treatment of type 2 diabetes and obesity.

2.6.7 Adipocyte as a drug discovery target

Adipocytes are the main storage site for excess energy in the form of triglycerides. Adipocytes are able to build network with the brain and peripheral tissues which assists food intake and energy expenditure. The presence of adipose tissue helps to sustain an insulin-sensitive state. In previous studies, development of adipose tissue was blocked at the embryonic stage of the rodent models. Such fatless mice became severely insulin-

University of Malaya

resistant with improper insulin-signaling in the liver and muscle (Moitra *et al*., 1998; Kim *et al*., 2000).

Even though diabetes and cardiovascular diseases are interrelated with fat mass, reports showed that only a few of the commercially available medications interfere with the adipose tissue directly. For example, adipocytes are targeted with chemically structured specificity by thiazolidine (TZD) a class of peroxisome proliferator activated receptor γ (PPARγ) (Nawrocki & Scherer, 2005). However, adipocytes contain complex metabolic network and overlapping functions which complicates the effort to target the adipocytes for treatment. For example, Adipokines are known as biomarkers that act as sensors of the body's metabolic status. These are hormonally active factors that influence metabolic processes such as lipid mobilisation, glucose production in the liver and glucose uptake in muscle cells. Therefore, there is avenue to explore adipocytes to develop new therapies that involves areas of adipocytes physiology and their therapeutic potential for drug development as shown in Table 2.2.

University of Malaya

Table 2.2: Areas of adipocyte physiology and their therapeutic potential drug development

Therapeutic target area	Putative drugs	Advantage	Disadvantage	Therapeutic potential
Angiogenesis within adipose tissue	Vascular endothelial growth factor (VEGF) peptide inhibitors.	Might inhibit growth of stromal tumours (e.g. breast cancer), might be effectively inducing weight loss.	Lipotoxicity; might result in hepatic steatosis and accumulation of lipids in muscle	High
Apoptosis of adipocytes		Might be effectively inducing weight loss	Might result in hepatic steatosis and accumulation of lipids in muscle	
Adiponectin administration	Recombinant protein	Acute and chronic insulin sensitisation	Requires large amounts of recombinant protein	Moderate
Leptin administration		Treatment of acquired and inherited leptin deficiency	Not useful to treat general obesity	High
Anti-inflammatory strategies in adipocytes	TZDs	Selective targeting of a major contributor of systemic inflammation	Weight gain when used in the context of PPARγ agonists	
Cholesterol synthesis within adipocytes	Statins	Might have a positive impact on signalling and the release of inflammatory cytokines	Might have a negative impact on insulin sensitivity	Unknown
Lipid metabolism	Antisense oligos against SCD1 or PTP1B	Might reduce fat mass and increase insulin sensitivity	Stability and treatment modality of antisense oligos	High
Glucocorticoid metabolism	11β-HSD1 inhibitors		Specificity: difficulty to target specifically to adipocytes	
ROS production	Antioxidants	Will improve insulin sensitivity		

(Nawrocki & Scherer, 2005)

University of Malaya

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

American Type Culture Collection, United States of America

Mouse 3T3-L1 cells line (preadipocytes)

Applied Biosystem, United States of America

High Capacity cDNA reverse transcription kit, RNAqueous® Micro kit, TaqMan® Gene Expression Master Mix, TaqMan® Gene Expression assays, MicroAmp™ optical 8-cap strip, MicroAmp™ fast 8-tube strip

Cayman Chemical Company, United States of America

Glucose Uptake Cell-Based Assay Kit

Flow Lab, Australia

Phosphate buffered saline (PBS)

ICN, United States of America

Dexamethasone (Dexa)

3-isobutyl-1-methylxanthine (IBMX)

Nas Agro Farm, Sepang, Selangor, Malaysia

Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde

Sigma-Aldrich, United States of America

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Minimum Essential Media (MEM), Dimethyl sulfoxide (DMSO), L-Glutamine, Penicillin, Streptomycin, Oil Red O powder

Thermo Fisher Scientific, Gibco, United Kingdom

Insulin

Zenbio, United States of America

Lipolysis kit

University of Malaya

3.2 Sample preparation

3.2.1 Preparation of fruiting bodies

Fruiting bodies of *P. giganteus* was obtained from a local farmer in Nas Agro Farm, Sepang, Malaysia. The identity of the commercially available *P. giganteus* was verified by experts at Mushroom Research Centre, University of Malaya, Kuala Lumpur, Malaysia. Mushrooms were sliced and freeze-dried for three days. The freeze dried mushrooms were then ground into a fine powder using a Waring blender. The powdered mushroom was kept at -20°C until further analysis.

3.2.2 Hot water extraction

To prepare the aqueous extract, 200 g of mushroom powder was weighed and soaked in distilled water (pH 7) at a ratio of 1:10 overnight at 27°C in a shaker. The next day, the soaked mushroom powder was double boiled in a water bath for 30 minutes. The boiled mushroom was left to cool and filtered using Whatman filter paper. The supernatant was freeze-dried and kept at -20°C until further analysis (Wong *et al*., 2011).

3.2.3 Methanol extraction

Mushroom powder (200 g) was weighed and soaked in methanol: water (8:2) for two days at 27°C in a shaker. The methanol-aqueous layer was then filtered using Whatman filter paper. The supernatant was evaporated using a rotary evaporator until the extract dried completely and kept at -20°C until further analysis (Wong *et al*., 2012).

3.2.4 Fractionation of methanol extracts

The methanol-aqueous supernatant was further partitioned with hexane at (1:1). The hexane soluble layer was filtered and evaporated. The hexane insoluble fraction was further fractionated with a mixture of ethyl acetate and water (1:1). Evaporation of the ethyl acetate layer gave rise to ethyl acetate fraction (Kanagasabapathy *et al*., 2011). The aqueous fraction was further extracted with n-butanol to yield butanol extract.

University of Malaya

3.3 Cell culture

The 3T3-L1 preadipocyte cell line was obtained from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin and streptomycin and 1% (v/v) of L-glutamine from Sigma respectively at 37°C in a humidified 5% CO_2 incubator.

University of Malaya

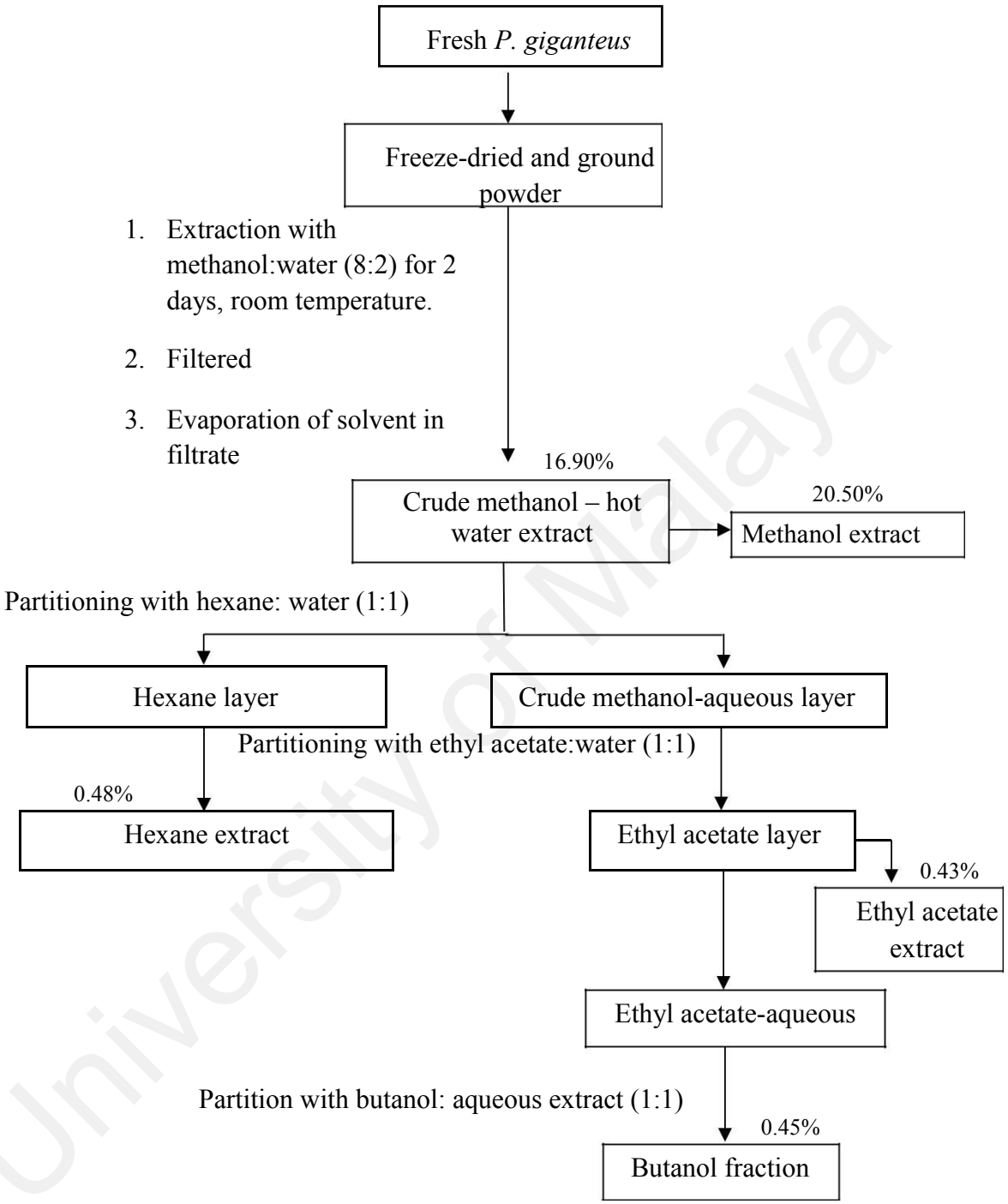


Figure 3.1: Flow chart showing yield of extracts and fractions (%) obtained after extraction and fractionation of freeze dried fruiting bodies of *P. giganteus*

University of Malaya

The medium was changed every three to four days. The cells were cultured in 75 cm^2 cell culture flask at a density of $3x10^3$ cells per cm^2. Prior to 70% confluence, the cells were harvested from culture flask using 0.25% trypsin-EDTA. Trypsin-EDTA dissolved fibronectin on the cell surface and therefore promoted cell detachment from the bottom of flask. However, prolonged trypsinisation was avoided to prevent cellular damage and death. ScepterTM Handheld Automated Cell Counter was used to count the cell.

3.3.1 Seeding of preadipocytes

Aliquots of 1 mL cell suspension were loaded into each well of a 24-well tissue culture plate with 20,000 cells per well. The cells were cultured in DMEM culture media in humidified air jacketed CO_2 incubator until the cells reached confluent state.

3.3.2 Differentiation of 3T3-L1 fibroblast cells into adipocytes

The cells were then induced to differentiate in DMEM differentiation media according to an established method (Madsen *et al*., 2003). For differentiation of 3T3-L1 fibroblast cells, confluent preadipocytes were left for post confluent stage for 48 hours (Defined as day 0). Differentiation was induced by incubating the cells with a differentiation media one (DM1) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone and 0.5 µM insulin (INS) in DMEM containing 10% (v/v) fetal bovine serum and incubated for 48 hours. On day two, the cells were cultured in differentiation media two (DM2) which is similar to DM1 but without 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone. From day two, the cells were maintained in DM2 until they were fully differentiated into mature adipocytes before using it for further studies.

As for fully matured adipocytes that were used to study adipogenic effect of mushroom extracts, the cells were induced to differentiate using DM1 as mentioned earlier. Subsequently, when the differentiation media was changed to DM2, instead of insulin, mushroom extracts were added to DM2 media at various concentrations. On day

University of Malaya

five, the media was discarded and replenished with new media. On day seven of differentiation, the media was discarded and cells were used for further analysis.

3.4 Effects of extracts and fractions of *P. giganteus* on proliferation of 3T3-L1 cells by Colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) Assay

3.4.1 Principles of MTT assay

The degree of cytotoxicity of *P. giganteus* extracts in 3T3-L1 preadipocytes was determined by the MTT assay which was carried out based on the method described by Mosmann (1983) which detects only viable cells. Viable cells reduce the dye (MTT) which is a yellow coloured tetrazolium salt to form insoluble purple formazan crystals. Formazan crystals can be solubilized by isopropanol and the amount of formazan formed is directly proportional to the number of viable cells. The colour formation can then be quantified using an Elisa reader. The inhibition or proliferation of the cells was calculated.

3.4.2 MTT assay

Preadipocytes of 3T3-L1 cells were seeded in 96-well tissue culture plate of 0.37 cm^2 growth area at a density of 1 X 10^3 cells per well; allowed to attach for 24 hours prior to the proliferation assay. The preadipocytes were incubated with various concentrations of *P. giganteus* extracts such as methanol, hexane, ethyl acetate, butanol and aqueous extracts. All the extracts were diluted using DMSO. After 48 hours of incubation at 37°C, MTT solution was added into each well. After four hours of incubation with MTT solution, the culture medium and MTT solution was carefully removed from the wells using fine pipette to ensure formazan formed was not aspirated along with the medium. Dimethyl sulfoxide (100%) was added into each well and the plate was kept on a shaker for five minutes to make sure all formazan crystals were dissolved. Finally, absorbance

University of Malaya

was measured using a microplate reader at 570 nm to obtain the percentage of viable cells. Cell viability, expressed as a percentage, was defined as the ratio of absorbance of cells incubated with sample to cells incubated with control. The percentage of cell viability was denoted as 100% for the control (cells in complete growth medium with the solvent that is used to dissolve the extracts).

The percentage of cell proliferation was calculated as follows;

$$\frac{A_{570} \text{ of sample}}{A_{570} \text{ of control}} \times 100\% = \% \text{ cell proliferation}$$

3.5 Effects of extracts and fractions of *P. giganteus* on lipogenesis (lipid accumulation) of 3T3-L1 cells by Oil Red O Staining

Oil Red O staining and quantitation of triglycerides content were performed as previously described (Lim *et al*., 2008) with some modifications. On day seven of differentiation, the cells in 24 well plates were rinsed by PBS twice. The cells were fixed with 10% (v/v) formaldehyde in isotonic solution for one hour. The cells were subsequently washed with PBS twice followed by 60% isopropanol. A 0.5% Oil Red O isopropanol solution was diluted with 1.5 volume of water, filtered and added to the fixed adipocyte monolayer for one hour (Lim *et al*., 2008). The Oil Red O stained cells were washed with PBS to remove unbound dye and was assessed microscopically using an Olympus CK-40 inverted microscope. The stained triglyceride droplets were extracted with isopropanol (750 ul per well) and quantified spectrophotometrically by measuring absorbance at 510 nm. The increase in percentage of adipogenesis of treated cells compared to the control was calculated as follow;

$$\% \text{ Increase in Adipogenesis} = \frac{A_{510nm} \text{ (sample assayed)}}{A_{510nm} \text{(Control)}} \times 100\%$$

University of Malaya

3.6 Effects of extracts and fractions of *P. giganteus* on glucose uptake of 3T3-L1 cells by fluorescence assay

3.6.1 Principles of glucose uptake based fluorescence assay

Caymen's Glucose Uptake Cell-based Assay kit provides a convenient tool for studying modulators of cellular glucose uptake. The kit employs 2-NBDG, a fluorescently-labelled deoxyglucose analog, as a probe for the detection of glucose taken up by cultured cells. Apigenin, a flavonoid that has been reported to be an inhibitor of glucose transport, is included as a control.

3.6.2 Measurement of glucose uptake

Cells were plated into 24-well plate at a density of 20,000 cells per well and incubated until the cells were fully differentiated into matured adipocytes (as mentioned in Methodology Section 3.3.2). The cells were treated with various concentrations of *P. giganteus* extracts, rosiglitazone (positive control) and DMSO (negative control) on day two. On day five, supernatant was discarded and the cells were treated with experimental compounds or vehicle control in glucose free culture medium containing 150 µg/ml 2-NBDG (fluorescently-labeled deoxyglucose analog). Apigenin was used as a positive control and it was diluted 1: 1000 in the culture medium as per instruction of assay kit protocol. After three hours of treatment, glucose uptake assay was performed according to the protocol of the assay kit. The amount of 2-NBDG taken up by cells was measured at wavelengths usually designed to detect fluorescein (excitation/emission = 485/535) (Manaharan *et al*., 2013).

University of Malaya

3.7 Effect of extracts and fractions of *P. giganteus* on lipid-mobilization (lipolysis) of 3T3-L1 cells by non-estrified free fatty acid and glycerol quantification kit

3.7.1 Principles of non-esterified free fatty acid and glycerol quantification kit

Lipolysis plays a central role in the regulation of energy balance. Lipolysis is a process in which triglyceride are hydrolysed into glycerol and free fatty acids. This process releases fatty acids into the bloodstream. The assessment of lipolytic activity is investigated through a coupled reaction to measure non-esterified fatty acids and glycerol released by adipocytes. The glycerol and free fatty acids can be quantified by a series of chemical modifications to yield an equal amount of hydrogen peroxide (H_2O_2) to glycerol or fatty acids which is measurable once modified into chromatic compounds. The concentration of glycerol or fatty acids detected is directly proportional to the increase in optical density.

3.7.2 Quantification of lipolysis

Cells were plated into 96-well plate at a density of 20,000 cells per well and incubated until the cells were fully differentiated into matured adipocytes (as mentioned in Methodology section 3.3.2). The cells were treated with various concentrations of *P. giganteus* extracts. Isoprotrenol was used as positive control meanwhile DMSO which was used to dissolve mushroom extracts was used as negative control. After four hours of treatment, lipolysis assay was performed according to the protocol of the assay kit. The absorbance reading was read at 550 nm using microplate reader. Two parameters were measured, namely glycerol and free fatty acids (FFA). A standard curve of glycerol and fatty acids was plotted. The fold of induction was calculated as stipulated below (Kanagasabapathy *et al*., 2012a):

$$\text{Fold Induction} = \frac{\text{glycerol / or FFA}_{\text{sample}}}{\text{glycerol / or FFA}_{\text{control}}}$$

University of Malaya

3.8 Assessment of gene expression using real-time reverse transcription polymerases chain reaction (Real-Time RT-PCR)

3.8.1 RNA extraction

Preadipocytes were seeded into 25 cm^2 culture flask containing 5 ml growth media. Preadipocytes were differentiated into adipocytes using the method described in Methodology section 3.3.2. The fully differentiated adipocytes were treated with ethyl acetate extract of 100 µg/ml. Insulin 10 µg/ml final concentration was used as positive control meanwhile DMSO 0.9% was used as negative control. After five days of incubation, the total RNA was isolated from the adipocytes using Ambion-RNAqueous Micro® kit. Briefly, the cells were lysed with lysis solution that disrupts cell membranes but capable of protecting the RNA from endogenous RNases. The cells were then homogenized by draining them through a small-bore needle syringe several times. The homogenate was then mixed with ethanol and centrifuged through a microfilter cartridge supplied with a silica-based membrane that selectively binds RNA. The impurities were removed by specific washing step and total RNA was eluted through an elution cartridge. Homogenates were kept ice-cold to prevent RNase activity. Purified RNA was used immediately for reverse transcription. (Kanagasabapathy *et al*., 2012a)

3.8.2 RNA purity & integrity

The purity of recovered total RNA was estimated by calculating the ratio of absorbance reading of 260 nm and 280 nm. Purified RNA with A_{260}/A_{280} ratio between 1.8 to 2.0 was further used to synthesize complementary DNA (cDNA) by polymerase chain reaction (PCR) approach. The concentration of RNA was calculated by the following formula:

$$\text{Total RNA (µg/ml)} = A_{260} \text{ X } 40 \text{ µg/ml X dilution factor X volume (ml)}$$

University of Malaya

The integrity of RNA was estimated using Agilent 2100 Bioanalyzer (Applied Biosystem, USA). RNA samples with RIN value of 8-10 were used in this study. (Appendix, Page 92-94)

Figure 3.2: Chip is filled with RNA sample for the assessment of RNA integrity

3.8.3 cDNA synthesis

Complementary DNA (cDNA) was synthesized by using the purified RNA. High capacity cDNA Reverse Transcription Kit was used for the reverse transcription (RT) of total RNA to single-stranded cDNA. RNA sample (18 µl) was mixed with 22 µl of High capacity cDNA Reverse Transcriptase which contained all the reagents (RT buffer, dNTP mix, random primers, Multiscribe reverse^{TM} transcriptase enzyme and nuclease free water). The mixture was mixed properly and short-spinned to bring down the contents and eliminate air bubbles in the tubes. The mixture was loaded into a thermal cycler (Eppendorf, USA) under optimized thermal cycling conditions as stipulated in Table 3.1.

University of Malaya

Table 3.1 Optimised thermal cycling conditions

Step	1	2	3	4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 sec	HOLD

3.8.4 Real-time RT-PCR

Reverse transcription PCR based assays are the common method used for confirming gene expression levels. High sensitivity and rapid real time quantification of fluorescence amplicon are made possible by using fluorogenic DNA binding chemicals (SyberDyes®) and fluorescence probes (TaqMan®). TaqMan® probes only bind to the specific complementary sequences within the target gene whereas SyberDyes® intercalate with any double stranded DNA. Therefore, TaqMan® probe was used in real-time PCR. A reporter dye and a quencher moiety at respective 5' and 3' ends were used to design the probe. Due to the closeness of the reporter and quencher, the fluorescence of the reporter is quenched. The probes are designed to anneal to target sequence within the forward and reverse PCR primers. During the extension cycle, the Taq polymerase with intrinsic 5' to 3' nuclease activity cleaves the reporter dye from the probe. Once released, the reporter emits the fluorescence signal. The generated signal is proportional to the amount of amplicon in each cycle of amplification; therefore the relative amount of template can be determined.

Reaction set up for all TaqMan® Gene Expression assay was performed according to the reaction set up instructions generated by the StepOne software (Ver 2.0, Applied Biosystem). TaqMan® Gene Expression Master Mix and assay mix consisting cDNA were prepared separately. TaqMan® Gene Expression Master Mix contains AmpliTaq Gold® DNA Polymerase (Ultra Pure), Uracil-DNA glycosylase, dNTPs (with dUTP),

University of Malaya

ROX™ Passive Reference and optimized buffer components. Assay Mix contains target primer. Reaction mix was prepared by mixing both Master Mix and assay mix in triplicate, whereby sterile ultrapure water was used for no template control reactions (NTC) or isolated cDNA for test. Endogenous control (also known as housekeeping genes) used in this study was eukaryotic 18S rRNA with FAM/MGB probe. All TaqMan® probe used in this investigation were labeled with FAM^{TM} reporter dye at the 5' end and a MGB quencher at the 3' end. The mixtures were then transferred into fluorescence compatible MicroAmp^{TM} Fast Reaction Tube Strips and capped with MicroAmp^{TM} Optical Cap Strips. The strips were centrifuged for about 15 sec before loading onto the real-time PCR thermal cycler (StepOnePlus^{TM} Real Time PCR System). All reagents and mixtures were kept on ice once thawed. The PCR conditions were; 1 cycle 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Table 3.2 shows the list of the genes and corresponding accession numbers investigated in this study.

General abbreviation of genes selected for this study and corresponding assay ID and accession number was obtained from Applied Biosystems website and NCBI database. Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan® probe mix. Accession number with 'Mm' is referred to as *'Mus musculus'*. All gene Expression Assay kits indicated are FAM/MGB probed.

