ANTIDIABETIC ACTIVITY OF *AQUILARIA MALACCENSIS* (AGARWOOD) LEAVES EXTRACTS

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ABSTRAK

Diabetes mellitus adalah disebabkan oleh penurunan pengambilan glukosa dalam darah oleh sel dan metabolisme. Objektif tesis ini adalah untuk mengkaji mengenai kesan aktiviti antidiabetes dari ekstrak metanol dan air dari daun *Aquilaria malaccensis*. Ia dijalankan melalui kaedah *in vitro* dan *in vivo*. Dalam kajian *in vitro*, dua kaedah enzimatik yang dijalankan adalah perencatan α-glucosidase dan α-amylase dimana kesan perencatan dari ekstrak metanol dan ekstrak air dengan kepekatan dari 100 hingga 1000µg/ml berbanding dengan Acarbose. Kajian toksik akut dan toksik sub-kronik telah dijalankan untuk melihat kesan toksik dengan dos maksimum 2g/kg selama 14 hari ke atas tikus, manakala dalam sub-kronik, dua kepekatan iaitu 250mg/kg dan 500mg/kg ekstrak telah diberi kepada tikus setiap hari selama 28 hari. Dalam kajian *in vivo* dengan menggunakan tikus diabetes yang diinduksi dengan streptozotocin (STZ) pemerhatian dibuat keatas kesan 500mg/kg *Aquilaria malaccensis* ekstrak metanol dan ekstrak air dalam menurunkan paras glukosa darah berbanding dengan Metformin. Ekstrak metanol dan ekstrak air *Aquilaria malaccensis* menunjukkan aktiviti perencatan α-glucosidase dengan nilai IC_{50} 428.92 dan 425.09µg/ml, berbanding IC_{50} Acarbose iaitu 402.06µg/ml. Begitu juga dengan aktiviti perencatan α-amylase, kedua-dua ekstrak ini menunjukkan nilai IC_{50} metanol dan ekstrak air adalah sebanyak 752.98 dan 771.53µg/ml berbanding Acarbose, 584.93µg/ml. Kajian ketoksikan akut *Aquilaria malaccensis* ekstrak metanol dan ekstrak air menunjukkan bahawa ia tidak menyebabkan kesan toksik pada tikus dimana dos maut (LD_{50}) untuk semua ekstrak adalah lebih tinggi daripada 2g/kg. Kajian sub-kronik dilakukan selama 28 hari di mana tikus *Sprague Dawley* jantan dewasa dirawat dengan ekstrak pada kepekatan 250mg/kg dan 500mg/kg. Serum darah dianalisis untuk profil buah pinggang dan fungsi hati tiada perubahan signifikan dan kekal dalam julat normal. Dalam kajian *in vivo* menggunakan tikus diabetes STZ, rawatan dengan ekstrak metanol dan air *Aquilaria malaccensis* dengan 500 mg/kg berat selama 5 hari menunjukkan penurunan yang signifikan dalam glukosa darah dengan peratusan kesan penurunan glukosa pada 57.08% dan 55.48% menurun masing-masing berbanding metformin, 68.79%. Analisis biokimia pada tikus diabetes yang dirawat dengan kedua-dua ekstrak menunjukkan bahawa ekstrak tidak meningkatkan kerosakan pada protein serum. Ia menunjukkan bahawa ekstrak ini tidak menghasilkan sebarang kerosakan yang ketara di dalam organ dalaman; hati dan buah pinggang. Secara keseluruhan ekstrak metanol dan ekstrak air *Aquilaria malaccensis* mempunyai potensi untuk menurunkan tahap glukosa darah tanpa membahayakan haiwan dan mempunyai potensi untuk digunakan sebagai maklumat tambahan dalam pengurusan diabetes mellitus dan boleh membangunkan standard perubatan untuk diabetes mellitus.

ABSTRACT

Diabetes mellitus is defines clinically by hyperglycaemia or an abnormal increased glucose uptake. The purpose of this study was to assess the possible inhibitory effects of methanolic and aqueous leaves extract of *A. malaccensis* against α-glucosidase and α-amylase activities at concentrations ranged from 100 to 1000µg/ml compared with Acarbose, a commercial drug used in the clinical management of diabetes. Acute and sub-chronic toxicity studies were conducted to observe any toxic effects of *A. malaccensis* leaves extracts by administrating maximum dose of 2g/kg body weight for 14 days and two concentrations of 250mg/kg and 500mg/kg body weight daily for 28 days, respectively. *In vivo* study was conducted using STZ-induced diabetic rats model to evaluate the effects of administrating 500mg/kg body weight of methanolic and aqueous *A. malaccensis* leaves extracts on blood glucose level compared with standard drug, Metformin. *A. malaccensis* methanolic and aqueous leaves extracts exhibited potent inhibitory effects against α-glucosidase activity with IC_{50} values of 428.92 and 425.09µg/ml, respectively, compared to Acarbose, with IC_{50} values of 402.06µg/ml. Similarly, methanolic and aqueous *A. malaccensis* extracts showed dose dependant inhibitory effects against α-amylase activity with IC_{50} values of 752.98 and 771.53µg/ml, respectively compared with Acarbose, with IC_{50} value of 584.93µg/ml. Acute toxicity study of *A. malaccensis* methanolic and aqueous leaves extracts showed that the extracts did not exhibit any toxic effect in rats and is therefore, likely to be safe for consumption; oral lethal dose (LD_{50}) recorded for all extracts was greater than 2g/kg body weight. Blood serum was analysed for kidney profile and liver function; changes of these values were insignificant and remained within normal range. *In vivo* study using STZ-induced diabetic rats treated with *A. malaccensis* methanolic and aqueous leaves extracts at concentration of 500mg/kg body weight for 5 days showed that blood glucose level was significantly decreased by a percentage of 57.08% and 55.48%, respectively, compared with metformin, 68.79%. Assessment of biochemical parameters in diabetic rats treated with both extracts showed that the extracts did not inflict damage to serum protein; the results suggest that these extracts do not cause significant damage to the internal organs, particularly liver and kidney. These results suggest that *A. malaccensis* methanolic and aqueous extracts can potentially lower the blood glucose level in diabetic individual without inflicting harmful side effects. The extracts can be potentially used as an adjunct in the management of diabetes mellitus as well as development of standardized phytomedicine for diabetes mellitus.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

^{o}C	degree Celcius
α	alpha
β	beta
%	percentage
IC_{50}	inhibitory concentration at 50%
µg/ml	microgram per millilitre
µM	micromolar
µg	microgram

LIST OF ABBREVIATIONS

Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CK	creatine kinase
CREA	creatinine
CTLA4	cytotoxic T-Lymphocyte Associated Protein 4
DH_2O	distilled water
DMSO	dimethylsulfoxide
DNS	3.5-dinitrosalicyclic acid
e.g.	for example
EHD	extremely high dose
g	gram
g/day	gram per day
G6PD	glucose-6-phosphate dehydrogenase
Glob	globulin
GLUT4	glucose transporter type 4
HbA1c	glycated haemoglobin
HD	high dose
HDL	high density lipoprotein
i.e.	for example
IL2Ra	interleukin 2 receptor subunit alpha
kg	kilogram
LD	low dose
LDH	lactate dehydrogenase
LDL	low density lipoprotein
mg	milligram
mg/kg	milligram per kilogram
mg/ml	milligram per millilitre
min	minutes
ml	millilitre

mmol	millimole
mmol/L	millimole per litre
NaCl	Sodium chloride
$NaCO_3$	Sodium carbonate
nm	nanometre
O.D	optical density
pH	potential of hydrogen (pH value)
PLT	platelet
pNPG	para-nitrophenol-α-D-glucopyranoside
PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
RBC	red blood cells
rpm	rotation per minutes
S.E.M	standard error of the mean
spp	subspecies
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TBil	total bilirubin
TC	total cholesterol
TG	triglyceride
TP	total protein
U	Unit
UA	uric acid
v/v	volume/volume
w/v	weight/volume
WBC	white blood cells

CHAPTER 1

INTRODUCTION

1.1 ANTIDIABETIC STUDY

Diabetes mellitus is a type of methanolic disorder characterized by hyperglycemia resulting from defects in insulin secretion and action. A major metabolic defect associated with diabetes is the failure of peripheral tissues in the body to utilize glucose properly, thereby resulting in chronic hyperglycemia. Control of plasma glucose concentration is vital to decrease the incidence and severity of long-term diabetic complications (Sanjay, 2013). Although the two types (Type 1and Type 2) of diabetes have distinct pathogeneses, hyperglycemia and various life-threatening complications resulting from long-term hyperglycemia are common to both.

Diabetes mellitus is classified into two major categories: type 1 diabetes (formerly known as insulin-dependent diabetes mellitus, or T1DM), and type 2 diabetes (formerly known as non-insulin dependent diabetes mellitus, or T2DM). Type 1diabetes is a disease that results from the body's failure to produce insulin, the hormone that "unlocks" the cells of the body, and consequently allow the glucose to enter and fuel the cells. Since glucose cannot enter the cells in the absence of insulin, it builds up in the blood and the cells of the body literally starve to death.

People with type 1 diabetes must take daily insulin injections and regularly monitor blood sugar levels. The risk of developing type 1 diabetes is higher than virtually all other severe chronic diseases of childhood. Dennis (2006) suggested that management of type 1 diabetes is best undertaken in the context of a multidisciplinary health team and requires continuing attention to many aspects, including insulin administration, blood glucose monitoring, meal planning, and screening for comorbid conditions and diabetes-related complications. Another type of diabetes is non-insulin

dependent, Type 2 diabetes, which results from the interaction between a genetic predisposition and behavioral and environmental risk factors (Yanling et al., 2014). Type 2 diabetes results from inability of the body to make enough or properly use insulin. Often type 2 diabetes can be controlled through diet and exercise, but sometimes these are not enough and either oral medications or insulin must be used.

According to *Malaysian Diabetes Association,* people with type 2 diabetes often develop the disease after age 30 but would not be aware of the disease until treated for one of its serious complications. The risk for type 2 diabetes increases with age. Studies have indicated that diabetes is generally under-reported on death certificates, particularly in the cases of older people with multiple chronic conditions such as heart disease and hypertension. Due to this, the toll of diabetes is believed to be much higher than officially reported.

1.2 PLANT OF INTEREST

To date, at least 40 plants worldwide have been documented as beneficial in the treatment of diabetes (Wesam et al. 2016). *A. malaccensis* is one of these beneficial plants, yet there is no report and detail systemic documentation onantidiabetic potential. Agarwood is considered the finest natural incense and has been used in many societies for their religious, cultural, and medicinal purposes for centuries. In Indonesia and Malaysia, it is known as ‘gaharu’, ‘jin-koh’ in Japan, ‘kritsana noi’ in Thailand, ‘chen hsiang’ in China, ‘agar’ in India, ‘chim-hyuang’ in Korea, ‘tram huong’ in Vietnam, and ‘oud’ in the Middle East (Ng et al., 2014). Agarwood tree has been known as ‘karas’ or ‘kekaras’, while ‘gaharu’ refers to fragrant wood in Malay language (Lim et al., 2010).

The economic interest in agarwood has always been directed towards its pathological, heavy and dense resin-impregnated wood, which develops in the tissues of the stem in response to mechanical stress of injury. Briefly, the resin could be formed through pathological, wounding and non-pathological mechanisms (Ng et al., 2014). These mechanisms have been the basis for inoculation or induction techniques to induce resin formation in cultivated agarwood trees, whereby the techniques often involve physical penetration into the trunk (wounding), insertion of a microbial, mainly fungal, concoction (pathology) and response of the tree towards the administered stress (non-

pathological). A method of producing agarwood resin by creating an artificial wound in the xylem of agarwood trees have been patented (Blanchette and van Beek, 2005).

The fragrant wood has been associated with religious history, rituals and ceremonies in Hinduism, Buddhism, Christianity, and Islam, whereas other materials from the agarwood tree have also been prominently used in traditional medicine practices of Southeast Asian communities, such as Chinese, Tibetan, Unani and Ayurvedic (Barden et al., 2000; Blanchette and van Beek, 2005). This ethnopharmacological evidence have stimulated the interest of scientific society to investigate these medicinal claims using modern tools. This is proved by the surge in number of scientific publications in recent years, particularly those describing the pharmacological actions of agarwood including its antidiabetic (Feng et al., 2011; Pranakhon et al. 2015), antiinflammatory (Kumphune et al., 2011; Rahman et al., 2012; Sattayasai et al., 2012; Zhou et al., 2008), anticancer (Dahham et al., 2016; Hashim et al., 2014), anti-depressant (Takemoto et al., 2008) and antioxidant (Dahham et al., 2016; Han and Li, 2012; Sattayasai et al., 2012; Kamonwannasit et al., 2013; Miniyar et al., 2008; Moosa, 2010; Nik Wil et al., 2014; Ray et al., 2014; Tay et al., 2014) activities.

Agarwood leaves extract has been found to possess antipyretic, laxative and antimicrobial activities (Zhou *et al*., 2008) and some studies have reported its remarkable anticancer activity (Dahham et al., 2016). There was a trial, which demonstrated that the long-term uptake of agarwood leaf tea by a diabetic patient resulted in decreased blood sugar level to normal condition. In Thailand, there was a report on a diabetic patient who had drank water infused with agarwood leaf instead of plain water for six months (Akrarapholchote, 2008). The patient's blood sugar decreased from 10.2 mmol/L to 7.1 mmol/L and further decreased to 6.49 mmol/L after an uptake of twice daily, in the morning and bedtime. This information reveals that agarwood has the potential antihyperglycemic properties.

1.3 PROBLEM STATEMENT

Diabetes is a chronic health problem with devastating, yet preventable consequences. It is evident that the cases of diabetes mellitus is increasing in many populations of both developed and developing country. Therefore, this research is to analyse and evaluate the new finding on the components of *A. malaccensis* leaves extracts which have therapeutic potential as antidiabetic agents and safe for human consumption. Malaysian *A. malaccensis* leaves extract in particular, has great potential as dietary supplement (antihyperglycaemic properties) that allows for flexibility in meal planning and helps in reducing the number of diabetic cases in worldwide population.

1.4 OBJECTIVES OF STUDY

The main objective of the study is to determine antidiabetic potential of *Aquilaria malaccesnsis* in lowering blood glucose of STZ-induced diabetic rats. This research has covered on extraction of bioactive compounds and analysis of respective antidiabetic properties. The specific aims of this study were:

1. to evaluate the in vitro antidiabetic potential of A. malaccensis leaves extracts
2. to determine both acute and sub-chronic toxicity of *A. malaccensis* leaves extracts in animal model
3. to investigate the effects of methanolic and aqueous *A. malaccensis* extracts *in vivo* using rat model (STZ-induced diabetic rats).

1.5 SCOPE OF STUDY

This review will include an overview of diabetes mellitus, type of diabetes and diabetic drugs, various herbal solutions for diabetic followed by a discussion of lowering blood glucose effects by Malaysian agarwood, *Aquilaria malaccensis* aqueous and methanolic extracts. This latter section will discuss on the effects of these extracts in toxicological and acute hyperglycaemic effects on streptozotocin-induced diabetic rats and its assessment in the clinical laboratory.

