ANTIOXIDANT AND ANTI HYPERGLYCAEMIC ACTIVITIES OF *Aquilaria sinensis* LEAVES (GAHARU)

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ANTIOXIDANT AND ANTI HYPERGLYCAEMIC ACTIVITIES OF *Aquilaria sinensis* LEAVES (GAHARU)

ABSTRACT

Aquilaria sinensis is commonly known as Gaharu belongs to the family of Thymelaeceae. It has been used as traditional medicine for diabetes treatment and possesses several other pharmacological activities. The objective of the present study is to determine the antioxidant and anti hyperglycaemic properties in the *A. sinensis* leaves. The methanolic leaves extract of *A. sinensis* was fractionated via solvent – solvent extraction using *n*-hexane, dichloromethane, butanol, chloroform, ethyl acetate and methanol aqueous to produce fractions. The TLC results of the plant extracts showed the presence of phenolic, terpenoid and alkaloid compounds. The total phenolic contents is the highest in the ethyl acetate extract at 1.69 ± 0.02 mg GAE/ g dry weight and the total flavonoid content highest in butanol fraction extract at 3.59 ± 0.07 mg QE / g dry weight. The fractions were subjected to the *in vitro* antioxidant assay. The results showed that ethyl acetate fraction exhibited the higher scavenging DPPH radicals activity with IC_{50} value of 0.10 ± 0.02 mg/ml, the highest ability of ferric reducing power activity at 0.37 ± 0.07 mmol/g extract , and good chelating of metal with IC_{50} < 0.05 ±0.01 mg/ml. However, methanol aqueous exhibits the highest scavenging of superoxide radical activity with IC_{50} value of 0.18 ± 0.01 mg/ml, nitric oxide radical scavenging activity with IC_{50} >1.6 ± 0.001 mg/ml. Based on the results obtained from the antioxidant assays, the fractions which showed good antioxidant activities were subjected to *in vitro* anti hyperglycaemic assay. Among all of the active fractions, ethyl acetate showed the highest inhibition of alpha amylase enzyme at 43.14% with IC_{50} value of >1.6 ± 0.04 mg/ml, inhibition of alpha glucosidase enzyme at 81.06% with IC_{50} value of < 1.6 ± 0.03 mg/ml, inhibition of glycogen phosphorylase at 17 % with

IC_{50} value of >1.6 ± 0.09 mg/ml and inhibition of haemoglobin glycosylation at 65.25 % with IC_{50} value of 0.87 ± 0.12 after 72 hours of incubation. However, all of the fractions showed poor inhibition on glycogen phosphorylase enzyme activity. The results from the LCMS analysis showed the presence of a bioactive compound, magniferin in aqueous fraction and tripteroside in ethyl acetate fraction. Those compounds are actively known to have the properties of anti hyperglycaemic and antioxidant. In conclusion, the leaves of the *A. sinensis* possessed antioxidant and anti hyperglycaemic activity that provide scientific evidence to support the claim of traditional medicinal or herbal medicinal in treating diabetes.

Keywords : antioxidant, anti hyperglycaemic, Gaharu, and Magniferin

ANTIOKSIDAN DAN ANTI HIPERGLICEMIA AKTIVITI *Aquilaria sinensis* DAUN (GAHARU)

ABSTRAK

Aquilaria sinensis dikenali sebagai Gaharu berasal dari keluarga Thymelaeceae. Ia telah digunakan sebagai ubat tradisional untuk rawatan diabetes dan memiliki beberapa aktiviti farmakologi yang lain. Objektif kajian adalah untuk menentukan sifat antioksidadan dan antidiabetik dalam daun *Aquilaria sinensis*. Ekstrak metanolik daun *A.sinensis* telah diekstrak secara fraksinasi pelarut dengan n-heksana, diklorometana, , butanol, kloroform, etil asetat dan methanol akueus. Keputusan TLC daripada ekstrak tumbuhan menunjukkan kehadiran sebatian fenol, terpenoid dan alkaloid. Jumlah kandungan fenolik tertinggi dalam ekstrak etil asetat pada 1.69 ± 0.02 mg GAE / g berat kering dan jumlah kandungan flavonoid tertinggi dalam ekstrak butanol pada 3.59 ± 0.07 mg QE/ g berat kering. Fraksi ekstrak daun dijalankan assei *in vitro* antioksidan. Hasil kajian menunjukkan bahawa ekstrak etil asetat memberikan penyerap radikal DPPH tertinggi pada IC_{50} 0.10 ± 0.02 mg/ml mg/ml, keupayaan tertinggi kuasa ferik penurunan pada 0.37 ± 0.07 mmol/g ekstrak dan pengkelat logam tertinggi pada IC_{50} < 0.05 ±0.01 mg/ml. Walau bagaimanapun, ekstrak metanol akueus menunjukkan hasil penyerap radikal superoksida tertinggi pada IC_{50} 0.18 ± 0.01 mg/ml, dan radikal nitrik oksida aktiviti pada IC $_{50}$>1.6 ± 0.01 mg/ml. Berdasarkan keputusan yang diperolehi dari assei antioksidan, ekstrak yang menunjukkan aktiviti antioksida terbaik dijalankan assei *in vitro* anti hyperglycaemic. Di antara semua ekstrak yang aktif, ekstrak ethil asestat menunjukkan nilai yang tertinggi dalam perencatan enzim alpha amylase pada 43.14% serta IC_{50} >1.6 ± 0.04 mg/ml, perencatan enzim alpha glucosidase pada 81.06% serta IC_{50} < 1.6 ± 0.03 mg/ml, perencetan enzim glikogen phosphorylase pada 17% serta IC_{50} of >1.6 ± 0.0.9mg/ml dan perencetan hemoglobin glikosilasi pada 65.25% serta IC_{50} 0.87± 7.68 selepas 72

jam pengeraman. Walau bagaimanapun, semua fraksi ekstrak menunjukkan perencatan yang lemah dalam aktiviti enzim glikogen phosphorylase. Keputusan daripada analisis LCMS, menunjukkan kehadiran sebatian bioaktif, magniferin dan tripteroside yang mempunyai sifat-sifat antioksidan dan anti hyperglycaemic. Kesimpulannya, ekstrak daun *A.sinensis* mempunyai aktikiti antioksidan dan antti hyperglycaemic yang memberikan bukti sokongan saintifik yang menyokong dakwaan pengamal tradisional ubatan herba dalam rawatan diabetis.