University of Malaya

Table 3.2: Genes and corresponding accession number and assay ID

No	Gene name and abbreviation	Assay ID	Accession number
1	Srebp	srebf1 Mm00550338_m1	NM_011480.3
2	Ppar	ppar$_\gamma$ Mm01184322_m1	NM_011146.3
3	glut 1	slc2a1 Mm_00441480_m1	NM_011400.3
4	glut 4	slc2a4 Mm_00436615_m1	NM_009204.2
5	PI3K	pik3r1 Mm_01282781_m1	NM001024955.2

3.8.5 Calculations

Comparative C_T method or $2^{-\Delta\Delta CT}$ method was used for quantification in this study. C_T value refers to the number of amplification cycles required for a significant increase in the reporter's fluorescence and it is referred to as threshold cycle. The passive reference dye, 6-carboxy-X-rhodamine (ROX^{TM}) was used in all real time PCR reactions to normalize fluorescent fluctuations and to compensate well-to-well variations including volume and concentration differences. The relative expression of the investigated genes was normalized with the endogenous control (18S rRNA). C_T values calculated are means of triplicate measurements. The calculations and formulas involved were as follows;

University of Malaya

$C_{T\ Target} - C_{T\ Endogenous\ control} = \Delta C_T$

$\Delta C_{T\ Sample} - \Delta C_{T\ calibrator} = \Delta\Delta C_T$

Relative fold change = $2^{-\Delta\Delta CT}$

Target = gene of interest

Endogenous control = a gene that is present at a stable amount in total RNA despite experimental conditions (18s rRNA).

Sample = treated sample

Calibrator = untreated sample or control

In this study, relative fold change lower than one has shown downregulated gene expression and relative fold change higher than one has been expressed as gene upregulation.

University of Malaya

CHAPTER 4

RESULTS AND DISCUSSION

Fresh fruiting bodies of *P. giganteus* were obtained from Nas Agro Farm, Sepang, Malaysia. The fruiting bodies (2 kg) were washed, sliced and freeze dried for 2-3 days. Once it has completely dried, the dried fruiting body was ground into fine powder using a blender. The ground sample was soaked in respective solvents as mentioned in methodology session 3.2. Fine powder of *P. giganteus* (50 g) was double-boiled, filtered and freeze-dried to produce 8.45 g of yellowish aqueous extract. On the other hand, to isolate the extracts into fractions, 200 g of finely powdered *P. giganteus* was soaked in a mixture of methanol and water at a ratio of 8:2. The soluble methanol layer was then separated and evaporated to obtain dark brown viscous methanol extract. The insoluble methanol-aqueous layer was further partitioned with a mixture of hexane and water at the ratio 1:1. The mixture formed soluble hexane layer and insoluble hexane-aqueous layer. Both hexane soluble and hexane-aqueous layers were filtered and evaporated to obtain yellowish brown and dark brown viscous extract respectively. Hexane-aqueous layer was further partitioned with a mixture of ethyl acetate and water at a ratio (1:1). A dark yellowish brown ethyl acetate soluble fraction was obtained after filtration and evaporation. Ethyl acetate-aqueous layer was partitioned further with a mixture of butanol and water at a ratio 1:1 to obtain dark brown viscous butanol fraction. Table 4.1 shows the yield of all *P. giganteus* extracts and fractions obtained after extraction and fractionation processes of freeze dried fruiting bodies.

University of Malaya

Table 4.1: Yield of all extracts and fractions of *P. giganteus* obtained after extraction and fractionation of freeze dried fruiting bodies.

Extracts/fractions	Weight (g)	Yield (%)
Aqueous	33.80	16.90
Methanol	40.92	20.50
Hexane	0.96	0.48
Ethyl acetate	0.86	0.43
Butanol	0.90	0.45

4.1 Effects of extracts and fractions of *P. giganteus* on proliferation of 3T3-L1 cells

MTT assay was performed after the 3T3-L1 preadipocyte cells were incubated with various extracts and fractions of *P. giganteus* for 48 hours. The cells were incubated in hot aqueous extract, methanol extract, hexane fraction, ethyl acetate fraction and butanol fraction of *P. giganteus* at varying concentrations.

Overall, the 3T3-L1 cells incubated in hot aqueous extract exhibited the highest percentage of proliferation at all concentrations. This was followed by butanol fraction, methanol extract, ethyl acetate and hexane fraction in descending order. Overall, the lowest proliferation was observed in 3T3-L1 cells incubated in hexane fraction. At 100 μg/ml of hexane fraction, the proliferation was significantly ($p < 0.05$) the lowest with 65.80% compared to control.

University of Malaya

As shown in Figure 4.1, the preadipocytes incubated with methanol extract and butanol fraction of *P. giganteus* showed a dose-dependent increase in proliferation. Among all concentrations tested, the preadipocytes incubated with 100 µg/ml of methanol extract and butanol fraction showed the highest ($p < 0.05$) percentage of proliferation with 114.00 ± 1.8% and 152.40 ± 4.2% respectively. The proliferation of preadipocytes incubated with butanol fraction was significantly ($p < 0.05$) high at all concentrations tested compared to control.

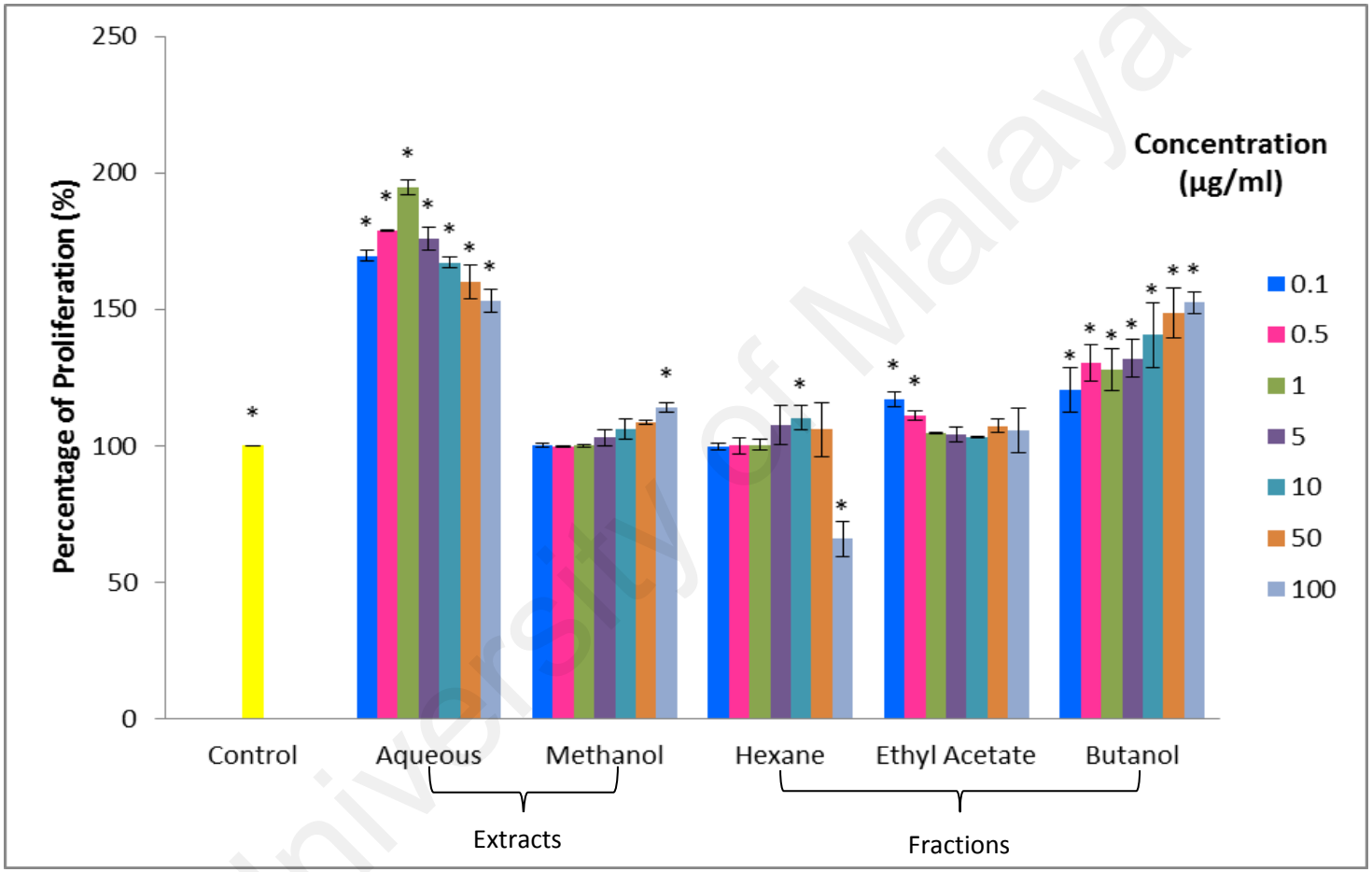
The preadipocytes incubated in aqueous extract shows the highest percentage of proliferation compared to other extracts and fractions, however the percentage of proliferation reduced dose dependently. The highest proliferation of 194.80 ± 2.8% was exhibited at one µg/ml and the lowest proliferation of 152.99 ± 4.1% was exhibited at 100 µg/ml. When the preadipocytes were incubated with hexane fraction, the highest proliferation of 110.30 ± 4.5% was caused by 10 µg/ml. As shown in Figure 4.1, the proliferation of preadipocytes incubated with ethyl acetate fraction showed proliferation of 117.00 ± 2.7% and 111.10 ± 1.6% at 0.1 and 0.5 µg/ml respectively. The proliferations of the cells were not significant at higher concentrations of ethyl acetate fractions.

Nutritional analysis that was conducted by Phan *et al*. (2012) had shown the presence of potassium in *P. giganteus*. It can be hypothesized that the proliferation obtained by the preadipocytes treated with hot aqueous extract, methanol and butanol fractions could be due to the presence of potassium. It has been demonstrated that potassium channels may be necessary for the normal proliferation and differentiation of adipocytes (Pappone & Ortiz-Miranda, 1993). The chemical constituents of hot aqueous and methanol extracts as well as butanol fraction of *P. giganteus* that are responsible for the proliferation of preadipocytes have not been explored yet. The hexane fraction of *P.*

University of Malaya

giganteus at 100 µg/ml suppressed proliferation of 3T3-L1 cells and this could be due to the presence of tyrosol and/or tyrosol derivatives. It has been reported that these compounds can be cytotoxic compounds which cause the arresting of cell cycle progression via the inhibition of DNA replication (Ahn *et al*., 2008). Ethyl acetate fraction of *P. giganteus* had shown mild proliferation at higher concentrations and this could be due to the presence of palmitate compounds, a product of fatty acid synthesis. Previous studies have reported that the preadipocytes in contact with fatty acids have demonstrated inclined inflammatory response that may have induced apoptosis of preadipocytes (Dordevic *et al.*, 2014; Shao-Ling *et al.*, 2009). The presence of palmitate compounds in the ethyl acetate extract may have caused inflammation and caused stress by modulating intracellular signal (Guo *et al.*, 2007). However, at lower concentrations, ethyl acetate fraction promoted the proliferation of preadipocytes because possibly due to the co-presence of other unsaturated fatty acids such as linoleic acid which may counteract the effect of palmitate (Wang *et al*., 2009). Moreover it is highly important to moderate the proliferation of preadipocytes otherwise preadipocytes can proliferate throughout life and increase the fat mass in the body. The increase in the mass of adipose tissue arises through the increase in cell size, an increase in cell number or both. Therefore the reduction of preadipocyte proliferation had been a target for developing anti-obesity agents. However little is explored about the mechanism of apoptosis in adipocytes to date (Nawrocki & Scherer, 2005).

University of Malaya



University of Malaya

Figure 4.1: The effect of hot water extract, methanol extract, hexane fraction, ethyl acetate fraction and butanol fraction of *P. giganteus* on the proliferation of 3T3-L1 preadipocytes. The mean absorbance of negative control (cells with medium only) was designated as 100%. Each value represents the mean ± SD and n=3; Values designated by (*) indicates significant ($p < 0.05$) difference compared to negative control.

4.2 Effects of extracts and fractions of *P. giganteus* on insulinemic activity of 3T3-L1 cells by lipogenesis (lipid accumulation) assay

After determining the proliferation range of the extracts and fractions via MTT Assay, the lipogenic activity of 3T3-L1 cells was determined by incubating the differentiation-induced matured adipocytes with the extracts and fractions of *P. giganteus.* Overall, methanol extract, hexane, ethyl acetate and butanol fractions have enhanced the lipogenic activity of the adipocytes except hot water extract. However, the lipogenic activity of the adipocytes have been significantly lower compared to positive control for all the extracts at all concentrations except the one incubated with 100 µg/ml of ethyl acetate fraction.

Figure 4.2 shows that lipogenic activity of 3T3-L1 adipocytes treated with hot water extract of *P. giganteus* was lower compared to other extracts and fractions. Even though the lipogenic activity increased in ascending order of the concentration, it was significantly lower compared to control (basal medium) and positive control (Insulin 100 µg/ml).

Incubation of adipocytes with methanol extract of *P. giganteus* increased lipogenesis in a dose-dependent manner from 1 µg/ml to 100 µg/ml. At the lowest concentration of 0.1 µg/ml, the lipogenesis was lower than negative control by 2.6%. Meanwhile, at the highest concentration of 100 µg/ml, lipogenic activity of 110.70 ± 3.8% was obtained. At this concentration, the lipogenic activity of the adipocytes was significantly ($p < 0.05$) higher than negative control but lower than positive control (insulin) by 26.0%.

Adipocytes incubated with hexane fraction at 100 µg/ml, showed the highest lipogenic activity of 113.20 ± 1.6% and it was significant ($p < 0.05$) compared to

University of Malaya

negative control. However, the lipogenesis caused by hexane fraction was significantly ($p < 0.05$) lower by 23.5% compared to positive control (insulin).

Figure 4.2 shows that the lipogenic acitivity of the adipocytes incubated with 100 µg/ml of ethyl acetate fraction has been significantly ($p < 0.05$) higher with 175.30 ± 2.7% compared to negative control. When the adipocytes were compared against positive control, lipogenesis was significantly ($p < 0.05$) higher by 38.6%. However, the cells incubated in the lower concentrations of this fraction was significantly ($p < 0.05$) lower than the positive control.

The lipogenic activity of adipocytes incubated with butanol fractions showed a dose-dependent increase. The highest lipogenic activity of 112.10 ± 2.0% was observed when adipocytes were incubated with 100 µg/ml of butanol fraction. At 100 µg/ml, the lipogenic activity was significantly higher than the negative control but was significantly lower by 24.6% compared to positive control.

Generally, methanol extract, hexane, ethyl acetate and butanol fractions increased the lipogenic activity of adipocytes in a dose-dependent manner. Among the concentrations used, the highest percentage of lipogenesis was attained by the cells incubated at the highest concentration of the extracts and fractions which was 100 µg/ml.

Moroney (2012) has reported that the hexane fraction of *P. giganteus* contains oleic acid, a monounsaturated fatty acid which is able to regulate lipogenic gene expression via PPAR-mediated process. However the lipogenic activity induced by hexane fraction was not as high as insulin and this was probably due to the presence of linoleic acid, a polyunsaturated fatty acid. Previously it was reported that linoleic acid counter-acted lipogenic activity by suppressing the lipogenic and glycolytic genes (Simon *et al*., 2005). In this study, it can be proposed that the hexane fraction of *P. giganteus*

University of Malaya

moderately stimulated lipogenesis due to the co-existance of oleic acid and linoleic acid. The lipogenic activity that was induced by ethyl acetate fraction of *P. giganteus* was reported to be significantly ($p < 0.05$) higher than the insulin. Hypothetically this could be attributed to the presence of nicotinamide in this extract as reported by Moroney, (2012). Nicotinamide can stimulate the differentiation of adipocytes by increasing the expression of adipogenic genes (Bai *et al*., 2008; Hsu & Yen, 2007). Moroney (2012) had also reported the presence of saturated (palmitic acid) or monounsaturated (methyl palmitate acid, methyl stearate, methyl linoleate and methyl mandelate) fatty acids in ethyl acetate fraction of *P. giganteus*. In previous studies, these compounds have stimulated lipogenesis (Clarke *et al*., 1997).

In current study the adipocyte differentiation caused by hot water extract of *P. giganteus* was significantly ($p < 0.05$) lower compared to control and this could be due to the presence of phenolic compounds such as caffeic acid, cinnamic acid, benzoic acid, coumaric acid, hydroxyl benzoic and hydroxyl cinnamic acids (Moroney, 2012). Several reports have shown that phenolic compounds inhibited lipid accumulation in adipocytes (Hsu & Yen, 2007; Hsu & Yen, 2008; Gosmann *et al*., 2012). The lipogenic activity of butanol fraction was most probably low due to the presence of hydroxybenzoic, a phenolic acid.

4.3 Effects of extracts and fractions of *P. giganteus* on lipolytic activity of 3T3-L1 cells

Fully differentiated adipocytes with accumulated lipid droplets were incubated with the extracts and fractions of *P. giganteus* and the extent of lipid mobilization was measured as stipulated in section 3.7. Figure 4.3 shows the effect of extracts and fractions of *P. giganteus* on the free fatty acid and glycerol release of matured 3T3-L1 adipocytes.

University of Malaya

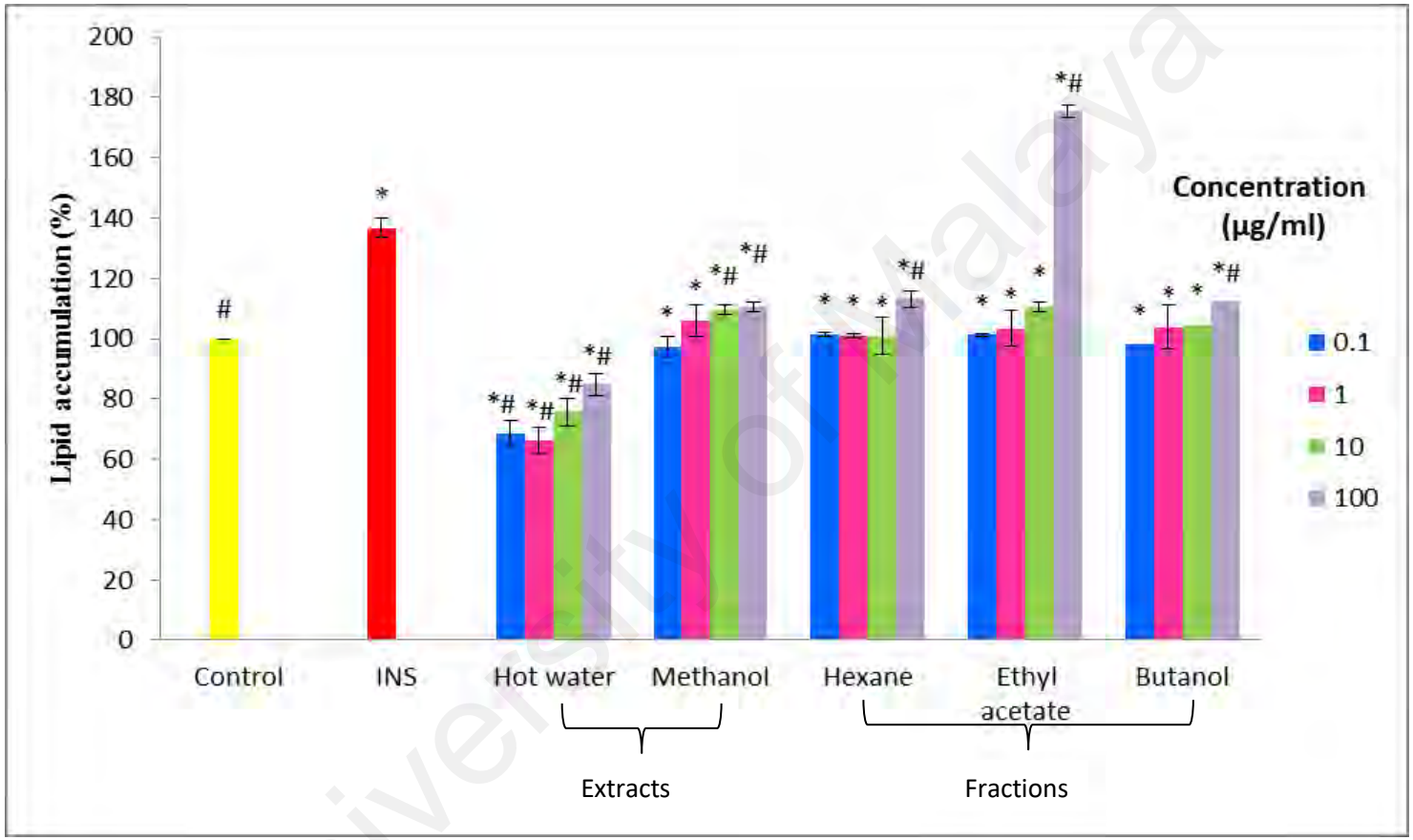


Figure 4.2: Effect of hot water extract and methanol extract, hexane fraction, ethyl acetate fraction and butanol fractions of *P. giganteus* on lipogenesis (lipid accumulation) in matured 3T3-L1 cells. Values expressed are based on lipogenesis of the 3T3-L1 cells incubated in extracts against control cells with (medium only). Each value represents the mean ± standard error and n=3; Values designated by (#) indicates significant ($p < 0.05$) difference compared to the Control (negative control). Values designated by (*) indicates significant ($p < 0.05$) difference compared to INS (positive control: insulin 100 µg/ml)

University of Malaya

The most free fatty acid release of adipocytes was observed at the highest concentrations of all the extracts and fractions of *P. giganteus*. However, no significant free fatty acid release was observed when adipocytes were incubated at lower concentrations. The free fatty acid release of the adipocytes was the highest when incubated with isoproterenol (positive control) followed by, ethyl acetate fraction, hexane fraction, hot water extract, butanol fraction and methanol extract.

The free fatty acid release caused by the ethyl acetate fraction was dose-dependent manner. The highest free fatty acid release of 3.40 fold was observed when the cells were incubated with 100 µg/ml of ethyl acetate fraction. The free fatty acid release was significantly ($p < 0.05$) higher by 2.40 fold compared to negative control. However, the effect of this fraction was significantly ($p < 0.05$) lower when compared to the positive control at all concentrations.

The free fatty acid release caused by hexane fraction was dose-dependent in ascending order. At 100 µg/ml the free fatty acid release was significantly ($p < 0.05$) higher by 1.40 fold compared to the negative control and lower by 2.00 fold compared to positive control respectively.

The adipocyte incubated with hot water and methanol extract caused mild increase in free fatty acid release only at higher concentrations when compared to negative control (basal medium). Whereas adipocytes incubated in butanol fraction had no significant ($p < 0.05$) effect on the release of free fatty acid release. Overall, the free fatty acid of all the extracts and fractions tested were significantly ($p < 0.05$) lower than isoproterenol which was the positive control.

Figure 4.3 also shows the lipolytic activity in terms of fold change of glycerol release by the adipocytes incubated with the extracts and fractions of *P. giganteus*. Overall, there

University of Malaya

was only mild release of glycerol from the adipocytes incubated with the extracts and fractions. In comparison to positive control, the glycerol release was significantly ($p < 0.05$) lower for all the extracts and fractions tested. The highest glycerol release of 1.20 fold has been reported when the adipocytes were incubated with 100 μg/ml of the ethyl acetate fraction. Meanwhile, in comparison to the positive control, glycerol release of the adipocytes has been significantly low at all concentrations of ethyl acetate fraction.

In this assay the lipolytic activity was measured by quantifying the free fatty acid and glycerol release after the accumulated triglycerides in the 3T3-L1 matured adipocytes cells were broken down. Lipolysis in adipocytes occurs at the surface of cytosolic lipid droplets whereby triglycerides that were accumulated in 3T3-L1 cells were hydrolyzed via the elevation of cyclic adenosine monophosphate (cAMP) which acts as second messenger to activate hormone sensitive lipase (HSL). Isoprotrenol, a non-specific β-adrenergic agonists is used as the positive control to confirm the lipolytic process via β-adrenergic receptors. In this study, hot water extract, hexane and ethyl acetate fractions of *P. giganteus* had significantly ($p < 0.05$) stimulated lipolytic activity. Isoprotrenol at 1000 μm, which was used as positive control, had significantly ($p < 0.05$) increased free fatty acid and glycerol release by 4.40 and 3.40 fold compared to experimental control. The lipolytic activity of hot water extract was most probably due to the presence of triterpenes (Hashim *et al*., 2011). However, the presence of phenolic acids in water extract might suppress the adipocyte lipolysis as reported by Ren *et al*. (2009). As for hexane fraction, the lipolytic activity was recorded as the highest at 100 μg/ml probably due to the presence of oleic acid and this is because the ester form of this compound was reported to enhance the basal and isoprotrenol-stimulated lipolysis by increasing the intracellular cAMP content (Fong, 1990). As reported by Kennedy (2010) the presence of linoleate compound in ethyl acetate fraction may have exhibited the highest lipolytic

University of Malaya

activity because it was reported that linoleate could enhance the lipolytic activity in 3T3-L1 cells.