CHAPTER 2

LITERATURE REVIEW

2.1 DIABETES MELLITUS

Diabetes mellitus is a chronic and systemic disease that occurs either due to insulin deficiency or insulin resistance or both (Anees, 2013). Insulin is a small peptide hormone consists of two polypeptide chains with 51 amino acids that regulate blood glucose levels by stimulating glucose uptake and transport in bloodstream (Newsholme, 2007). Major signs and symptoms of diabetes include hyperglycaemia, polyuria (frequent urination), polydipsia (excessive thirst), increased hunger, weight loss, fatigue, blurred vision, frequent infections, slow-healing wounds, vomiting and stomach pain. Diabetes mellitus may lead to long-term complications such as cardiovascular and Alzheimer's diseases; kidney failure (diabetic nephropathy) and nerve dysfunction (diabetic neuropathy); blindness due to damage of retinal blood vessels (diabetic retinopathy); diabetic foot syndrome; increased susceptibility to skin problems and other infections. Diabetes mellitus can be further classified into Type 1 Diabetes Mellitus (T1DM), Type 2 Diabetes Mellitus (T2DM) and gestational diabetes (Hu, 2011).

2.1.1 Type 1 Diabetes

According to Centers for Disease Control and Prevention (CDC), there are more than 13, 000 young people who are diagnosed with T1DM annually, especially among younger populations (less than 30 years old). T1DM is a chronic autoimmune disorder that does not fit into the pattern of simple inheritance and may be considered complex (multifactorial), with possible interactions between genetic susceptibility and environmental factors (Atkinson, 2012). Individual autoimmune system may destroy or

damage the beta-cells (β-cells) in the islets of Langerhans of pancreas in order to reduce and eventually eliminate the production of insulin. On rare but increasing occasions, both T1DM and T2DM are diagnosed in the same patients.

2.1.2 Type 2 Diabetes

T2DM is the most common metabolic disease worldwide that makes up about 90% of total diabetes incidence, with the rest being T1DM and gestational diabetes. Based on epidemiological statistics by International Diabetes Federation (IDF), 387 million people in the world suffered from this disease in 2014 and there has been no definitive cure for T2DM due to the lack of understanding on pathogenesis of insulin resistance. National Health and Morbidity Survey (NHMS) reported that the prevalence of diabetes in Malaysia has increased by 31.0% over the past five years, from 11.6% in 2006 to 15.2% in 2011 (Feisul and Azmi, 2013). Hu (2011) reported that a number of risk factors associated with the development of T2DM have been identified including age, ethnicity, genetics, dietary intake, overweight and inactive lifestyle The number of people suffer from T2DM worldwide is expected to increase from 135 million to more than 300 million by 2025 (Hu, 2011).

Insulin resistance is a hallmark of T2DM and plays an important role in the pathogenesis of the disease (DeFronzo, 2010). Insulin resistance is defined as a reduced response of target tissues of skeletal muscle, adipose tissue and liver to insulin (DeFronzo and Tripathy, 2009). There is impairment of glucose transport and insulin signalling in target tissues of patients with insulin resistance that cause the release of free fatty acids, hormones and inflammatory markers from the adipose tissue (Sesti, 2006). Although β-cell failure has mainly been associated with T2DM, insulin resistance is believed to be the primary defect that leads to T2DM prior to β-cell failure (DeFronzo and Tripathy, 2009). Insulin resistance in skeletal muscle would hinder glucose disposal and induce hyperinsulinemia, thus precede the development of T2DM. The risk factors of insulin resistance include dysglycemia, dyslipidaemia, obesity, hypertension, hyperinsulinemia and lack of physical activity. Meanwhile, the potential mechanisms for regulating glucose metabolism in the skeletal muscles are glycogen

synthase, hexokinase and the major insulin-stimulated glucose transporter GLUT4 (Pessin & Saltiel, 2000).

2.1.3 Destruction of beta-pancreatic/ beta cells

Pancreatic β-cells are found within the islets of Langerhans, which make up 65-80% of the total islet cells. The primary function of β-cell is to synthesize and secrete insulin hormone into the blood stream to normalize hyperglycaemia that occurs after meal ingestion or when the blood glucose levels get too high. However, progressive β-cells destruction by autoimmune processes may lead to development of T1DM. T1DM arises following the autoimmune destruction of insulin-producing pancreatic β-cells. The autoantibodies together with major histocompatibility complex as well as macrophage, T-lymphocytes and β-lymphocytes, are responsible for the immune response and destruction of pancreatic islet cell (Atkinson, 2012). The autoimmune attack on β-cells will restrain insulin secretion and impair the functions of insulin. However, autoimmunity may not be the primary cause for T1DM. Genetic factor seems to play an important role in autoimmunity that further contributes to T1DM which involve genes such as HLA, insulin, PTPN22, IL2R and CTLA4 (Noble & Erlich, 2012). Environmental factors such as enterovirus infection, cow's milk protein and vitamin D insufficiency may also lead to T1DM development (Knip & Simell, 2012).

2.1.4 Alloxan and streptozotocin

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) and streptozotocin [2-deoxy-2-(3-(methyl-3-nitrosoureido)-d-glucopyranose] are two diabetogenic drugs that are commonly used to induce insulin-dependent diabetes in experimental animal model due to the selective destruction of the insulin-producing pancreatic β-cells. It is accumulated in β-cells, interfering with insulin secretion that eventually kill the β-cells through different mechanisms. Alloxan induces oxidative stress that kills β-cells, while streptozotocin contributes to DNA damage and subsequent cell death in mammalian cells through selective cytotoxic effects on pancreatic β-cells (Lenzen, 2008; King, 2012). Diabetes induced by alloxan results in blood glucose levels comparable to that of diabetic condition after total pancreatectomy; therefore, the test using plant extract in a

severely alloxan-diabetic animal with significant hypoglycaemia must be conducted through a different mechanism.

Meanwhile, induction of diabetes mellitus using streptozotocin may take a longer time; experiment carried out at suitable time intervals after administration of the agent would give insights into the mechanism of hypoglycaemic action of tested plant extract - whether the effects have been rendered by stimulation of β-cells or some other mechanisms (Lenzen, 2008; King, 2012). Therefore, streptozotocin is primarily used for reproducible induction of diabetes in experimental animals, owing to its chemical properties and greater stability compared to alloxan.

It has been hypothesized that a lot of Malaysian medicinal plants (*Momordica charantia, Parkia speciosa, Phyllanthus niruri Linn, Trigonella foenum-graecum, Zingiber zerumbet, Anacardium occidentale, Centella asiatica, Cinnamomum verum, Clinacanthus nutans, Cosmos caudatus, Ficus deltoidea, Gynura procumbens, Orthosiphon stamineus* and *Andrographis paniculata*) have an excellent medicinal effect in controlling plasma glucose level in alloxan- and streptozotocin- animal models with minimal side effects (Mustaffa *et al*., 2011)

2.1.5 Diabetic drug and side effects

Insulin was introduced as a therapeutic drug, which has improved the quality and life expectancy of diabetic patients. Insulin therapy is carried out through injection as normal digestion process interferes with insulin function if it is taken orally. Current methods of insulin delivery include using syringes, continuous subcutaneous insulin infusion and insulin pens. The use of insulin is essential for the treatment of T1DM. In T2DM, it is reserved for patients with severe hyperglycaemia with ketonemia or ketonuria; newly diagnosed diabetics; or those who do not respond to treatment with diet, exercise, oral hypoglycaemic agents and the anti-hyperglycaemic action of insulin sensitizers (Nora, 2016). The commonly used types of insulin are fast-acting, short-acting, intermediate-acting, long-acting, ultra-long acting and their combinations. However, patients with T2DM may develop resistance to insulin therapy.

Metformin is an oral antidiabetic drug of the biguanide class and has been the drug of choice for the treatment of adults with T2DM due to lesser side effects produced from its use. Metformin is sold in the form of 500 and 850 mg tablets with the maximum dosage of 2.5-3.0 g/day, which should be administered after meals to minimize gastrointestinal side effects (Bouchoucha et al., 2011). Metformin lowers blood glucose levels and glycosylated haemoglobin, as well as improves the lipid profile by decreasing triglyceride levels, LDL-cholesterol and increasing HDL-cholesterol. This drug increases glucose uptake in muscle and inhibits lipolysis and release of free fatty acids in adipocytes. Furthermore, metformin improves insulin action in the liver by reducing hepatic glucose production and stimulates GLUT4 translocation at the cellular level (Jung et al., 2012). The common side effects of metformin include diarrhoea, nausea, vomiting, flatulence and lactic acidosis.

Another class of drugs used in the treatment of T2DM is sulfonylureas (Reddy, 2000) that include the sulfonylureas of first generation (e.g. chlorpropamide, acetohexamide, tolazamide and tolbutamide), second generation (e.g. glibenclamide, glipizide, gliclazide) and third generation (glimepiride). Sulfonylureas have long been established in the treatment of diabetes and it was the first oral glucose-lowering medication to be introduced into clinical practice. Sulfonylureas act as insulin secretagogues that exert their main action on islet β cells, stimulate insulin secretion and thereby, reduce the plasma glucose concentration (Lorenzati et al., 2010). Therefore, sulfonylureas are useful only in patients with fewer β cell functions by stimulating the release and responsiveness of β cells (Lorenzati et al., 2010). Sulfonylureas was found to lower the HbA1c and blood glucose concentrations significantly (Hirst et al., 2013). They are the most effective in patients whose weight is normal or slightly increased, while insulin therapy works best for patients who are underweight, losing weight, or ketotic.

2.2 ANTIDIABETIC ACTIVITES

2.2.1 Various herbal solution for diabetic

There has been numerous research conducted to investigate the potential use of plant extracts as alternative to synthetically produced drug used in the treatment of diabetes (Abbas, 2018; Jafarnejad et al., 2017; Sinha, Pratap, & Varma, 2018). Traditionally, various plants including the species of *Aquilaria* are used in the treatment of diabetes and therefore, has sparked great interest in the development of plant-based drug (Md Zaki, Yusoff, Alwi, & Ku Hamid, 2015). There are at least 400 plant species have been reported for anti-diabetic potential, which involved identification, characterization and isolation of bioactive compounds that could potentially act as therapeutic agent. Most of the research have been focusing on the discovery of potent inhibitors of enzymes associated with diabetic conditions (Lee et al., 2016). Recent upsurge in demand for a natural remedy is one of the factors that has driven the progress made in bioactivity screening and pharmacological investigations of various plant extracts.

Recently, the leaves extract of *Carica papaya* was reportedly produce anti-hyperglycaemic effects by increasing the level of plasma insulin in alloxan-induced diabetic mice (Sinha et al., 2018). The study suggested the possible insulinogenic activity of the extract, which stimulate the secretion of insulin by beta cells. The preliminary phytochemical screening revealed the presence of alkaloids, glycosides, saponins, phytosterols, tannins and amino acids in the leaves extract. The seed of *Nigella sativa*, or commonly known as fennel flower, demonstrated anti-diabetic potential in recent study (Abbas, 2018). Similar to *C. papaya*, the decrease in blood glucose level of STZ-induced diabetic mice was observed possibly due to stimulation of insulin secretion by *N. sativa* seed extract. Another study was reported on the potential antidiabetic activities of *Zinger officinale*, a member of ginger family (Jafarnejad et al., 2017). The extract, reportedly lower the blood glucose level by exerting beneficial effects on lipid profile, which consequently enhanced cellular sensitivity towards insulin in diabetic and hyperlipidemic subjects.

2.3 ANTIDIABETIC POTENTIAL OF *AQUILARIA MALACCENSIS*

2.3.1 *Aquilaria malaccensis*

Aquilaria (or Agarwood) is a genus in the family of Thymeleceae and class of Magnoliopsida. The name of the species is derived from Greek word '*Aquila'* that means eagle. Aquilaria has adapted to live in various habitats including those that are rocky, sandy or calcareous; well-drained slopes and ridges; and land near swamps. This tree grows particularly in rain forest of Indonesia, Thailand, Malaysia, Cambodia, Laos, Northern India, Philippines and Borneo.

There are four species of agarwood available in Malaysia including *A. malaccensis, A. hirta, A. rostrata* and *A. beccariana*. It was reported that *A. malaccensis* is the major producer of agarwood oil in Malaysia (Mohamed, 2010). This tree has been widely used for many purposes. *A. malaccensis* wood has been used as tonic, stimulant, diuretic and flatulence relief while the grated wood has been used in preparation of traditional medicine to treat smallpox and illness during and after childbirth.

The tree is also harvested for its highly valuable fragrant wood called "gaharu" or "agarwood", which is produced upon pathological infection or mechanical wounding of the tree. In Malaysia and Indonesia, this resin is mainly produced by the tree of the genus Aquilaria, of which *A. malaccensis* is known as the main species to produce agarwood or gaharu. To date, the resinous wood or oil extracted from the resin-impregnated trees is extremely valuable since it is highly regarded for its use in Buddhist and Islamic cultural activities; Japanese incense ceremonies, and as an important ingredient in many traditional medicines.

Aroma of agarwood comes from dark-coloured resin that accumulates inside stems and roots of the tree. Resin act as chemical barrier against fungal and insect attacks that cause injury to the tree – a reaction known as primary defence mechanism. Agarwood is highly prized in both local and foreign markets because of its precious use in traditional medicines and other products (Barden *et al.*, 2000; Naef, 2011). Many parts of this plant including the leaf, skin, seed, wood and root are valuable for their

medicinal properties. Despite numerous ethnopharmacological evidence, most of the times Aquilaria is highly sought after for its fragrant resin rather than for the health benefits rendered by other parts of the plant. These include antioxidant, anti-inflammatory (Zhou, 2008), antihyperglycemic (Pranakhon, 2015) and antimicrobial properties of the plant that can be potentially explored for development of diverse medicinal purposes.

2.3.2 Agarwood traditional uses

Its use as ingredient in various preparations of traditional prescriptions since ancient times has been compiled in numerous classic medical records such as Traditional Chinese Medicine (TCM), Unani System, Ayurvedic and many others (Hashim, Kerr, Abbas, & Mohd Salleh, 2016). These traditional use of agarwood leaves extract as medicine has garnered interests from various research communities to study its potential for modern applications in pharmaceutical and alternative medicines. Most of the recent research has been focusing on characterizing the active compounds present in agarwood leaves which may contribute to its biological activities as commonly claimed (Khalil, Rahim, Taha, & Abdallah, 2013). The toxicology of agarwood leaves has also been studied to investigate on its potential acute and chronic toxicity effects and assess its safety for human use (Ghan, Chin, Thoo, Yim, & Ho, 2016).

Chinese, Japanese, Taiwanese and Hong Kong people are among the cultures that have been frequently associated with the practice of using agarwood leaves as prime ingredient in preparation of traditional herbal teas. High quality agarwood leaves are fused with other herbs in preparations of herbal drinks, believed to be effective in treating various diseases including diabetes (Pranakhon, Pannangpetch, & Aromdee, 2011). Apart from the herbal drinks, agarwood leaves have also been processed into pastes and pills, and taken orally as traditional medicine to treat various health disorders. These practices are still retained in some cultures and advancement in pharmaceutical industry has allowed for the leaves to be processed into various forms of dietary supplements via a more sophisticated and well-regulated processes. However, there is still limited medical evidence available to prove the medicinal properties of agarwood leaves.