Kata kunci : antioksidan, anti hyperglycaemic, Gaharu dan Magniferin

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LIST OF SYMBOLS AND ABBREVATIONS

%	:	percentage
±	:	plus minus
α - amylase	:	alpha amylase
α - glucosidase	:	alpha glucosidase
µl	:	microlitre
DMSO	:	dimethyl sulfoxide
DPPH	:	2,2 – diphenyl 1-1 picrylhydrazyl
EDTA	:	Ethylene –diamine-tetraacetic acid
ELISA	:	Enzyme –linked immunosorbent assay
FRAP	:	Ferric reducing Antioxidant power
G6P	:	Glucose-6-Phosphate
HCl	:	hydrochloric acid
IC_{50}	:	half maximal inhibitory activity
mg	:	milligram
ROS	:	reactive oxygen species
Rpm	:	revolution per time
SD	:	standard deviation
SEM	:	standard error of the mean
TLC	:	Thin layer chromatography
UV	:	Ultraviolet

CHAPTER 1

INTRODUCTION

Diabetes is a chronic metabolic disease, which is characterized by hyperglycemic condition from impaired secretion of insulin. It is most commonly present with glucose intolerance and defective of insulin action (Sicree *et al.,* 2006). Diabetes becomes principal cause of major morbidity and mortality along with multiple biochemical impairments associated with complications (Xie *et al.,* 2011). Over the recent years, diabetes has become leading major cause of death in the world affecting more than 360 million individual globally and this figure is expected to increase more by the year of 2030 as per the report by the International Diabetes Federation. The type 2 diabetes currently comprises about 90% of all diabetic cases globally especially in Asia countries. This disease leads to numerous other complications such as coronary heart diseases, kidney failures and liver dysfunction. Although good dietary intake lifestyle and regular exercise manage the disease, the success rate in impeding of diabetic complication is still low.

However, till date, there is no effective and promising cure for diabetes although it might be able to be considerably controlled through proper diet and regular exercise. The currently available synthetic drugs require the combination of more drugs in order to maintain the glycemic condition in most cases.

The widely- available synthetic anti-diabetic drugs that have been used as treatment eventually leads to numerous complications and undesirable side effects to the patient. In addition, synthetic drugs unable to afford by members of the rural communities due to the high cost (Baily *et al.,* 2000).

In conjunction to the limitations of available synthetic drugs and to overcome the increasing prevalence of diabetes, researchers were prompted to find an alternative anti-diabetic remedies. In specific, consideration was given to medicinal plants and herbs that are used for traditional healer and as anti hyperglycaemic remedies in the hope of discovering the new source of alternative medicine. Based on previous findings, several medicinal plants have were found to have hypoglycemic properties using *in vitro* and *in vivo* experimental studies**.** The natural hypoglycemic compounds from medicinal plant might be an effective to the synthetic drugs and they be ingested through daily dietary intake (Christina *et al.,* 2012).

Selective plants do exhibits α-glycosidase and α-amylase inhibitory activity and hampering the absorption of glucose and inhibiting carbohydrate-hydrolyzing enzyme. This could be a good strategy to regulate the elevated post- prandial blood glucose level in Type 2 diabetic patients. Nevertheless, researchers (Toddler, 1994) have intensively studied the discovery of enzyme inhibitors from medicinal plant. Apart from anti-diabetic compounds identification ,the presence of antioxidants properties may also be considered as an alternative for the treatment (Skyrmejones *et al.,* 2000). Based on previous studies, researchers have proven that hyperglycemic condition in diabetic patients do causes an increased level of free radicals which induces oxidative stress and reduces antioxidant defenses (Brownlee, 2001). Most medicinal plants and herbs possess antioxidant properties and are able to combat disease related to oxidative stress and act as free radical scavengers due to the presences of bioactive compounds.

Recently, researchers have focused on the search of the effective natural inhibitors and one of is *A. sinensis* leaves, which is also known as gaharu or agarwood (Xing *et al*., 2012). *A. sinensis* is widely distributed in the region of South China and Asian countries especially Thailand and Malaysia.

The resin of this plant has been used as incense as well as for traditional analgesic and sedative medicine. The leaves of *A. sinensis* have traditionally used as anti-inflammatory due to laxative properties. Based on recent studies, it has proven that the plant has inhibitory effects against α-glucosidase activity and chemical constituents were studied. However, to our best knowledge, there is no reports on the evaluation of antioxidant and anti hyperglycaemic properties extensively on this plant with *in vitro* assays. Therefore, the gaharu medicinal plant was selected for this study.

The objectives of study were to :

a. To separate and determine the phytochemical bioactive compound of *A. sinensis* leaves.

b. Evaluate the antioxidant activities of *A. sinensis* leaves.

c. Determine *in vitro* anti hyperglycaemic properties of *A. sinensis* leaves.

CHAPTER 2

LITERATURE REVIEW

2.1 Brief History of Diabetes Mellitus

Diabetes is a name that was originally derived from a Greek words by Greek Physician, Aretaeus at the early century of 30-90 CE. The word „mellitus" which means honey sweet was added to the term „diabetes" making it „Diabetes Mellitus" by a physician, Thomas Willis in 1675 after rediscovering the presences of excess sugar in urine where the first discovery was made by the ancient Indians (Ahmed, 2002).

2.2 Diabetes

Diabetes Mellitus is a long term and chronic metabolic disease, which causes significant mortality and morbidity rate all over the world. The prevalence of diabetes increases due to the practice of poor dietary lifestyle and reduced physical activity. The disease is characterized by chronic hyperglycemia, disorders of carbohydrate, lipid and protein metabolism which results from defects in insulin secretion by the pancreatic β cells. It is mainly caused by the incapability of pancreas to produce sufficient insulin or the inability of the body to utilize the insulin, which then causes elevated concentration of glucose in the blood. The diagnosis for diabetes is mostly suggested with presenting symptoms such as glycosuria and blood test for HbA1c and Oral Glucose Tolerance Test (OGTT). The OGTT criteria that defines diabetes according to The World Health Organization (WHO) is by the results of fasting plasma glucose >7 mmol/L and post pradianal 2 hours of plasma glucose of >11.1 mmol/L.

Diabetes Mellitus is also known as heterogeneous group of disorders where certain distinct diabetic phenotypes are characterized into specific or overlapping pathogenesis. In this case, Diabetes mellitus is classified into 3 major types which Type 1 diabetes, Type 2 diabetes and Gestational diabetes (Leslie, 1997)

2.2.1 Type 1 diabetes

The Type 1 diabetes is also known as insulin –dependent diabetes mellitus (IDDM) which is caused by the destruction of insulin secreting pancreatic β-cells. However, Type 1 diabetes accounts to about 5% to 10 % of all cases of diabetes and known as juvenile-onset diabetes. Individuals suffering from IDDM are mostly infants and children. The most common risk factor for this disease includes immunological, genetic, and environmental factors (Kukreja & Maclaren, 1999)

Type 1 diabetes is also characterized by the absolute absence of insulin secretion, which results into auto-immune β-cell destruction in pancreas. There are several markers that are responsible for this destruction which includes insulin antibodies (IAAs), islet cell autoantibodies (ICAs), tyrosine phosphatase 1A-2,1 A-2α autoantibodies, and glutamic acid decarboxylase autoantibodies (GAD_{65}). The autoantibodies are initially detected in hyperglycemic condition and are presents in more than 85 % of diabetic cases.