4.4 Effects of extracts and fractions of *P. giganteus* on glucose uptake of 3T3-L1 cells

Subsequently, the extracts and fractions with the moderate proliferation and high lipogenesis and lipolysis activity were selected for assessment of glucose uptake activity by 3T3-L1 adipocytes. The extracts that fulfilled the above stipulated criteria were namely methanol extract, hexane and ethyl acetate fractions of *P. giganteus*. Figure 4.4 shows that the adipocytes incubated with methanol extracts caused significant ($p < 0.05$) inhibition of glucose uptake, especially at 10 μg/ml and 100 μg/ml compared to control (basal medium). At lower concentrations of 0.1 and 1 μg/ml, there was mild inhibition of glucose uptake.

As shown by Figure 4.4, the inhibition of glucose uptake was mild in adipocytes incubated with 0.1, 1 and 10 μg/ml of hexane fractions. However, at 100 μg/ml of hexane fraction, the glucose uptake activity has been significantly ($p < 0.05$) higher by 135.40 ± 12.7% compared to negative control.

The adipocytes that were incubated with ethyl acetate fraction of *P. giganteus* have shown a dose dependent increase of glucose uptake activity. The highest uptake of glucose has been obtained when the adipocytes were incubated with ethyl acetate fraction at 100 μg/ml with a glucose uptake of 136.20 ± 10.5%. In comparison to positive control, the glucose uptake activity of adipocytes incubated with ethyl acetate fraction at 100 μg/ml was higher by 3.4%.

In conclusion, ethyl acetate fraction showed a dose-dependent increase in glucose uptake activity with the highest glucose uptake activity at a concentration of 100 μg/ml.

University of Malaya

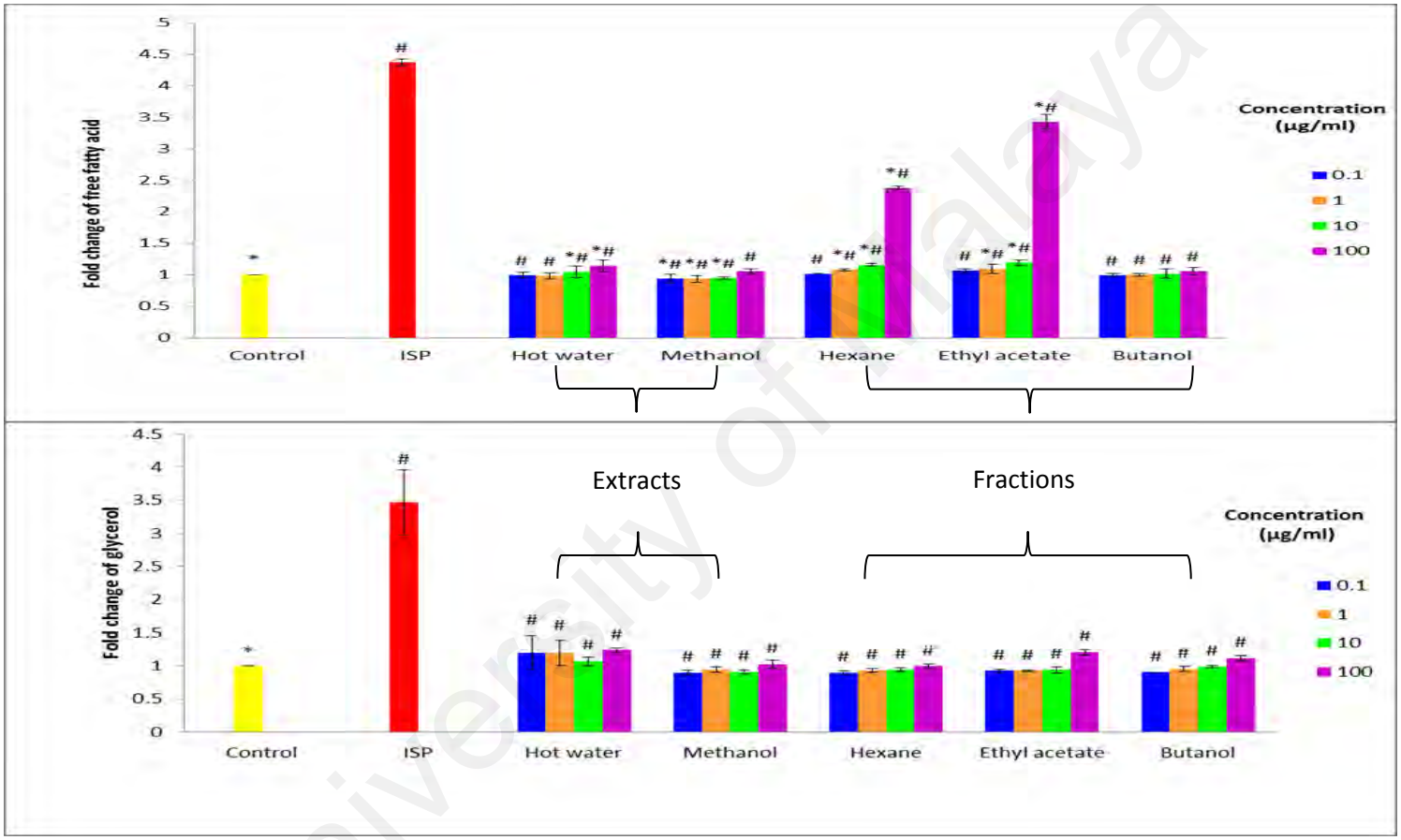


Figure 4.3: The effect of *P. giganteus* (hot water and methanol extract, hexane, ethyl acetate and butanol fraction) on the free fatty acid and glycerol release of matured 3T3-L1 adipocytes. The mean absorbance of negative control (cells with medium only) is designated as 1 fold increase. Each value represents the mean ± standard error and n=3; Values designated by (*) indicates significant ($p < 0.05$) difference compared to Control (negative control). Values designated by ($^{\#}$) indicates significant ($p < 0.05$) difference compared to ISP (positive control: isoprotrenol 100 µg/ml).

University of Malaya

Methanol extract caused inhibition of glucose uptake at all concentrations and hexane fraction showed mild increase as the concentration increased. The presence of both saturated and unsaturated fatty acids such as palmitic acid and oleic acid in the ethyl acetate fraction may have contributed to the enhanced transportation of glucose into the 3T3-L1 cells. The type of fatty acids present influences the efficiency of glucose transportation. Based on previous reports, both saturated and unsaturated fatty acids were shown to enhance the transportation of glucose (Mukherjee *et al*., 1980; Joost *et al*., 1985; Lomeo *et al*., 1986). However, Shechter *et al*. (1984), reported that unsaturated fatty acids are more effective than the saturated fatty acids. In addition to that, as reported by Moroney (2012), the presence of monounsaturated (methyl palmitate acid, methyl stearate, methyl linoleate and methyl mandelate) fatty acids in ethyl acetate fraction of *P. giganteus* could have also stimulated the transportation of glucose.

4.5 The molecular mechanism of insulin-like and or insulin sensitizing effect of ethyl acetate fraction of *P. giganteus* in 3T3-L1 cells

To further characterize the effects of ethyl acetate fraction, the fraction at 100 µg/ml was selected to be tested upon adipocyte differentiation. Quantitative real-time PCR was used to examine the expression of adipocyte transcriptional regulators, the signal molecules and the glucose transporters.

Thus this part of the study, attempts to increase the understanding of how ethyl acetate fraction possibly regulates the underlying mechanism responsible for adipocyte differentiation, glucose uptake and lipogenesis. Rosiglitazone 1 µM was used as a positive control.

University of Malaya

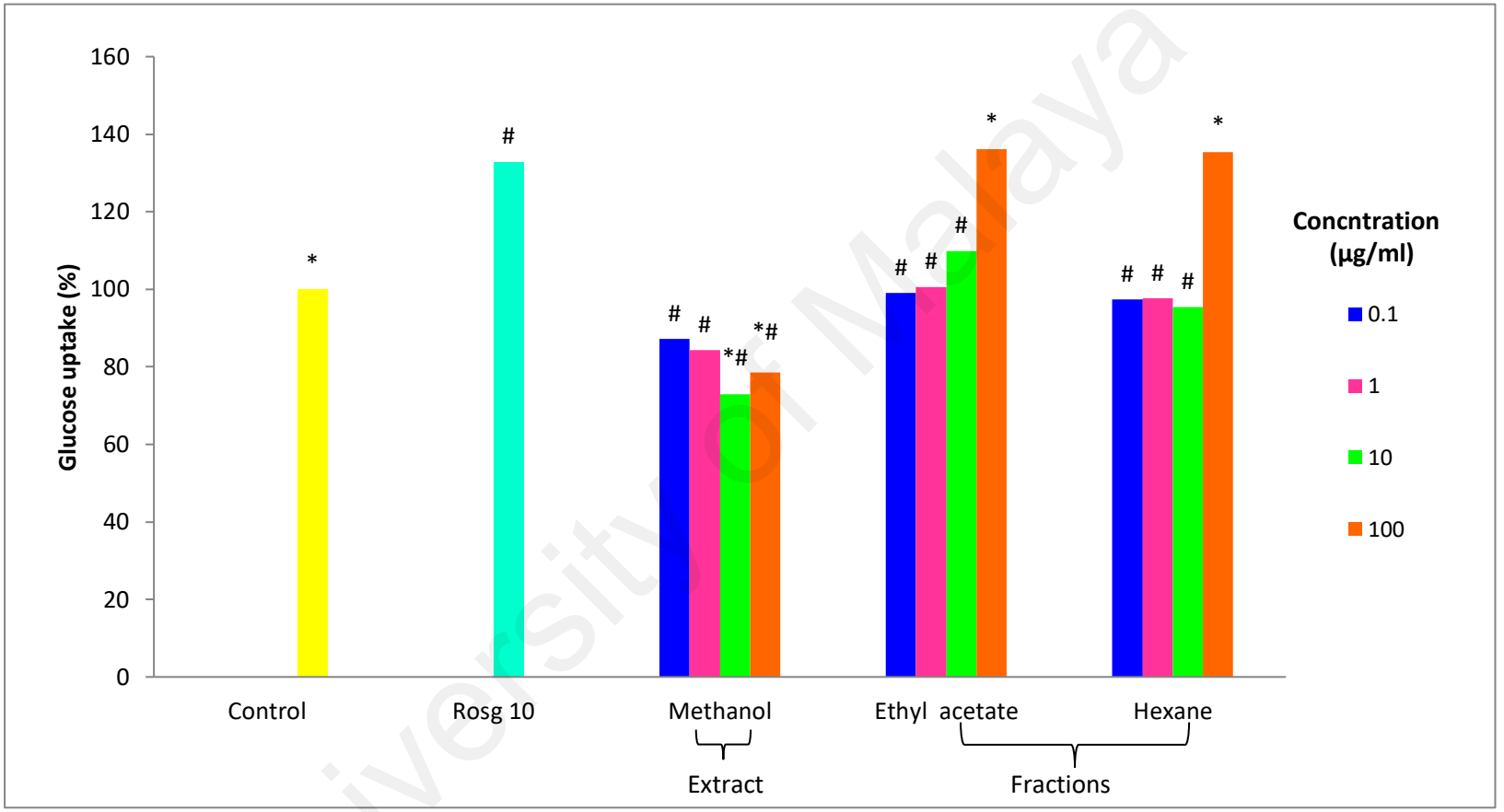


Figure 4.4: Effect of methanol extract, hexane and ethyl acetate fraction on glucose uptake activity in 3T3-L1 adipocytes. Cells incubated with Control (medium only) served as negative control and Rosg 10 (rosiglitazone 10 µm) served as positive control. Data expressed in mean ± standard error, n=3. Values designated by (*) indicates significant ($p < 0.05$) different compared to the negative control. Values designated by (#) indicates significant ($p < 0.05$) different compared to the positive control.

University of Malaya

Fig 4.5 shows that the adipocytes that were incubated with ethyl acetate fraction of *P. giganteus* significantly ($p < 0.05$) upregulated the expression of both PPARγ and SREBP, the key adipogenic transcription factor in 3T3-L1 cells, by 3.19 and 2.27 fold respectively.

The expression of phosphatidylinositol 3-kinase/Akt (PI3K), which is a downstream signalling cascade of insulin receptor substrates, was significantly ($p < 0.05$) upregulated by 2.45 and 2.31 when incubated with ethyl acetate fraction and rosiglitazone respectively. Next, the effect of ethyl acetate fraction on the expression of glucose transporters was investigated. The gene expressions of Glut 1 and Glut 4 were significantly ($p < 0.05$) increased by 2.96 and 3.61 fold in cells treated with ethyl acetate fraction whereas rosiglitazone caused an increase of 6.34 and 4.28 fold respectively. These results demonstrated that ethyl acetate fraction can enhance adipocyte differentiation by upregulating the expressions of PPARγ, SREBP, PI3K, Glut 1 and Glut 4.

In order to elucidate the differentiation mechanism of 3T3-L1 cells, the relative expression of selected genes involved in adipocyte transcriptional regulators, the signal molecules and the glucose transporters were examined. The potential of ethyl acetate fraction of *P. giganteus* was demonstrated as a novel PPARγ agonist that has induced adipogenesis via upregulation of PPARγ, the master gene of adipogenesis. Activation of PPARγ is accompanied by activation of SREBP, being the two most crucial adipogenic transcription factors. Expressions of these two factors are enhanced during the differentiation process which is important for the maturation of adipocyte. SREBP and PPARγ may have synergistically activated the downstream adipocyte specific gene expression including insulin signaling pathway via phosphorylation of PI3K as well as glucose transporters; glut 1 and glut 4 (Gregoire *et al*., 1998).

University of Malaya

The results have shown that ethyl acetate fraction may have inhibitory activity against type 2 diabetes as it acted as an insulin sensitizer activating insulin signaling pathway. The adipogenic genes were activated with the co-presence of insulin and ethyl acetate fraction in the differentiation media. Rosiglitazone was used as a positive control because it is an anti-diabetic drug in the thiazolidinedione class of drugs which works as an insulin sensitizer by binding to the PPARγ receptor in adipocytes making the cells more responsive to insulin. The regulation of PPARγ gene transcription is based on the conformational change that occurs as a result of ligand binding onto the heterodimers of PPARγ with retinoid X receptor (RXR). Presence of ligands is crucial for the expression of PPARγ which thereafter causes differentiation of preadipocytes. The ligands available for this receptor in pharmaceutical industry are the antidiabetic thiazolidinedione (TZD) drugs, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-deoxy-PGJ2) and certain polyunsaturated fatty acids. TZDs are well known potent antihyperglycemic agents which plays integral role in enhancing the insulin sensitivity in peripheral tissues (Górniak, 2014).

It has been reported that the biological ligands of PPARγ is bound with low affinity and therefore there is vast avenue for exploration of exogenous ligands. These ligands are likely to be fatty acid derivatives or certain polyunsaturated fatty acids such as linoleic acid and arachidonic acid which have the affinity for the activation of PPARγ. The content of linoleic acid in ethyl acetate fraction of *P. giganteus* may function as a dietary source of exogenous ligands that induces preadipocyte differentiation as direct ligands for PPARγ (Kim *et al*., 1998). Previous report showed that, emodin, one of the main active components in the root and rhizome of *Rheum palmatum* L, exhibited a very high binding affinity to PPARγ (Yang *et al*., 2007). Previous studies have shown that polyunsaturated fatty acids are less effective in increasing adipocyte numbers, but are more potent in inducing adipocyte differentiation by acting as direct ligands for

University of Malaya

PPARγ (Fernyhough *et al.*, 2007). Similarly, the ethyl acetate fraction that contains polyunsaturated fatty acids displayed only mild effect on proliferation but had significantly enhanced differentiation of 3T3-L1 cells. Alternatively, transcriptional targets of PPARγ might also be resulted from other mechanism such as up-regulation of signalling cascades to activate PPARγ. A study conducted by Joo *et al*. (2010) confirmed that an aqueous extract of *Inonotus obliquus* has stimulated 3T3-L1 adipocyte differentiation by enhancing signalling cascades activating PPARγ. In another study, it was shown that nobiletin, a polymethoxylated flavone found in certain citrus fruits, activated cAMP–responsive element binding protein (CREB) and extracellular signal-regulated kinase (ERK) which enhanced the adipogenesis through activation of signalling cascades including C/EBPβ and PPARγ (Saito *et al*., 2007). Further investigation is highly recommended to identify and purify the compounds that act as ligands of PPARγ in ethyl acetate fraction of *P. giganteus.*

Ethyl acetate fraction of *P. giganteus* has enhanced the expression of SREBP-1c which may have activated the expression of PPARγ. Previous studies have shown that, besides activating the promoters to induce transcription of PPARγ 1 and 3, SREBP-1c could also induce secretion of some lipid molecules which act as an endogenous ligand for PPARγ (Shimano, 2001). However, polyunsaturated fatty acids such as linoleic acid may also suppress the expression of SREBP-1c (Yoshikawa *et al*., 2002).

It has been elucidated that PPARγ is capable of direct modulation of the insulin-signaling pathway through the up-regulation of insulin receptor substrate 1 and 2 (IRS), PI3K and eventually the signalling cascade glut 4. After insulin binds to the extracellular subunit, the intracellular subunit tyrosine kinase domain is activated. Consequently, IRS protein activates PI3K/Akt pathway. This pathway regulates glut 4 relocation to cell surface for glucose transportation into the cells (Okada *et al*., 1994). In this study we have shown for the first time that ethyl acetate fraction of *P. giganteus*

University of Malaya

had significantly ($p < 0.05$) upregulated PI3K. Previously, it has been reported that beta glucans had enhanced the activity of PI3K/Akt through several receptors (Hsu *et al*., 2002; Chen & Seviour, 2007). Some of those receptors are known as Dectin-1, complement receptor 3, lactosylceramide, scavenger and toll like receptors (Brown, 2006; Chen & Seviour, 2007).

The other mechanism that likely confirmed the finding that ethyl acetate fraction of *P. giganteus* had enhanced differentiation of 3T3-L1 adipocytes was the elevation of glucose transporter genes. Gene expression of glucose transporters, glut 1 and glut 4 was higher in 3T3-L1 cells incubated with co-presence of insulin and ethyl acetate fraction compared to 3T3-L1 cells incubated with only differentiation media, insulin or ethyl acetate fraction respectively, clarifying the function of the fraction as an insulin sensitizer (Appendix, Page 91). The ligands of PPARγ are known to increase glucose transport via direct association with glucose transporters in insulin sensitive tissues (Choi *et al*., 2009; Ribon *et al*., 1998). Both glut 1 and glut 4 are among the genes directly involved in the translocation of glucose into 3T3-L1 adipocytes regulated by PPARγ ligands (Fernyhough *et al*., 2007). Once the adipocytes have matured into a lipid-accumulating cell, glut 4 plays a major role in the metabolic function of adipocytes allowing the glucose transportation into the cell after signalled by insulin. In the basal state, glut 4 is cycled slowly between the plasma membrane and intracellular compartments. Most of the transporter is present in vesicular compartments within the cell interior. Glut 4 is translocated to the plasma membrane in the presence of insulin causing an increase in glucose transportation. PPARγ regulates the expression of glut 4 at the initial stage of adipocyte development and leads to subsequent events of lipid metabolism (Scheepers *et al*., 2004). Even though PPARγ and glut 4 genes may be expressed under different cellular mechanisms and pathways, they jointly enhance the differentiation of most adipose-type cells.

University of Malaya

In this study, incubation of 3T3-L1 adipocytes in ethyl acetate fraction of *P. giganteus* showed increased uptake of glucose which was hypothesized as a result of enhanced expression of glut 4 genes. Previously, Joo *et al*. (2010) had reported that the expression of glut 4 protein level in 3T3-L1 adipocytes was increased by aqueous extract of *Inonotus obliquus* via the upregulation of glut 4 expression which was regulated by PPARγ.

Another important finding that was elucidated via this study was the upregulation of glut 1 gene expression in 3T3-L1 cells by the ethyl acetate fraction of *P. giganteus.* As shown in Figure 4.5, 100 µg/ml of ethyl acetate fraction of *P. giganteus* had significantly ($p < 0.05$) increased the glut 1 gene expression. Commonly available agents that activate Glut 1 gene expression in adipocytes are namely prostaglandin F2 alpha (Chiou & Fong, 2004), mitogen-activated kinase (Yamamato *et al.*, 2000), RNA-binding protein Hel-N1 (Jain *et al*., 1997), and insulin sensitizing agent, Troglitazone (Tafuri, 1996). In addition to that, previous studies have shown that PI3K and Akt acts as a mediator of glut 1 expression (Tong *et al*., 2002; Wang & Proud, 2006). Whereby, the elevation of glut 1 expression is directly correlated to the enhanced expression of PI3K.

University of Malaya

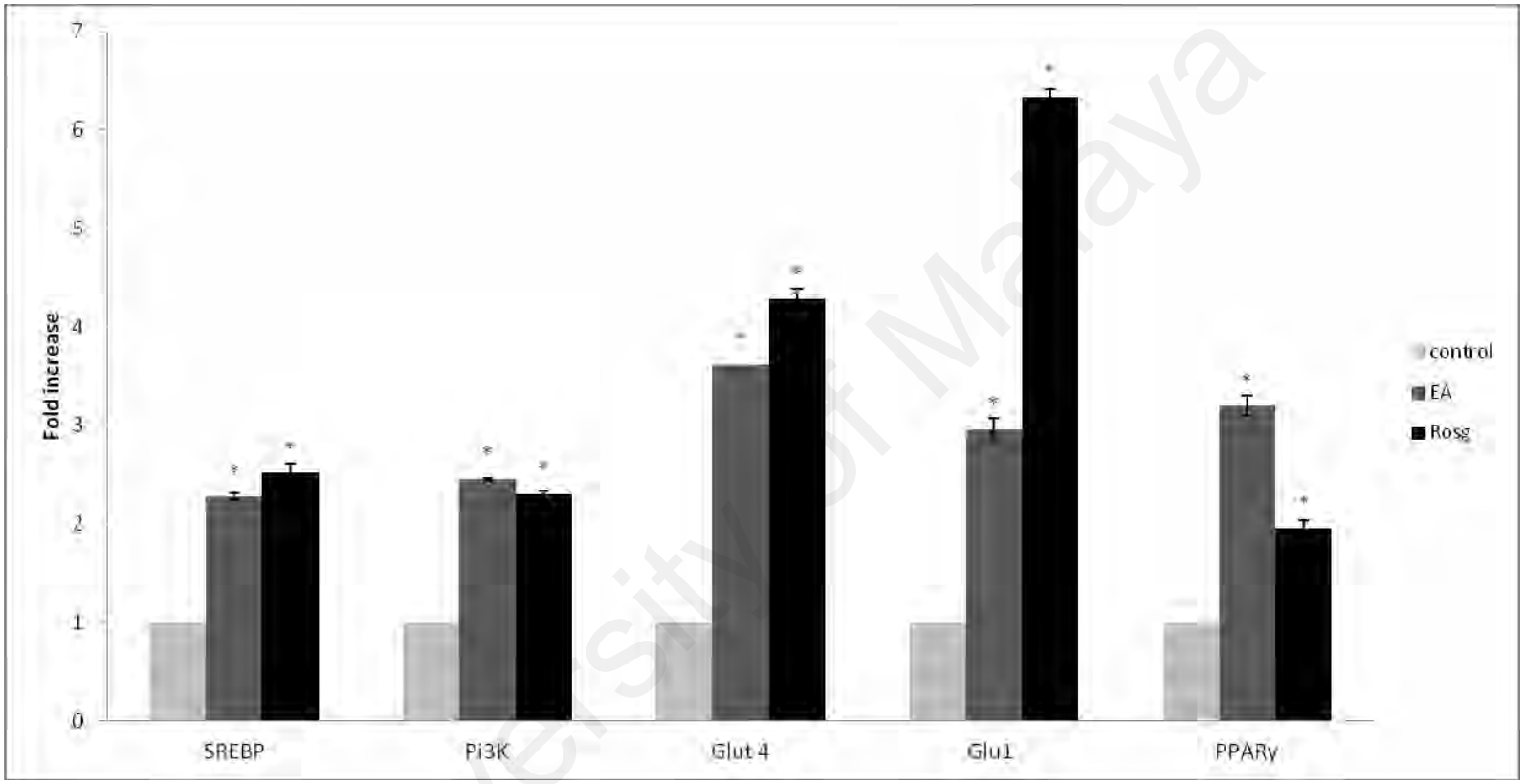


Figure 4.5: Effects of EA (ethyl acetate) fraction (100 µg/ml) of *P.giganteus* on the expression of adipocyte specific genes such as SREBP, PPARγ, PI3K, glut 1 and glut 4. Cells treated with Control (medium only) served as negative control and Rosg (Rosiglitazone 10 µg/ml) served as positive control. Data is expressed as mean ± standard error, n=3. Values designated by (*) indicates significant difference ($p < 0.05$) compared to negative control.