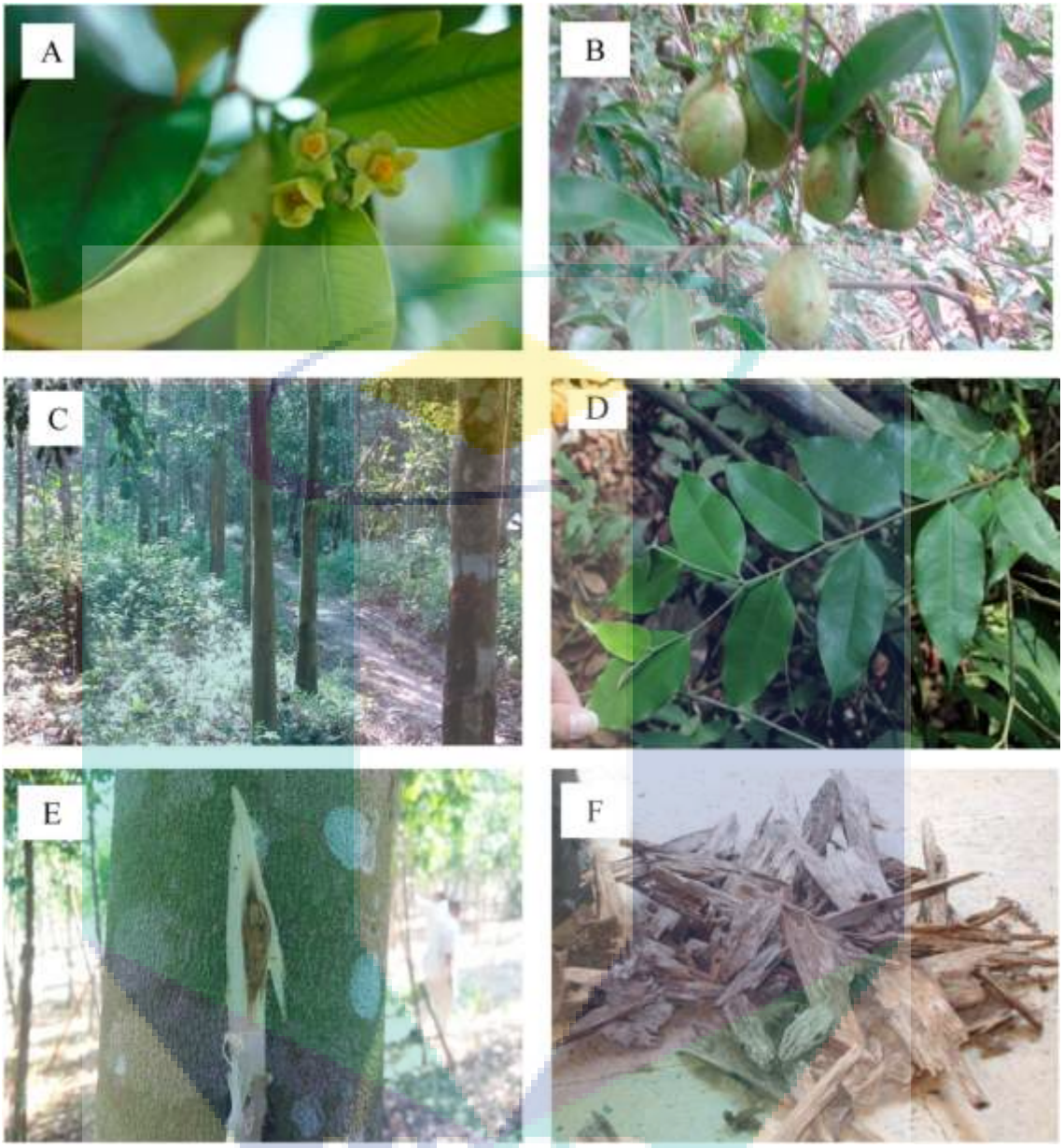

Figure 1.1 *A. malaccensis* (A) flowers, (B) fruits, (C) trees from a plantation in Bangi, Selangor, (D) leaves, (E) agarwood (resin) formation and (F) resin-impregnated wood chips.

2.3.3 Clinical trials on antidiabetic potential

Currently, most of scientific investigations on potential anti-diabetic activity of *A. malaccensis* leaves extract have not yet progressed to clinical stage. However, there are promising results generated from few pre-clinical studies to evaluate the effects of leaves extract from several species of *Aquilaria* including *A. malaccensis* on blood glucose level in animal models (Manoka, Sungthong, Sato, Sugiyama, & Sato, 2016; Pranakhon et al., 2011). These studies have reported on the lowering of blood glucose level in streptozotocin (STZ)-induced rats following oral treatment using leaves extract of *Aquilaria*. The significance of anti-hyperglycaemic effects produced by the extracts was proved statistically against the activities of widely used commercial anti-diabetic drug, Acarbose. These findings may provide the basis to the traditional uses of *Aquilaria* leaves in alternative treatment of diabetes.

Several studies have also reported on enhanced glucose uptake by adipose tissues (Pranakhon et al., 2011) and skeletal muscles (Said & Kamaluddin, 2016) in rats with type 2 diabetes. The condition is characterized by insulin resistance in diabetic patient, which is due to functional impairment in tissues glucose transport system, resulting in accumulation of glucose in blood. As skeletal muscle is the primary tissue responsible for glucose uptake in postprandial state, stimulating plasma glucose uptake by these tissues has been one of the key strategy in the treatment of diabetes. Improvement in insulin activities by *Aquilaria* leaves extract may have occurred via several mechanisms: activation of AMP-activated protein kinase (AMPK) may improve the activity of liver insulin and increase in tissues sensitivity towards insulin through expression of GLUT4 in tissues (Wang et al., 2018). GLUT4 plays important role in homeostasis of glucose in adipose, muscle and heart tissues to maintain the glucose level in blood.

On the other hand, an observational study on anti-diabetic potential of *Aquilaria* leaves was reported by Pranakhon et al. (2011). The study reported on a singular case of anti-hyperglycaemic effects of *Aquilaria* leaves in a diabetic patient, who consumed the leaves-infused water for 6-month long. However, the report may be considered as statistically insignificant as there was no further systematic investigation has been

carried out to prove the observation. While oral consumption of *Aquilaria* leaves extract may be proved safe according to investigation at pre-clinical level, further toxicology tests via systematic clinical trials should be carried out to prove its safety for human consumption. Despite promising findings, there are still limited scientific and clinical evidence available to prove the anti-diabetic potential of *Aquilaria* leaves extract.

2.3.4 Bioactive compound isolated from *Aquilaria*

There have been many studies done to identify and characterize the bioactive compounds present in the leaves of *Aquilaria* species. Non- and semi-volatile constituents such as alkaloids, tannins, flavonoids, phenolic compounds (Said & Kamaluddin, 2016), steroids and saponins (Nik Wil, Mhd Omar, Awang@Ibrahim, & Tajuddin, 2014) are the chemical groups that are commonly found in all species of *Aquilaria* including *A. malaccensis*. The most common volatile groups found in the leaves of *Aquilaria* include phytol, squalene, n-hexadecanoic acid, octadecatrienoic acid, terpenoids (Adam et al., 2018; Wang et al., 2018). Depending on factors such as species, origin and method of extraction, the composition of these chemical compounds may vary from one another (Lee et al., 2016). Among these compounds, flavonoids, alkaloids, saponins and phenolic constituents have been numerously associated with anti-hyperglycaemic activity which contribute to the anti-diabetic property of the leaves extract.

A study conducted showed the positive correlation between the total phenolic contents in the leaves of *A. malaccensis* and inhibitory activity against the enzyme, α-amylase (Md Zaki et al., 2015). The finding provides the scientific rationale, which may support the anti-diabetic potential of *A. malaccensis* leaves. In another study, several single compounds namely aquilarisinin, aquilarisin, hypolaetin 5-*O*-β-d-glucuronopyranoside, aquilarixanthone, mangiferin, iriflophenone 2-*O*-α-l-rhamnopyranoside, iriflophenone 3-*C*-β-d-glucoside and iriflophenone 3,5-*C*-β-d-diglucopyranoside were isolated from the leaves extract of *Aquilaria* (Feng, Yang, & Wang, 2011). Bioactivity screening showed that these compounds could inhibit the activity of α-glucosidase and thus, may have the potential to lower the blood glucose level in diabetic patient. However, further pharmacological investigation should be

carried out to elucidate the mechanisms involved and evaluate the associated potentials and risks of using *Aquilaria* leaves extract as natural anti-diabetic agent.

2.4 *IN-VITRO* ANTIDIABETIC POTENTIAL OF *A. MALACCENSIS*

Several scientific investigations have reported on anti-hyperglycaemic activity of agarwood leaves from various *Aquilaria* species including *A. malaccensis* (Hashim et al., 2016; Li, Yin, Yang, Tang, & Wei, 2012; Pranakhon et al., 2011). The findings have supported the anti-diabetic potential of agarwood leaves. As there is extremely limited report on anti-hyperglycaemic activity of other plant parts of *A. malaccensis*, most of discussions regarding anti-diabetic potential of agarwood refer to the potential use of its leaves extract. Investigations on agarwood leaves extract showed that bioactive compounds present in the leaves may exhibit anti-hyperglycaemic activities through two mechanisms of actions: the bioactive compounds may induce the production of insulin or mimic the actions of insulin to lower the blood glucose level.

Previous studies have showed that leaves extract of *Aquilaria malaccensis* remarkably inhibited the activity of α-amylase and α-glucosidase, which represented antidiabetic potential of the extract at cellular level (Hashim et al., 2016; Yunus, Md Zaki, & Ku Hamid, 2015). α-amylase is the enzyme present in saliva and pancreatic juice, which hydrolyses alpha bonds of large alpha-linked polysaccharides such as carbohydrates during digestion process. The process yields the smallest sugar unit that is glucose, which will be used for cellular activities. Similarly, α-glucosidase presents in the brush border of small intestines breaks down carbohydrates into glucose, by hydrolysing the terminal non-reducing-linked α-glucose residues. Activities of these enzymes result in the increase of blood glucose level and therefore, inhibition of their activities is the common strategy in most treatments of diabetes type 2 (Riyaphan et al., 2017).

2.4.1 Alpha (α)-amylase inhibitory assay

Alpha (α)-amylase is a prominent enzyme found in saliva and pancreatic juice (Afifi et al., 2008). Initially, partial digestion by the salivary α-amylases results in the degradation of larger insoluble polymeric substrates into shorter oligomers.

Subsequently, these molecules are further hydrolysed in the gut, by pancreatic α-amylases into maltose, maltotriose and small malto-oligosaccharides, which would eventually be broken down into glucose. On the other hand, α-glucosidase is a membrane bound enzyme located on the epithelium of small intestine and acts by catalysing the cleavage of starch and disaccharides to form glucose (Manohar et al., 2002).

2.4.2 Alpha (α)-glucosidase inhibitory assay

Inhibition of α-amylase and α-glucosidase can retard the uptake of dietary carbohydrates and suppress post-prandial hyperglycaemia in diabetic condition (Matsui et al., 2006). Inhibitors of α-amylase and α-glucosidase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion (Kazeem et al., 2013). Several inhibitors of α-amylase and α-glucosidase have been isolated from medicinal plants to serve as potential alternative drug with increased potency and lesser adverse effects than the currently commercially available synthetic drugs for diabetes (Matsuda et al., 2002).

2.5 *IN VIVO* ASSESSMENTS

2.5.1 Toxicology

Toxicology is a branch of science that investigate the adverse effects of chemical on living organisms. The investigation is composed of observation and reporting on symptoms, mechanisms, detection and treatment of a particular toxic substance in relation to its poisonous effects (Lyoussi, Cherkaoui Tangi, Morel, Haddad, & Quetin-Leclercq, 2018). Depending on the design and objective of the experiment, there are different types of toxicological tests that can be carried out, which include acute, sub-chronic and chronic toxicity tests. The main differences between each test are the length of experiment and the dosage of test substance used. Generally, the minimum length of experiment for acute, sub-chronic and chronic toxicity tests is 14 days, 28 days and 2 months, respectively. Acute toxicity study serves as the initial step in assessing the potential health hazards of the test substance (OECD, 2001).

According to OECD guidelines, the dosages used in acute toxicity study must be in the range that cause less than 50% but not 0% and more than 50% but not 100% mortality. Each group in acute toxicity test is orally and parenterally administered with a single dose of test substance. Moderate doses of test substance are used in acute toxicity and doses that are expected to cause lethality are avoided in the experiment. Sub-chronic and chronic toxicity tests are carried out by daily administration of test substance to several groups of experimental animal in graduated doses for a specified period (OECD, 2008). At the end of sub-chronic and chronic toxicity tests, all experimental animals will be sacrificed and necropsied for further physical, haematological and clinical biochemistry analysis. According OECD, the dose of 2000 mg/kg is expected to produce evident toxicity in tested animals and therefore used as limit dose in toxicity test. Any doses higher than 2000 mg/kg are only permitted for substances that have low acute toxicity but pose danger to human populations.

2.5.1.1 Biochemical markers for kidney toxicity evaluation

Among the most common biochemical markers used to evaluate toxicity of a substance towards human health are creatinine and blood urea. Creatinine and urea are the wastes produced from muscle activities and breakdown of proteins, respectively. In normal condition, these wastes will be removed from the body by kidney. Kidney malfunction due to causes such as exposure to toxic substances would result in accumulation of these wastes in the blood. Therefore, creatinine and urea are the ideal organ-specific biochemical markers to detect organ damage or as an indicator to the presence and extent of organ damage in toxicological studies. According to Huggett, Kimerle, Mehrle, & Bergman (2018), the presence of these biomarkers can also be measured by increased concentrations of enzymes such as creatinine phosphokinase in specific organ.

2.5.1.2 Biochemical markers for liver toxicity evaluation

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein and bilirubin are commonly monitored in clinical practice and elevation in their concentrations may indicate liver malfunction as in the

case of toxicity (Huggett et al., 2018). In toxicological studies, these molecules are used as organ-specific biochemical markers that make up the liver profile to detect any damaging effects in liver function. ALP is a membrane-bound enzyme that helps to break down protein in the liver. Alteration in its level may affect the membrane permeability and cause impairment in the transport of metabolites (Ike, Arome, Affiong, & Chimere, 2016; Shamban, Patel, & Williams, 2014). Elevation of ALP normally reflects various liver diseases which may have been caused by cholestatic disorder, cirrhosis, hepatitis or infiltrative liver diseases. On the other hand, AST and ALT are part of transaminases group in liver cells, which are released into the blood stream following the damage in hepatocyte as in the various liver associated diseases.

The total protein concentration in blood is normally measured by the level of albumin and globulin (Ike et al., 2016). High level of albumin usually reflects dehydration of protein while the low level may indicate infections, liver diseases, ulcers and in some cases, kidney diseases. Globulin is antibody produced to curb infections and therefore, elevation in its level may indicate chronic inflammation, kidney infection, stress, liver diseases and parasite infestations. In the case of decreased in globulin level, there could due to hepatic DNA and RNA disorders following exposures to toxic substances (Shalan, Mostafa, Hassouna, El-Nabi, & El-Refaie, 2005). Bilirubin is a waste product from degradation of aged red blood cells, which is normally excreted by liver. Accumulation of bilirubin in blood may indicate liver dysfunction, which makes bilirubin of the important biochemical markers in toxicological studies. Measurement of these biochemical markers as parameters in toxicological investigation on plant extract are still relevant as reported in recent studies (Kadhim, Musa, Abed, Mijbil, & Aziz, 2018; Lyoussi et al., 2018).

2.5.2 Antidiabetic activity on STZ-induced diabetic rats

In order to gain insight into the underlying antidiabetogenic mechanisms of medicinal plant extracts, biochemical parameters such as glucosidase activity, G6PD activity, hepatic and skeletal glycogen stores and restoration of functional β-cells will be estimated in *in vivo* murine model. As afore mentioned, glucosidase plays an important role in the digestion of dietary carbohydrates. Inhibition of α-glucosidase in diabetic rats may retard the utilization of dietary carbohydrates and subsequently reduce postprandial

hyperglycaemia levels (Matsui et al., 2006). Glucose-6-phosphate dehydrogenase (G6PD) is an important enzyme that helps in regulating the glucose metabolism via pentose phosphate pathway (Bhat et al., 2011). Besides, hepatic and skeletal muscles glycogen synthesis and storage were found impaired in diabetic rats (Huang et al., 2000; Bhat et al., 2011).