In addition, genetic risk factor is highly associated with human leukocyte antigen (HLA) locus class II in Type 1 diabetes. Apart from HLA loci, about 40 non-HLA polymorphisms is also associated with the pathogenesis of Type 1 diabetes and is analyzed through genome-wide association studies (Nokoff & Rewers, 2013).

There are few extrinsic factors that causes dysfunction of beta cell which includes viruses such as mump virus and coxsackie virus B4, chemical agents, and destructive cytotoxins and antibodies.

Besides that, underlying genetic defect which has a role in the replication of beta cells and plays a function may predispose to beta cell failure. In minority cases, patients with Type 1 diabetes have no evidence of autoimmunity and therefore, the Type 1 diabetes is classified as idiopathic diabetes. The pathophysiology of Type 1 diabetes which is shown in the Figure 2.1, involving hypothalamus, beta cell, adrenal gland and adipose tissue. It is characterized by complete insulin deficiency and strongly inherited.

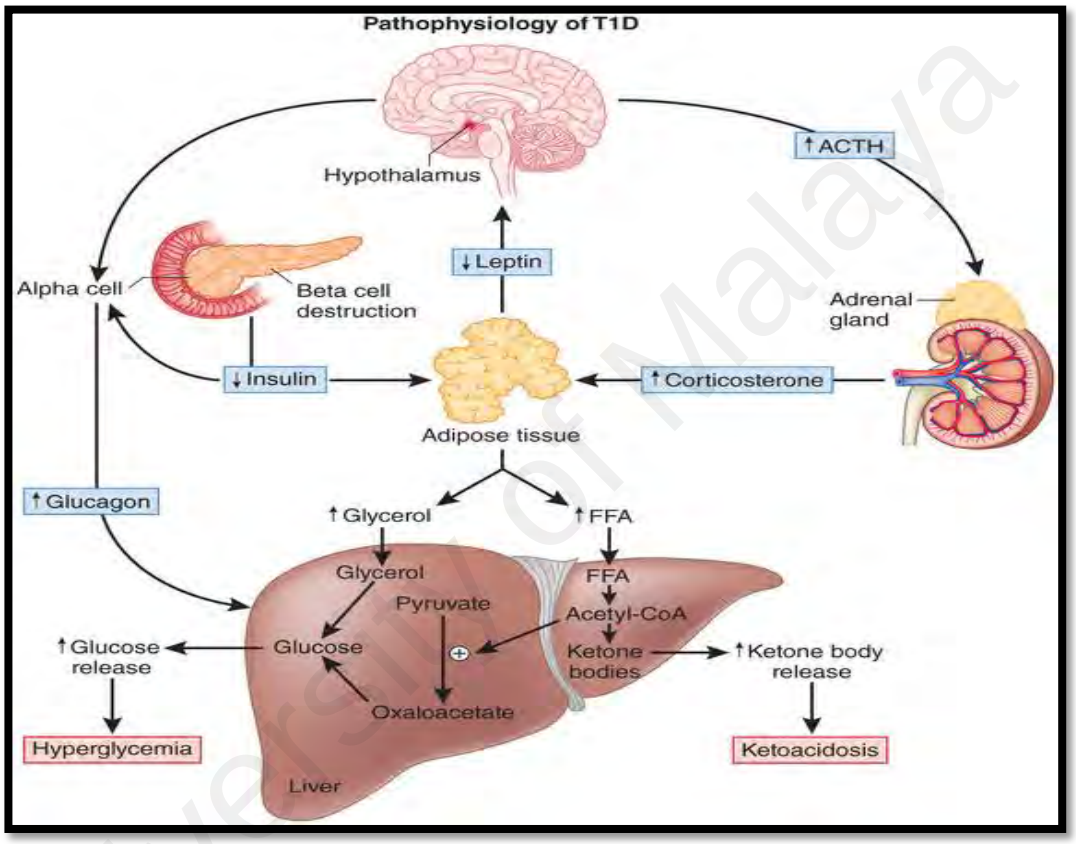

Figure 2.1 : The pathophysiology of Type 1 diabetes (Bettina & Samuel, 2014)

Besides hyperglycemia criteria, diabetic ketoacidosis is also an indication to Type 1 diabetes. It is caused by decreased level of glucose utilization and increases level of protein and lipid breakdown in order to compensate the body energy demand (Daneman, 2006). The prolonged lipid catabolism results in the accumulation of acetyl CoA which is associated to imbalance homeostatic mechanism such as body temperature and pH. However, untreated symptoms would lead to coma or death from ketoacidosis. Main features of Type 1 diabetes consist of polyuria, polyphagia, polydipsia, abdominal pain, weight loss and lethargy. As a treatment, insulin therapy is the most predominant treatment and typically used to manage the disease. In spite of lower prevalence of Type 1 diabetes, most severe diabetic case that leads to death mainly caused by Type 2 diabetes.

2.2.2 Type 2 diabetes

Type 2 diabetes is most commonly known as non-insulin dependent and accounts nearly 90% of all diabetic cases. It is largely associated with severe obesity and low physical activities in individuals. This disease is categorized as a polygenic disorder as individuals with this disease have an excessive hepatic glucose production, deficiency in insulin secretion or insulin resistance and failure of pancreatic β-cells (Ahmed, 2006). The disease does develop due to an unexpected increase in resistance against the insulin body unable to produce efficient amount of insulin to counter the resistance. Hence, it results to an elevated blood glucose concentration, leading to numerous complications. The pathophysiology of Type 2 diabetes, which is shown in Figure 2.2, describes the condition that take place in liver, beta cell and muscles and leads to diabetic condition.