University of Malaya

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Adipocyte differentiation is a very complex process that is triggered and promoted by coordinated signals of growth factors, cytokines and hormones. However, impaired adipocyte differentiation may cause complications such as insulin resistance, hyperlipidemia and type 2 diabetes (Staels *et al*., 2005; Maeda *et al*., 2001). Literature shows that the lack of properly differentiated adipocytes during excess intake of glucose is one of the many causes of type 2 diabetes.

In this study, the newly domesticated mushroom in Malaysia, *P. giganteus* was selected to evaluate its potential anti-diabetic activity. This study provides the first evidence on the effect of fruiting body extracts and fractions of *P. giganteus* on the proliferation, lipogenesis, glucose uptake and lipolysis of 3T3-L1 adipocytes. Subsequently, the gene expression that lies beneath was investigated. In this research, the freeze dried fruiting bodies of *P. giganteus* were extracted with water and methanol and further partitioned with water, hexane, ethyl acetate and butanol to separate the high molecular weight and low molecular weight compounds.

None of the *P. giganteus* extracts and fractions exhibited any cytotoxic effects on 3T3-L1 preadipocytes. Methanol extract and butanol fraction stimulated lipogenesis but did not exert any significant effect on lipolysis. Hexane fraction stimulated both lipogenesis and lipolysis activity in 3T3-L1 differentiated adipocytes. However, ethyl acetate fraction caused mild proliferation of 3T3-L1 but significantly enhanced 3T3-L1 adipocytes differentiation. Glucose uptake by the adipocytes was also stimulated by ethyl acetate fraction. Taken together, the findings in this study show that ethyl acetate fraction (100 μg/ml) of *P. giganteus* promoted both lipogenic and lipolytic activities 3T3-L1 adipocytes.

University of Malaya

To investigate the underlying mechanism of ethyl acetate fraction, gene expressions of several key molecular markers was investigated. Ethyl acetate fraction upregulated the expression of PPARγ, SREBP, PI3K, glut 1 and glut 4 which makes the fraction a good potential agent for prevention of hyperglycemia. Besides controlling the total mass of fat, ethyl acetate fraction also ensured the appropriate differentiation of adipocytes by upregulating SREBP and PPARγ transcription activity. In addition, upregulation of PI3K induces the sensitivity of insulin towards its target which is the adipocytes. Eventually, the glucose transportation is facilitated by the upregulation of the glucose transporters, both Glut 1 and Glut 4. Therefore, ethyl acetate fraction may serve as potential insulin sensitizers without increasing the body weight gain since the lipolysis of the adipocytes is also stimulated with the treatment of ethyl acetate fraction. To confirm this further, the expressions of gene relevant to the lipolytic activity of 3T3-L1 cell model will need to be investigated in future.

Glucose metabolism is strongly related to subtle balance between lipogenesis and lipolysis. Insulin increases glucose uptake into adipocytes which stimulates lipogenesis and inhibits the free fatty acid and glycerol release into the blood circulation (DeFronzo, 2013). Ethyl acetate fraction of *P. giganteus* acts as an insulin-mimicking agent at a lower concentration by stimulating lipogenesis in adipocytes but mimics epinephrine in enhancing lipolysis in the same cells at a higher concentration.

With regards to that, this study had extended recent findings that some mushroom extracts can have both lipogeneic and lipolytic activity. Since the ethyl acetate fraction of *P. giganteus* had promoted lipogenesis, glucose uptake and lipolysis in 3T3-L1 cells, the function of the extract can be related to the Metformin role, a biguanide compound used as anti-diabetic drug in clinical practice (Gregorio *et al.*, 1996). Effect of Metformin was mediated by changes in metabolic variables such as decreased insulin resistance and weight loss. Studies reported that Guanide, a known hypoglycemic

University of Malaya

substance related to the biguanide class of oral antidiabetic drugs, had been detected in edible mushroom of the *Pleurotus* spp and might be responsible for the anti-diabetic effect (Badole *et al*., 2007).

In summary, the results prove that *P. giganteus* may act as an insulin-sensitizer which then reduces the insulin-resistance. Moreover, the capability of ethyl acetate fraction of *P. giganteus* as the potential anti-diabetic and anti-obesity has been validated in this study. The results provided here undoubtedly show that the *P. giganteus* have valuable medicinal potential which may be explored further for the benefit of mankind.

University of Malaya

REFERENCES

Abdullah, N., Ismail, S. M., Aminudin, N., Shuib, A. S., & Lau, B. F. (2011). Evaluation of selected culinary-medicinal mushrooms for antioxidant and ACE inhibitory activities. *Evidence-Based Complementary and Alternative Medicine*, *2012*.

Agrawal, R. P., Chopra, A., Lavekar, G. S., Padhi, M. M., Srikanth, N., Ota, S., & Jain, S. (2010). Effect of oyster mushroom on glycemia, lipid profile and quality of life in type 2 diabetic patients. *Australian Journal of Medical Herbalism*, *22*(2), 50.

Ahn, E. Y., Jiang, Y., Zhang, Y., Son, E. M., You, S., Kang, S. W., ... Kim, D. K. (2008). Cytotoxicity of p-tyrosol and its derivatives may correlate with the inhibition of DNA replication initiation. *Oncology Reports*, *19*(2), 527-534.

Akehi, Y., Anzai, K., Katsuta, H., Yoshida, R., Ohkubo, K., Yamashita, T., ... Ono, J. (2008). Adverse effects of obesity on β-cell function in Japanese subjects with normal glucose tolerance. *Obesity Research and Clinical Practice*, *2*(3), 195-202.

Alam, N., Amin, R., Khan, A., Ara, I., Shim, M. J., Lee, M. W., ... Lee, T. S. (2009). Comparative effects of oyster mushrooms on lipid profile, liver and kidney function in hypercholesterolemic rats. *Mycobiology*, *37*(1), 37-42.

Allweiss, P., Cleeman. J., Cutler. J., Malozowski, S., Nicholas, A., Rock, M., Rosenstock, J., Vigersky. R., & Zettervall, D. (2006). Diabetes Medications Supplements- Working together to manage diabetes. Retrieved from http://chfs.ky.gov/nr/rdonlyres/a37166cc-3b65-4111-9477-869a076e8348/0/ppodmedicationinfo07.pdf

Arner, P., & Östman, J. (1974). Mono-and diacylglycerols in human adipose tissue. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *369*(2), 209-221.

Anand, A., & Chada, K. (2000). *In vivo* modulation of Hmgic reduces obesity. *Nature Genetics*, *24*(4), 377-380.

Badole, S. L., & Bodhankar, S. L. (2007). Interaction of aqueous extract of *Pleurotus pulmonarius* (Fr.) Quel-champ. with acarbose in alloxan induced diabetic mice. *Interaction*, *5*(3), 157-166.

University of Malaya

Badole, S. L., Patel, N. M., Thakurdesai, P. A., & Bodhankar, S. L. (2008). Interaction of aqueous extract of *Pleurotus pulmonarius* (Fr.) Quel-Champ. with glyburide in alloxan induced diabetic mice. *Evidence-Based Complementary and Alternative Medicine*, *5*(2), 159-164.

Bai, L., Pang, W. J., Yang, Y. J. & Yang, G. S. (2008). Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes. *Molecular and Cellular Biochemistry*, *307*(1-2), 129-140.

Bailey, C. J., Day, C., & Leatherdale, B. A. (1986). Traditional treatments for diabetes from Asia and the West Indies. *Practical Diabetes International*, *3*(4), 190-192.

Bickel, P. E., Tansey, J. T., & Welte, M. A. (2009). PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1791*(6), 419-440.

Brown, G. D. (2006). Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nature Reviews Immunology*, *6*(1), 33-43.

Chang, L., Chiang, S. H., & Saltiel, A. R. (2004). Insulin Signaling and the Regulation of Glucose Transport. *Molecular Medicine*, *10*(7-12), 65-71.

Chen, J., & Seviour, R. (2007). Medicinal importance of fungal β-(1→ 3),(1→ 6)-glucans. *Mycological Research*, *111*(6), 635-652.

Cheung, C.K. 1996. The Hypocholestrolemic effect of two edible mushrooms: *Auricularia auricular* (Tree-ear) and *Tremella fuciformis* (White Jelly-Leaf) In hypocholestrolemic rats. *Journal of Nutrition Research, 16(10),* 1721-1725.

Chiou, G. Y., & Fong, J. C. (2004). Prostaglandin F< sub> 2α</sub> increases glucose transport in 3T3-L1 adipocytes through enhanced GLUT1 expression by a protein kinase C-dependent pathway. *Cellular Signalling*, *16*(4), 415-421.

Chorvathova, V., Bobek, P., Ginter, E., & Klvanova, J. (1992). Effect of the oyster fungus on glycaemia and cholesterolaemia in rats with insulin-dependent diabetes. *Physiological Research/Academia Scientiarum Bohemoslovaca*, *42*(6), 175-179.

Choi, S. S., Cha, B. Y., Lee, Y. S., Yonezawa, T., Teruya, T., Nagai, K., … Woo, J. T. (2009). Magnolol enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. *Life Sciences*, *84*(25), 908-914.

University of Malaya

Cimerman, N.G. (1999). Medicinal value of the genus *Pleurotus* (Fr.) P. Karst. (Agaricales s.i., Basidiomycetes). *International Journal of Medicinal Mushroom*, *1*(1), 69-80.

Clarke, S. D., Turini, M., & Jump, D. (1997). Polyunsaturated fatty acids regulate lipogenic and peroxisomal gene expression by independent mechanisms. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *57*(1), 65-69.

Coleman, R. A., & Lee, D. P. (2004). Enzymes of triacylglycerol synthesis and their regulation. *Progress In Lipid Research*, *43*(2), 134-176.

Deepalakshmi, K., & Mirunalini, S. (2011). Therapeutic properties and current medical usage of mushroom: *Ganoderma lucidum*. *International Journal of Pharmaceutical Sciences and Researches, 2*(8), 1922-1929.

DeFronzo, R. A., Eldor, R., & Abdul-Ghani, M. (2013). Pathophysiologic approach to therapy in patients with newly diagnosed type 2 diabetes. *Diabetes Care*, *36* (Supplement 2), S127-S138

Diagnosis and Classification of Diabetes Mellitus. (2009). *Diabetes care*, *31*(1), S62-S67. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2613584 /pdf/S62.pdf

Dobson, M. (1776). Nature of urine in diabetes. *Medical Journal of Obstetric & Gynecology, 5*, 298-310.

Dordevic, A. L., Konstantopoulos, N., & Cameron-Smith, D. (2014). 3T3-L1 preadipocytes exhibit heightened monocyte-chemoattractant protein-1 response to acute fatty acid exposure. *PLoS ONE 9(6): e99382*.

Duncan, R. E., Ahmadian, M., Jaworski, K., Sarkadi-Nagy, E., & Sook, H. (2007). Regulation of Lipolysis in Adipocytes. *Annual Review of Nutrition*, *27*, 79-101.

Eik, L. F., Naidu, M., David, P., Wong, K. H., Tan, Y. S., & Sabaratnam, V. (2011). *Lignosus rhinocerus* (Cooke) Ryvarden: A medicinal mushroom that stimulates neurite outgrowth in PC-12 cells. *Evidence-Based Complementary and Alternative Medicine*, *2012*.

Fernyhough, M. E., Okine, E., Hausman, G., Vierck, J. L., & Dodson, M. V. (2007). PPARγ and GLUT-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. *Domestic Animal Endocrinology*, *33*(4), 367-378.

University of Malaya

Fong, J. C. (1990). The effect of chronic fatty acid treatment on lipolysis in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications*, *171*(1), 46-52.

Fonseca-Alaniz, M. H., Takada, J., Alonso-Vale, M. I. C., & Lima, F. B. (2007). Adipose tissue as an endocrine organ: from theory to practice. *Jornal De Pediatria*, *83*(5), S192-S203.

Fröjdö, S., Vidal, H., & Pirola, L. (2009). Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1792*(2), 83-92.

Garber, A. J. (2006). Premixed insulin analogues for the treatment of diabetes mellitus. *Drugs*, *66*(1), 31-49.

Gosmann, G., Barlette, A. G., Dhamer, T., Arçari, D. P., Santos, J. C., de Camargo, E. R., ... Ribeiro, M. L. (2012). Phenolic compounds from mate (*Ilex paraguariensis*) inhibit adipogenesis in 3T3-L1 preadipocytes. *Plant Foods For Human Nutrition*, *67*(2), 156-161.

Green, H., & Kehinde, O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. *Cell*, *1*(3), 113-116.

Gregorio, F., Manfrini, S., Testa, I., & Filipponi, P. (1996). Metformin treatment in elderly type II diabetic patients. *Archives of Gerontology and Geriatrics*, *22*, 261-270.

Gregoire, F. M., Smas, C. M., & Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews*, *78*(3), 783-809.

Grygiel-Górniak, B. (2013). Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications--a review. *Nutrition Journal*, *13*, 17-17.

Guo, W., Wong, S., Xie, W., Lei, T., & Luo, Z. (2007). Palmitate modulates intracellular signaling, induces endoplasmic reticulum stress, and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. *American Journal of Physiology-Endocrinology and Metabolism*, *293*(2), E576-E586.

Hashim, P., Sidek, H., Helan, M. H. M., Sabery, A., Palanisamy, U. D., & Ilham, M. (2011). Triterpene composition and bioactivities of Centella asiatica. *Molecules*, *16*(2), 1310-1322.

University of Malaya

Herman, M. A., & Kahn, B. B. (2006). Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. *Journal of Clinical Investigation*, *116(7)*, 1767-1775.

Hsu, M. J., Lee, S. S., & Lin, W. W. (2002). Polysaccharide purified from *Ganoderma lucidum* inhibits spontaneous and Fas-mediated apoptosis in human neutrophils through activation of the phosphatidylinositol 3 kinase/Akt signaling pathway. *Journal of Leukocyte Biology*, *72*(1), 207-216.

Hsu, C. L., & Yen, G. C. (2007). Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. *Journal of Agricultural and Food Chemistry*, *55*(21), 8404-8410.

Hsu, C. L., & Yen, G. C. (2008). Phenolic compounds: evidence for inhibitory effects against obesity and their underlying molecular signaling mechanisms. *Molecular Nutrition and Food Research*, *52*(1), 53-61.

Hu, S. H., Wang, J. C., Lien, J. L., Liaw, E. T., & Lee, M. Y. (2006). Antihyperglycemic effect of polysaccharide from fermented broth of *Pleurotus citrinopileatus*. *Applied Microbiology and Biotechnology*, *70*(1), 107-113.

Jain, R. G., Andrews, L. G., McGowan, K. M., Pekala, P. H., & Keene, J. D. (1997). Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT1) expression in 3T3-L1 adipocytes. *Molecular and Cellular Biology*, *17*(2), 954-962.

James, D. E., & Piper, R. C. (1994). Insulin resistance, diabetes and the insulin-regulated trafficking of GLUT-4. *Journal of Cell Biology*, *126*(5), 1123-1126.

Jiskani, M. M. (2001). Energy potential of mushrooms. *The DAWN Economic and Business Review*, 15-21.

Jones, E. G., Hyde, K. D., & Sabaratnam, V. (Eds.). (2007). *Malaysian Fungal Diversity*. Mushroom Research Centre, University of Malaya.

Joo, J. I., Kim, D. H., & Yun, J. W. (2010). Extract of Chaga mushroom (*Inonotus obliquus*) stimulates 3T3-L1 adipocyte differentiation. *Phytotherapy Research*, *24*(11), 1592-1599.

Joost, H.F. & Stenfelder, H.J. 1985. Insulin-like stimulation of glucose transport in isolated adipocytes by fatty acids. *Biochemical and Biophysical Research Communications, 128*, 1358-1363.

University of Malaya

Kanagasabapathy, G., Malek, S. N. A., Kuppusamy, U. R., & Vikineswary, S. (2011). Chemical composition and antioxidant properties of extracts of fresh fruiting bodies of *Pleurotus sajor-caju* (Fr.) Singer. *Journal of Agricultural and Food Chemistry*, *59*(6), 2618-2626.

Kanagasabapathy, G. (2012a*). Identification and the evaluation of chemical constituents of Pleurotus Sajor-Caju (FR.) Singer extracts in lipid metabolism and glucose homeostasis*. (Doctoral dissertation). University of Malaya. Malaysia.

Kanagasabapathy, G., Kuppusamy, U. R., Malek, S. N. A., Abdulla, M. A., Chua, K. H., & Sabaratnam, V. (2012b). Glucan-rich polysaccharides from *Pleurotus sajor-caju* (Fr.) Singer prevents glucose intolerance, insulin resistance and inflammation in C57BL/6J mice fed a high-fat diet. *BMC Complementary and Alternative Medicine*, *12*, 261-261.

Karunarathna, S. C., Yang, Z. L., Raspé, O., Ko Ko, T. W., Vellinga, E. C., Zhao, R. L., ... Hyde, K. D. (2012). *Lentinus giganteus* revisited: new collections from Sri Lanka and Thailand. *Mycotaxon*, *118*(1), 57-71.

Kennedy, A., Martinez, K., Schmidt, S., Mandrup, S., LaPoint, K., & McIntosh, M. (2010). Antiobesity mechanisms of action of conjugated linoleic acid. *The Journal of Nutritional Biochemistry*, *21*(3), 171-179.

Kersten, S. (2001). Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Reports*, 2(4), 282-286.

Khan, M.A. (2010). *Nutritional composition and hypocholestrolemic effect of mushroom: Pleurotus sajor-caju and Pleurotus florida.* LAP Lambert Academic Publishing GMBH & Co. KG: SaarbrÜcken Germany, pp 1-11.

Kim, J. B., Wright, H. M., Wright, M., & Spiegelman, B. M. (1998). ADD1/SREBP1 activates PPARγ through the production of endogenous ligand. *Proceedings of The National Academy of Sciences*, *95*(8), 4333-4337.

Kim, J. K., Gavrilova, O., Chen, Y., Reitman, M. L., & Shulman, G. I. (2000). Mechanism of insulin resistance in A-ZIP/F-1 fatless mice. *Journal of Biological Chemistry*, *275*(12), 8456-8460.

Kim, D. H., Yang, B. K., Jeong, S. C., Hur, N. J., Das, S. U. R. A. J. I. T., Yun, J. W., ... Song, C. H. (2001). A preliminary study on the hypoglycemic effect of the exo-polymers produced by five different medicinal mushrooms. *Journal of Microbiology and Biotechnology*, *11*(1), 167-171.

University of Malaya

Kim, Y.W., Kim, K.H., Choi, H.J. & Lee, D.S. (2005). Anti-diabetic activity of β-glucans and their enzymatically hydrolyzed oligosaccharides from *Agaricus blazei*. *Biotechnology Letters*, *27*, 483-487.

Kim, J. I., Kang, M. J., Im, J., Seo, Y. J., Lee, Y. M., Song, J. H., ... Kim, M. E. (2010). Effect of king oyster mushroom (*Pleurotus eryngii*) on insulin resistance and dyslipidemia in db/db mice. *Food Science and Biotechnology*, *19*(1), 239-242.

Krishna, S., & Usha, P. T. A. (2009). Hypoglycaemic effect of a combination of *Pleurotus ostreatus*, *Murraya koenigii* and *Aegle marmelos* in diabetic rats. *Indian Journal of Animal Sciences*, *79*(10), 986-987.

Kuppusamy, U. R., & Das, N. P. (1994). Potentiation of β-adrenoceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes. *Biochemical Pharmacology*, *47*(3), 521-529.

Le Lay, S., Lefrère, I., Trautwein, C., Dugail, I., & Krief, S. (2002). Insulin and sterol-regulatory element-binding protein-1c (SREBP-1C) regulation of gene expression in 3T3-L1 Adipocytes identification of CCAAT/enhancer-binding protein β an SREBP-1C target. *Journal of Biological Chemistry*, *277(38)*, 35625-35634.

Lee, S.S., Chang, Y.S. & Noraswati, M.N.R. (2009). Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. *Forest Ecology Management*, *257*(10), 2062–2065.

Lee, J., Miyazaki, M., Romeo, G. R., & Shoelson, S. E. (2014). Insulin receptor activation with transmembrane domain ligands. *Journal of Biological Chemistry*, *289*(28), 19769-19777.

Leney, S. E., & Tavaré, J. M. (2009). The molecular basis of insulin-stimulated glucose uptake: signalling, trafficking and potential drug targets. *Journal of Endocrinology*, *203*(1), 1-18.

Letchuman, G. R., Wan Nazaimoon, W. M., Wan Mohamad, W. B., Chandran, L. R., Tee, G. H., Jamaiyah, H., & Ahmad Faudzi, Y. (2010). Prevalence of diabetes in the Malaysian National Health Morbidity Survey III 2006. *Medical Journal of Malaysia*, *65*(3), 180-186.

Li,W.L., Zheng, H.C., Bukuru, J. and Kimpe, N.D. (2004). Natural medicines used in the traditional Chinese medical system for therapy of Diabetes Mellitus. *Journal of Ethnopharmacology, 92, 1-21.*

University of Malaya

Li, N., Li, L., Fang, J. C., Wong, J. H., Ng, T. B., Jiang, Y., ... Lv, P. Y. (2012). Isolation and identification of a novel polysaccharide–peptide complex with antioxidant, anti-proliferative and hypoglycaemic activities from the abalone mushroom. *Bioscience Reports*, *32*(3), 221-228.

Lim, S.L., Chai, J.W. & Kupusamy, U.R. (2008). Evaluation of *Syzygium jambolanum* methanolic leaf extract for insulin-like properties. *Research Journal of Biological Sciences. 3 (99),* 1109-1114.

Lomeo, F., Kokher, M.A. & Dandona, P. (1986). Unsaturated fatty acids potentiate insulin on adipocytes. *Journal of Lipid Research, 25*, 511-513.

Madsen, L., Petersen, R. K., Sørensen, M. B., Jørgensen, C., Hallenborg, P., Pridal, L., ... Berge, R. K. (2003). Adipocyte differentiation of 3T3-L1 preadipocytes is dependent on lipoxygenase activity during the initial stages of the differentiation process. *Biochemical Journal*, *375*(3), 539-549.

Maeda, N., Takahashi, M., Funahashi, T., Kihara, S., Nishizawa, H., Kishida, K., & Matsuzawa, Y. (2001). PPARγ ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes*, *50(9)*, 2094-2099.