Insulin may improve glycogen synthesis and storage by stimulating glucose uptake and activating glycogen synthase through insulin signalling pathways (Alan, 2001). Glycogen depletion may increase the insulin-stimulated glucose uptake but increase in insulin sensitivity can restore the glycogen content (Jensen et al., 2011). Moreover, some medicinal plant extracts could restore the functional β-cells by stimulating the β-cells to secrete insulin in streptozotocin-induced diabetic mice and thus, improve glucose metabolism towards the re-establishment of normal blood glucose control (Bhat et al., 2011). Therefore, it is crucial to measure these biochemical parameters in diabetic rats in order to study the antidiabetic effects of medicinal plants. Thus far, no study has been conducted on the antidiabetogenic mechanism of *A. malaccensis in vivo* in animals.

2.5.2.1 Haemotological assessment in diabetes mellitus

In the treatment of diabetes, measuring the level of haematological parameters namely red blood cells (RBCs), platelet, white blood cells (WBCs) and haemoglobin is important to evaluate the effectiveness of and safety of the treatment. These parameters are used as there have been reports on alteration of these haematological components in diabetic patients and animals compared to normal group (Ogugua Vic, Obiora, I. Egba, & Robert Ike, 2017; Uko et al., 2013). In some cases, RBCs, WBCs and platelet dysfunction were reported in diabetic condition (Biadgo, Melku, Mekonnen Abebe, & Abebe, 2016). Exposure of RBC to high level blood glucose in hyperglycaemia condition in diabetic individual results in glycation of hemoglobin, prothrombin, fibrinogen, and other proteins that are involved in blood clotting mechanisms (Biadgo et al., 2016). Consequently, the level of RBCs and haemoglobin may drop in diabetic condition as in the case of STZ-induced rats. Increase in the levels of RBCs in the

treatment of diabetes may signify increased erythropoietin (EPO), the hormone produced in the kidney to enhance RBC production through erythropoiesis.

Elevation in the level of WBCs and abnormal platelets conditions are the common haematological condition reported in diabetic patient (Biadgo et al., 2016). WBCs play a major role in destruction of pathogens at infection sites, removal of foreign substances and debris generated from cell death and injury. In diabetic condition, which is often characterized by inflammation in cellular structure, the levels of peripheral WBCs such as basophils, eosinophils, and neutrophils increase as part of the natural damage control in the body. On the other hand, platelet count and mean platelet volume (MPV) are among the most commonly used parameter to measure the reactivity of platelet in diabetic conditions (Buch, Kaur, Nair, & Jain, 2017). Insulin resistance in STZ-induced diabetic rats may experience platelet hyper reactivity, which results in increased coagulation, impaired fibrinolysis, and endothelial dysfunction that leads to diabetic complications. The MPV in diabetic condition was reported larger in diabetic condition as the larger platelets are known to be more reactive than the smaller ones (Biadgo et al., 2016). Therefore, measuring these parameters is vital to evaluate the effects of proposed treatment on the common haematological symptoms in diabetic conditions.

2.5.2.2 Lipid profile abnormalities in diabetes mellitus

The body uses cholesterol to help build the cells and hormones. Too much cholesterol in the blood can result in built-up along the inside of the arterial walls, forming a plaque. Large-size plaque increases the chances of having heart attack or stroke (Genest et al. 2003). High-density lipoprotein (HDL), also known as 'good cholesterol' helps remove fat from the body by binding with the fat in the bloodstream and carrying it back to liver for disposal. Another form of cholesterol is the low-density lipoprotein (LDL), also called 'bad cholesterol' does not aid in transportation of fat out of the body, instead it deposits fat onto the vessel wall to be transported to other parts of the body. Triglycerides are the types of fat, which are produced in the liver from the excess carbohydrates that are not in use for energy generation. Only small amounts of triglycerides are found in the blood.

Dyslipidaemia is a condition associated with type 2 diabetes, which is characterized by a combination of increased serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and decreased in high density lipoprotein (HDL). These different types of lipids are the most commonly used markers to evaluate the lipid profile in diabetic individual. The build-up of TG and LDL, along the arterial wall in diabetic condition may lead to atherosclerosis and the associated cardiovascular diseases and various inflammatory reactions. It was reported that the oral administration of Aquilaria leaves extract had lowered the TC and TG in diabetic individual Bursill, Abbey, & Roach (2007). While the elevation in TG may not always result in diabetes, the condition may signify insulin malfunction, which condition often referred to as pre-diabetes. The condition occurs as the result of cellular insensitivity towards insulin due to increased TG level. HDL is a lipoprotein responsible in transporting lipid in the body and decrease in its activity in diabetic condition impairs lipid transportation, which often results in various cardiovascular diseases (Jafarnejad et al., 2017).

CHAPTER 3

MATERIAL & METHODOLOGY

3.1 Plant Material

Leaves of *A. malaccensis* were collected in December 2011 from Malaysia's tropical forest and plantations, assisted by officers of Malaysian Timber Industry Board (MTIB) and Universiti Putra Malaysia (UPM) for species identification purposes (voucher no. SK 2422/14).

3.2 Preparation of Plant Extract

The fresh leaves of *A. malaccensis* were dried at room temperature for 30 days. The dried leaves were pulverized into powder and subjected to solvent extraction using methanol and hot aqueous extraction. For methanolic extract, the dried leaves powder of *A. malaccensis* (1000 g of sample, dry weight) was extensively extracted using methanol (solvent) at the ratio of sample to solvent, 1:20 at room temperature for 48 hours and filtered through a Whatman No. 1 filter paper. The filtrate was then concentrated at 60^{o}C using rotary vacuum evaporator to separate the solvent from crude extract. The total yield of 200 g of oily crude extract was kept in universal bottle and stored at 4^{o}C until further use.

Aqueous extract was prepared through immersion of 1000 g (dry weight) of powdered extract in 20 liter (w/v) of distilled boiled water which was removed from the heat source and allowed to infuse for 15 minutes. The suspension was filtered through a Whatman No. 1 filter paper and subjected to freeze-dry to obtain a dry powder. The total yield of 100 g of crude extract was kept in universal bottle and stored at 4^{o}C until further use.

3.3 *In-vitro* Antidiabetic Activity

The test samples of *A. malaccensis* leaves extracts were freshly prepared at concentration ranged from 200 to 1000µg/ml (w/v). Methanolic extracts were dissolved in 10% dimethylsulfoxide (DMSO) while aqueous extracts were dissolved in distilled water.

3.3.1 α-glucosidase Inhibitory Assay

An α-glucosidase inhibition assay method from Kim et al. (2001) was adopted with minor modification. All reagents were pre-incubated for 15 min at 37ºC in water bath. Inhibitory activity of the enzyme, α-glucosidase was determined by incubating 30 µl of α-glucosidase enzyme solution (1 U in 67mM potassium phosphate buffer, pH 6.9, 37ºC) in 30µl of various concentrations of Acarbose (as a positive control) and plant extracts (ranged from 100 µg/ml to 1000 µg/ml) at 37ºC for 60 min. Afterwards, 30µl of the *para*-nitrophenol-α-D-glucopyranoside (pNPG) solution (10 mM pNPG in 67mM potassium phosphate buffer, pH 6.9, 37ºC) was added and incubated for another 10 min at 37ºC and the reaction was stopped by the addition 90 µl of 100 mM $NaCO_3$. Finally, the reaction mixtures were cooled under the running tap water and absorbance was measured at 405 nm by using spectrophotometer. α-glucosidase inhibition activity was expressed as percentage of inhibition and calculated using the following equation:

$$\% \text{ inhibition} = \frac{(\text{Enzyme activity of control} - \text{Enzyme activity of extract})}{\text{Enzyme activity of control}} \times 100$$

Controls incubation represented 100% enzyme activity and was conducted by replacing extracts with vehicles (30µl DMSO and distilled water respectively) under similar experimental conditions. For blank incubation, enzyme solution was replaced with buffer solution. Experiments were performed in triplicates.

3.3.2 α-amylase Inhibitory Assay

Enzyme inhibition activity for α-amylase was determined according to the method reported by Apostolidis (2007) with minor modifications. 100 µl of various concentrations of Acarbose (as a positive control) and plant extracts (ranged from 200 µg/ml to 1000 µg/ml) were incubated in 100 µl of 20 mM sodium phosphate buffer (with 6.7 mM sodium chloride, pH 6.9) containing α-amylase at concentration of 2 U/ml at 25°C for 30 min. After pre-incubation, 100 µl of 1% (w/v) starch solution in 20 mM sodium phosphate buffer, pH 6.9 were added. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 200 µl of 96 mM 3.5-dinitrosalicyclic acid (DNS) colour reagent and incubated at 85°C in a water bath for 5 min and cooled to room temperature. The generation of maltose is quantified by the reduction of 3, 5 dinitro salicylic acid to 3-amino-5- nitro salicylic acid. This reaction was measured at 540 nm by using spectrophotometer. α-amylase inhibition activity was expressed as percentage of inhibition and calculated using the following equation:

$$\% \text{ inhibition} = \frac{(\text{Enzyme activity of control} - \text{Enzyme activity of extract})}{\text{Enzyme activity of control}} \times 100$$

Controls incubation represented 100% enzyme activity and was conducted by replacing extracts with vehicles (100µl DMSO and distilled water respectively) under similar experimental condition. For blank incubation, enzyme solution was replaced with buffer solution. Experiments were performed in triplicates.

3.3.3 Calculation of 50% Inhibitory Concentration (IC_{50})

The concentration of the plant extract required to scavenge 50% of the radicals (IC_{50}) were calculated by using the percentage of scavenging activities at five different concentrations of extract. Formula for Percentage inhibition (I %) is as follows:

$$I\ \% = (Ac\text{-}As)/Ac\ X\ 100$$

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

3.4 Animal Test

Animal testing was conducted with animal ethics no: 20180810/R/MAEC00046 in Malaysia Agriculture Research and Development Institute, MARDI Serdang. Adult male *Sprague-Dawley* rats aged 6 to 8 weeks and weighed between 200 to 250 g were kept in their cages for 7 days prior to the start of dosing to allow for acclimatization to laboratory conditions. The animals were caged at 22 ^{o}C (± 3^{o}C) and fed with standard laboratory pellet and provided with water *ad libitium.*

3.4.1 Acute Oral Toxicity – Fixed Dose Procedure

A total of 48 adult male *Spraque Dawley* rats aged from 6 to 8 week-old were used. The animals were randomly selected, marked to permit individual identification and group-caged by doses. Acute oral toxicity studies were carried out for methanolic and aqueous extracts of both species using methodology described in The Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2001)

3.4.1.1 Experimental design

The animals were randomly divided into groups of with different treatments (control, low dose-LD, high dose-HD and extremely high dose-EHD) of six rats each. Rats were fasted prior to dosing (food but no water) and withheld overnight. The extract was administered at 1ml/100g body weight in a single dose through gavage using a stomach tube. Following the period of fasting, rats were weighted and extract was administered. Food were deprived for further 3 to 4 hours after treatment.

Animal were observed individually after dosing during the first 30 min and periodically during the first 24 hours; special attention was given during the first 4 hours for emergence of any toxicological symptoms and continued daily monitoring during the next 14 days for any mortality. Changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous systems and behaviour pattern were observed throughout the experiment.

Table 3.1 Experimental design of acute oral toxicity study for 14 days.

TREATMENT	GROUP	CONCENTRATION mg/kg body weight
Distilled water	Control (aqueous)	-
***A. malaccensis* aqueous extracts**	Low dose	250
	High dose	1000
	Extremely high dose	2000
10% DMSO	Control (methanol)	-
***A. malaccensis* methanolic extracts**	Low dose	250
	High dose	1000
	Extremely high dose	2000

The animals were sacrificed under chloroform euthanasia on the day 15th. Blood was collected in two different tubes: a tube containing anticoagulant, potassium oxalate and sodium fluoride for plasma; another tube containing no anticoagulant for serum separation. Whole blood was then centrifuged at 3000 rpm for 10 minutes using refrigerated centrifuge at 4^{o}C to remove red blood cells and recover the serum. Serums were separated and collected using dry autoclaved micropipette and store at -80^{o}C for analyses. The analyses were completed within 24 hours of sample collection.

3.4.2 Sub-chronic toxicity: Repeated Dose 28-days Oral Toxicity

A total of 36 adult male *Spraque Dawley* rats aged from 6 to 8-week old that weighed between 200 to 250g were used. The animals were randomly selected, marked to permit individual identification and group-caged by doses. Sub-chronic toxicity studies of methanolic and aqueous extracts were carried out on both species using repeated dose of 28-day oral toxicity study in rodents according to guideline for testing of chemicals described in The Organization of Economic Co-operation and Development (OECD) (OECD, 2008).

3.4.2.1 Experimental design

The animals were randomly divided into two groups with different treatments (control, low dose-LD and high dose-HD) of six animals each.

Table 3.2 Experimental design of sub-chronic oral toxicity for 28 days.

TREATMENT	GROUP	CONCENTRATION mg/kg body weight
Distilled water	Control (aqueous)	-
A. malaccensis aqueous extracts	Low dose	250
	High dose	500
10% DMSO	Control (methanol)	-
A. malaccensis methanolic extracts	Low dose	250
	High dose	500

The rats were fasted prior to dosing with different concentrations of extracts for a period of 28 days. Extracts were administered to the rats by gavage using stomach tube, 1ml/100g body weight. Observation was made during the 28 days to detect delayed occurrence of or recovery from toxicity. All rats were weighed once a week and food consumption (feed residue) was measured twice a week. Morbidity and mortality of the rats were observed.

After 28 days, the rats were sacrificed by euthanasia. Blood was collected in two different tubes: a tube containing anticoagulant, potassium oxalate and sodium fluoride for plasma; another tube containing no anticoagulant for serum separation. Whole blood was then centrifuged at 3000 rpm for 10 minutes using refrigerated centrifuge at 4^{o}C to remove red blood cells and recover the serum. Serums were separated and collected using dry autoclaved micropipette and store at -80^{o}C for analyses. The analyses were completed within 24 hours of sample collection.

3.4.3 Antidiabetic activity of the extracts in STZ-induced diabetic rats

A total of 36 Male Sprague-Dawley rats (200-280g) were purchased and maintained in an air-conditioned room (25±1^{o}C) with 12-hour light/ 12-hour dark cycle and fed with standard diet and water *ad libitium*. Rats were acclimatized for 7 days before starting the experiment. Rats were diabetes-induced by a single subcutaneous injection of streptozotocin (STZ) (55 mg/kg body weight), dissolved in sterile commercial NaCl solution (normal saline). After induction, rats were normally fed with standard diet and allowed to drink 10% glucose solution. After 7 days of STZ injection, blood was collected from rat tail to determine fasting blood glucose level using glucometer. Only the rats with fasting blood glucose over 7.0 mmol/L, were considered diabetic and used for the experiments.

3.4.3.1 Experimental design

The animals were randomly divided into groups of six animals each after the diabetes induction.

Table 3.3 Experimental design of antidiabetic test on STZ-induced diabetic rats.

TREATMENT	GROUP	CONCENTRATION mg/kg body weight
Distilled water	Control (aqueous)	-
Aquilaria malaccensis aqueous extracts	Low dose	250
	High dose	500
10% DMSO	Control (methanol)	-
Aquilaria malaccensis methanolic extracts	Low dose	250
	High dose	500

Blood was obtained by snipping the tail of rat using sharp needles and the blood glucose levels were monitored by using a single strips touch glucometer at 2 hours interval (2, 4, 6 hours) after oral administration of extract and the monitoring continued until day 5th. The tail of the rat was sterilized with alcohol prior to snipping.