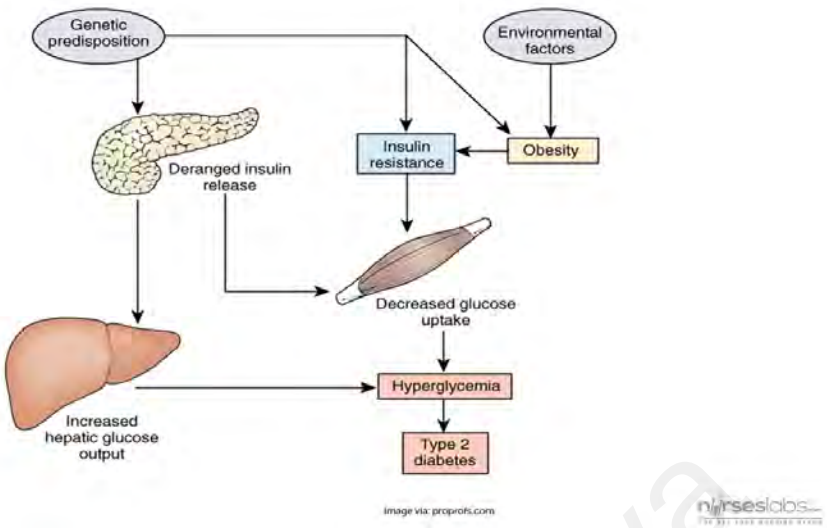

Figure 2.2 : The pathophysiology of diabetes Type 2 (Marianne, 2016)

The interaction of genetic and environmental factor contributes to the development of Type 2 diabetes. Insulin resistance and dysfunction of β-cells falls under the genetic risk factor category (DeFronzo, 2009). Resistance of insulin do develop in Type 2 diabetes when the body is unable to produce sufficient amount of insulin to cope the elevated blood glucose concentration. However, there are several risk factor that involves in the development of Type 2 diabetes, which comprises of insulin resistance, obesity, and oxidative stress.

a) Insulin resistance

Insulin mainly affects the glucose metabolism both directly and indirectly. The insulin receptors are mainly available in insulin-sensitive organs, which include liver, kidney, adipose tissue and muscles. Insulin signaling activation is depend upon the binding of insulin to insulin receptors which then helps in the suppression of gluconeogenesis in the liver and kidney, helps in glucose uptake through the process of translocation of glucose transporter-4(GLUT-4) from inner membrane to plasma membrane and inhibition of fatty acid from being released into circulation (Meyer *et al.,*1998).

However, resistance of insulin do develop in Type 2 diabetes when the body unable to produce sufficient amount of insulin to cope the elevated blood glucose concentration. The impaired insulin mediated glucose uptake results in insulin resistance. The endogenous glucose is elevated in Type 2 diabetes leading to hyperglycemia condition. The cause of insulin resistance is mainly the down regulation of insulin receptors and acquired factors such as obesity and oxidative stress.

b) Obesity

A complex mechanism is involved in obese individuals that cause insulin resistance where it comprises of non-esterified fatty acids (NEFA), cytokines, and circulating hormones. Large adipocytes are formed when there is an increase in mass of stored triglycerides in adipose tissue. The large adipocyte resists the insulin action and impedes the breakdown of lipids. Hence, an elevated level of glycerol and NEFA occurs, stimulating the insulin resistance in liver and adipose tissue (De Feo *et al.,*1989). At the early stage of diabetes, insulin resistances are neutralized with hyperinsulinemia by maintaining the normal glucose tolerance. However, the worst case of the impaired glucose tolerance takes places when the insulin resistance increases or insulin secretory decreases or both happening at the same time.

c) Oxidative stress

Researches are still being made to understand the concept and involvement of oxidative stress in pathogenesis of insulin resistance. However, there is a finding stated that a reactive oxygen species (ROS) which is H_2O_2, to have the tendency to weaken insulin stimulation and glucose transport activity that leads to insulin resistance. Besides that, research studies have also shown that stress-activated serine kinase able to inhibit its function and activate ROS with insulin resistance (Dokken *et al.,* 2008).

2.2.3 Gestational Diabetes Mellitus (GDM)

It is usually defines as a condition of glucose intolerance which takes place onset of pregnancy. GDM develops in a small proportion of pregnant women which accounts to about 3 % - 5 % diabetic cases. It do increases the risk of pre-eclampsia, high blood pressure and depression. GDM do develop when there is deficiency of insulin secretion due to insulin resistance condition. It is commonly occurs during the 3rd trisemester of pregnancy and has higher risk to develop type 2 diabetes. However, it may improve its condition of disappear after delivery phase. In a worse case, gestational diabetes is able to damage the health of fetus or mother and it can be develop into type 2 diabetes after delivery phase (Mayfield, 1998).The pathophysiology of GDM are briefly described in Figure 2.3, with the impaired condition faced by each phase of pregnancy.

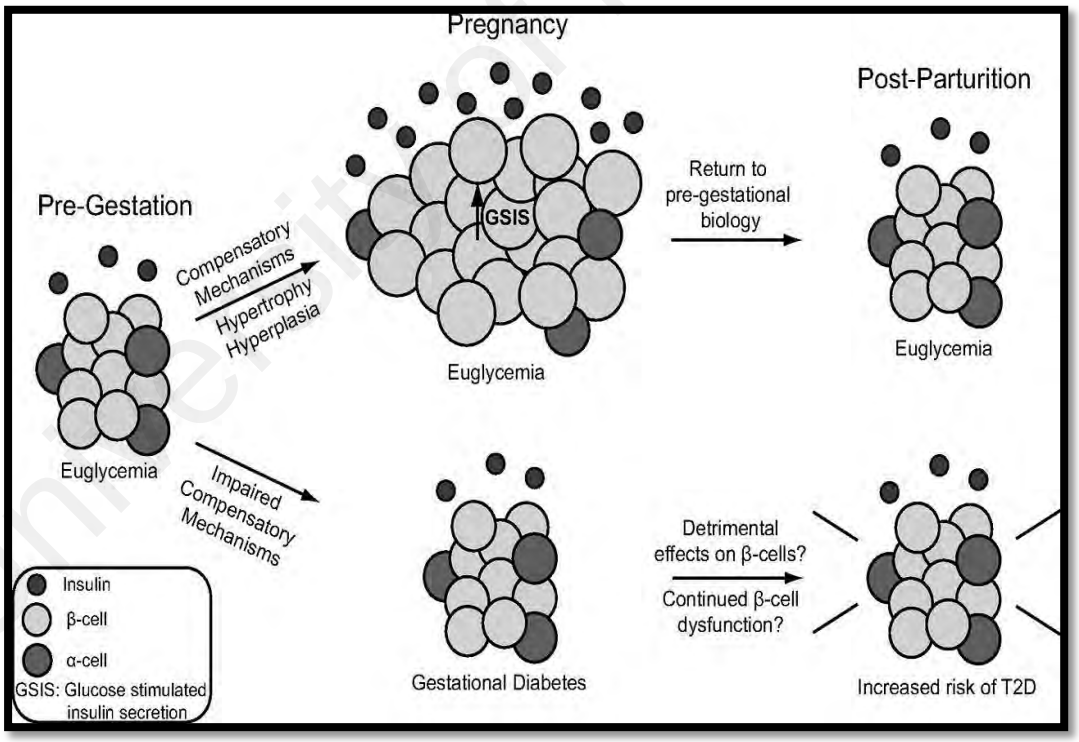

Figure 2.3 The pathophysiology of Gestational Diabetes Mellitus (Raymond & Maureen, 2013).