Mahajna, J., Dotan, N., Zaidman, B. Z., Petrova, R. D., & Wasser, S. P. (2008). Pharmacological values of medicinal mushrooms for prostate cancer therapy: the case of *Ganoderma lucidum*. *Nutrition and Cancer*, *61*(1), 16-26.

Manaharan, T., Ming, C. H., & Palanisamy, U. D. (2013). *Syzygium aqueum* leaf extract and its bioactive compounds enhances pre-adipocyte differentiation and 2-NBDG uptake in 3T3-L1 cells. *Food Chemistry*, *136*(2), 354-363.

Maregesi, S. M., Pieters, L., Ngassapa, O. D., Apers, S., Vingerhoets, R., Cos, P., ... Vlietinck, A. J. (2008). Screening of some Tanzanian medicinal plants from Bunda district for antibacterial, antifungal and antiviral activities. *Journal of Ethnopharmacology*, *119*(1), 58-66.

Martin, S., & Parton, R. G. (2006). Lipid droplets: a unified view of a dynamic organelle. *Nature Reviews Molecular Cell Biology*, *7*(5), 373-378.

Martin, K. R., & Brophy, S. K. (2010). Commonly consumed and specialty dietary mushrooms reduce cellular proliferation in MCF-7 human breast cancer cells. *Experimental Biology and Medicine*, *235*(11), 1306-1314.

University of Malaya

Matthaei, S., Stumvoll, M., Kellerer, M. & Haring, H.U. (2000). Pathopysiology and pharmacological treatment of insulin resistance. *Endocrine Review, 21*(6), 585-618.

Mayell, M. (2001). Maitake extracts and their therapeutic potential-a review. *Alternative Medicine Review*, *6*(1), 48-60.

Md. Asaduzzaman Khan & Mousumi Tania (2012). Nutritional and Medicinal importance of *Pleurotus* Mushrooms: An Overview. *Food Reviews International*. *28(3)*, 313-329.

Miller, B. R., Nguyen, H., Jia-Haur Hu, C., Lin, C., & Nguyen, Q. T. (2014). New and emerging drugs and targets for type 2 diabetes: reviewing the evidence. *American Health and Drug Benefits*, *7*(8).

Moitra, J., Mason, M. M., Olive, M., Krylov, D., Gavrilova, O., Marcus-Samuels, B., & Reitman, M. L. (1998). Life without white fat: a transgenic mouse. *Genes and Development*, *12*(20), 3168-3181.

Moreno-Navarrete, J. M., & Fernández-Real, J. M. (2012). Adipocyte Differentiation. *Adipose Tissue Biology*, 17-38.

Morimoto, C., Tsujita, T., Sumida, M., & Okuda, H. (2000). Substrate-dependent lipolysis induced by isoproterenol. *Biochemical and Biophysical Research Communications*, *274*(3), 631-634.

Moroney, S. (2012*). Chemical constituents in the fruiting bodies of Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde extracts by GC-MS and LC-MS/MS*. (Master's thesis). University of Malaya. Malaysia.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55-63.

Mukherjee, S. P., Mukherjee, C., Yeager, M. D., & Lynn, W. S. (1980). Stimulation of glucose transport and oxidation in adipocytes by fatty acids: evidence for a regulatory role in the cellular response to insulin. *Biochemical and Biophysical Research Communications*, *94*(2), 682-689.

Narayan, K.M., Gregg, E.W., & Fagot-Campagna, A. (2000). Diabetes – a common, growing, serious, costly, and potentially preventable public health problem. *Diabetes Research and Clinical Practice, 50*, S77-84.

University of Malaya

National Institute of Diabetes and Digestive and Kidney Diseases (2013). *Pancreatic islet transplantation.* Retrieved on 30th May 2016 from http://www.niddk.nih.gov/health-information/health-topics/Diabetes/pancreatic-islet transplantation/Documents/Pancreatic_Islet_Transplantation_508.pdf

Perrini, S., Natalicchio, A., Laviola, L., Belsanti, G., Montrone, C., Cignarelli, A., & Giorgino, F. (2004). Dehydroepiandrosterone stimulates glucose uptake in human and murine adipocytes by inducing GLUT1 and GLUT4 translocation to the plasma membrane. *Diabetes*, *53*(1), 41-52

National Health and Morbidity Survey (2015). *Non-communicable diseases risk factors & other health problems* (Vol 2). Retrieved on 30th May 2016 from http://www.iku.gov.my/images/IKU/Document/REPORT/nhmsreport2015vol2.pdf.

Nawrocki, A. R., & Scherer, P. E. (2005). Keynote review: the adipocyte as a drug discovery target. *Drug Discovery Today*, *10*(18), 1219-1230.

Organisation for Economic Co-operation and Development Consensus Documents. (2006). *Safety Assessment of Transgenic Organisms* (Vol 1). Retrieved on 16th August 2016 from http://dx.doi.org/10.1787/9789264095380-14-en

Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., & Ui, M. (1994). Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *Journal of Biological Chemistry*, *269*(5), 3568-3573.

Okuno, A., Tamemoto, H., Tobe, K., Ueki, K., Mori, Y., Iwamoto, K., ... Yazaki, Y. (1998). Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *Journal of Clinical Investigation*, *101*(6), 1354.

Pappone, P. A., & Ortiz-Miranda, S. I. (1993). Blockers of voltage-gated K channels inhibit proliferation of cultured brown fat cells. *American Journal of Physiology-Cell Physiology*, *264*(4), C1014-C1019.

Patlak, M. (2002). New weapons to combat an ancient disease: treating diabetes. *The FASEB Journal*, *16*(14), 1853e-1853e.

University of Malaya

Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., & Okada, S. (1999). Molecular Basis of Insulin-stimulated GLUT4 Vesicle Trafficking. Location! Location! Location! *Journal of Biological Chemistry*, *274*(5), 2593-2596.

Phan, C. W., Wong, W. L., David, P., Naidu, M., & Sabaratnam, V. (2011). *Pleurotus giganteus* (Berk.) Karunarathna & KD Hyde: Nutritional value and in vitro neurite outgrowth activity in rat pheochromocytoma cells. *BMC Complementary and Alternative Medicine*, *12*, 102-102.

Phan, C., Wong, W., David, P., Naidu, M., & Sabaratnam, V. (2015). Therapeutic Potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases: diversity, metabolite, and mechanism. *Critical Reviews in Biotechnology, 35(3)*, 355-368.

Poulos, S. P., Dodson, M. V., & Hausman, G. J. (2010). Cell line models for differentiation: preadipocytes and adipocytes. *Experimental Biology and Medicine*, *235*(10), 1185-1193.

Ravishankar, B., & Shukla, V. J. (2007). Indian systems of medicine: a brief profile. *African Journal of Traditional, Complementary and Alternative Medicines*, *4*(3), 319-337.

Ren, N., Kaplan, R., Hernandez, M., Cheng, K., Jin, L., Taggart, A. K., ... Cai, T. Q. (2009). Phenolic acids suppress adipocyte lipolysis via activation of the nicotinic acid receptor GPR109A (HM74a/PUMA-G). *Journal of Lipid Research*, *50*(5), 908-914.

Ribon, V., Johnson, J. H., Camp, H. S., & Saltiel, A. R. (1998). Thiazolidinediones and insulin resistance: peroxisome proliferatoractivated receptor γ activation stimulates expression of the CAP gene. *Proceedings of the National Academy of Sciences*, *95*(25), 14751-14756.

Rosen, E. D., & Spiegelman, B. M. (2001). PPARγ: a nuclear regulator of metabolism, differentiation, and cell growth. *Journal of Biological Chemistry*, *276*(41), 37731-37734.

Saito, T., Abe, D., & Sekiya, K. (2007). Nobiletin enhances differentiation and lipolysis of 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications*, *357*(2), 371-376.

Saltiel, A. R., & Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*, *414*(6865), 799-806.

University of Malaya

Schäffler, A., Schölmerich, J., & Büchler, C. (2005). Mechanisms of disease: adipocytokines and visceral adipose tissue - emerging role in intestinal and mesenteric diseases. *Nature Clinical Practice Gastroenterology and Hepatology*, *2*(2), 103-111.

Scheepers, A., Joost, H. G., & Schurmann, A. (2004). The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. *Journal of Parenteral and Enteral Nutrition*, *28*(5), 364-371.

Shechter, Y., & Henis, Y. I. (1984). Cis-unsaturated fatty acids induce both lipogenesis and calcium binding in adipocytes. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *805*(1), 89-96.

Shen, W., Wang, Z., Punyanita, M., Lei, J., Sinav, A., Kral, J. G., & Heymsfield, S. B. (2003). Adipose tissue quantification by imaging methods: a proposed classification. *Obesity Research*, *11*(1), 5-16.

Shepherd, P., Withers, D., & Siddle, K. (1998). Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochemical Journal*, *333*(3), 471-490.

Shi, Y., & Burn, P. (2004). Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. *Nature Reviews Drug Discovery*, *3*(8), 695-710.

Shimano, H. (2001). Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Progress in Lipid Research*, *40*(6), 439-452.

Silva, D. D. D., Rapior, S., Hyde, K. D., & Bahkali, A.H. (2012). Medicinal mushrooms in prevention and control of diabetes mellitus. *Fungal Diversity*, *56*(1), 1-29.

Simon, E., Macarulla, M. T., Fernandez-Quintela, A., Rodriguez, V. M., & Portillo, M. P. (2005). Body fat-lowering effect of conjugated linoleic acid is not due to increased lipolysis. *Journal of Physiology and Biochemistry*, *61*(2), 363-369.

Sobieralski, K., Siwulski, M., Lisiecka, J., Jędryczka, M., Sas-Golak, I., & Frużyńska-Jóźwiak, D. (2012). Fungi-derived β-glucans as a component of functional food. *Acta Scientiarum Polonorum-Hortorum Cultus*, *11*(4), 111-128.

Spiegelman, B. M., & Flier, J. S. (2001). Obesity and the regulation of energy balance. *Cell*, *104*(4), 531-543.

University of Malaya

Staels, B., & Fruchart, J. C. (2005). Therapeutic roles of peroxisome proliferator–activated receptor agonists. *Diabetes*, *54*(8), 2460-2470.

Stamets, P. (2000). *Growing gourmet and medicinal mushrooms* (Vol 3). Berkeley: Ten Speed Press.

Stolar, M.W. & Chilton, R.J. (2003). Type 2 diabetes, cardiovascular risk and the link to insulin resistance. *Clinical Therapy Supply*, *25*, B4-B31.

Stoops, J. K., Arslanian, M. J., Oh, Y. H., Aune, K. C., Vanaman, T. C., & Wakil, S. J. (1975). Presence of two polypeptide chains comprising fatty acid synthetase. *Proceedings of the National Academy of Sciences*, *72*(5), 1940-1944.

Sussman, M. A., Völkers, M., Fischer, K., Bailey, B., Cottage, C. T., Din, S., & Quijada, P. (2011). Myocardial AKT: the omnipresent nexus. *Physiological Reviews*, *91*(3), 1023-1070.

Tafuri, S. R. (1996). Troglitazone enhances differentiation, basal glucose uptake, and Glut1 protein levels in 3T3-L1 adipocytes. *Endocrinology*, *137*(11), 4706-4712.

Tilburt, J. C., & Kaptchuk, T. J. (2008). Herbal medicine research and global health: an ethical analysis. *Bulletin of the World Health Organization*, *86*(8), 594-599.

Tong, H., Imahashi, K., Steenbergen, C., & Murphy, E. (2002). Phosphorylation of glycogen synthase kinase-3β during preconditioning through a phosphatidylinositol-3-kinase–dependent pathway is cardioprotective. *Circulation Research*, *90*(4), 377-379.

Udugama, S. & Wickramaratna, K. (1991). *Artificial production of naturally occurring Lentinus giganteus (Uru Paha), a Sri Lankan edible mushroom*. Horticultural Crop Research and Development Institute (HORDI). Retrieved from https://www.doa.gov.lk/HORDI/index.php/en/dowenload-2

Vaidya, H. B., Ahmed, A. A., Goyal, R. K., & Cheema, S. K. (2013). Glycogen phosphorylase-a is a common target for anti-diabetic effect of iridoid and secoiridoid glycosides. *Journal of Pharmacy and Pharmaceutical Sciences*, *16*(4), 530-540.

Vats, V., Grover, J. K., & Rathi, S. S. (2002). Evaluation of anti-hyperglycemic and hypoglycemic effect of *Trigonella foenum-graecum Linn*, *Ocimum sanctum Linn* and *Pterocarpus marsupium Linn* in normal and alloxanized diabetic rats. *Journal of Ethnopharmacology*, *79*(1), 95-100.

University of Malaya

Vinicor, F. (1998). The public health burden of diabetes and the reality of limits. *Diabetes Care*, *21*(Supplement 3), C15-C18.

Wang, X., & Proud, C. G. (2006). The mTOR pathway in the control of protein synthesis. *Physiology*, *21*(5), 362-369.

Wang, S., Li, Y., Wen, Y., Chen, Y., Na, L., Li, S., & Sun, C. (2009). Curcumin, a potential inhibitor of up-regulation of TNF-alpha and IL-6 induced by palmitate in 3T3-L1 adipocytes through NF-kappaB and JNK pathway. *Biomedical and Environmental Sciences*, *22*(1), 32-39.

Wasser, S.P. & Weis, A.I. (1999). Medicinal properties of substances occurring in higher basidiomycetes mushrooms: current perspectives (Review). *International Journal of Medicinal Mushrooms*, *1*, 31-62.

Wilcox, G. (2005). Insulin and insulin resistance. *Clinical Biochemist Reviews*, *26*(2), 19-39.

Wild, S., Roglic, G., Green, A., Sicree, R. & King, H. (2004). Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care, 27*(5), 1047-1053.

Wilsie, L. C., Chanchani, S., Navaratna, D., & Orlando, R. A. (2005). Cell surface heparan sulfate proteoglycans contribute to intracellular lipid accumulation in adipocytes. *Lipids in Health and Disease*, *4*(1), 1.

Wong, K. H., Naidu, M., David, P., Abdulla, M. A., Abdullah, N., Kuppusamy, U. R., & Sabaratnam, V. (2011). Peripheral nerve regeneration following crush injury to rat peroneal nerve by aqueous extract of medicinal mushroom *Hericium erinaceus* (Bull.: Fr) Pers.(Aphyllophoromycetideae). *Evidence-Based Complementary and Alternative Medicine*, *2011*.

Wong, W., Abdulla, M. A., Chua, K., Kuppusamy, U.R., Tan, Y. & Sabaratnam, V. (2012). Hepatoprotective effects of *Panus giganteus* (Berk.) Corner against thioacetamide-(TAA-) induced liver injury in rats. *Evidence-based Complementary and Alternative Medicine*, *2012*.

World Health Organization (2013). *Traditional medicine strategy 2014-2023*. Retrieved on 5th September 2016 from http://apps.who.int/iris/bitstream/10665/92455/1/9789241506090_eng.pdf

University of Malaya

Yamamoto, Y., Yoshimasa, Y., Koh, M., Suga, J., Masuzaki, H., Ogawa, Y., ... Nakao, K. (2000). Constitutively active mitogen-activated protein kinase kinase increases GLUT1 expression and recruits both GLUT1 and GLUT4 at the cell surface in 3T3-L1 adipocytes. *Diabetes*, *49*(3), 332-339.

Yang, B. K., Ha, J. Y., Jeong, S. C., Jeon, Y. J., Ra, K. S., Das, S., ... Song, C. H. (2002). Hypolipidemic effect of an exo-biopolymer produced from submerged mycelial culture of Auricularia polytricha in rats. *Biotechnology Letters*, *24*(16), 1319-1325.

Yang, X., Jansson, P. A., Nagaev, I., Jack, M. M., Carvalho, E., Sunnerhagen, K. S., ... Smith, U. (2004). Evidence of impaired adipogenesis in insulin resistance. *Biochemical and Biophysical Research Communications*, *317*(4), 1045-1051.

Yang, Y., Shang, W., Zhou, L., Jiang, B., Jin, H., & Chen, M. (2007). Emodin with PPARγ ligand-binding activity promotes adipocyte differentiation and increases glucose uptake in 3T3-Ll cells. *Biochemical and Biophysical Research Communications*, *353*(2), 225-230.

Yin, W., Mu, J., & Birnbaum, M. J. (2003). Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis In 3T3-L1 adipocytes. *Journal of Biological Chemistry*, *278*(44), 43074-43080.

Yoshikawa, T., Shimano, H., Yahagi, N., Ide, T., Amemiya-Kudo, M., Matsuzaka, T., ... Iizuka, Y. (2002). Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *Journal of Biological Chemistry*, *277*(3), 1705-1711.

Yuan, Z., He, P., Cui, J. and Takeuchi, H. (1998). Hypoglycemic effect of water soluble polysaccharide from *Auricularia auricula-judae* Quel. On genetically diabetic KK-A^Y mice. *Journal of Bioscience, Biotechnology and Biochemistry, 62* (10), 1898-1903.

Zaini, A. (2000). Where is Malaysia in the midst of the Asian epidemic of diabetes mellitus? *Diabetes Research and Clinical Practice*, *50*, S23-S28.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, *372*(6505), 425-432.

University of Malaya

LIST OF PUBLICATIONS AND PAPERS PRESENTED

1) Isi-Cited publication

Paravamsivam, P., Heng, C. K., Malek, S. N. A., Sabaratnam, V., Ravishankar Ram, M., & Kuppusamy, U. R. (2016). Giant Oyster Mushroom *Pleurotus giganteus* (Agaricomycetes) Enhances Adipocyte Differentiation and Glucose Uptake via Activation of PPARγ and Glucose Transporters 1 and 4 in 3T3-L1 Cells. *International Journal of Medicinal Mushrooms*, *18*(9), 821-831.

2) Poster Presentation

Paravamsivam, P., Heng, C. K., Malek, S. N. A., Sabaratnam, V., & Kuppusamy, U. R. (2013). Ethyl acetate extract of *Pleurotus giganteus* (berk.) Karunarathna & K.D. Hyde enhances adipocyte differentiation and glucose uptake by inducing peroxisome proliferator-activated receptor in 3T3-L1 cells. Paper presented at the 3rd Asia-Korea Conference on Science and Technology, Sentosa Island, Singapore.

University of Malaya

International Journal of Medicinal Mushrooms, 18(9): 821–831 (2016)

Giant Oyster Mushroom *Pleurotus giganteus* (Agaricomycetes) Enhances Adipocyte Differentiation and Glucose Uptake via Activation of PPARγ and Glucose Transporters 1 and 4 in 3T3-L1 Cells

Puvaneswari Paravamsivam,[1,2] Chua Kek Heng,[1,3] Sri Nurestri Abdul Malek,[1,2] Vikineswary Sabaratnam,[1,2] Ravishankar Ram M,[3] & Umah Rani Kuppusamy[1,3,*]

[1]Mushroom Research Centre (MRC), Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia; [2]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia; [3]Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

*Address all correspondence to: Umah Rani Kuppusamy, Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia; umah@um.edu.my

ABSTRACT: The edible mushroom *Pleurotus giganteus* was tested for its effect on adipocyte differentiation and glucose uptake activity in 3T3-L1 cells. The basidiocarps of *P. giganteus* were soaked in methanol to obtain a crude methanol extract and then fractionated to obtain an ethyl acetate extract. In this study, cell proliferation was measured using an MTT assay, lipid accumulation using an Oil Red O assay, and glucose uptake using a fluorescence glucose uptake assay. Gene expression was measured via real-time polymerase chain reaction analysis with TaqMan primer. Ethyl acetate extract significantly enhanced adipogenic differentiation and glucose uptake in 3T3-L1 adipocytes via the expression of sterol regulatory element-binding protein, peroxisome proliferator-activated receptor γ, and phosphatidylinositol 3-kinase/Akt. Glucose uptake was facilitated by the highly expressed glucose transporters Glut1 and Glut4. Taken together, these results suggest that *P. giganteus* ethyl acetate extract has an insulin-sensitizing effect on adipocytes and has potential as an adjuvant for the management of type 2 diabetes.

KEY WORDS: adipocyte, glucose transporters, glucose uptake, medicinal mushrooms, *Pleurotus giganteus*, type 2 diabetes mellitus

ABBREVIATIONS: 2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; **Dexa**, dexamethasone; **DM**, differentiation medium; **DMEM**, Dulbecco's modified Eagle's medium; **DMSO**, dimethyl sulfoxide; **ELISA**, enzyme-linked immunosorbent assay; **FBS**, fetal bovine serum; **IBMX**, 3-isobutyl-1-methylxanthine; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **PBS**, phosphate-buffered saline; **PCR**, polymerase chain reaction; **PI3K**, phosphoinositide 3-kinase; **PPARγ**, peroxisome proliferator-activated receptor γ; **SREBP**, sterol regulatory element-binding protein

I. INTRODUCTION

Type 2 diabetes mellitus is rapidly increasing in Malaysia and worldwide.[1] It is generally characterized by insulin resistance, insulin insensitivity, and insufficient uptake of glucose. Adipocytes are one of the target tissues specialized to synthesize triglycerides during periods of an abundant food supply and to mobilize them via lipolysis when there is an energy deficit.[2] Abnormal adipocyte differentiation affects glucose and lipid metabolism, and is the main cause of insulin resistance.

Adipocyte differentiation is a complex process that involves the induction and suppression of a large number of genes, including peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding protein (SREBP), which lead to morphological changes and lipid accumulation within the cells.[3] PPARγ is the master regulator of adipocyte differentiation, which enhances the conversion of preadipocytes into insulin-sensitive adipocytes. Mature adipocytes can be measured by lipid accumulation, changes in cell morphology, and the expression of adipogenic genes.[4] SREBP is the

University of Malaya

transcriptional factor that interplays with PPARγ by producing endogenous ligands that are necessary for the activation of PPARγ.[5] In addition, activation of PPARγ in mature adipocyte stimulates other genes involved in the insulin signaling cascade, such as phosphoinositide 3-kinase (PI3K), and the glucose transporters Glut1 and Glut4.[6,7]

In addition, basal and insulin-stimulated glucose uptake in adipocytes play a crucial role in glucose metabolism. Glut1, which is located in the plasma membrane of cells, is responsible for maintaining a basal rate of glucose uptake. Insulin binds to the insulin receptor on the plasma membrane of adipocytes and thereby activates the signaling cascade involving the PI3K gene, which eventually causes translocation of Glut4 from intracellular stores to the plasma membrane.[8] Although different hypoglycemic drugs have been synthesized for the treatment of diabetes mellitus, many drugs are likely to cause adverse effects to the host; hence, interest has been diverted to natural substances.[9] The search for natural substances exhibiting potential antihyperglycemic and or antidiabetic properties is ongoing in the field of nutritional research.

Traditionally, mushrooms have been used as a major component of folk medicine to promote health and longevity.[10] The curative effects claimed by folk medicine for many mushrooms have been validated by recent scientific research.[11] The giant oyster mushroom, *Pleurotus giganteus* (Berk.) Karun. & K.D. Hyde (=*Lentinus giganteus*, *Panus giganteus*, Pleurotaceae, Agaricomycetes), is one of the largest edible mushrooms that have been treated as special foods since ancient times.[12,13] Confusion in the nomenclature of the mushroom was eventually solved by transferring *Lentinus giganteus* to *P. giganteus* based on morphological and molecular evidence.[14] In Malaysia, *P. giganteus* is consumed by indigenous peoples[12]; it is locally known as "*cendawan seri pagi*" (morning glory mushroom). Commercial cultivation of this mushroom, though new to Malaysia, is very popular in Northern Thailand[14] and China.[15] However, limited reports describe the potential medicinal properties of *P. giganteus*. Nutritional analysis conducted by Phan et al.[16] showed that *P. giganteus* contained carbohydrates, dietary fiber, potassium, and phenolic compounds. *P. giganteus* significantly ($P < 0.05$) stimulated neurite outgrowth[16] and had a hepatoprotective effect in rats.[15] In the present study, a crude methanol extract and an ethyl acetate extract, which was fractionated from the crude methanol extract of *P. giganteus* basidiocarps, were used to investigate the mushroom's effect on adipogenic differentiation of 3T3-L1 adipocytes.