The animals were sacrificed by euthanasia after day 5. Liver, kidney and pancreas were removed, washed with cold saline, patted dry, preserved in 10% (v/v) formalin in buffer form and weighed for recording purposes. Blood was collected from the dorsal aorta in two different tubes: a tube containing anticoagulant, potassium oxalate and sodium fluoride for plasma; and another tube containing no anticoagulant for serum separation. Whole blood was then centrifuged at 3000 rpm for 10 minutes using refrigerated centrifuge at 4^{o}C to remove red blood cells and recover the serum. Serums were separated and collected using dry autoclaved micropipette and store at -80^{o}C for analyses. The analyses were completed within 24 hours of sample collection.

3.4.4 Biochemistry test

A clinical biochemistry experiment was performed on samples of blood to determine major toxic effects in tissues and specifically on kidney and liver. Blood was collected in the blood tubes containing potassium oxalate and sodium fluoride as anticoagulants and serum were immediately separated by centrifugation at 3500 *g* for 10 minutes within 2 hours after blood collection. Estimation of serum cholesterol, triglycerides, high density lipoprotein (HDL), total protein levels, creatinine and urea were also evaluated in control and diabetic rats for lipid profiling and determination of toxic effects on kidney function. Biochemistry tests were carried out at MARDI, Selangor, Malaysia

3.5 Statistical analysis

All results were expressed as mean ± standard error of the mean (S.E.M). Statistical analysis was performed by SPSS. Differences between groups were analysed by one-way analysis of variance (ANOVA) and the value of $P<0.05$ would be considered statistically significant. All *in vitro* antidiabetic assays were performed in triplicates while that of *in vivo* antidiabetic assays in six replicates.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 In vitro Test

One of the approaches used in medication to treat diabetes is by retarding absorption of glucose through inhibition of carbohydrate-hydrolysing enzymes, α-amylase and α-glucosidase, in the digestive organs (Widharna et al. 2010). Inhibition of these enzymes has been found to reduce the high postprandial blood glucose peaks in diabetic individual. The α-glucosidase inhibitors retard the digestion of carbohydrates and eventually slow down the absorptions. Therefore, one of the therapeutic approach for reducing postprandial blood glucose levels in patient with diabetes mellitus is by preventing absorption of carbohydrate after food intake (Narkhede, 2011). The α-amylase inhibitors act as anti-nutrient that obstructs the digestion and absorption of carbohydrates.

One of the established α-glucosidase and α-amylase inhibitor is Acarbose isolated from microorganism, which we used in this *in vitro* test as a control; use of Acarbose in reducing postprandial hyperglycemia was proven (Kim et al. 2004). Acarbose delays digestion of complex carbohydrates and disaccharides to absorbable monosaccharides by reversibly inhibiting α-glucosidase and α-amylase within the intestinal brush border, thereby attenuating postprandial blood glucose peaks (Narkhede, 2011).

4.1.1 *In vitro* α-amylase and α-glucosidase inhibitory study

The α-glucosidase inhibition of Acarbose and both *A. malaccensis* methanolic and aqueous extracts was evaluated by determining the α-glucosidase inhibitory activity

using 4-Nitrophenyl-β-D-glucopyranosiduronic acid (pNPG) as the reaction substrate. *In vitro* inhibitory tests of α-glucosidase showed that methanolic and aqueous extract of *A. malaccensis* exhibited inhibitory activity against α-glucosidase. Based on percentages of inhibition at different concentrations of extracts (200, 400, 600, 800 and 1000 µg/ml) showed in Table 4.1, the inhibition was dose dependent. The highest concentration (1000 µg/ml) of methanolic and extracts showed the highest percentage of inhibition that was nearly 79.33% and 71.65%, respectively as compared to Acarbose, 87.71%. The percentage of inhibition varied from 18-79% for methanolic extract and 16-71% for aqueous extract. The 50% inhibitory concentration (IC_{50}) of methanolic and aqueous extracts of *A. malaccensis* was 608.27µg/ml and 682.13µg/ml, respectively; the values were significantly different to that of positive control using Acarbose, with IC_{50} value of 548.85µg/ml (Table 4.1).

Whereas, the α-amylase inhibition of Acarbose and both *A. malaccensis* methanolic and aqueous extracts was evaluated by determining the α-amylase inhibitory activity using 1% starch solution as the reaction substrate. The enzyme-substrate reaction is stopped by heat inactivation; DNS is mainly used in detecting or quantifying the alpha amylase activity. Reducing sugars produced by alpha amylase reacts with DNS and produce ANS which absorb the light at 540nm. *In vitro* α-amylase inhibitory tests demonstrated that methanolic and aqueous extracts of *A. malaccensis* exhibited inhibitory activity against the enzyme. The calculated percentages of inhibition at 200, 400, 600, 800 and 1000 µg/ml concentrations depict a dose dependent inhibition activity (Table 4.1). Thus, the highest concentration (1000 µg/ml) of methanolic and aqueous extracts resulted in the highest percentage of inhibition: 61.26% and 55.75%, respectively as compared to Acarbose, 70.40%. The percentage of α-amylase inhibition by methanolic and aqueous extracts varied from 15-61% and 11-55%, respectively. The 50% inhibitory concentrations of methanolic and aqueous extracts of *A. malaccensis* were found to be 772.80µg/ml and 846.02µg/ml, respectively; the values were significantly different to that of the standard Acarbose with IC_{50} value of 661.38µg/ml (Table 4.1).

Both enzymes inhibition test revealed that, both methanolic and aqueous extracts of *A. malaccensis* inhibited the activity of α-amylase and α-glucosidase in a

dose-dependent manner. At lower concentrations of extracts, the inhibition was marginal; however, significant inhibitory response was evident as concentrations increased. There was no great difference in the inhibition between aqueous and methanol extracts as all were inhibited ($p = 0.578$). However, IC_{50} of methanol extracts of *A.malaccensis* in this study performed better than the aqueous extracts. These findings given that most antidiabetic active components in plant matter are saturated organic molecules, which are non-polar. Additionally, despite water being more polar solvent than methanol, the phytochemical profile of an extract determines polarity of the compounds being extracted in a given solvent (Khalil et al., 2013). Hence, for active lipophilic constituents that are not extracted in water extract, methanol extraction would provide more consistent antidiabetic activity compared to those that are extracted in water.

Several major groups of plant products with antidiabetic activities have been identified in *in vitro* study and according to Said and Kamaluddin (2016), particularly valuable class of health-enhancing plant compounds is flavonoids. These are polyphenolic molecules with properties that include reduction of blood-lipids and glucose (Atoui, 2005). This study suggests that *A. malaccensis* methanolic and aqueous extracts has potential therapeutic value for the treatment of diabetes via acting as α-amylase and α-glucosidase inhibitors by improving hepatic glucose and carbohydrate metabolism, suppressing oxidative stress, and preventing inflammation in diabetic rats.

The efficacy of *A. malaccensis* associated with anti-hyperglycaemic could be due to the presence of flavonoids, alkaloids, saponins and phenolic constituents (Lee et al., 2016), along with synergistic effect of multiple compounds such as aquilarisinin, aquilarisin, hypolaetin 5-O-β-d-glucuronopyranoside, aquilarixanthone, mangiferin, iriflophenone 2-O-α-l-rhamnopyranoside, iriflophenone 3-C-β-d-glucoside and iriflophenone 3,5-C-β-d-diglucopyranoside were isolated from the leaves extract of *Aquilaria* (Feng, et al., 2011). These bioactivity screening showed that these compounds could inhibit the activity of both enzymes and thus, have the potential to lower the blood glucose level in diabetic rats. Consistent with findings in this study, the methanolic extracts isolated flavonoid compounds that were effective inhibit against the enzyme, α-amylase (Md Zaki et al., 2015) as well as several single compounds inhibits the activity of α-glucosidase (Lee et al., 2016).

In addition, efficacy of plants can be affected by other factors. For instance, differences in combinations of secondary metabolites such as phenolic compounds, tannins, alkaloids and steroids in different solvents used for extraction that also have contributed to observed variations (Simon et al., 2015). Accordingly, methods of extracting these compounds from source material include solvent extraction processes that utilize solvent polarity as a major separation technique. These methods applied in this experiment by using water extraction (aqueous) and organic solvent extraction (methanol).

In this experiment, extraction solvents differed in the rates of their activity; aqueous extracts had less inhibition compared to methanol (Table 4.1). Methanol is a good solvent for lipid, particularly the membrane-associated lipid because of the higher polarity compared to neutral lipids, thus resulting of oily crude extract in methanol extraction methods. Water-cosolvent system with 10% DMSO was used to dissolve this oily *A. malaccensis* methanolic crude extract before further dilution.

These results also indicate that although aqueous extraction might be popular, it is not necessarily the most effective method. However, higher potency of the *A. malaccensis* aqueous extracts against methanolic extract would be advantageous to the locals since water is readily available and easy to handle. It is the ease of handling and use that makes water the most used solvent for bioactive compounds in traditional remedy preparations. Nevertheless, the activity demonstrated by the methanol extracts gives an indication of their potential as useful bioactive substances.

To conclude, the inhibition rate for α-glucosidase was close to that of acarbose, and the inhibition rate for α-amylase was obviously lower than that of acarbose. This indicated that *A. malaccensis* was a strong inhibitor for α-glucosidase with mild inhibitory activity against α-amylase. The inhibition of α-glucosidase, together with α-amylase by *A. malaccensis* methanolic and aqueous extracts, considered an effective strategy for the control of diabetes by diminishing the absorption of glucose. Therefore, we deduce that these results generally show antidiabetic potential of methanolic and aqueous extracts of *A. malaccensis,* which would be further verified through *in vivo* treatment of STZ-induced diabetic rats.

Table 4.1 Inhibitory activity of methanolic and aqueous extracts of *A. malaccensis* and Acarbose against α-glucosidase and α-amylase.

Sample	Concentration (µg/ml)	α-glucosidase		α-amylase	
		% Inhibition	IC_{50} (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Acarbose (Standard drug)	200	21.59±1.60	548.85	20.18±.25	661.38
	400	39.18±2.01		31.67±4.06	
	600	59.29±3.15		49.84±2.92	
	800	71.37±3.64		61.58±2.92	
	1000	87.71±.52		70.40±1.39	
Aqueous	200	16.15±1.31	682.13*	11.40±1.56	846.02*
	400	23.50±.92		28.18±2.48	
	600	47.90±.50		33.93±1.58	
	800	60.23±1.08		50.17±.39	
	1000	71.65±.87		55.75±2.09	
Methanol	200	18.31±.60	608.27*	15.69±3.91	772.80*
	400	30.21±1.31		25.44±1.92	
	600	52.88±1.12		41.25±1.74	
	800	67.49±.74		53.81±.31	
	1000	79.33±.96		61.26±3.16	

The IC_{50} value was define as the concentration of inhibitor that requires inhibiting 50% of activity of target enzyme under assay conditions. All experiments were carried out in triplicates and values are expressed as mean ± SEM. A value less than 0.05 (P<0.05) was considered to have significantly different compared with control experiment using one-way ANOVA by Tukey's post-test and indicated by *.

4.2 Acute Toxicity Study (14 days)

Toxicological screening is essential to observe possible adverse effects resulting from the exposure, which is necessary to initiate the clinical evaluation of investigational products or substances to minimize variance, bias and potential false positives and false negatives. Preclinical evaluation should be established which includes the study of toxicity of investigational products on the target organ; relationship between the dose and response; any relevant effects and potential complications on human's health arising due to treatment.

Acute toxicity study was conducted to determine adverse effects that occurred following oral administration of a single dose of extracts to obtain information on biological activity and mechanism of action of the extracts. In acute toxicity study,

normal healthy male rats were treated with methanolic and aqueous *A. malaccensis* extracts at different doses: control (vehicle); low dose (LD) of 250mg/kg body weight; high dose (HD) of 1000mg/kg body weight; and extremely high dose (EHD) of 2000mg/kg of body weight. All tested animals were observed for 14 days.

A. malaccensis aqueous and methanolic extracts did not cause mortality in male rats up to a dose of 2g/kg body weight. No signs of gross behavioural and significant visible toxicity symptoms manifested in treated rats even at this dose. There were also no significant differences in body weight gain of treated rats when compared to that of control groups as shown in Table 4.2. The result shows that the body weight of treated rats was generally normal; the treatment with extracts did also not affect their body temperature and stool output. The weight of internal organs of treated rats had not significantly deviated from normal except for liver as compared to that of normal groups. The weight of liver in rats treated with aqueous *A. malaccensis* extract at concentration of 250 mg/kg and 500 mg/kg was significantly decreased when compared with control group (Table 4.3). Nonetheless, the decrease in weight could be deemed a slight change, which might be due to variation in size of internal organs of the animals (Bailey, 2004). In general, all tested animals were healthy as judged by the colour of fur and body surface, nature and frequency of movement, loss of reflex and normal respiratory pattern.

From the results, both *A. malaccensis* aqueous and methanolic extracts are safe for consumption even at high dose and do not exert acute toxicity. These results suggest that the oral lethal dose (LD_{50}), as it was defined as statistically derived dose that administered in an acute toxicity test to cause death in 50% of the treated animal (Walum, 1998), in this study was greater than 2g/kg of body weight.

Table 4.2 Body weight of rats for acute toxicity study of *A. malaccensis* methanolic and aqueous extracts.

Group	Body weight (g)		
	Baseline Day 0	**Final Day 14**	**Weight gain on day 14**
A. malaccensis aqueous extract			
Control	195.44±3.51	206.40±3.90	10.96±1.85
Low dose	188.35±2.76	200.13±2.15	11.78±1.53
High dose	180.44±2.52	192.40±2.41	11.95±1.40
Extremely high dose	184.01±1.42	195.42±4.85	11.41±3.90
A. malaccensis methanol extract			
Control	185.37±2.51	195.66±2.08	10.29±1.81
Low dose	189.24±5.30	201.71±6.69	12.47±3.38
High dose	191.29±4.80	203.58±6.03	12.29±1.87
Extremely high dose	189.61±4.29	201.80±5.31	12.19±3.62

Weight gain on day 14 was calculated as difference between animal weights at Day 14 with Day 0. Values expressed are mean ± S.E.M of six animals. There were no significant differences at (P<0.05). Low dose = 250mg/kg, high dose = 1000mg/kg and extremely high dose = 2000mg/kg.

Table 4.3 Organ weight of rats for acute toxicity study of *A. malaccensis* methanolic and aqueous extracts.

Group	Organ weight (g)		
	Liver	**Kidney**	**Spleen**
A. malaccensis Aqueous extract			
Control	6.65±0.19	1.46±0.03	0.48±0.04
Low dose	6.01±0.08*	1.40±0.08	0.41±0.04
High dose	6.05±0.23*	1.47±0.03	0.41±0.03
Extremely high dose	6.42±0.40	1.49±0.05	0.43±0.03
A. malaccensis Methanol extract			
Control	6.12±0.33	1.40±0.05	0.43±0.03
Low dose	6.22±0.27	1.43±0.05	0.44±0.04
High dose	6.15±0.44	1.45±0.04	0.42±0.03
Extremely high dose	6.68±0.42	1.46±0.05	0.44±0.03

Values expressed were mean ± S.E.M of six animals. A P-value less than 0.05 (P<0.05) was considered as significantly different compared with control group using one-way ANOVA by Tukey's post-test and indicated by *. Low dose = 250mg/kg, high dose = 1000mg/kg and extremely high dose = 2000mg/kg.

4.3 Sub-chronic Toxicity Study (28 days)

Sub-chronic toxicity is a consequence of the persistent or progressively deteriorating function of cells, organs or multiple organ systems, resulting from long-term exposure to a chemical. The acute toxicity study demonstrates that the LD_{50} of aqueous and methanolic *A. malaccensis* extracts may be higher than 2g/kg body weight. The finding suggests that the extracts would be used at the limit dose tested for the assessment and evaluation of sub-chronic toxicity by using the repeated doses for 28 days.