2.2.4 Complications of Diabetes Mellitus

Diabetes complications have proved to be major and dominant causes of morbidity and mortality around the world. In addition, considering the high prevalence of Type 2 diabetes cases, an individual affected by Type 2 diabetes faced more complications. Complications induced by Type 2 diabetes accounts to nearly more than 70 % of the diabetic cases around the world. Studies have shown that patients with diabetes are prone to cardiovascular disease such as myocardial infarction, diabetic retinopathy which could lead to blindness, and to renal related disease such as kidney failure.

The complications are categorized as microvascular complication and macrovascular complications. Microvascular complications include diabetic nephropathy, diabetic retinopathy. Meanwhile, macrovascular complications are more commonly related to cardiovascular disorders, cerebrovascular and peripheral diseases.

The pathophysiology of complications in Type 2 diabetes includes excess of sorbitol formation through polyol pathway, accumulation of advancend glycation end product (AGE), and activation protein kinases C (Takayanagi *et al.,* 2011). In a hyperglycemic condition, the increased glucose level involves the process called autoxidation and produces free radicals which lead to damage of pancreatic cells and development of long term complications (Weiss & Sumpio*,* 2006). However, the entire pathophysiology pathway leads to the induction of free radicals and oxidative stress.

2.3 Free radical and oxidative stress

Free radicals are atoms or group molecules with unpaired number of electron. It is mainly derived from oxygen and nitrogen species, which is also known as reactive oxygen species (ROS), and reactive nitrogen species (RNS). Due to the unstable condition of atoms, free radicals search for pairing electron and takes up electron from another stable molecule in turn which would become free radicals. Free radicals do cause oxidation by interfering with the normal physiological process of cells and leading to necrosis or cell death (Paul *et al.,* 2015).

Oxidative stress, defined as imbalance condition of production of free radicals and the body‟s inability counteract through neutralization by antioxidants. The excess generation of free radicals causes the antioxidants to be inactive, making the equilibrium of free radical and antioxidant to shift into favor of stress. The imbalance production of free radicals and scavenging of free radical system leads to oxidative stress. Oxidative free radicals comprises of superoxide, hydrogen peroxide and hydroxyl radical implicates in pathophysiology of ischemia and cellular injuries which is shown in Figure 2.4.

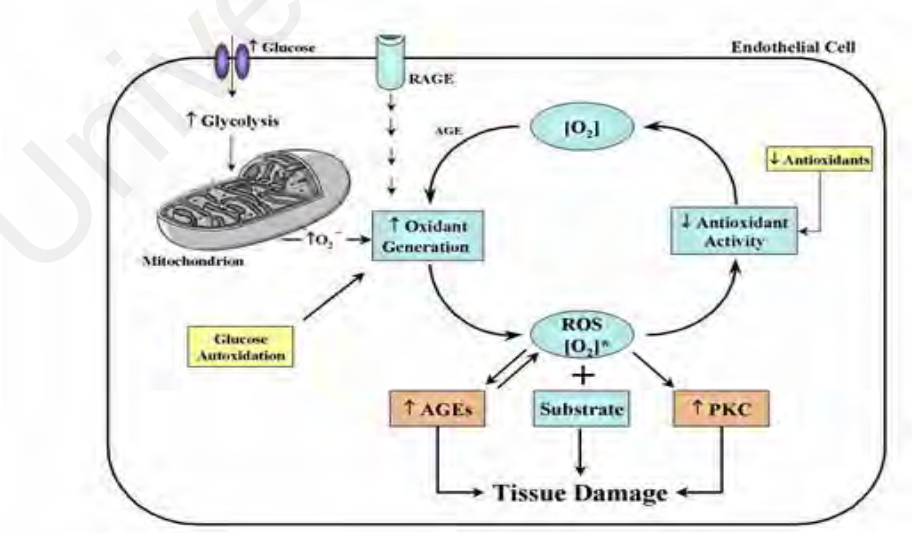

Figure 2.4 The effect of free radicals and oxidative stress (Paul *et al.,* 2015).

2.3.1 Oxidative stress and Type 2 diabetes

In a hyperglycemic condition, the elevated level of blood glucose contributes to the production of oxygen –free radicals (OFR) and further cause‟s cellular damage. Oxidative stress plays a vital role in the pathogenesis of diabetic complications. In Type 2 diabetes, hyperglycemic condition do induces excess generation of free radicals and oxidative stress through multiple pathways, which includes glucose oxidation, increased metabolic flux of polypol pathway, increased production of advanced glycation end product (AGE) and activation of protein kinases C.

i. Glucose autoxidation

It is known as single-hyperglycemic unifying mechanism, which is involved in pathogenesis of diabetic complication. The excess of glucose is stored in diabetic cells and glucose is being oxidized concomitant with overdrive of TCA cycle. These results are increased the electron donors, which are NADH and $FADH_2$ into the electron transport chain (ETC). Outcome of the process lead to increase in voltage gradient across the mitochondrial membrane until reached a critical state of threshold and blocked the electron transfer inside complex III. Therefore, the electrons are regress and backed up by coenzyme Q where it donates electron to molecular oxygen and thereby generates overproduction of superoxide. The excess of superoxide unable to be neutralized by the mitochondrial SOD and induces oxidative stress in Type 2 diabetes (Brownlee, 2005).

ii. Increased metabolic flux of polypol pathway

In the polypol pathway, toxic reductase are reduced into inactive alcohols by normal aldose reductase. However, when the glucose concentration is elevated, glucose is reduced to sorbitol and further oxidized into fructose. The aldose reductase consumes cofactor NADH for the reduction of glucose to sorbitol.

Increased polypol pathway under diabetic situation often leads to high levels of intracellular sorbitol which eventually causes oxidative stress. Meanwhile, the cofactor NADH do causes increase in polypol pathway by reviving the endogenous antioxidant, reduced glutathione (Obrosova, 2005).

iii. Increased production of advanced glycation end product (AGE)

The accumulation and high production of AGE precursor are closely related to type 2 diabetes complications. The AGE is known as a group of compounds that were formed from non-enzymatic covalent bonding aldehyde or reducing sugar of ketone groups. The formation gives an end product of free amine groups on protein, lipid, or nucleic acid. Meanwhile in hyperglycemic condition, high glucose causes elevated production of AGEs and consequently overproduction of free radicals which unable to scavenged and neutralized by endogenous antioxidant. The unbalanced condition leads to oxidative stress. Besides that, the AGE percursors have the capability to to modify the circulating proteins into albumin that leads to production of inflammatory cytokines and oxidative stress.

iv. Increased of Protein Kinase C activation

Protein kinase C is a serine kinase which plays an essential role in signal transduction and responds to neuronal, growth and hormonal stimuli in body. In a normal physiological condition, activation of PKC takes place through pathways that produces diacyl glycerol (DAG). However, an increase in metabolic flux of glycolysis and elevated production of DAG takes place in hyperglycemic condition. Upon the activation of PKC, the generation of ROS is increased through the source of NADPH oxidase.