II. MATERIALS AND METHODS

A. Mushroom Material and Chemicals

P. giganteus basidiocarps were obtained from Nas Agro Farm (Sepang, Selangor, Malaysia). Murine preadipocyte cells (3T3-L1) were purchased from the American Type Culture Collection (Manassas, VA; catalogue no. CL-173). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), minimum essential medium, fetal bovine serum (FBS), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (Dexa), L-glutamine, penicillin and streptomycin, and Oil Red O powder were purchased from Sigma. Insulin was purchased from Gibco. A kit to quantify nonesterified free fatty acids and glycerol was purchased from Zenbio. A glucose uptake cell-based assay kit was obtained from Cayman Chemical (item no. 600470).

B. Extraction of *P. giganteus* Basidiocarps

The basidiocarps were sliced, weighed, freeze-dried (Alpha 1-4 LD plus freeze dryer; Martin Christ Gefriertrocknungsanlagen GmbH), and ground to a powder using a commercial blender (Waring). For the crude methanol extract, mushroom powder was soaked in methanol and water at a 4:1 (v/v) ratio for 6 consecutive days. The solvent was changed every 2 days and removed using Whatman filter paper. The pooled filtrates were evaporated to dryness using a rotary evaporator under reduced pressure and kept at −20°C until further analysis.[17] Organic layers were separated using separating funnel. The crude

extract was defatted with hexane and fractioned with a mixture of ethyl acetate and water (1:1) to yield an ethyl acetate–soluble fraction. Evaporating the solvent from the ethyl acetate layer produced an ethyl acetate extract.

C. Cell Culture

3T3-L1 preadipocyte cells from the American Type Culture Collection were maintained in DMEM containing 10% FBS, 1% penicillin and streptomycin, and 1% L-glutamine at 37°C in a humidified 5% carbon dioxide atmosphere in an incubator. The medium was changed every 2 days. To differentiate, preadipocytes were seeded in culture flasks and were maintained until 48 hours after confluence. Differentiation was induced by incubating the cells with a differentiation medium (DM) 1 containing 0.5 mM IBMX, 1 µM Dexa, and 5 µg/mL insulin in DMEM containing 10% FBS and incubated for 48 hours (defined as day 0). Subsequently, after 48 hours the cells were cultured in DM2, which is similar to DM1 but without IBMX and Dexa. Mushroom extracts were dissolved in 0.9% DMSO to obtain final concentrations of 0.1, 1, 10, and 100 µg/mL. The extracts were filtered through 0.2-µm-pore syringe filters before adding them to the cell culture. The cells were pretreated with methanol and ethyl acetate extract for 2 days.

D. Cell Viability and Cytotoxicity Assay

Cell viability was determined by the conversion of MTT to formazan. Approximately 1×10^3 cells were seeded on a 96-well plate and incubated at 37°C overnight in a humidified environment of 5% carbon dioxide and 95% air. After incubation, the cells were treated with 0.1–100 µg/mL methanol and ethyl acetate extracts of *P. giganteus*. Subsequently, 10 µL of sterilized MTT (5 mg/mL) in PBS was added to each well and incubated for 4 hours. The supernatant was removed, and 200 µL of DMSO was added to each well to dissolve the MTT formazan (blue color) at the bottom of the wells. After 5 minutes, absorbance was measured at 550 nm with an enzyme-linked immunosorbent assay (ELISA) microplate reader (Biotech Instruments). Cells incubated in medium only, without mushroom extracts, were used as the negative control.[18]

E. Oil Red O Quantitative Assay

Oil Red O staining and quantitation of triglyceride content were performed as described previously,[19] with some modifications. Differentiated adipocytes were treated with methanol and ethyl acetate extracts of *P. giganteus* for 5 days. On day 7, cells in 24-well plates were rinsed with PBS twice and fixed with 10% formalin solution for 1 hour. Oil Red O isopropanol solution (60% [v/v]) was prepared by diluting the stock with distilled water. The diluted and filtered solution was added to the fixed adipocyte monolayer and kept aside for 1 hour. Cells were washed with water and dried. Images were captured using an Olympus CK-40 inverted microscope (at magnification of ×200). Subsequently, the stained triglyceride droplets were dissolved in isopropyl alcohol and the absorbance was read at 510 nm using an ELISA microplate reader (Biotech Instruments). The amount of staining of 3T3-L1 adipocytes with Oil Red O is directly proportional to the extent of differentiation. The optical densities of the Oil Red O–eluted solutions were indicators of the degree of adipocyte differentiation. Intracellular lipids stained with Oil Red O were extracted from the cells and quantified as previously described. In this study, to investigate the effect of the methanol and ethyl acetate extracts on adipocyte differentiation, matured 3T3-L1 cells were treated with the extracts after 2 days of the induction of differentiation.

F. Glucose Uptake via Fluorescence Assay

Glucose uptake into 3T3-L1 adipocytes was measured according to a modified method.[20] Matured adipocytes that were cultured in 24-well fluorescence plates, were treated with *P. giganteus* ethyl acetate extract on day 5. The differentiation

University of Malaya

medium was removed and replaced with glucose and serum-free medium containing 150 μg/mL 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), with the extracts. The control cultures were treated in basal medium (0.9% DMSO). The cells that were treated with insulin (10 μg/mL) served as the positive control. The cells were incubated for 3 hours. Subsequently, the cells were washed with PBS to remove free 2-NBDG. The fluorescence retained in cell monolayers was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Results are expressed as relative fluorescence units.

G. RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from 3T3-L1 cells that were treated with *P. giganteus* ethyl acetate extract using an Ambion RNAqueous Micro kit (Applied Biosystems). The purity and quality of recovered total RNA was estimated by calculating the ratio of absorbance at 260 and 280 nm using an ELISA microplate reader. The integrity of RNA was estimated using an Agilent 2100 Bioanalyzer (Applied Biosystems). RNA from each sample was reverse-transcribed to complementary DNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), which contained all the reagents (reverse transcription buffer, deoxynucleotide triphosphate mix, random primers, Multiscribe reverse transcriptase enzyme, and nuclease free water). After complementary DNA synthesis, quantitative real-time polymerase chain reaction (PCR) was performed using TaqMan Gene Expression Master Mix and assay mix (Applied Biosystems), according to the manufacturer's protocol. Each assay mix contained corresponding primers tagged with a probe for each targeted gene. The mixture was then loaded into the real-time PCR thermal cycler (StepOnePlus Real-Time PCR System). The PCR conditions were 1 cycle at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 second and 60°C for 1 minute. TaqMan probes were used in the real-time PCR reaction, and all the probes were labelled with FAM reporter dye at the 5′ end and a minder groove binder quencher at the 3′ end. The primers used for the PCR amplifications were predesigned by Applied Biosystems with the individual assay identifiers: srebf1 (Mm00550338_m1), PI3K (Mm01282781_m1), glut4 (Mm00436615_m1), glut1 (Mm00441480_m1), and ppar$_\gamma$ (Mm01184322_m1). The endogenous control (also known as housekeeping genes) used in this study was eukaryotic 18S ribosomal RNA with a FAM/MGB probe. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to measure gene expression.[21]

H. Statistical Analysis

All experimental data are expressed as the mean ± standard deviation of triplicate assays and were statistically analyzed with 1-way analysis of variance in which post hoc analysis was used to determine the significant difference between groups using Duncan multiple range tests. Statistical significance was accepted at $P < 0.05$, and SPSS statistical software (version 18; IBM Corp., Armonk, NY) was used for all statistical analyses.

III. RESULTS

A. Effect of Crude *P. giganteus* Methanol and Ethyl Acetate Extracts on 3T3-L1 Cell Proliferation

The MTT assay was performed to determine the viability of 3T3-L1 preadipocytes in the presence of crude methanol and fractionated ethyl acetate extracts of *P. giganteus* (Fig. 1). Cell viability was denoted as 100% for the control (i.e., cells in complete growth medium with solvent [0.9% DMSO] used to dissolve the methanol and ethyl acetate extracts). There was mild dose-dependent growth of 3T3-L1 cells incubated in methanol crude extract (Fig. 1). Methanol extract induced a significant ($P < 0.05$) percentage of preadipocyte proliferation at 10, 50, and 100 μg/mL: 106.27%, 108.59%, and 113.96%, respectively. Incubation of the 3T3-L1 cells with the ethyl acetate extract significantly stimulated proliferation at 0.1 and 0.5 μg/mL, but no changes were observed at higher doses.

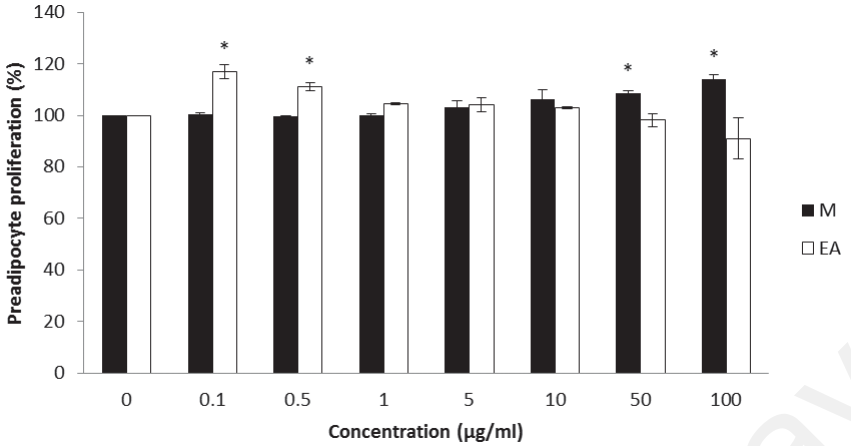


FIG. 1: The effects of methanol (M) and ethyl acetate (EA) extracts of *Pleurotus giganteus* on 3T3-L1 cell proliferation. The mean absorbance of the negative control (medium with cells and vehicle) was designated as 100%. Each value represents the mean ± standard error (n = 3). *Significant difference of methanol and ethyl acetate extracts compared with control ($P < 0.05$).

B. Effect of Crude *P. giganteus* Methanol and Ethyl Acetate Extracts on Lipogenesis (Lipid Accumulation) of 3T3-L1 Cells

The staining of 3T3-L1 cells with Oil Red O has been commonly used to visualize macroscopically adipocytes from cultures of preadipocyte cell lines. Figure 2 shows accumulated lipid droplets stained with Oil Red O and visualized under a microscope 8 days after the induction of differentiation. Figure 3 shows that treatment of 3T3-L1 cells with both extracts enhanced adipocyte differentiation in a dose-dependent manner, at doses ranging from 0.1 to 100 µg/mL. When the cells were incubated with methanol and ethyl acetate extracts at 10 and 100 µg/mL, a significant ($P < 0.05$) percentage of lipid accumulation was quantified. At 10 and 100 µg/mL, methanol extract induced 109.47% and 110.70% lipid accumulation, respectively. At the same concentrations, the ethyl acetate extract induced 110.51% and 175.34% of lipid accumulation, respectively. No significant ($P < 0.05$) amount of lipid accumulation was observed during the incubation of 3T3-L1 cells with lower concentrations of the methanol and ethyl acetate extracts.

C. Effects of *P. giganteus* Ethyl Acetate Extract on 2-NBDG Uptake in 3T3-L1 Cells

The ethyl acetate extract, which significantly ($P < 0.05$) enhanced lipid accumulation in 3T3-L1 cells, was further assessed for its insulin-like activity in stimulating glucose uptake. Insulin stimulates

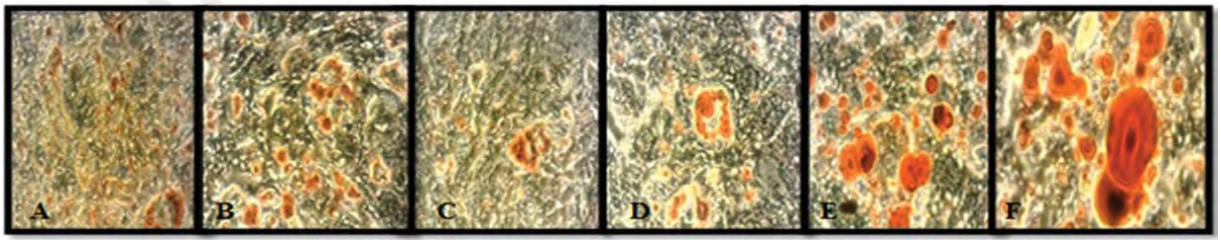


FIG. 2: Representative photomicrographs (at magnification of ×200) showing triglyceride droplets in mature adipocytes stained with Oil Red O dye. Shown here are cells in medium only (A) and in insulin 10 µg/mL (B), and cells that have been treated with *Pleurotus giganteus* ethyl acetate extract at 0.1 (C), 1 (D), 10 (E), and 100 µg/mL (F).

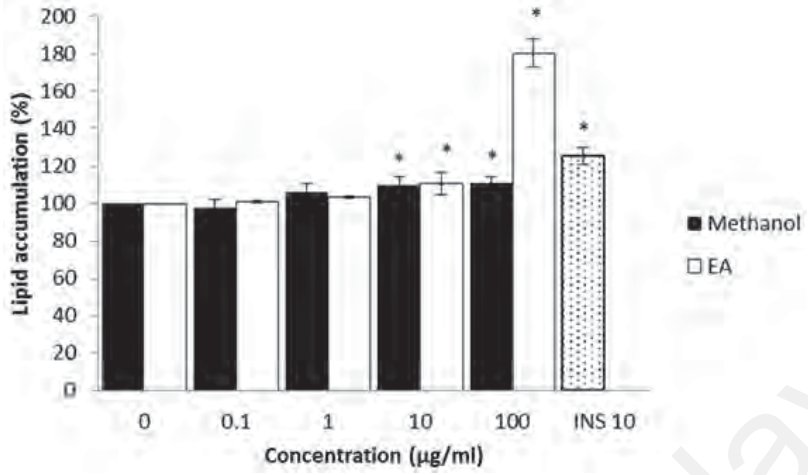


FIG. 3: Effect of methanol and ethyl acetate (EA) extracts of *Pleurotus giganteus* on lipogenesis (lipid accumulation) in mature 3T3-L1 cells. Insulin at 10 µg/mL (INS 10) was used as the positive control. Values expressed were based on the lipogenic effect of the extracts on 3T3-L1 cells against the control (without treatment). Each value represents the mean ± standard deviation (n = 3). *Significant effect compared with the negative control ($P < 0.05$).

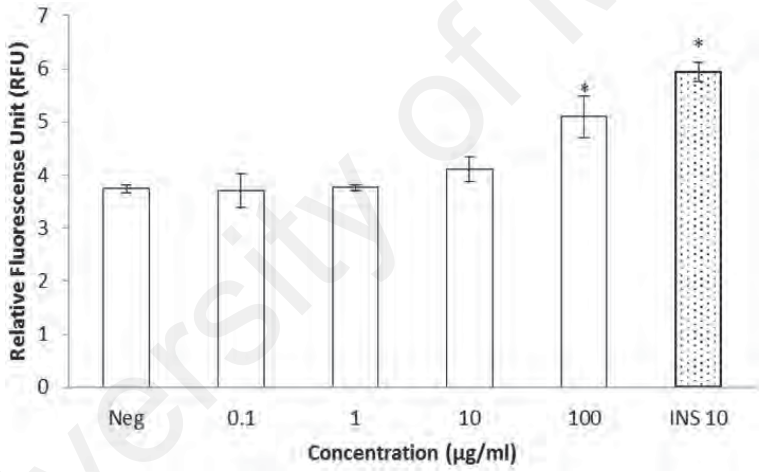


FIG. 4: Effect of the *Pleurotus giganteus* ethyl acetate extract on 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose uptake by mature 3T3-L1 cells. Cells treated with the solvent (0.9% dimethyl sulfoxide) served as the negative control (Neg) and insulin 10 µg/mL (INS 10) served as the positive control. Data are expressed as means ± standard deviations (n = 3). *Significant effect compared with the negative control ($P < 0.05$).

glucose uptake in adipocytes by binding to insulin receptors within the cell, leading to the translocation of Glut4 into the plasma membrane. In this study, differentiated 3T3-L1 cells were incubated with the ethyl acetate extract at 0.1, 1, 10, 100 µg/mL, and insulin (10 µg/mL) was used as the positive control. Figure 4 shows that 2-NBDG uptake (glucose uptake) increased in a dose-dependent manner in differentiated 3T3-L1 cells that were treated with the ethyl acetate extract at doses ranging from 0.1 to 100 µg/mL. The maximum dose tested (100 µg/mL) induced glucose uptake of 5.1 relative fluorescence units ($P < 0.05$). The 3T3-L1 cells that were treated with insulin (10 µg/mL) stimulated significant ($P < 0.05$) glucose uptake compared with the control, as expected.

University of Malaya

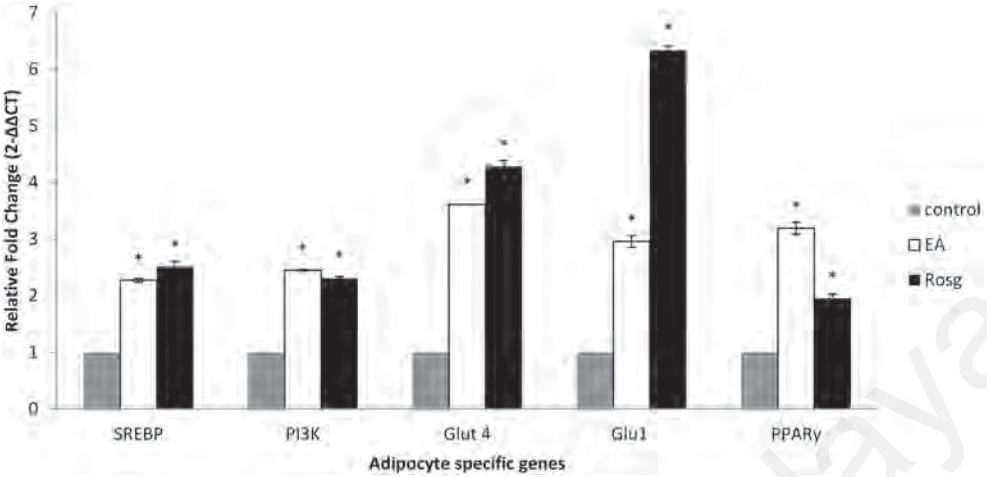


FIG. 5: Effects of *Pleurotus giganteus* ethyl acetate extract (EA) on the expression of adipocyte-specific genes. Cells treated with the solvent (0.9% dimethyl sulfoxide) served as the negative control and rosiglitazone (Rosg; 1 μm) served as the positive control. The cells were treated with 100 μg/mL EA. Data are expressed as means ± standard deviations (n = 3). Significant effect compared with the negative control ($P < 0.05$). Glut, glucose transporter; mRNA, messenger RNA; SREBP, sterol regulatory element-binding protein; PI3K, phosphatidylinositol 3-kinase; PPARγ, peroxisome proliferator-activated receptor γ.

D. Effects of Ethyl Acetate Extract on the Expression of Adipocyte Transcriptional Regulators, Signal Molecules, and Glucose Transporters

To characterize further the effects of the ethyl acetate extract, the 100 μg/mL of the extract was selected to be tested for adipocyte differentiation. Quantitative real-time PCR was used to examine the expression of adipocyte transcriptional regulators, signal molecules, and glucose transporters. Thus this part of the study attempts to increase the understanding of how the ethyl acetate extract possibly regulates the underlying mechanisms responsible for adipocyte differentiation, glucose uptake, and lipid accumulation. Rosiglitazone 1 μM was used as the positive control. Figure 5 shows that the adipocytes that were treated with the *P. giganteus* ethyl acetate extract significantly ($P < 0.05$) upregulated the expression of both PPARγ and SREBP (by 3.19- and 2.27-fold, respectively). The expression of PI3K/Akt, which is a downstream signaling cascade of insulin receptor substrates, was significantly ($P < 0.05$) upregulated by 2.45- and 2.31-fold when incubated with the ethyl acetate extract and rosiglitazone, respectively. Next, the effect of the ethyl acetate extract on the expression of glucose transporters was investigated. The gene expression of Glut1 and Glut4 was significantly ($P < 0.05$) increased by 2.96- and 3.61-fold, respectively, in cells treated with the ethyl acetate extract, whereas rosiglitazone caused increases of 6.34- and 4.28-fold, respectively. These results demonstrate that the ethyl acetate extract can enhance adipocyte differentiation by upregulating the expression of PPARγ, SREBP, PI3K, Glut1, and Glut4.

IV. DISCUSSION

This study provides what is, to our knowledge, the first evidence that the ethyl acetate extract of *P. giganteus* promotes insulin sensitivity and increases the expression of SREBP and PPARγ, a key adipogenic transcription factor in 3T3-L1 cells. The extract activated the insulin signaling pathway by increasing PI3K and Glut4. Basically, impaired adipocyte differentiation may cause complications such as insulin resistance, hyperlipidemia, and type 2 diabetes.[22] It has also been reported that the deposition of triglyceride and free fatty acids in tissues comprising cells other than adipocytes, such as liver, muscle, and pancreas, has a negative effect on insulin sensitivity.[23] This implies that adipose

tissue itself functions as an attractive avenue to treat complications related to diabetes.[24]

Mushrooms generally contain biologically active compounds that are reported to have hypoglycemic effects by reducing blood glucose concentrations.[25] Ongoing studies have shown that *P. giganteus* contains bioactive secondary metabolites—namely, fatty acids such as palmitic acid, fatty acid methyl esters such as linoleic acids methyl esters, phenolics such as caffeic acid and cinnamic acids, and sterols such as ergosterol and neoergosterol (unpublished data). The total phenolics present in the mushroom extracts were positively correlated to the antioxidant activity (free radical scavenging, ferric-reducing power, and lipid peroxidation inhibition). *P. giganteus* could potentially be used in a well-balanced diet and as a source of dietary antioxidants to promote neuronal health and antihyperglycemic activity.[26,27] The crude methanol extract of *P. giganteus* promoted mild proliferation of 3T3-L1 preadipocytes, but the ethyl acetate extract moderately reduced the percentage of proliferation (based on 3 separate experiments). The presence of palmitate compounds may have inhibited the proliferation of mouse 3T3-L1 preadipocytes by modulating the intracellular signal that induces endoplasmic reticulum stress.[28] However, the presence of other unsaturated fatty acids such as linoleic acid may counteract the effect of palmitate.[29] Mild to moderate inhibition of preadipocyte proliferation may be favorable; otherwise preadipocytes can potentially proliferate throughout life and increase fat mass in the body. An increase in the mass of adipose tissue arises through an increase in cell size, in cell number, or both. Therefore the reduction of preadipocyte proliferation had been a target for developing antiobesity agents. As an index of adipogenic differentiation, lipid accumulation was measured using Oil Red O because lipid droplets produced in the adipocytes' cytoplasm are selectively stained with Oil Red O dye. In this study the crude methanol and fractioned ethyl acetate extracts promoted lipid accumulation in a dose-dependent manner. At 100 μg/mL, the ethyl acetate extract significantly ($P < 0.05$) enhanced lipid accumulation. Nicotinamide was previously reported to stimulate adipocyte differentiation by increasing the expression of adipogenic genes.[30] Moreover, the stimulation of lipogenesis may be attributed to the presence of saturated (palmitic acid) or monounsaturated (methyl palmitate acid, methyl stearate, methyl linoleate, and methyl mandelate) fatty acids found in the *P. giganteus* ethyl acetate extract.[31] In this study the ethyl acetate fraction showed more lipogenesis activity compared with the crude methanol extract. This could be attributed to the presence of different compounds and the above-mentioned fatty acids, which were concentrated in the fraction as a result of the fractionation process.