In this study, we used 250mg/kg and 500mg/kg of aqueous and methanolic *A. malaccensis* extracts to feed normal healthy male rats for 28 days. The concentrations used are in the suitable range for sub-chronic toxicity study (Udem et al. 2009) and the period of 28 days is scientifically accepted for toxicity study of long-term feeding (Bartsch et al. 2005). The study was conducted for 28 days as minimum period of treatment and any significant changes were observed and recorded. Number of animals used was determined based on 3R principles, 'Reduction'. We therefore decided on n=6 as a minimum and sufficient number for informative results in this experiment.

The results from sub-chronic toxicity study showed evident normal behaviour among the treated rats. No lethality was recorded in any of the groups subjected to both experiments using the dose of 250mg/kg and 500mg/kg body weight. There was no significant difference recorded in body weight gain or internal organ weight of treated rats when compared to that of control group as shown in Tables 4.4 and Table 4.5, respectively. Body weight was generally normal in all treated rats.

Clinical biochemical tests were performed on blood samples in order to evaluate any toxic effects on liver and kidney. The results were summarized in Table 4.6. Significant decrease ($P \leq 0.05$) in AST, creatinine and total bilirubin was observed in male rats treated with 250/mg/kg/day and 500mg/kg/day of aqueous *A. malaccensis* extract as compared to that of control; on contrary, there was significant increase ($P \leq 0.05$) in total protein concentration in the same treated rats (Table 4.6). Meanwhile, rats treated with 250mg/kg/day and 500mg/kg/day of methanolic *A. malaccensis* extract

experienced significantly decrease ($P \leq 0.05$) of all serum concentration parameters which included AST, ALT, ALP, creatinine, urea and total bilirubin; the total protein concentration was however, increased significantly ($P \leq 0.05$) in comparison to that of control group.

Table 4.4 Body weight of rats for sub-chronic toxicity study of *A. malaccensis* methanolic and aqueous extracts.

Group	Body weight (g)		
	Initial Day 0	**Final Day 28**	**Weight gain on day 28**
A. malaccensis aqueous extract			
Control	264.83±6.30	337.40±12.42	72.57±13.82
Low dose	264.99±13.49	343.40±15.82	78.41±23.18
High dose	257.36±5.42	332.31±12.81	74.94±10.09
A. malaccensis methanol extract			
Control	270.79±5.82	343.76±11.90	72.97±12.35
Low dose	237.27±10.02	326.48±12.52	89.21±10.07
High dose	260.75±11.73	345.54±12.72	84.79±9.81

Weight gain on day 28 was calculated as difference between animal weights at Day 28 with Day 0. Values expressed were mean ± S.E.M of six animals. Low dose = 250mg/kg and high dose = 500mg/kg.

Considering that parameters in clinical biochemical tested were considered minor and fell within the normal range for index of kidney and liver function, the result showed that the extracts did not leave toxicity effects on the kidneys and liver. Generally, oral dosages of 250mg/kg and 500mg/kg body weight of *A. malaccensis* aqueous and methanolic extracts did not cause either acute or sub-chronic toxicities in rats. Therefore, we decided on the cut-off dosage of 500mg/kg body weight for effective dose (ED_{50}) in animal study, which is expected to be $1/4^{th}$ of LD_{50}

Table 4.5 Organ weight of rats for sub-chronic toxicity study of *A. malaccensis* methanolic and aqueous extracts.

Organ Weight	AQUEOUS EXTRACTS			METHANOL EXTRACTS		
	Control	Low dose	High dose	Control	Low dose	High dose
Lung	1.78±0.07	1.71±0.08	1.79±0.19	1.78±0.08	1.78±0.13	1.71±0.10
Heart	1.02±0.05	1.02±0.03	1.00±0.04	1.00±0.05	1.07±0.04	1.10±0.05
Liver	10.49±0.76	10.61±0.24	10.96±0.63	10.72±0.77	10.13±0.77	10.94±0.54
Spleen	0.55±0.03	0.56±0.04	0.60±0.01	0.53±0.05	0.62±0.02	0.58±0.03
Kidney	2.37±0.14	2.67±0.13	2.51±0.11	2.55±0.13	2.50±0.15	2.80±0.10
Testis	2.99±0.08	2.94±0.13	2.94±0.18	2.93±0.15	2.81±0.23	2.99±0.05
Brain	1.82±0.06	1.84±0.09	1.85±0.08	1.80±0.02	1.84±0.03	1.82±0.05
Thymus	0.50±0.06	0.55±0.04	0.50±0.01	0.50±0.02	0.47±0.01	0.47±0.07
Thyroid	0.67±0.04	0.71±0.03	0.68±0.03	0.70±0.03	0.71±0.04	0.73±0.03
Adrenal	0.04±0.01	0.04±0.004	0.04±0.01	0.06±0.01	0.05±0.01	0.05±0.01

Values expressed were mean ± S.E.M of six animals. There were no significant differences at $P<0.05$. Low dose = 250mg/kg and high dose = 500mg/kg.

Table 4.6 Clinical biochemistry test on blood samples for sub-chronic toxicity study of *A. malaccensis* aqueous and methanolic extracts.

Group	Serum levels						
	AST (U/L)	**ALT (U/L)**	**ALP (U/L)**	**CREA (µmol/L)**	**UREA (mmol/L)**	**TP (g/L)**	**TBIL (µmol/L)**
Normal range	74 – 143	18 – 78	62 – 230	17- 70	5 - 7.497	56 – 76	3.42 -9.40
Aquilaria malaccensis aqueous extracts							
Control	144.00±6.16	75.57±6.62	153.33±9.90	57.67±3.13	6.50±0.32	78.68±0.98	9.45±0.62
Low dose	134.50±7.27*	71.13±4.08	151.33±17.08	55.50±2.14*	6.26±0.18	83.20±1.68*	5.061±0.29*
High dose	135.00±8.90*	72.68±4.25	152.67±6.58	55.67±3.42*	6.36±0.23	81.92±1.38*	6.70±0.54*
Aquilaria malaccensis methanol extracts							
Control	141.83±5.81	74.52±2.50	160.67±14.70	56.83±1.40	6.45±0.23	79.73±2.37	8.57±0.43
Low dose	106.17±5.40*	64.53±5.07*	135.00±7.52*	53.83±1.78*	5.95±0.20*	85.08±1.01*	4.35±0.36*
High dose	110.00±4.40*	66.65±2.94*	141.83±8.66*	54.00±0.97*	6.12±0.11*	84.70±5.04*	4.55±0.33*

AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, CREA: creatinine, TP: total protein, and TBIL: total bilirubin. Values expressed were mean ± S.E.M of six animals. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with control group using one-way ANOVA by Tukey's post-test and indicated by *. Low dose = 250mg/kg and high dose = 500mg/kg.

Source of normal range: Giknis et al. (2008).

4.4 STZ-Induced Diabetic Study

The limitations, side effects and cost of the currently available oral antidiabetic agents to control blood glucose have spurred the interest among the scientists to develop novel natural antidiabetic agents with lesser side effects. Peng et al. (2005) stated that many plant species used in traditional medicines are known for their hypoglycaemic effects albeit insufficient scientific verification. Previous study found that there was a significant decrease in glucose serum of diabetic rats after receiving 50 mg/kg *Teucrium polium* extract for a month (Shahraki et al. 2007). Similarly, it has been reported that the antidiabetic action of *T. polium* that caused significantly decreased in plasma glucose level in streptozotocin-induced (STZ-induced) hyperglycaemic rats after 6 weeks of consecutive oral treatment (Esmaeili and Yazdanparast, 2007).

In this study, we used STZ-induced diabetic rats to assess the antidiabetic activity of methanolic and aqueous extracts of *A. malaccensis*. The predetermined dosage of these extracts was 500mg/kg body weight, which was used in this experiment. Streptozotocin (STZ) was chosen to induce diabetes in rats rather than alloxan since the former is known for its selective destruction to pancreatic β-cells and is less toxic than alloxan while maintaining a diabetic condition.

For positive control, oral hypoglycaemic drug metformin with optimal dosage of 500mg/kg body weight was used in this model. Metformin is a dimethylbigunide oral hypoglycemic drug derived from guanide, a hypoglycaemic active compound isolated from *Galega officinalis* or French Lilac, a medicinal plant used for centuries in Europe for diabetes treatment (Musi, 2002). Metformin is widely prescribed for patients with type 2 diabetes, for which it is known to be able to inhibit hepatic glucose production and acts as an insulin sensitizer in isolated skeletal muscle of insulin-resistant patients (Hawley, 2002).

4.4.1 Blood glucose effects

Overproduction of glucose in bloodstream through excessive hepatic glycogenolysis and gluconeogenesis is one of the fundamental cause of hyperglycemia and diabetes mellitus. Increase in blood glucose concentration is an important characteristic in diabetic studies. The effects of oral extracts administered on blood glucose level from day 1 until day 5 in STZ-induced diabetic rats are depicted in Figure 4.1. The initial blood glucose levels of diabetic rats selected for the study were in the range of 7.0 to 12.0mmol/L. The experimentally induced diabetes had significantly increased (P<0.05) the fasting blood glucose level by 3-fold from initial reading.

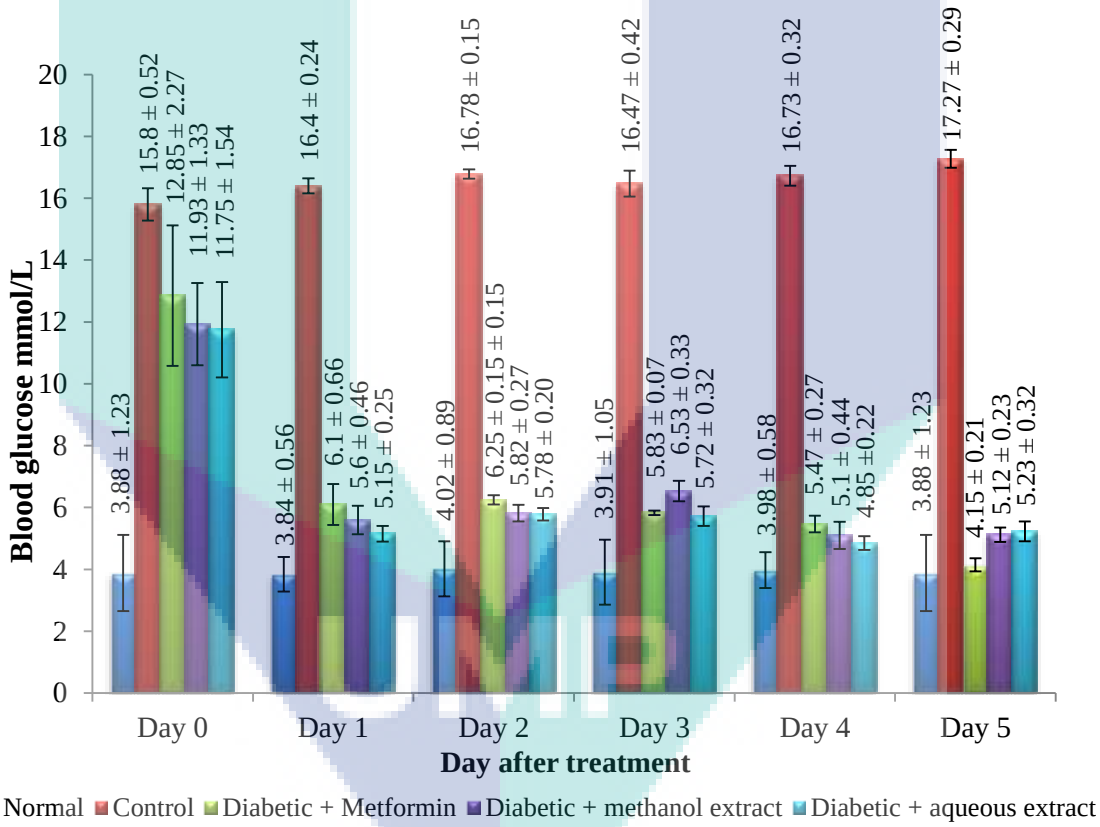

Figure 4.1 Effects of *A. malaccensis* aqueous and methanolic extracts on blood glucose of the STZ-induced rats in after 5-day treatment. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significant difference compared with diabetic control using one-way ANOVA by Tukey's post-test and indicated by *. (n=6 rats in each group).

Table 4.7 Values on effects of *A. malaccensis* aqueous and methanolic extracts on blood glucose of the STZ-induced rats in after 5-day treatment.

Group	Blood Glucose (mmol/L)					
	Initial	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Normal group	3.88±1.23	3.84±0.56	4.02±0.89	3.91±1.05	3.98±0.58	3.88±1.23
Untreated Diabetic Group	15.80±0.52	16.40±0.24*	16.78±0.15*	16.47±0.42*	16.73±0.32*	17.27±0.29*
Diabetic + metformin	12.85±2.27	6.10±0.66	6.25±0.15	5.83±0.07	5.47±0.27	4.15±0.21
Diabetic + Methanol extract	11.93±1.33	5.60±0.46	5.82±0.27	6.53±0.33	5.10±0.44	5.12±0.23
Diabetic + Aqueous extract	11.75±1.54	5.15±0.25	5.78±0.20	5.72±0.32	4.85±0.22	5.23±0.32

Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significant difference compared with diabetic control using one-way ANOVA by Tukey's post-test and indicated by *. (n=6 rats in each group)

The results showed that treatment with aqueous and methanolic *A. malaccensis* extracts for five days could reduced the fasting blood glucose to normal level, demonstrating good glycemic control that characterized antidiabetic properties (Figure 4.1). Treatment with methanolic and aqueous leaves extracts of *A. malaccensis* at the dosage of 500mg/kg body weight for 5 days exhibited a significantly decreased (P<0.05) in blood glucose of STZ-induced diabetic rats as compared to untreated diabetic and normal control groups. Blood glucose level in induced-diabetic rats dropped dramatically on day 1 after oral administration of *A. malaccensis* extracts and continued to decrease until day 5, the result of which comparable to metformin.

As shown in Figure 4.1, there is significant differences (P<0.05) between blood glucose level in the healthy normal male rats and untreated diabetic rats (diabetic control). In diabetic control groups, the animals were not treated with any extracts, fed with standard diet and allowed to drink 10% glucose solution similar to treated group. According to Figure 4.1, there was significant reduction in blood glucose level of treated rats on day 5 after treatment with 500mg/kg of methanolic and aqueous *A. malaccensis* compared to untreated diabetic group (5.12+0.23 and 5.23+0.32 vs. 16.27+0.29 mmol/L, respectively, $P \leq 0.05$). Similarly, it was observed that the blood glucose level was significantly decreased with the treatment of metformin (4.15+0.21 mmol/L). However, the decrease in blood glucose level in rats treated with metformin was relatively less significant than those treated with *A. malaccensis* extracts when compared to the diabetic control group (Figure 4.1). One of the possible reasons for the increase in blood glucose level with the passage of time is the metabolism of active ingredients contained in the extract, which have been responsible for the hypoglycemic effects.

It was found through this study that the methanolic extract demonstrated more pronounced antidiabetic activities than the aqueous extract with the percentage of blood glucose-lowering effect of 57.08% and 55.48% respectively; the results were almost similar to that of metformin, 68.79%. Therefore, the results obtained in STZ-induced diabetic rats indicated that at the dosage of 500 mg/kg body weight, the methanolic and aqueous *A. malaccensis* extracts are able to lower the blood glucose levels as compared to the normal and untreated diabetic groups. Furthermore, we found that the blood

glucose lowering effect in STZ-induced diabetic rats treated with methanolic extracts of *A. malaccensis* was almost similar to that of metformin.