2.3.2 Antioxidant & oxidative stress

Antioxidant has the capabilities to scavenge free radicals and inhibits oxidation of molecules. Oxidation undergoes the reaction of transferring electrons from oxidizing agents and contributes to the generation of free radicals. Hence, the chain reactions lead to cellular damage and causes complications. However, antioxidant has the ability to terminate the chain reaction to take place by removing the intermediates of free radicals and inhibit the oxidation process. It has the ability to counteract and scavenger the free radicals as well capable to prevent oxidative damage to take place in cellular level. Antioxidants often play a role as reducing agents. The antioxidant activities of the phenolic compounds were mainly on the redox properties, to act as reducing agents, donors of the free radical initiating element and chelating metal ions.

The antioxidants have classified in two major classes, which consist of enzymatic and non-enzymatic (Lee *et al.,* 2014). The enzymatic antioxidant are mainly produced endogenously and the non-enzymatic antioxidant, produced in exogenously. Based on the previous studies by Hue *et al*. (2012), antioxidants are divided into 2 categories, which are known as primary and secondary antioxidants.

i. Primary antioxidants

The antioxidants are mainly having the properties of stabilizing the free radicals and act as scavengers by donating hydrogen atom or electrons. It has two main mechanism including the hydrogen atom transfer (HAT) and single electron transfer (SET). The HAT method measures the ability of an antioxidant to suppress free radicals by acting as hydrogen donor. Examples of HAT based methods are oxygen radical absorbance capacity (ORAC) and total peroxyl radical trapping antioxidant (TRAP) assay.

Meanwhile, SET method is based primarily detects on the capability of a potential antioxidant to reduce any compound by transferring one electron (Prior *et al.,* 2005). The reduction of oxidant showed in Figure2.5 indicates the degree of color changes which correlated with the concentration of antioxidant presents in the sample. Examples of SET assays are DPPH, FRAP, Folin-ciocalteu ,TEAC, and CUPRAC assays.

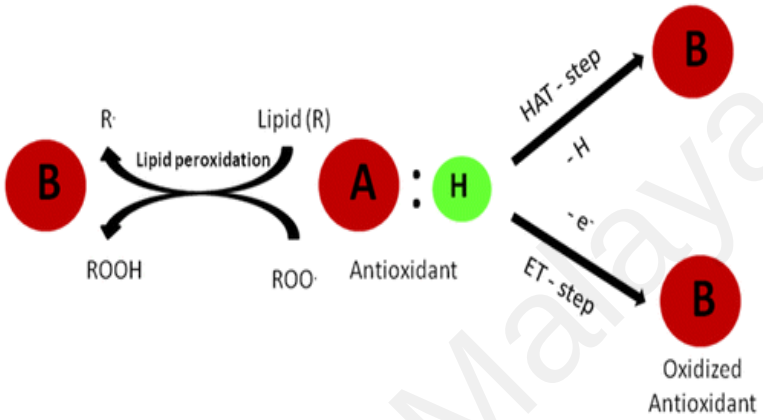

Figure 2.5 : The mechanism of HAT and SET in neutralizing free radicals (Vajragupta *et al.,* 2004).

ii. Secondary antioxidants

The antioxidants have the ability to quench and suppress the generation of free radicals and prevents oxidative damage from taking place. Secondary antioxidants referred as hydroperoxide decomposer that decomposes hydroperoxides into non-reactive products. It‟s often used with the combination of primary antioxidants to achieve neutralization effects.

2.3.3 Natural antioxidants

The natural antioxidants primarily presents in plants. Meanwhile, research studies on novel identification of natural antioxidant compound with effective antioxidant properties and non-toxic has extensively focused on past few years. There are several commercial available natural antioxidants, which include ascorbic acid (Vitamin C), tocopherol (vitamin E), and carotenoids. Meanwhile, natural substance such as alkaloids, flavonoids, enzymes, organic compounds and protein hydrolyzes has the properties of a potential antioxidant (Gupta & Sharma, 2006).

Phenolic compound commonly known as heterogenous group of secondary metabolite in plant and have potential in counteract oxidative damage. These compounds were produced onset of the response process against pathogens on plants. According to Mathew and Abraham (2006), plant phenolics are known as multifunctional which includes scavenger of free radicals, metal chelators, singlet oxygen quenchers and able to act as reducing agents. Phenolic compound comprises of five sub-groups which includes flavonoids, tannins, phenolic acids, diferuloylmethane, and stilbenes. The antioxidant activities of phenolic compounds are mainly due to the redox reactions.

Flavonoids is plant metabolite and polyphenolic molecules which consists of 15 carbon atoms. It is best known for anti-inflammatory and anticarcinogenic properties (Pinent *et al.,* 2008).Researchers have proved that tannins and flavonoids are the secondary metabolites found in plant are the best natural source of antioxidants by preventing destruction of β cells and diabetes induced ROS formation.

2.4 Management and treatment of Type 2 Diabetes

2.4.1 Oral antidiabetic drugs

The chronic hyperglycemic condition can lead to complex complication and can be prevented or delayed by achieving a well-maintained plasma glucose level. The current goal of treatment is to maintain the fasting blood glucose between the range of 4.5 mmol and 6.6 mmol along with $HbA1_c$ levels lower than 7. The most common oral hypoglycemic drugs are including sulfonylureas, metformin, alpha glucosidase inhibitors and thiazolidinediones (TZDs). However, in a severe case of hyperglycemia, patients are usually given insulin injections to improvise the insulin action.

a) Insulin secetagogeous :

Includes sulfonylureas and meglitinides. Both drugs stimulate insulin secretion by binding to sulfonylurea receptor (SUR) onto pancreatic β-cells. It induces insulin secretion by blocking ATP –dependent potassium channels.

b) Biguanides :

Includes metformin and phenformin. Both drug functions as it inhibit the hepatic glucose production by the activation of AMP-activated protein kinases (AMPK). It does improve glucose tolerance and lowers the postprandial plasma glucose levels.

c) α-glucosidase inhibitor :

It functions as inhibitor of enzyme that is responsible for the conversion of disaccharides to monosaccharaides. Blood glucose is reduced by delaying digestion and absorption of complex carbohydrate. The inhibitor does inhibit enzyme activities such as α-amylase and α-glucosidase which are responsible for the hydrolyzation of polysaccharide to glucose.

d) Glycogen phosphorylase inhibitor :

Glycogen phosphorylase is a functional enzyme that catalyses glycogen to glucose-1-phosphate and further metabolized to glucose. Glucose that are released from glycogen degradation contributes to elevated level of hepatic glucose. Therefore, inhibition of GP enzyme leads to reduced hepatic glucose production, and thus helps to decrease in blood glucose levels. Currently available GP inhibitors includes corosolic acid and ingliforib.