At 100 μg/mL the *P. giganteus* ethyl acetate fraction exerted a significant ($P < 0.05$) percentage of glucose uptake. The presence of both saturated and unsaturated fatty acids, such as palmitic acid and oleic acid, in extracts may have contributed to the enhanced transportation of glucose into the 3T3-L1 cells. The efficiency of glucose transportation is strongly affected by the type of fatty acids present. It was previously reported that unsaturated fatty acids are more effective than saturated fatty acids.[32] Conversely, others found that saturated fatty acids also stimulate glucose uptake.[33]

The relative expression of 5 selected genes involved in adipocyte transcriptional regulation, signal molecules, and glucose transporters were examined. This study provides initial evidence of upregulation of the expression of SREBP and PPARγ by the *P. giganteus* ethyl acetate extract of. This study also demonstrated that *P. giganteus* extract activates the insulin signaling pathway via phosphorylation of PI3K, followed by the upregulation of Glut4, a downstream gene that is responsible for the translocation of vesicles from the cytoplasm to the cell membrane in order to facilitate the intake of glucose. The results suggest that the ethyl acetate extract may have inhibitory activity against type 2 diabetes by acting as an insulin sensitizer that activates the insulin signaling pathway and eventually improves adipogenic differentiation. Rosiglitazone, an antidiabetic drug in the thiazolidinedione drug class, was used as a positive control because it works as an insulin sensitizer by binding to the PPARγ receptor. PPARγ is a ligand-dependent nuclear receptor transcription factor that makes cells

more responsive to insulin, and thus efficiently converts preadipocytes into differentiated adipocytes. In addition to commercially available ligands such as thiaizolidinedione, certain polyunsaturated fatty acids such as linoleic acid and arachidonic acid also have an affinity for PPARγ activation.[34] Therefore the presence of linoleic acid in the *P. giganteus* ethyl acetate extract may not only act as a dietary source of exogenous ligands that induce adipocyte differentiation, but it is also less efficient in increasing adipocyte numbers.[24] In addition, it has been suggested that PPARγ promotors are induced by SREBP, which then activate the transcription of PPARγ 1 and 3. SREBP could also induce the secretion of some lipid molecules that may act as endogenous ligands for PPARγ.[35] In this study adipocytes that were treated with the *P. giganteus* ethyl acetate extract stimulated significant upregulation of the SREBP gene (by 2.27-fold), whereas rosiglitazone stimulated expression 2.51-fold compared with the basal control. Therefore it is feasible to speculate that the contents of the *P. giganteus* ethyl acetate extract may act as a direct ligand to PPARγ and may stimulate the production of endogenous ligands by upregulating the expression of SREBP.[35]

PPARγ also modulates the insulin signaling pathway through the upregulation of insulin receptor substrates 1 and 2, PI3K, and protein kinase B (Akt).[36] Phosphorylated Akt facilitates glucose uptake in adipocytes by allowing Glut4 located in intracellular storage vesicles to translocate to the plasma membrane.[37] In this study PI3K was shown to be significantly ($P < 0.05$) upregulated (by 2.5-fold) compared with the control. In addition, the other possible mechanism underlying the finding that the ethyl acetate extract enhanced glucose transport in 3T3-L1 adipocytes is the increased expression of both Glut1 and Glut4, which are among the genes directly involved in the translocation of glucose into 3T3-L1 adipocytes, regulated by PPARγ ligands.[38] The expression of Glut4 is regulated by PPARγ at the initial stage of adipocyte development and leads to the subsequent events of lipid metabolism.[39]

V. CONCLUSIONS

P. giganteus enhanced adipocyte differentiation by increasing PPARγ transcription activity. In addition, *P. giganteus* stimulated basal and insulin-stimulated glucose transport in 3T3-L1 adipocytes via the enhanced expression of Glut1 and Glut4. To our knowledge, this is the first evidence of the effect of *P. giganteus* on adipocyte differentiation and glucose uptake.

ACKNOWLEDGMENTS

This research was supported by a Kuala Lumpur High Impact Research Grant UM-MOHE UM.C/625/1/HIR/MOHE/SC/02 (HIR F00002-21001) and a postgraduate grant (PV007/2012A), both from the University of Malaya.

REFERENCES

1. Ahmad NS, Ramli A, Islahudin F, Paraidathathu T. Medication adherence in patients with type 2 diabetes mellitus treated at primary health clinics in Malaysia. Patient Prefer Adherence. 2013;17(7):525–30.
2. Fonseca-Alaniz MH, Takada J, Alonso-Vale MIC, Lima FB. Adipose tissue as an endocrine organ: from theory to practice. J Pediatr (Rio J). 2007;83(5):S192–203.
3. Li H, Xiao L, Wang C, Gao J, Zhai Y. Epigenetic regulation of adipocyte differentiation and adipogenesis. J Zhejiang Univ Sci B. 2010;11(10):784–91.
4. Saben J, Thakali KM, Lindsey FE. Distinct adipogenic differentiation phenotypes of human umbilical cord mesenchymal cells dependent on adipogenic conditions. Exp Biol Med (Maywood). 2014;239(10):1340–51.
5. Nora A, Jurgen B. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. Pharmacol Rev. 2008;60(3):311–57.
6. Watson RT, Kanzaki M, Pessin JE. Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr Rev. 2004;25(2):177–204.
7. Herman MA, Kahn BB. Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. J Clin Invest. 2006;116(7):1767–75.
8. Habtemichael EN, Alcázar-Román A, Rubin BR, Grossi LR, Belman JP, Julca O, Löffler MG, Li H, Chi NW, Samuel VT, Bogan JS. Coordinated regulation of vasopressin inactivation and glucose uptake by action of TUG protein in muscle. J Biol Chem. 2015;290(23):14454–61.
9. Chang ST, Wasser SP. The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. Int J Med Mushrooms 2012;14(2):95–134.

University of Malaya

10. Wasser SP. Medicinal mushroom science: history, current status, future trends, and unsolved problems. Int J Med Mushrooms. 2010;12(1):1–16.
11. Eik LF, Naidu M, David P, Wong KH, Tan YS, Sabaratnam V. Lignosus rhinocerus (Cooke) Ryvarden: a medicinal mushroom that stimulates neurite outgrowth in PC-12 cells. Evid Based Complement Alternat Med. 2012;2012: 320308.
12. Lee SS, Chang YS, Noraswati MNR. Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. For Ecol Manag. 2009;257(10):2062–5.
13. Lindequist U. The merit of medicinal mushrooms from a pharmaceutical point of view. Int J Med Mushrooms. 2013;15(6):517–23.
14. Karunarathna SC, Yang ZL, Ko TWK, Vellinga EC, Zhao R, Bahkali AH. Lentinus giganteus revisited: new collections from Sri Lanka and Thailand. Mycotaxonomy. 2011;118:57–71.
15. Wong WL, Abdulla MA, Chua K, Kuppusamy UR, Tan Y, Sabaratnam V. Hepatoprotective effects of Panus giganteus (Berk.) Corner against thioacetamide- (TAA-) induced liver injury in rats. Evid Based Complement Alternat Med. 2012;2012:170303.
16. Phan C, Wong W, David P, Naidu M, Sabaratnam V. Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde: nutritional value and in vitro neurite outgrowth activity in rat pheochromocytoma cells. BMC Complement Altern Med. 2012;12:102.
17. Kanagasabapathy G, Malek SNA, Kuppusamy UR, Vikineswary S. Chemical composition and antioxidant properties of extracts of fresh fruiting bodies of Pleurotus sajor-caju (Fr.) Singer. J Agric Food Chem. 2011;59(6):2618–26.
18. Borra RC, Lotufo MA, Gagioti SM, Barros FM, Andrade PM. A simple method to measure cell viability in proliferation and cytotoxicity assays. Braz Oral Res. 2009;23(3):255–62.
19. Kanagasabapathy G, Chua KH, Malek SN, Vikineswary S, Kuppusamy UR. AMP-activated protein kinase mediates insulin-like and lipo-mobilising effects of β-glucan-rich polysaccharides isolated from Pleurotus sajor-caju (Fr.), Singer mushroom, in 3T3-L1 cells. Food Chem. 2014;145:198–204.
20. Manaharan T, Ming CH, Palanisamy UD. Syzygium aqueum leaf extract and its bioactive compounds enhances pre-adipocyte differentiation and 2-NBDG uptake in 3T3-L1 cells. Food Chem. 2013;136:354–63.
21. Kanagasabapathy G, Kuppusamy UR, Malek SNA, Abdulla MA, Chua KH, Sabaratnam V. Glucan-rich polysaccharides from Pleurotus sajor-caju (Fr.) Singer prevents glucose intolerance, insulin resistance and inflammation in C57BL/6J mice fed a high-fat diet. BMC Complement Altern Med. 2012;12:261.
22. Patel P, Abate N. Body fat distribution and insulin resistance. Nutrients. 2013;5(6):2019–27.
23. Grinspoon S, Carr A. Cardiovascular risk and body-fat abnormalities in HIV-infected adults. N Engl J Med. 2005;352(1):48–62.
24. Staels B, Fruchart JC. Therapeutic roles of peroxisome proliferator-activated receptor agonist. Diabetes. 2005;54(8):2460–70.
25. Fernyhough ME, Okine E, Hausman G, Vierck JL, Dodson MV. PPARγ and Glut-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. Domest Anim Endocrinol. 2007;33(4):367–78.
26. Lo HC, Wasser SP. Medicinal mushrooms for glycemic control in diabetes mellitus: history, current status, future perspectives, and unsolved problems (review). Int J Med Mushrooms. 2011;13(5):401–26.
27. Chia-Wei P, Pamela D, Yee-Shin T, Murali N, Kah-Hui W, Kuppusamy UR, Vikineswary S. Intrastrain comparison of the chemical composition and antioxidant activity of an edible mushroom, Pleurotus giganteus, and its potent neuritogenic properties. Sci World J. 2014;2014:378651.
28. Lin S-Y, Chen Y-K, Yu H-Y, Barseghyan GS, Asatiani MD, Wasser SP, Mau J-L. Comparative study of contents of several bioactive components in fruiting bodies and mycelia of culinary-medicinal mushrooms. Int J Med Mushrooms. 2013;15(3):315–23.
29. Guo W, Wong S, Xie W, Lei T, Luo Z. Palmitate modulates intracellular signalling, induces endoplasmic reticulum stress and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. Am J Physiol Endocrinol Metab. 2007;293(2):E576–86.
30. Wang SL, Li Y, Wen Y, Chen YF, Na LX, Li ST. Curcumin, a potential inhibitor of up-regulation of TNF-α and IL-6 induced by palmitate in 3T3-L1 adipocytes through NF-kappa B and JNK pathway. Biomed Environ Sci. 2009;22(1):32–9.
31. Bai L, Pang WJ, Yang YJ, Yang GS. Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes. Mol Cell Biochem. 2008;307(1–2):129–40.
32. Kamphorst JJ, Cross JR, Fan J. Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. Proc Natl Acad Sci USA. 2013;110(22):8882–7.
33. Jan FCG, Joost JFP, Luiken AB. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol Rev. 2010;90(1): 367–417.
34. Grosso G, Galvano F, Marventano S, Malaguarnea M, Bucolo C, Drago F, Craci F. Omega-3 fatty acids and depression: scientific evidence and biological mechanisms. Oxid Med Cell Longev. 2014;2014:313570.
35. Benedetti E, Galzio R, D'Angelo B, Cerù MP, Cimini A. PPARs in human neuroepithelial tumors: PPAR ligands as anticancer therapies for the most common human neuroepithelial tumors. PPAR Res. 2010;2010:401–27.
36. Pawlac M, Lefevre P, Staels B. Molecular mechanism of PPARα action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. J Hepatol. 2015;62(3):720–33.

37. Chang L, Chiang SH, Saltiel AR. Insulin signalling and the regulation of glucose transport. Mol Med. 2004; 10(7–12):65–71.
38. Françoise K, Bo J, Jing Y, Geoffrey DH. Insulin signalling meets vesicle traffic of GLUT4 at a plasma-membrane-activated fusion step. Cell Metab. 2005;2(3): 179–89.
39. Im SS, Kwon SK, Kim TH, Kim HI, Ahn YH. Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. IUBMB Life. 2007;59(3):134–45.

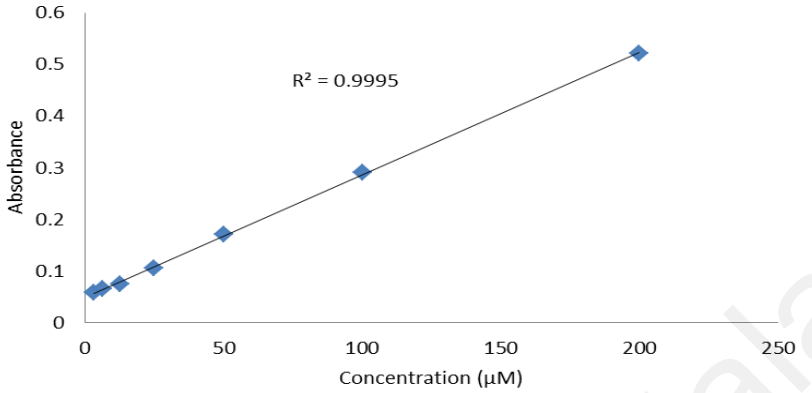
University of Malaya

University of Malaya

APPENDICES

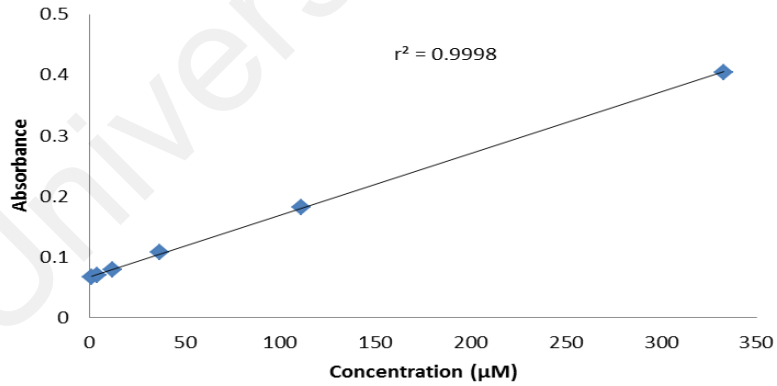
Appendix A: Analytical techniques

1. Determination of glycerol content (lipolysis assay)



$$\text{Glycerol content (µM)} = \frac{y - 0.0495}{0.0024}$$

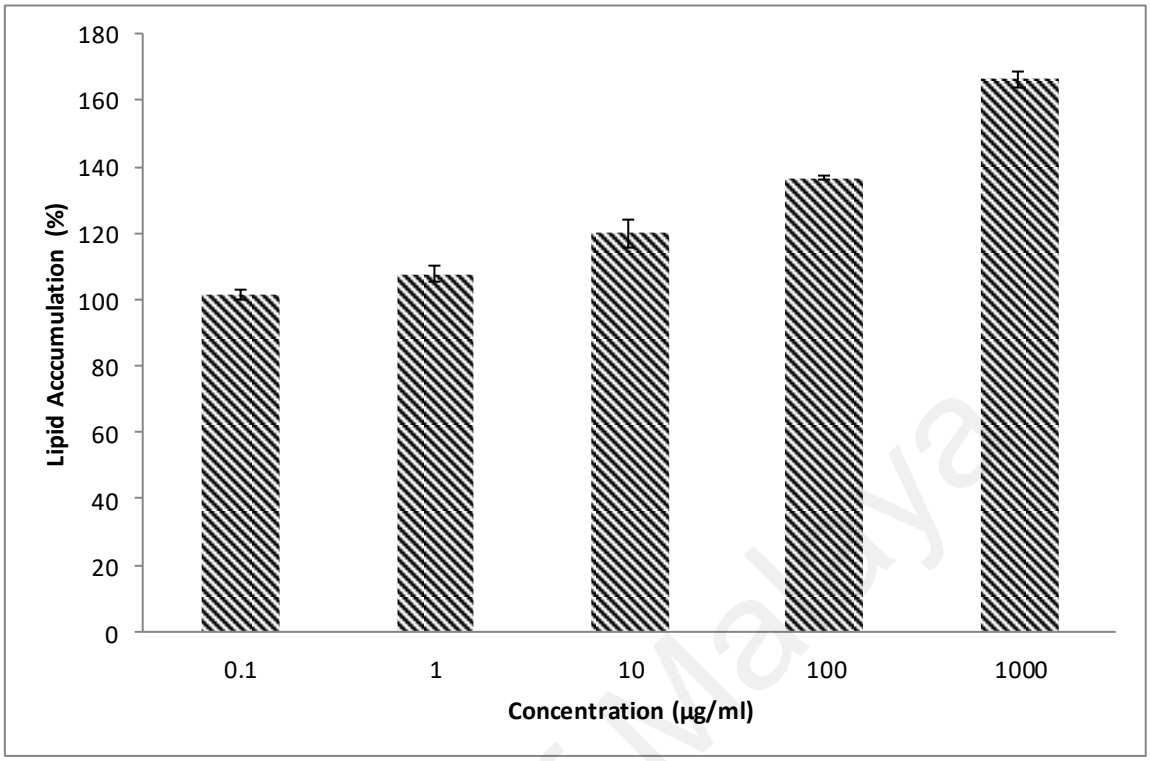
2. Determination of fatty acids content (lipolysis assay)



$$\text{Fatty acids content (µM)} = \frac{y - 0.0668}{0.001}$$

University of Malaya

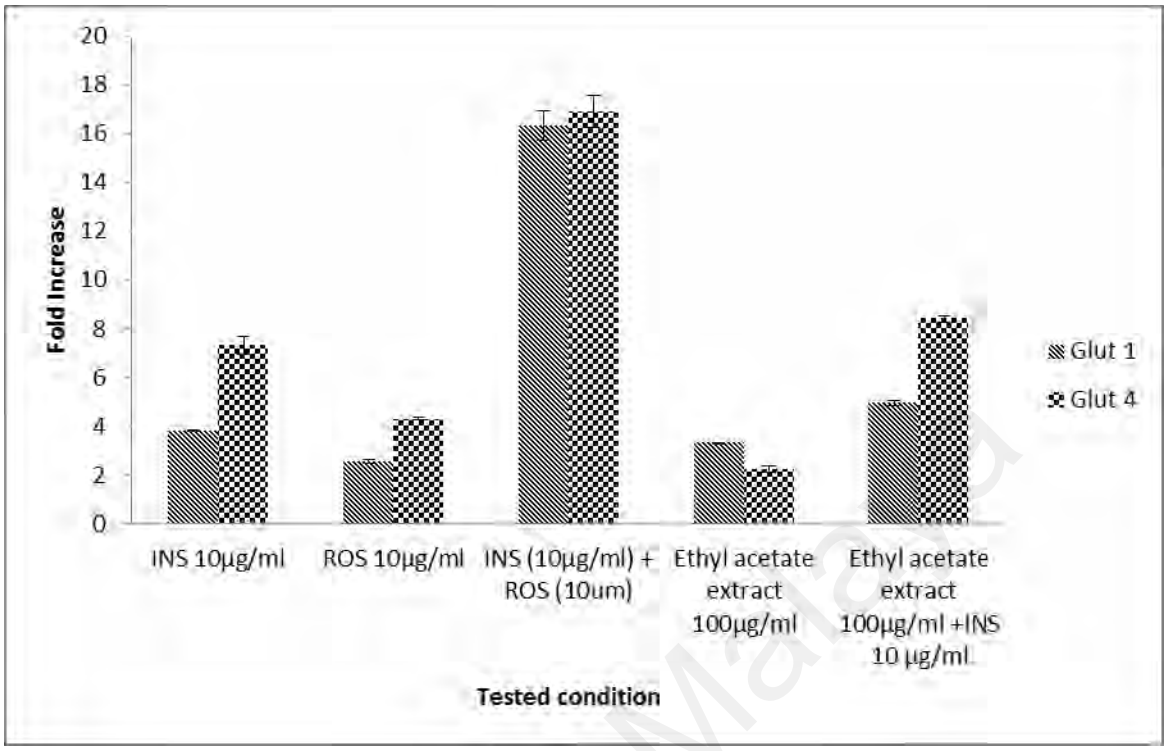
3. The Percentage (%) of lipogenesis of 3T3-L1 mature adipocytes



The percentage (%) of lipogenesis in 3T3-L1 mature adipocytes treated by Insulin at various concentrations (from 0.1µg/ml to 1000 µg/ml)

University of Malaya

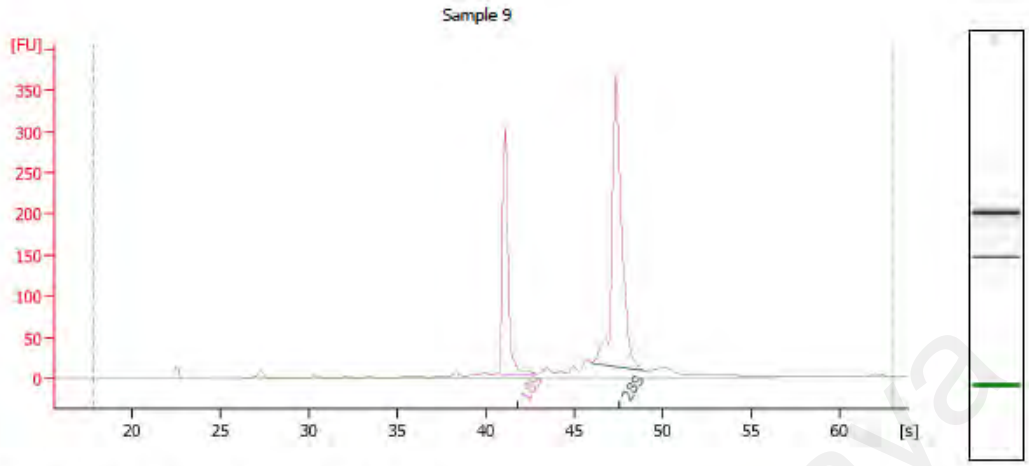
4. Gene expression of glut 1 and glut 4 in various treatment condition



Effects of ethyl acetate fraction (100µg/ml) of *P. giganteus* on the expression of glucose transporter genes, glut 1 and glut 4. Cells treated with basal medium served as negative control. Insulin (10µg/ml) and Rosiglitazone (10µg/ml) individually and in combination served as positive control. The fourth bar indicates gene expression of cells with the presence of only ethyl acetate fraction (100µg/ml). The fifth bar indicates gene expression of cells with co-presence of ethyl acetate fraction (100µg/ml) and insulin (10µg/ml).