4.4.2 Body weight effects

Hyperglycemia induced by STZ was indicated by the severe loss of body weight (Khaleel et al., 2015). However, diabetic rats fed by *A. malaccensis* extracts showed an increase in body weight. In contrary, group of diabetic rats without extracts administrated revealed a decrease in body weight (Table 4.7).

Initially, the body weight reduction was obvious in all STZ-induced diabetic rats where there was significantly decreased ($p>.05$) in body weight gain of diabetic rats as compared to normal rats. It was observed that untreated STZ-induced diabetic rats experienced significant weight loss throughout the experiment in contrast to significant ($P>0.05$) increase in body weight of those treated with extracts and metformin. This suggests that metformin and both aqueous and methanolic *A. malaccensis* extracts had significantly prevented weight loss in diabetic rats. Meanwhile, there was significant loss in body weight of untreated diabetic rats when compared to the final weight of normal rats in control group (Table 4.7)

Table 4.8 Body weight of STZ-induced diabetic rats after treatment with *A. malaccensis* aqueous and methanolic extracts.

Group	Body weight (g)		
	Initial Day 1	**Final Day 5**	**Weight gain on day 5th**
Normal Group	272.38±14.75	284.64±13.80	12.25
Diabetic (control)	247.48±9.14	238.96±9.77	(-) 8.52
Diabetic + Metformin	253.97±12.55*	265.76±12.39*	11.79
Diabetic + methanol extract	249.01±6.24*	260.44±5.13*	11.43
Diabetic + aqueous extract	281.49±9.50*	292.65±9.38*	11.16

Weight gain values represent change (loss or gain) in body weight compared with initial body weight. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P\leq0.05$) was considered as significant difference compared with diabetic control using one-way ANOVA by Tukey's post-test and indicated by *. (n=6 rats in each group).

Therefore, both methanolic and aqueous *A. malaccensis* extracts and metformin treatments significantly prevented weight loss. Improvement in body weight of the treated group may be attributed to the increase in metabolic activity of their body system. Biochemically, when diabetic, no glucose go into the cells hence increases fat use. This results may suggest that *A. malaccensis* extracts have increased glucose uptake into the cells which then improved body weight of the rats.

Meanwhile, decrease in body weight of patients with diabetes mellitus has always been attributed to gluconeogenesis i.e., catabolism of proteins and fats, which is associated with the characteristic loss of body weight due to increased muscle wasting and loss of tissue proteins (Shirwaikar et al., 2006).

4.4.3 Organ weight effects

The effects of treatment with *A. malaccensis* extracts on organ weight (kidney, liver and pancreas) in STZ-induced diabetic rats were recorded in Table 4.8.

Table 4.9 Organ weight of STZ-induced diabetic rats after treatment with *A. malaccensis* aqueous and methanolic extracts.

Group	Organ weight (g)		
	Kidney	**Liver**	**Pancreas**
Normal Group	1.86±0.14	7.89±0.76	0.84±0.03
Diabetic (control)	1.81±0.03	10.19+0.4*	0.86±0.04
Diabetic + Metformin	1.88±0.07	7.98±0.26	0.88±0.05
Diabetic + methanol extract	1.81±0.12	7.72±0.60	0.81±0.05
Diabetic + aqueous extract	1.89±0.10	7.65±0.41	0.88±0.03

Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with control group using one-way ANOVA by Tukey's post-test and indicated by *(n=6 rats in each group).

In this study, there was significant increase in weight of liver of untreated diabetic rats as compared to groups of normal rats and rats treated with extracts. This may be an indication of liver inflammation (Diego, 2009) due to few mechanisms: increase in serum bilirubin levels and marker enzymes; and reduction in albumin and globulin concentration.

4.4.4 Haematology effects

The effects of treatment with methanolic and aqueous *A. malaccensis* extracts on haematology were studied through measurement of red blood cell (RBC), haemoglobin, platelet and white blood cell (WBC) in STZ-induced diabetic rats and recorded in Table 4.9.

Table 4.10 Haematological values in STZ-induced diabetic rats after treatment with *A. malaccensis* aqueous and methanolic extracts.

Group	Haematology Value			
	RBC ($x 10^{12}$/L)	**Haemoglobin (g/L)**	**Platelet ($x 10^9$/L)**	**WBC ($x 10^9$/L)**
Normal range	7.0 - 10.5	11.0 – 19.2	500 – 1400	10.2 – 3.8
Normal Group	7.58±0.97	14.77±0.19	1162.00±57.65	11.59±0.46
Diabetic (control)	6.89±1.25	13.47±2.33	1055.17±142.81	12.62±0.34
Diabetic + Metformin	7.32±0.42*	14.28±0.11*	1146.50±70.55*	11.60±0.35
Diabetic + methanol extract	7.14±1.21*	14.88±1.81*	1184.00±103.35*	11.17±0.43
Diabetic + aqueous extract	7.27±0.11*	14.10±0.86*	1122.83±185.01*	10.91±0.40

RBC: red blood cells, and WBC: white blood cells Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's post test and indicated by * (n=6 rats in each group).

Source of normal range: Giknis et al. (2008).

According to Table 4.9, significant reduction ($P<0.05$) of tested haematological parameters was observed in untreated diabetic rats with the exception of white blood cell, which values increased. Changes in haematological profile are common in diabetic state (Perez, 2006). Reduction in the concentrations of RBC, haemoglobin and platelet, and increment in concentration of WBC observed in untreated diabetic rats indicated impairment in haematological function. Administration with *A. malaccensis* extracts reversed this abnormal haematological condition to normal, the measured for the values which were almost the same to that of metformin and normal groups. In general, however, the difference in these values between the different treatment groups were considered minor. Most importantly, the alteration of haematological values and white blood cell count were insignificant and remained within the normal range (Inala, 2002).

4.4.5 Clinical biochemistry effects

Clinical chemistry effects in STZ-induced diabetic rats were studied. The content of creatinine (CREA), urea and uric acid (UA); serum protein: total bilirubin (TBil), total protein (TP), albumin (ALB) and globulin (GLOB); serum enzymes: aspartate aminotransferase (ASP), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatine kinase(CK); and serum cholesterol: total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL), in blood samples were recorded as shown in Table 4.10, Table 4.11, Table 4.12 and Table 4.13, respectively.

4.4.5.1 Kidney Profile

Kidney is the major excretory organ, which function is profiled to examine the safety of tested substance (Sun et al. 2012). Kidney damages are indicated by measuring the level of three metabolites excreted from kidney to bloodstream: urea, creatinine and uric acid. Insulin deficiency and consequent inability of glucose to reach hepatic tissues stimulate gluconeogenesis as an alternative route for glucose supply (Dale et al., 2002). This route is sustained by increased proteolysis which releases free glycogenic amino acids into the plasma, which would then deaminated in the liver, resulting in consequential increase of urea in the blood. Creatinine derived from the breakdown of muscle creatine and its concentration in serum is proportional to the body muscle mass. The amount of creatinine is usually constant and thus, easily excreted by the kidneys; elevation in the level of creatinine indicates diminished renal function (Sushrut et al., 2009). Meanwhile, uric acid is the major metabolic product of purine metabolism and its elevation in the serum signifies kidney impairment.

Creatinine, urea and uric acid were identified parameters in assessment of kidney profile in STZ-induced diabetic rats as outlined in Table 4.10. The results show that there was significantly increased ($P \leq 0.05$) in the level of serum urea, creatinine and uric acid concentrations of untreated diabetic rats (Table 4.10). These are indication of kidney damage, which was probably due to reduced functional capacity in kidney as reflected by the increased level of serum alcium ion, creatinine, urea and uric acid in STZ-induced diabetic rats. Administration of *A. malaccensis* extracts had a positive

impact on kidney function index of STZ-induced diabetic rats by significantly reduced the levels of creatinine, urea and uric acid as compared to normal group, thereby conferring protection against impairment due to diabetes. However, such changes in values compared between extracts and species were minor and the values remained within the normal range.

Table 4.11 Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts on serum creatinine, urea and uric acid in normal and STZ-induced diabetic rat for kidney profile.

Group	Serum level		
	Creatinine (µmol/L)	**Urea (mmol/L)**	**Uric Acid (µmol/L)**
Normal range	17.68 – 70.72	5.35 - 7.497	147.75 – 356.88
Normal group	52.33±1.67	5.87±0.18	248.67±9.65
Diabetic (control)	59.33±3.21	6.20±0.44	294.67±11.94
Diabetic + Metformin	53.00±3.04*	5.90±0.18*	252.17±14.34*
Diabetic + methanol extract	54.50±2.97*	5.91±0.47*	260.83±8.96*
Diabetic + aqueous extract	53.50±1.02*	5.90±0.20*	255.17±25.49*

Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's test and indicated by * (n=6 rats in each group).

Source of normal range: Giknis et al. (2008).

4.4.5.2 Liver Profile

Liver is the primary organ for detoxification and distribution of drugs (John, 2014). Liver damage can be measured through analysis of parameters, which are protein profile: total protein (TP), total bilirubin (TBIL), albumin and globulin; and enzyme profile: aspartate aminotransferase (AST) alanine aminotransferase (ALT) and alkaline phosphatase (ALP). These parameters for liver profiling were assessed in STZ-induced diabetic rats as shown in Table 4.11.

The total serum protein test measures the total amount of protein in the blood. During diabetes, there is increased protein catabolism with flowing of amino acids into the liver, to feed gluconeogenesis (Marion, 2000). This accelerated proteolysis of uncontrolled diabetes occurs due to insulin deficiency. Similar phenomenon might have

accounted for the decrease in total protein content in untreated STZ-induced diabetic rats. Administration of *A. malaccensis* extracts to diabetic rats had significantly inhibited proteolysis caused by insulin deficiency and thus, increased the level of total proteins to almost similar level rendered by treatment with metformin. As mentioned in subtopic of 4.4.3 on organ weight effects, increase in weight of liver was observed in untreated diabetic rats, which might indicate liver inflammation (Diego, 2009); increase in serum levels of bilirubin and marker enzymes and reduced albumin and globulin concentrations might have accounted for increase in weight of liver.

Protein profile in STZ-induced diabetic rats involved two major groups of protein: albumin and globulin, which are the mixtures of protein molecules that are useful for assessing the health of the liver. Albumin, which is manufactured in liver, is the major carrier protein that circulates in the bloodstream while globulins are larger proteins responsible for immunologic responses (Amy, 2006). The low concentration of serum albumin and globulin signifies chronic damage to the liver due to infection (Theodore, 2005). Therefore, reduction in serum albumin and globulin levels in untreated diabetic rats indicated diminished synthetic function of the liver. Oral administration of methanolic and aqueous *A. malaccensis* extract, however, restored the albumin and globulin levels to normal, which results were almost similar to that of metformin (standard drug used as a positive control) as shown in Table 4.11.

Bilirubin is the major product from the breakdown and destruction of old red blood cells. It is an important metabolic product with biological and diagnostic values (Amy, 2006). It is removed from the body by the liver; hence, it is a good indicator to the health status of liver. Elevated serum bilirubin level observed in diabetic rats treated with methanolic and aqueous *A. malaccensis* extracts (Table 4.11) might be due to liver disease. Treatment with *A. malaccensis* extract was able to reverse this condition in diabetic rats by lowering the bilirubin level to normal condition. All data obtained in respect to liver function indices indicated the absence of any significant liver damage following the treatment with both methanolic and aqueous *A. malaccensis* extracts in diabetic rats.

Table 4.12 Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts on blood serum in normal and STZ-induced diabetic for liver function profile.

Group	Serum level			
	TBIL (µmol/L)	**TP (g/L)**	**ALB (g/L)**	**GLOB (g/L)**
Normal range	3.42 -9.40	56 – 76	38 -48	15 – 25
Normal Group	4.79±0.11	72.90±1.93	48.28±1.63	25.05±0.46
Diabetic (control)	5.20±0.10	60.18±0.61	36.88±0.58	22.72±0.79
Diabetic + Metformin	4.89±0.06*	73.90±1.20*	48.47±0.45*	24.60±0.80
Diabetic + methanol extract	4.98±0.08*	72.45±2.57*	47.40±0.53*	26.20±0.73
Diabetic + aqueous extract	4.96±0.11*	73.12±1.73*	47.75±0.78*	26.18±0.96

TBIL: total bilirubin, TP: total protein, ALB: albumin and GLOB: globulin. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's post-test and indicated by * (n=6 rats in each group).

Source of normal range: Giknis et al. (2008).

ALP is made mostly in the liver and bone with few made in intestine and kidney. The liver makes more ALP than other organs. ALP is often employed to assess the integrity of plasma membrane and endoplasmic reticulum (Shahjahan et al., 2004), while GGT is a membrane-localized enzyme that plays a major role in glutathione metabolism in the liver (Gjin, 2017). Damage of the liver is reflected in increased activity of these two enzymes in the serum, probably due to leakage in altered cell membrane structure. Therefore, increase in serum ALP in untreated diabetic rats signified damages in plasma membrane while decrease in its value in diabetic rats treated with *A. malaccensis* extracts signified the reverse effects which was almost similar to that of metformin (Table 4.12).

The transaminases (AST and ALT) are well-known enzymes used as biomarkers to predict possible toxicity effects on the liver (Rahman, 2001). ALT is found mainly in the liver with smaller amount found in the kidney, heart, muscles and pancreas. ALT normally present at low concentration in blood. However, when the liver is damaged, it releases ALT into the bloodstream, which leads to significant increase in ALT levels. Meanwhile, AST is normally found in RBC, liver, heart, muscle tissues, pancreas and kidney. AST is also present at low concentration in blood under normal condition.

When body tissue and organ such as liver and heart is damaged, additional AST is secreted out into the bloodstream, resulting in increased AST level. Thus, the amount of AST in blood is proportional to the extent of the tissue damage.

Serum ALT and AST are useful indices for identifying inflammation and necrosis of the liver (Shivaraj et al., 2009). Elevation in serum activities of both transaminases as observed in diabetic rats indicated damage in the liver cells (Yakubu et al., 2003). According to Table 4.12, elevated AST and ALT levels in diabetic rats were significantly declined after treatment with methanolic and aqueous *A. malaccensis* extracts. Oral administration of *A. malaccensis* extracts attenuated the elevated activities of investigated enzymes in diabetic rats comparable to the effects rendered by metformin. This may be an indication of nontoxic nature and protective action of the extracts in reversing the damaging effects on liver due to diabetes.

Lactate dehydrogenase (LDH) was measured to check for tissue damage while creatine kinase (CK) was measured as an indication to muscle damage. When there was tissue or muscle damage, more LDH and CK were released into bloodstream. However, the result in Table 4.12 shows that the changes in these values were minor and remained within the normal range, which indicated there was no damage done in muscle and other tissues.

Table 4.13 Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts on enzymes in normal and STZ-induced diabetic rats for liver function profile.

Group	Serum level				
	AST (U/L)	**ALT (U/L)**	**ALP (U/L)**	**LDH (U/L)**	**CK (U/L)**
Normal range	74 – 143	18 – 78	62 – 230	140 – 280	60 – 174
Normal group	104.00±5.82	55.62±2.86	168.33±9.54	211.00±28.99	128.17±7.05
Diabetic (control)	135.617±4.81	81.10±5.51	198.50±22.43	218.67±13.09	134.33±8.61
Diabetic + Metformin	105.83±7.19*	56.63±1.69*	176.17±16.90*	217.50±36.26	110.50±9.15
Diabetic + methanol extract	110.83±4.31*	57.45±0.67*	185.33±16.86*	228.33±16.39	144.67±10.12
Diabetic + aqueous extract	109.83±5.04*	57.18±2.41*	178.17±17.62*	218.83±10.48	121.83±9.81

AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, LDH: lactate dehydrogenase, and CK: creatine kinase. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's post-test and indicated by * (n=6 rats in each group).