Table 2.1 : The synthetic oral antidiabetic drug and side effects

Oral antidiabetic agents	**Side effects**
Sulfonylureas	• Initiates the release of insulin even in the state of low glucose level which leads to hypoglycemia. • Weight gain • Develops skin rashes and hyponatreamia
Thuazolidinediones (TZDs)	• Causes anemia • Unsuitable to be used for patients with hepatic impairments. • Weight gain and deteriorate with insulin resistance
Biguanides	• Metformin – gastrointestinal discomfort which include nausea, bloating, abdominal pain and diarrhoea. • Weight loss & risk of lactic acidosis

In spite of having several oral antidabetic drugs available as management for this disease however; none are free from side effects to the individual. Therefore, new search and development of optimal therapeutic are encouraged in order to manage diabetes more effectively (Acharya & Sivastara, 2008). The current oral agents do gives side effects to the patients which including nausea, diarrhea, weight gain, nerve problems, hypoglycemia at a higher dosages and lactic acidosis (Bailey, 2000).

2.4.2 Antioxidant as treatment for diabetes

There are several studies proving the mechanisms that are involved in the β-cell damage that mostly contributed to the oxidative stress. In Type 2 diabetes, various type of free radicals which include ROS, hydroxyl, superoxide and nitric oxide radicals are mainly involved in the induction of oxidative stress which induces pancreatic β-cell destruction and activation of major pathways underlying the diabetic complications such as glycation and sorbitol pathways. Meanwhile, the activities of the antioxidants enzymes catalase, superoxide dismutase and gluthaionine peroxide reduce in diabetics along with impaired antioxidant defenses mechanism (Laight *et al.,* 2000). The antioxidant enzymes do decreased in diabetic patients and it is believed that antioxidant treatment would give a better and effective treatment. Instead of using insulin as a diabetic treatment for patients, antioxidants might also be considered as one of the alternative way for the treatment (Skyrme *et al.,* 2000). Studies have proved that plants were used as the traditional remedies as it is rich in polyphenolic content and good effective scavenger of free radicals.

Antioxidants that are found abundantly in plants and herbs do help in managing complication caused by diabetes. Researchers have proved that tannins and flavonoids are the secondary metabolites found in plant are the best natural source of antioxidants by preventing destruction of β cells and diabetes induced ROS formation. Therefore, it would a best strategy to manage the diabetes with the pants which able to show good enzyme inhibitory and has good antioxidant activity. In order to discover novel type of antioxidants, researchers are still keen finding for sources that has the effective replacement for treatment of diabetes.

2.5 Medicinal plants

Medicinal plants and herbs are traditionally used since the ancient time to treat diseases. The World Health Organization (WHO) has deduced that the effectiveness of modern medicine can never be progressed unless it is complemented with any alternative medicines such as traditional herbal medicine. The organization has also urged to utilize natural medicinal plant resources to achieve the premier goal for health care treatment. Meanwhile, the pharmacological activities of medicinal plants are accredited to the presence of secondary plant metabolites, which is found in few species of plants. The secondary metabolites are often serves as defensive compound, mechanical support, and as growth factor for the plant. There are a few of the secondary plant metabolites with medicinal properties including alkaloids, phenolic, flavonoids, terpenoids, and glycosides (Heinrich *et al.,* 2004)

i. Alkaloid

It is known as organic bases, which consist of nitrogen in a heterocyclic ring. The presence of compound in medicinal plants acts as pain reliever, analgesic, stimulant and antimalarial.

ii. Phenolic

It is the largest group of phytochemicals and consists of several dietary which including polyphenols and flavonoids. The flavonoid compound has the capability to control the gene expression of antioxidant enzymes and involves in pro-oxidant activity.

iii. Terpenoids

It is known as an isoprenoids which is the largest group of plant secondary metabolite. Terpenoid compound helps in wound scaling, defense and thermotolerance in plants (Bruneton,1999). The pharmacological properties of the compound are anti-inflammatory, anti-hypertensive, anti-bacterial and antioxidant activity.

2.5.1 Medicinal plants as source for anti hyperglycaemic medicine

In developing countries, people whom suffering from diabetes are more used to the access of insulin and hypoglycemic agents. This ultimately causes a decline in the utilization of medicinal plants and herbs. However, in the recent years, there has been a resurgence of interest in finding better pharmaceutical approach especially natural products as medicinal plants (Haq, 2004). The cause of the renewed interest in medicinal plants is believed to be due to several factors such as the side effects of oral hypoglycemic agents, high cost of synthetic antidiabetic drugs and high secondary failure rates in diabetes (Gurib, 2006).

Medicinal plants have been suggested as good source of anti hyperglycaemic and treatment for diabetes since the ancient time. The anti hyperglycaemic properties in medicinal plants are accredited to the presence of phytochemicals such as alkaloids, terpenoids, polyphenols and flavonoids. Based on previous researches, nearly 800 medicinal plants were found to possess anti hyperglycaemic properties. Most of the medicinal plants have given promising results in maintaining normal level of glucose level, improvising the secretion of insulin, increasing the sensitivity of hepatic cells, and increasing the glucose uptake in adipose cells.

However, the current focus for the anti hyperglycaemic research is to develop hypoglycaemic agents, which are safe at any dosage and free of negative side effects on the patients. Medicinal plant does have their active chemical compounds that are able to demonstrate activity in treatment of various diseases. Studies have proved that plants were used as the traditional remedies as it is rich in polyphenolic content and good effective scavenger of free radicals. The antioxidant properties in plant are capable to act synergistically with hyperglycemic condition by exerting anti hyperglycaemic actions. Antioxidants that found abundantly in plants and herbs do help in managing complication caused by diabetes.

Simultaneously, retarding and delaying the absorption of glucose also known as one of the therapeutic approach in diabetes. A good control of post prandial hyperglycemia can takes place through the inhibition of carbohydrate hydrolyzing enzyme, including glycogen phosphorylase, α-glucosidase, and α-amylase. The metabolic action of the enzymes plays key role in degrading complex carbohydrates and produces end- product of glucose. The inhibitory of glycogen phosphorylase enzyme are capable of blocking the catalyzation and release of glucose.