University of Malaya

5. The integrity of cDNA (Ethyl acetate fraction)



Overall Results for sample 9 : **Sample 9**

RNA Area:	995.1	RNA Integrity Number (RIN):	10 (B.02.07)
RNA Concentration:	378 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1.9	Result Flagging Label:	RIN:10

Fragment table for sample 9 : **Sample 9**

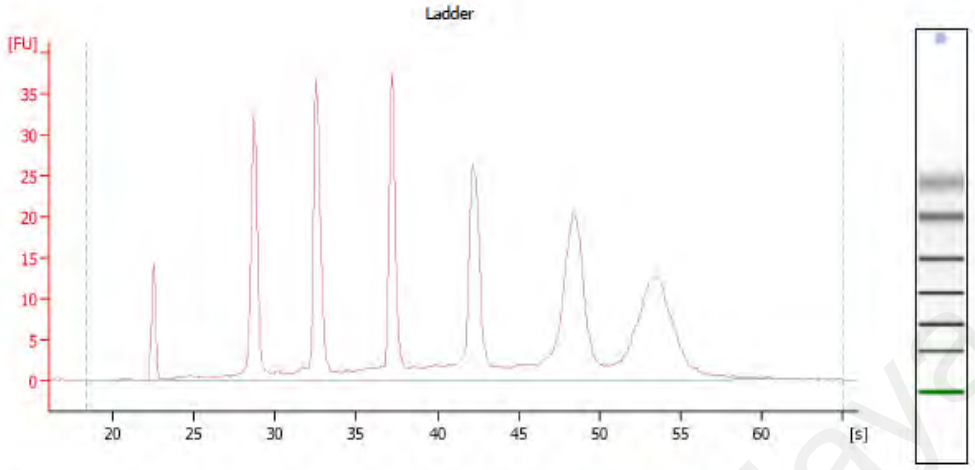
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.76	42.81	251.3	25.3
28S	45.96	49.06	476.9	47.9

6. The purity of cDNA

Samples	Purity	Concentration
Control	2.172	342.722
Rosiglitazone	2.109	489.68
Ethyl acetate fraction	2.133	398.301

University of Malaya

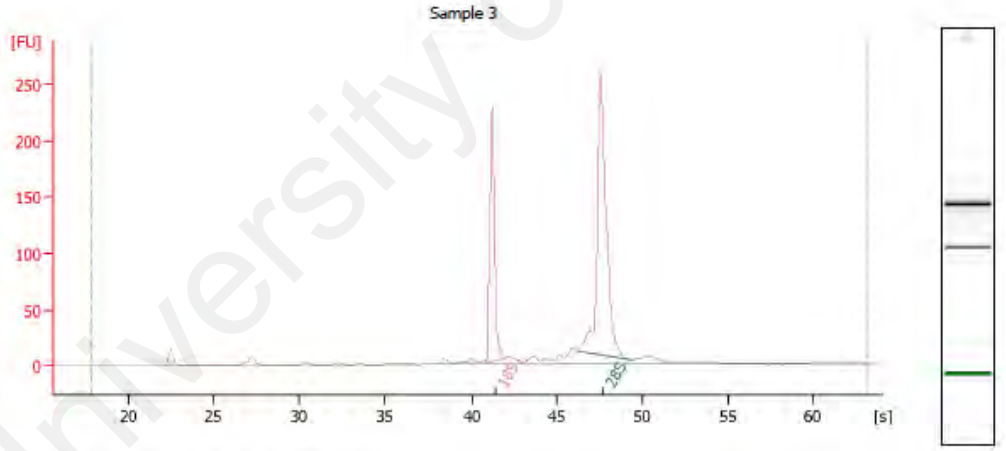
7. The integrity of cDNA (Ladder)



Overall Results for Ladder

RNA Area:	395.2	Result Flagging Color:	
RNA Concentration:	150 ng/µl	Result Flagging Label:	All Other Samples

8. The integrity of cDNA (control)



Overall Results for sample 3 : Sample 3

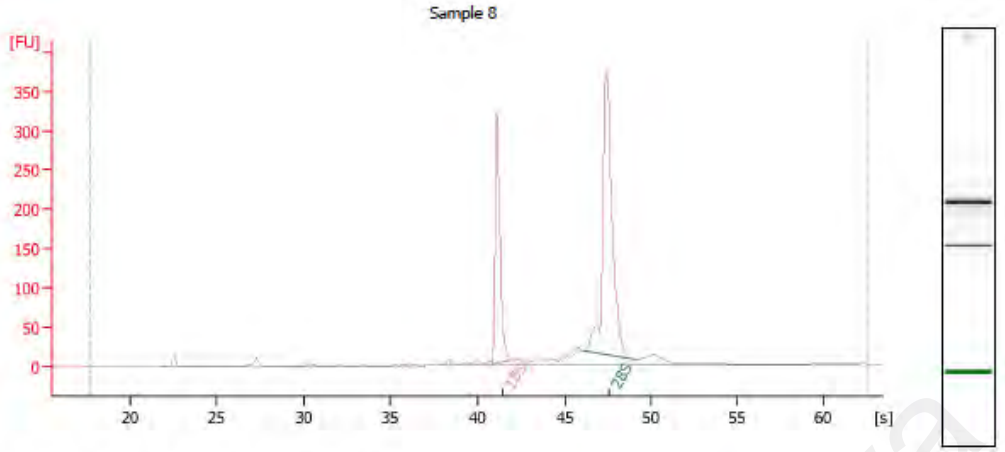
RNA Area:	689.3	RNA Integrity Number (RIN):	10 (B.02.07)
RNA Concentration:	262 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2.0	Result Flagging Label:	RIN:10

Fragment table for sample 3 : Sample 3

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.88	41.98	172.1	25.0
28S	46.14	49.34	337.5	49.0

University of Malaya

9. The integrity of cDNA (Rosiglitazone)



Overall Results for sample 8 : Sample 8

RNA Area:	1,018.6	RNA Integrity Number (RIN):	10 (B.02.07)
RNA Concentration:	387 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1.9	Result Flagging Label:	RIN:10

Fragment table for sample 8 : Sample 8

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.79	42.06	251.6	24.7
28S	46.04	49.12	484.3	47.5

University of Malaya

Appendix B: DATA AND STATISTICAL TABLES

Table 1: ANOVA for MTT Assay **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	97754.390	35	2792.983	122.348	.000
Within Groups	1643.630	72	22.828		
Total	99398.020	107			

Descriptives

Mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
					Lower Bound	Upper Bound			
Negative Control	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00	
Aqueous(1.0µg/ml)	3	194.7766	2.79937	1.61622	187.8226	201.7307	192.16	197.73	
Aqueous(5.0µg/ml)	3	175.7388	4.29704	2.48090	165.0644	186.4133	171.96	180.41	
Methanol (1.0µg/ml)	3	99.9447	.68923	.39793	98.2326	101.6568	99.45	100.73	
Methanol (5.0µg/ml)	3	102.9456	2.88694	1.66677	95.7741	110.1172	101.06	106.27	
Hexane (1.0µg/ml)	3	100.3148	2.02385	1.16847	95.2873	105.3423	98.85	102.62	
Methanol (5.0µg/ml)	3	107.7234	7.17557	4.14282	89.8983	125.5485	99.82	113.83	
Ethyl acetate (1.0µg/ml)	3	104.5915	.43324	.25013	103.5152	105.6677	104.13	104.99	
Ethyl acetate (5.0µg/ml	3	104.0258	2.69335	1.55501	97.3351	110.7164	100.97	106.04	
Butanol (1.0µg/ml)	3	127.9348	7.59393	4.38436	109.0704	146.7991	122.61	136.63	

University of Malaya

Butanol (5.0µg/ml)	3	132.0539	6.94014	4.00689	114.8137	149.2942	124.17	137.24
Aqueous(0.1µg/ml)	3	169.6220	1.80142	1.04005	165.1470	174.0970	167.63	171.13
Aqueous(0.5µg/ml)	3	178.7629	.20619	.11904	178.2507	179.2751	178.56	178.97
Aqueous(10.0µg/ml)	3	167.0790	1.87088	1.08015	162.4315	171.7266	165.36	169.07
Aqueous(50.0µg/ml)	3	160.1375	6.13149	3.54002	144.9060	175.3689	153.61	165.77
Methanol (0.1µg/ml)	3	100.2808	.81065	.46803	98.2670	102.2946	99.75	101.21
Methanol (5.0µg/ml)	3	99.7020	.16094	.09292	99.3022	100.1018	99.54	99.87
Methanol (10.0µg/ml)	3	106.2723	3.80503	2.19684	96.8201	115.7246	101.99	109.28
Methanol (50.0µg/ml)	3	108.5913	.91144	.52622	106.3271	110.8554	107.69	109.51
Hexane (0.1µg/ml)	3	99.5954	1.35002	.77943	96.2418	102.9491	98.10	100.73
Hexane (0.5µg/ml)	3	100.1796	2.96771	1.71341	92.8074	107.5518	97.13	103.05
Hexane (10.0µg/ml)	3	110.2833	4.45559	2.57243	99.2150	121.3516	105.14	113.01
Hexane (50.0µg/ml)	3	105.9722	10.01246	5.78070	81.0998	130.8445	94.43	112.35
Ethyl acetate (0.1µg/ml	3	116.9794	2.67478	1.54428	110.3348	123.6239	114.26	119.61
Ethyl acetate (0.5µg/ml	3	111.1338	1.56333	.90259	107.2503	115.0173	109.77	112.84
Ethyl acetate (10.0µg/ml	3	103.0744	.40916	.23623	102.0580	104.0908	102.65	103.47
Ethyl acetate (50.0µg/ml	3	89.0499	1.57160	.90736	85.1459	92.9540	87.44	90.58
Butanol (0.1µg/ml)	3	120.3838	8.03365	4.63823	100.4271	140.3405	115.11	129.63
Butanol (0.5µg/ml)	3	130.3942	6.65277	3.84098	113.8678	146.9206	125.10	137.86
Butanol (10.0µg/ml)	3	140.5435	11.77079	6.79587	111.3032	169.7837	131.74	153.91
Butanol (50.0µg/ml)	3	148.6515	8.94044	5.16177	126.4422	170.8607	138.49	155.29
Aqueous(100.00µg/ml)	3	152.9897	4.12371	2.38083	142.7458	163.2336	148.87	157.11
Methanol (100.0µg/ml)	3	113.9636	1.82134	1.05155	109.4391	118.4880	111.88	115.26
Hexane (100.0µg/ml)	3	65.8447	6.25115	3.60910	50.3160	81.3734	59.02	71.30
Ethyl acetate (100.0µg/ml	3	76.4540	3.24963	1.87617	68.3815	84.5266	74.58	80.21
Butanol (100.0µg/ml)	3	152.3913	4.15468	2.39870	142.0705	162.7121	149.67	157.17

University of Malaya

	Total	108	121.6217	30.47874	2.93282	115.8078	127.4357	59.02	197.73	
Model	Fixed Effects			4.77789	.45975	120.7052	122.5382			
	Random Effects				5.08537	111.2979	131.9456			923.38479

Table 2: ANOVA for Oil Red O Assay **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29200.967	21	1390.522	115.803	.000
Within Groups	528.336	44	12.008		
Total	29729.303	65			

Descriptives

Mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
					Lower Bound	Upper Bound			
Negative Control	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00	
Insulin (10.0µg/ml)	3	124.1098	7.31493	4.22328	105.9385	142.2811	117.95	132.20	
Aqueous(1.0µg/ml)	3	66.2804	3.22883	1.86417	58.2595	74.3012	62.58	68.54	
Methanol (1.0µg/ml)	3	106.0357	4.40028	2.54050	95.1048	116.9666	101.23	109.88	
Hexane (1.0µg/ml)	3	101.0386	3.78338	2.18434	91.6401	110.4370	97.26	104.82	
Ethyl acetate (1.0µg/ml	3	103.4222	.60393	.34868	101.9220	104.9225	102.82	104.03	
Butanol (1.0µg/ml)	3	103.8899	4.26945	2.46497	93.2840	114.4958	99.62	108.16	

University of Malaya

Aqueous(0.1µg/ml)	3	68.4879	2.10293	1.21413	63.2639	73.7118	66.06	69.70	
Aqueous(10.0µg/ml)	3	75.7174	3.61342	2.08621	66.7412	84.6937	72.52	79.64	
Methanol (0.1µg/ml)	3	97.3937	4.53298	2.61712	86.1331	108.6542	93.00	102.06	
Methanol (10.0µg/ml)	3	109.4650	4.63762	2.67753	97.9445	120.9855	104.12	112.35	
Hexane (0.1µg/ml)	3	101.4837	3.33828	1.92736	93.1909	109.7764	98.15	104.82	
Hexane (10.0µg/ml)	3	100.8160	1.11276	.64245	98.0518	103.5803	99.70	101.93	
Ethyl acetate (0.1µg/ml	3	101.1932	.64525	.37253	99.5904	102.7961	100.67	101.91	
Ethyl acetate (10.0µg/ml	3	110.5184	6.19576	3.57712	95.1273	125.9095	104.48	116.86	
Butanol (0.1µg/ml)	3	98.1025	.32866	.18975	97.2860	98.9189	97.91	98.48	
Butanol (10.0µg/ml)	3	104.7438	1.13852	.65732	101.9156	107.5721	103.61	105.88	
Aqueous(100.0µg/ml)	3	84.7130	2.81619	1.62593	77.7172	91.7088	82.62	87.91	
Methanol (100.0µg/ml)	3	110.6996	3.77167	2.17757	101.3302	120.0689	106.58	113.99	
Hexane (100.0µg/ml)	3	113.2047	1.57892	.91159	109.2825	117.1270	111.50	114.61	
Ethyl acetate (100.0µg/ml	3	175.3397	2.70224	1.56014	168.6270	182.0525	173.78	178.46	
Butanol (100.0µg/ml)	3	112.1442	1.97197	1.13852	107.2456	117.0429	109.87	113.28	
Total	66	103.1272	21.38630	2.63247	97.8698	108.3847	62.58	178.46	
Model Fixed Effects			3.46520	.42654	102.2676	103.9869			
Model Random Effects				4.59005	93.5817	112.6728			459.50486

University of Malaya

Table 3: ANOVA for fatty acid release in lipolysis assay **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49.733	21	2.368	841.764	.000
Within Groups	.124	44	.003		
Total	49.857	65			

Descriptives

Mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
					Lower Bound	Upper Bound			
.00	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00	
2.00	3	4.3726	.05030	.02904	4.2476	4.4975	4.32	4.42	
111.00	3	.9826	.05271	.03043	.8517	1.1136	.95	1.04	
121.00	3	.9347	.04949	.02857	.8118	1.0576	.88	.98	
131.00	3	1.0776	.01224	.00707	1.0471	1.1080	1.07	1.09	
141.00	3	1.0939	.07482	.04320	.9080	1.2797	1.03	1.18	
151.00	3	.9531	.04066	.02347	.8521	1.0541	.93	1.00	

University of Malaya

	1101.00	3	.9957	.04581	.02645	.8819	1.1094	.95	1.04	
	1110.00	3	1.0435	.09130	.05271	.8167	1.2703	.94	1.11	
	1201.00	3	.9347	.06963	.04020	.7617	1.1077	.86	.99	
	1210.00	3	.9469	.01870	.01080	.9005	.9934	.93	.97	
	1301.00	3	1.0163	.00000	.00000	1.0163	1.0163	1.02	1.02	
	1310.00	3	1.1571	.01837	.01060	1.1115	1.2028	1.14	1.18	
	1401.00	3	1.0653	.03240	.01870	.9848	1.1458	1.03	1.09	
	1410.00	3	1.1918	.04636	.02677	1.0767	1.3070	1.14	1.22	
	1501.00	3	.9014	.00000	.00000	.9014	.9014	.90	.90	
	1510.00	3	.9859	.01408	.00813	.9509	1.0209	.97	1.00	
	11100.00	3	1.1435	.09436	.05448	.9091	1.3779	1.08	1.25	
	12100.00	3	1.0531	.04242	.02449	.9477	1.1584	1.03	1.10	
	13100.00	3	2.3816	.03061	.01767	2.3056	2.4577	2.35	2.41	
	14100.00	3	3.4286	.11809	.06818	3.1352	3.7219	3.34	3.56	
	15100.00	3	1.1127	.04225	.02440	1.0077	1.2176	1.07	1.15	
	Total	66	1.3533	.87580	.10780	1.1380	1.5686	.86	4.42	
Model	Fixed Effects			.05304	.00653	1.3401	1.3665			
	Random Effects				.18943	.9594	1.7472			.78848

University of Malaya

Table 4: ANOVA for glycerol release in lipolysis assay **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	120.093	21	5.719	343.794	.000
Within Groups	.732	44	.017		
Total	120.825	65			

Descriptives

Mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
					Lower Bound	Upper Bound			
.00	3	1.000000	.0000000	.0000000	1.000000	1.000000	1.0000	1.0000	
2.00	3	7.462730	.4899361	.2828648	6.245661	8.679798	6.9695	7.9493	
111.00	3	1.194872	.1861051	.1074478	.732561	1.657182	.9846	1.3385	
121.00	3	.938967	.0406585	.0234742	.837966	1.039968	.9155	.9859	
131.00	3	.929577	.0281690	.0162634	.859602	.999553	.9014	.9577	
141.00	3	.924883	.0081317	.0046948	.904682	.945083	.9155	.9296	
151.00	3	.953052	.0406585	.0234742	.852050	1.054053	.9296	1.0000	

University of Malaya

	1101.00	3	1.194872	.2575870	.1487179	.554990	1.834753	1.0462	1.4923	
	1110.00	3	1.066667	.0621762	.0358974	.912212	1.221121	1.0000	1.1231	
	1201.00	3	.896714	.0354452	.0204643	.808663	.984764	.8592	.9296	
	1210.00	3	.906103	.0293192	.0169275	.833270	.978936	.8732	.9296	
	1301.00	3	.896714	.0293192	.0169275	.823881	.969547	.8732	.9296	
	1310.00	3	.938967	.0325268	.0187793	.858166	1.019768	.9014	.9577	
	1401.00	3	.924883	.0215144	.0124214	.871438	.978327	.9014	.9437	
	1410.00	3	.938967	.0452754	.0261397	.826497	1.051437	.8873	.9718	
	1501.00	3	.901408	.0000000	.0000000	.901408	.901408	.9014	.9014	
	1510.00	3	.985915	.0140845	.0081317	.950928	1.020903	.9718	1.0000	
	11100.00	3	1.235897	.0320256	.0184900	1.156341	1.315454	1.2000	1.2615	
	12100.00	3	1.023474	.0650536	.0375587	.861872	1.185076	.9859	1.0986	
	13100.00	3	.995305	.0354452	.0204643	.907254	1.083356	.9577	1.0282	
	14100.00	3	1.206573	.0430289	.0248427	1.099683	1.313462	1.1690	1.2535	
	15100.00	3	1.112676	.0422535	.0243951	1.007712	1.217640	1.0704	1.1549	
	Total	66	1.301328	1.3633943	.1678222	.966164	1.636492	.8592	7.9493	
Model	Fixed Effects			.1289734	.0158755	1.269333	1.333323			
	Random Effects				.2943589	.689175	1.913481			1.9006926

University of Malaya

Table 5: ANOVA for glucose uptake assay (methanol extract) **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14046.112	5	2809.222	13.679	.000
Within Groups	2464.362	12	205.363		
Total	16510.474	17			

Descriptives

Mean

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
.00		3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00	
.10		3	87.1429	4.94872	2.85714	74.8496	99.4362	81.43	90.00	
1.00		3	84.2857	9.89743	5.71429	59.6991	108.8723	72.86	90.00	
2.00		3	156.1039	23.89469	13.79561	96.7462	215.4616	128.57	171.43	
10.00		3	72.8571	8.57143	4.94872	51.5645	94.1498	64.29	81.43	
100.00		3	78.5714	21.57096	12.45400	24.9862	132.1567	55.71	98.57	
Total		18	96.4935	31.16415	7.34546	80.9959	111.9911	55.71	171.43	
Model	Fixed Effects			14.33051	3.37773	89.1341	103.8530			

University of Malaya

Descriptives

Mean

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
	.00	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00	
	.10	3	87.1429	4.94872	2.85714	74.8496	99.4362	81.43	90.00	
	1.00	3	84.2857	9.89743	5.71429	59.6991	108.8723	72.86	90.00	
	2.00	3	156.1039	23.89469	13.79561	96.7462	215.4616	128.57	171.43	
	10.00	3	72.8571	8.57143	4.94872	51.5645	94.1498	64.29	81.43	
	100.00	3	78.5714	21.57096	12.45400	24.9862	132.1567	55.71	98.57	
	Total	18	96.4935	31.16415	7.34546	80.9959	111.9911	55.71	171.43	
Model	Fixed Effects			14.33051	3.37773	89.1341	103.8530			
	Random Effects				12.49271	64.3800	128.6071			867.95300

Table 6: ANOVA for glucose uptake assay (hexane extract) **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5351.966	5	1070.393	10.738	.000
Within Groups	1196.238	12	99.686		
Total	6548.204	17			

Descriptives

University of Malaya

Mean

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
	.00	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00	
	.10	3	97.3434	3.29914	1.90476	89.1478	105.5389	94.74	101.05	
	1.00	3	97.6374	8.63624	4.98613	76.1838	119.0910	88.42	105.54	
	2.00	3	132.6316	17.23232	9.94909	89.8241	175.4390	113.68	147.37	
	10.00	3	95.4386	7.39344	4.26861	77.0723	113.8049	88.42	103.16	
	100.00	3	135.4386	12.68992	7.32653	103.9151	166.9621	122.11	147.37	
	Total	18	109.7483	19.62622	4.62594	99.9884	119.5081	88.42	147.37	
Model	Fixed Effects			9.98431	2.35333	104.6208	114.8757			
	Random Effects				7.71144	89.9254	129.5711			323.56888

Table 7: ANOVA for glucose uptake assay (ethyl acetate extract) **(between concentrations)**

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2971.041	5	594.208	16.038	.000
Within Groups	444.614	12	37.051		
Total	3415.655	17			

University of Malaya

Descriptives

Mean

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
Model	.00	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00	
	.10	3	99.0447	8.36980	4.83231	78.2530	119.8364	90.11	106.70	
	1.00	3	100.5881	1.27621	.73682	97.4178	103.7584	99.36	101.91	
	2.00	3	109.7663	.21282	.12287	109.2376	110.2950	109.55	109.98	
	10.00	3	109.8344	6.38813	3.68819	93.9654	125.7034	103.18	115.92	
	100.00	3	136.1758	10.47718	6.04900	110.1491	162.2026	127.39	147.77	
	Total	18	109.2349	14.17466	3.34100	102.1860	116.2838	90.11	147.77	
	Fixed Effects			6.08697	1.43471	106.1089	112.3609			
	Random Effects				5.74557	94.4654	124.0043			185.71903

University of Malaya

Table 8: ANOVA for PPARγ gene expression with treatment of ethyl acetate fraction 100µg/ml

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.261	2	3.630	670.376	.000
Within Groups	.032	6	.005		
Total	7.293	8			

Descriptives

Mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
2.00	3	3.1942	.10392	.06000	2.9360	3.4523	3.08	3.28
3.00	3	1.9572	.07380	.04261	1.7739	2.1405	1.88	2.03
Total	9	2.0505	.95481	.31827	1.3165	2.7844	1.00	3.28

University of Malaya

Table 9: ANOVA for SREBP gene expression with treatment of ethyl acetate fraction 100µg/ml

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.971	2	1.985	606.614	.000
Within Groups	.020	6	.003		
Total	3.990	8			

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
2.00	3	2.2731	.03300	.01905	2.1911	2.3550	2.24	2.31
3.00	3	2.5139	.09343	.05394	2.2818	2.7460	2.41	2.59
Total	9	1.9290	.70625	.23542	1.3861	2.4719	1.00	2.59

University of Malaya

Table 10: ANOVA for Pi3k gene expression with treatment of ethyl acetate fraction 100µg/ml

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.829	2	1.915	4419.012	.000
Within Groups	.003	6	.000		
Total	3.832	8			

Descriptives

mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
2.00	3	2.4507	.01308	.00755	2.4182	2.4832	2.44	2.46
3.00	3	2.3053	.03360	.01940	2.2219	2.3888	2.28	2.34
Total	9	1.9187	.69210	.23070	1.3867	2.4507	1.00	2.46

University of Malaya

Table 11: ANOVA for glut 1 gene expression with treatment of ethyl acetate fraction 100µg/ml

ANOVA

Mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43.705	2	21.852	3759.480	.000
Within Groups	.035	6	.006		
Total	43.740	8			

Descriptives

Mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
2.00	3	2.9619	.10582	.06110	2.6990	3.2248	2.84	3.03
3.00	3	6.3359	.07899	.04561	6.1397	6.5321	6.28	6.43
Total	9	3.4326	2.33826	.77942	1.6353	5.2300	1.00	6.43

University of Malaya

Table 12: ANOVA for glut 4 gene expression with treatment of ethyl acetate fraction 100µg/ml

ANOVA

mean

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18.055	2	9.028	2360.876	.000
Within Groups	.023	6	.004		
Total	18.078	8			

Descriptives

mean

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
2.00	3	3.6137	.00396	.00229	3.6038	3.6235	3.61	3.62
3.00	3	4.2827	.10703	.06180	4.0169	4.5486	4.17	4.38
Total	9	2.9655	1.50326	.50109	1.8100	4.1210	1.00	4.38

University of Malaya