Source of normal range: Giknis et al. (2008).

4.4.5.3 Lipid Profile

Insulin has important role in metabolism of lipid and lipoprotein and alteration of lipid metabolism, which are common in diabetic individual (Bruno, 2015). The total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol are the parameters tested to establish lipid profile in STZ-induced diabetic rats as outlined in Table 4.13.

Total cholesterol, triglycerides and LDL in STZ-induced diabetic rats had significantly increased, whereas the treatment with *A. malaccensis* extracts had significantly lowered the level of these indicators to condition that was almost similar to that of positive control, metformin (Table 4.13). Decrease in triglyceride level in STZ-induced diabetic rats treated with *A. malaccensis* extracts may indicate increased mobilization of lipids from the tissues. The effects of these extracts on controlled mobilization of serum triglycerides, cholesterol and phospholipids were presumably mediated by controlling the tissue metabolism and improving the level of insulin secretion and action.

Table 4.14 Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts in normal and STZ-induced diabetic for lipid profile.

Group	Serum level			
	TG (mmol/L)	**TC (mmol/L)**	**HDL (mmol/L)**	**LDL (mmol/L)**
Normal range	1.03 -3.36	< 1.6949	2.59-3.34	1.036 – 1.554
Normal Group	0.69±0.07	1.80±0.04	1.32±0.02	2.87±0.13
Diabetic (control)	1.25±0.04	2.54±0.16	1.01±0.06	3.38±0.16
Diabetic + Metformin	0.68±0.03*	1.77±0.12*	1.36±0.03*	2.58±0.15*
Diabetic + methanol extract	0.63±0.11*	1.77±0.09*	1.39±0.03*	2.33±0.11*
Diabetic + aqueous extract	0.60±0.02*	1.76±0.05*	1.41±0.07*	2.15±0.13*

TG: triglyceride, TC: total cholesterol, HDL: high density lipoprotein, LDL: low density lipoprotein. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($P \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's post-test and indicated by * (n=6 rats in each group).

Source of normal range: Giknis et al. (2008).

We also observed that the level of HDL-cholesterol was significantly lower in STZ-induced diabetic rats (Table 4.13). However, in both treatment using methanolic and aqueous extracts of *A. malaccensis*, it was observed that these adverse effects were normalized in an almost similar manner to that of metformin, possibly by controlling the hydrolysis of certain lipoproteins and selective uptake and metabolism by different tissues. Increase in concentration of total cholesterol, LDL-cholesterol and reduced HDL-cholesterol as observed in diabetic condition are frequently associated with increased risk of myocardial infarction (Mediene et al. 2008). Treatment of diabetic rats with *A. malaccensis* extracts elevated HDL-cholesterol and reduced LDL cholesterol levels, which indicated the reduced risk of myocardial infarction. Hypertriglyceridemia is a characteristic condition observed in diabetic rats, indicated by elevated triglyceride level and is an independent risk factor for cardiovascular disease (George, 2007). However, the results obtained from this study, show that treatment with *A. malaccensis* extracts have prevented the elevation of triglycerides, signifying intact and undamaged myocardial membrane. Abnormalities in lipid profile are very common in diabetic rats. This study suggests that the extracts of *A. malaccensis* could be used in diabetic treatment to improve lipid metabolism, by which the correction of hyperglycaemia could be associated to hypotriglyceridaemia effects.

CHAPTER 5

CONCLUSION & RECOMMENDATIONS

A. malaccensis leaves extract has potential as dietary supplement that may be useful for allowing flexibility in meal planning and thus, can be a promising source of antidiabetic agent. *In vitro* study showed that the aqueous and methanolic *A. malaccensis* extracts have potential antidiabetic properties; the extracts had significantly inhibited α-amylase and α-glucosidase activities, which are important in carbohydrates digestion and glucose absorption (activity of intestinal enzymes). The results suggest that methanolic extract of *A. malaccensis* is more efficient than aqueous extract in inhibiting α-amylase and α-glucosidase enzymes *in vitro*. It was observed in toxicity study that the aqueous and methanolic *A. malaccensis* extracts were nontoxic and did not cause mortality even at the highest single oral dosage of 2g/kg body weight. Sub-chronic toxicity study also demonstrated that both extracts did not cause mortality and generally not harmful to kidney and liver, thus safe for consumption. *In vivo* study in STZ-induced diabetic rats showed that the aqueous and methanolic *A. malaccensis* extracts rendered significant reduction in blood glucose level in a dose dependent manner. The methanolic extract produced a more pronounced antidiabetic activity than that of aqueous extract with percentages of blood glucose lowering effects of 57.08% and 55.48%, respectively, almost similar to that of metformin, 68.79%. Assessment on diabetic rats treated with extracts showed that the extracts did not damage the proteins and lipid and thus, it is suggested that these extracts did not cause any significant damage to internal organs namely liver and kidney. Therefore, the data produced from this study would be beneficial for future clinical works in correcting hyperglycemia and alleviating the adverse effects of diabetes mellitus using *A. malaccensis* extracts to enhance antioxidant defences system, considering that the antioxidant components have also been explored as an alternative treatment of diabetes. Therefore, further study is highly necessary to identify effective components contained in *A. malaccensis* extracts.

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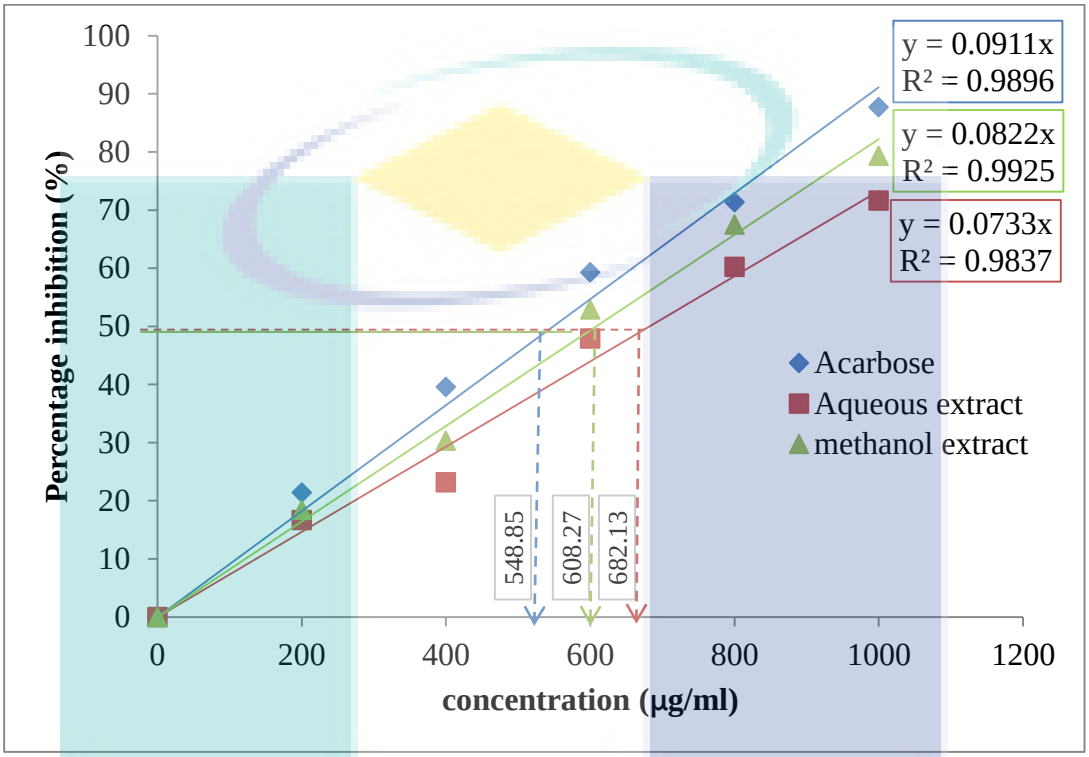
APPENDIX A

STANDARD CURVE OF *IN VITRO ALPHA*-GLUCOSIDASE INHIBITORY ASSAY



i. IC_{50} value for Acarbose;

y = 0.0911x

50 = 0.0911x

x = 548.85

IC_{50} = 548.85µg/ml

ii. IC_{50} value for aqueous extract;

y = 0.0733x

50 = 0.0733x

x = 682.13

IC_{50} = 682.13µg/ml

iii. IC_{50} value for methanol extract;

y = 0.0822x

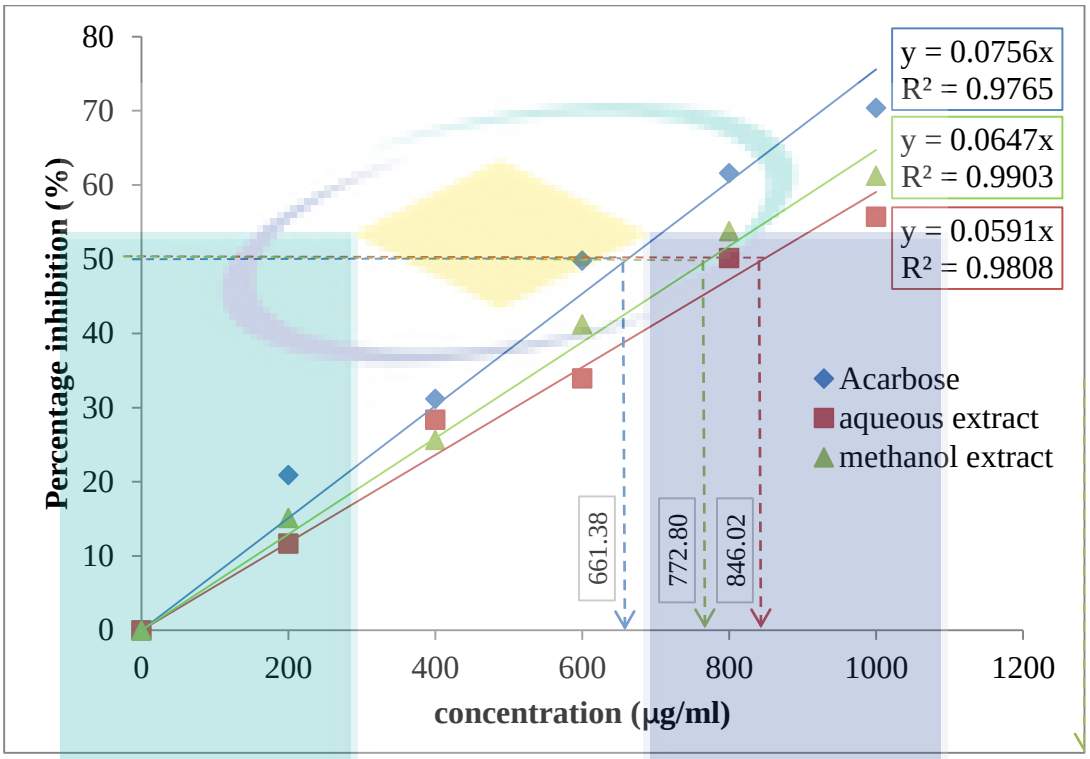
50 = 0.0822x

x = 608.27

IC_{50} = 608.27µg/ml

APPENDIX B

STANDARD CURVE OF *IN VITRO ALPHA*-AMYLASE INHIBITORY ASSAY



i. IC_{50} value for Acarbose;

y = 0.0756x

50 = 0.0756x

x = 661.38

IC_{50} = 661.38µg/ml

ii. IC_{50} value for aqueous extract;

y = 0.0591x

50 = 0.0591x

x = 846.02

IC_{50} = 846.02µg/ml

iii. IC_{50} value for methanol extract;

y = 0.0647x

50 = 0.0647x

x = 772.80

IC_{50} = 772.80µg/ml

APPENDIX C

LIST OF PUBLICATIONS

1. Zulkifle, N.L., Mhd Omar, N.A., Shaari, M.R. and Tajuddin, S.N. 2018. Acute and Sub-Chronic Toxicity Study of *Aquilaria malaccensis* Leaves Extract in Sprague-Dawley Rats. *Chemistry of Advanced Materials*, 3(1), published on March, 1st 2018.
2. Zulkifle, N.L., Mhd Omar, N.A. and Tajuddin, S.N. 2013. Antioxidant and Antidiabetic Activities of Malaysian Aquilaria spp. Leaves Extracts, *National Conference on Industry-Academia Initiatives in Biotechnology*.
3. Zulkifle, N.L., Mhd Omar, N.A. and Tajuddin, S.N. 2013. Antioxidant and Antidiabetic Activities of Malaysian Aquilaria spp. Leaves Extracts, *Creative, Innovative, Technology in Research Exhibition CITREX* (BRONZE).
4. Zulkifle, N.L., Mhd Omar, N.A. and Tajuddin, S.N. 2013. Antidiabetic Activities of Malaysian Aquilaria spp. Leaves Extract, *International Conference for Postgraduate Exhibition*.
5. Zulkifle, N.L., Mhd Omar, N.A. and Tajuddin, S.N. 2012. Antidiabetic Activities of Malaysian Aquilaria spp. Leaves Extract, *National Conference Postgraduate Research*.

APPENDIX D

JOURNAL

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Original paper

Acute and Sub-Chronic Toxicity Study of Aquilaria Malaccensis Leaves Extract in Sprague-Dawley Rats

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Abstract

Aquilaria malaccensis (*A. malaccensis*) is a woody plant of Thymeleceae family, mostly distributed in the Southeast Asian rainforest. Due to its pharmacological properties, it can potentially be used in the treatment of various health disorders. This work aims to evaluate acute and sub-chronic toxicity of *A. malaccensis* methanol leaves extract in adult male Sprague-Dawley rats. Acute toxicity study was carried out by administering *A. malaccensis* extract to groups of six adult male Sprague-Dawley rats by oral gavage at dosages of 250, 1000 and 2000 mg/kg body weight; sub-chronic study was carried out at dosages of 250 and 500 mg/kg body weight for 28 consecutive days. Hematology and biochemistry tests were carried out to investigate potential toxic effects on liver and kidney. No morbidity, mortality and physical or behavior abnormality were observed in rats treated at all tested dosages in acute and sub-chronic toxicity studies; body and organs weight of rats in treated groups and control groups were similar in both toxicity studies. Hematological and biochemical parameters slightly but significantly decreased as compared to that of the control group. The results suggest that oral effective dose (ED50) and lethal dose (LD50) of *A. malaccensis* extract are 500 mg/kg and greater than 2000 mg/kg body weight, respectively.

APPENDIX E

ANIMAL ETHICS APPROVAL

KEMENTERIAN PERTANIAN DAN INDUSTRI ASAS TANI

INSTITUT PENYELIDIKAN DAN KEMAJUAN PERTANIAN MALAYSIA
Malaysian Agricultural Research and Development Institute
Pusat Penyelidikan Sains Ternakan
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Reference No. : MDI/AS/FL/521/008
Date : 7 September 2018

Nur Liyana Zulkifle
K18183
Green Technology Programme
Agrobiodiversity & Environment Research Centre
MARDI, Serdang
SELANGOR

APPLICATION OF ANIMAL ETHIC APPROVAL FOR RESEARCH PROPOSAL

The above matter is kindly referred.

Congratulations.

Please be informed that the research proposal that you sent to Animal Ethic Committee, has been approved by the Committee Members on the 10th August 2018 (Friday).

The approval reference is as below:

No.	Project Title	Approval Reference
1.	Antidiabetic Activities of *Aquilaria malaccensis* (Agarwood) Leaf Extracts.	**20180810/R/MAEC00046**

Upon receiving this approval reference, you may proceed with your research works. Please remind that:

1. You must notify the Committee in writing regarding any alteration to the project
2. You must notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability of the project
3. At all times you are responsible for the ethical conduct of your research
4. Please fill up Animal Carcass Disposable Form after project completed and return to AEC Secretariat