Rapid degradation of starch of pancreatic alpha amylase enzyme causes elevated level of postprandial hyperglycemia and diabetic complication. Hence, alpha amylase inhibitor is an effective strategy for the treatment of postprandial hyperglycemic conditions. The inhibitor of alpha amylase functions to inhibit the hydrolysis of alpha bonds to maltose as discussed by Lonkisch *et al.* (1998).

The alpha glucosidase enzyme takes over the degradation of maltose to glucose into the bloodstream. Therefore, inhibitor of alpha glucosidase allows the reduction of dissacharide hydrolysis into absorbable monosaccharide and decrease the absorption of glucose.

Although alpha glucosidase inhibitor with sugar based is commercially available as oral hypoglycemic drug, it do causes gastrointestinal side effects and undergoes tedious process of slowing down the degradation of carbohydrate According to Zen *et al.(*2014) compounds as terpenes, alkaloids, flavonoids, phenols,sterides, and compounds with functional motif from medicinal plants, have shown potency as alpha glucosidase inhibitors.

Discovery of natural inhibitors have created a great interest in research and development. Natural inhibitor consisting of abundant secondary metabolite compounds and promising biological activities, are capable of treating hyperglycemic conditions.

Hence, researchers have effectively focused on the search of anti hyperglycaemic compound along with natural antioxidants from plant sources which includes berries (Boath *et al.,* 2012), muscadine (You *et al.,* 2012) and cowpeas (Sreerama *et al.,* 2012).

Meanwhile, poor blood glucose level often causes increase of glycated hemoglobin in bloodstream associated along with complications. The glycated hemoglobin causes generation of free radicals in the blood cells and oxidative stress by cellular damage. The parameter of HbA1c often been measured as it reflects the average amount of glucose been attached in hemoglobin. High glycated hemoglobin majorly leads to multiple severe complications, which includes formation of atheroma and plaques through inflammatory reactions. Hence, natural inhibitor of hemoglobin glcosylation would be able to inhibit the bind of glucose and hemoglobin in hyperglycemic condition. It would be able to reduce the risk of complication in diabetes and maintain the blood glucose level (Megha *et al.,* 2013).

However, scientific findings on enzyme inhibitors based on medicinal plants are still limited and insufficient. Therefore, it would a best strategy to manage the diabetes with the pants which able to show good enzyme inhibitory and has good antioxidant activity. In order to discover more novel type of antioxidants, researchers are still keen finding for sources that has the effective replacement for treatment of diabetes.

2.5.2 Studied plant- *Aquilaria sinensis*

A. sinensis belongs to the genus of *Aquilaria* species and Thymelaeceae family. *A. sinensis,* also known by the name *A. agallocha* or *Gilg Lour* is widely researched and highly distribution especially in China.

This plant is well known for its production of fragnant non-wood product which is commonly known as agarwood or gaharu. It is the most precious plant resource, which produces agarwood and peculiar medicinal plant.

The native of the plant is Southeast Asia and grows particularly in the rainforest area and semi evergreen monsoon forest up to altitudes of 400 m. Meanwhile, there are several undergoing projects in some countries in southeast Asia promote the cultivation of *Aquilaria* trees artificially to produce agarwood in a sustainable manner to overcome the depletion of the plant.

Researchers has reported that the *Aquilaria* species or most commonly known as agarwood do have pharmacological activities which includes the ability to decrease hypersensitivity, antipyretic, anti-asthmatic and anti-inflammatory (Zhou *et al*., 2008). It has a strong antibacterial activity on *Salmonella typhii* and have proved on inhibition of *Mycobacterium tuberculosis* process. The volatile oil of the plant has the capability to function as good pain relief and as an anesthetic. Study by Wei *et al. (*2016) describes the agarwood tree do play an important role in traditional Chinese medicine as analgesic, clinical sedative, anti-emetic effects, and also as incense for religious ceremonies. According to Huda *et al.* (2009), *Aquilaria malaccenis* one of the *Aquilaria* species have revealed the presences of bioactive compounds such as alkaloid, flavonoids, terpenoids and saponins. The findings from Nurul *et al.*(2015) proved that the leaves of *A. malaccenis* capable in inhibiting the α-amylase and able to provide a rationale for treatment of diabetes.

However, phytochemical findings from *A. sinenis* leaves are very limited. In this current study mainly focused to separate and study the bioactive compound in the species for determination of the anti hyperglycaemic potential and antioxidant properties of the *A. sinensis* leaves.

Figure 2.6: Tree of *A. sinensis*

Figure 2.7: The leaves of *A. sinensis*

CHAPTER 3

METHODOLOGY

3.1 Plant materials

The leaves of *Aquilaria sinensis* were collected from Ladang RAL Plantation Sdn Bhd,Kuala Kangsar. The plant samples were authenticated at the herbarium unit of Institute of Biological Science, University Malaya.

3.2 Apparatus and equipment

Laboratory centrifuge and pH meter, ELISA microplate reader (TECAN Sunrise) from Laboratory of Biohealth Science, Institute of Biohealth Science, University Malaya. LC-MS Analyzer from IPPP,University Malaya

3.3 Chemicals and reagents

3.3.1 Solvents

SIGMA-Aldrich brands of methanol*, n*-hexane, chloroform, ethyl acetate, *n*-butyl alcohol, dichloromethane, sulphuric acid, hydrocholoric acid, acetate acid, acetone, ethanol, HPLC grade water**,** glacial acetic acid.

3.3.2 Thin layer Chromatography Media

TLC aluminium Silica Gel 60 F_{254} sheets purchased from Merck Chemical, Malaysia. Folin-Ciocalteu reagent, Vanillin, Bismuth nitrate and Potassium iodide.

3.3.3 LC-MS parameter and solvent

The column that been used for analysis is Phenomenex Aqua C18-50 mm × 2.0 mm × 5 uM with buffer of water and acetonitrile

3.3.4 Total Phenolic content (TPC) and Total flavonoid content(TFC) assays

SIGMA-Aldrich brands of Folin-Ciocalteu reagent, Sodium carbonate,Gallic acid,Sodium nitrite,Aluminium chloride, Sodium hydroxide, and Quercetin.

3.3.5 Antioxidant assays

DPPH assay: 2,2,-diphenyl-1-picrylhydrazyl DPPH (Sigma-Aldrich) and Ascorbic acid

Ferric reducing antioxidant power (FRAP) assay: sodium acetate, TPTZ (2,4,6-tri[2-pyridyl]-s-triazine),Ferric(III) chloride, Ferrous sulphate.

Metal chelating assay: Ferric (II) chloride, Ferrozine, EDTA-Na_2

NORSA assay: Potassium dihydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4),Sodium chloride, Griess reagent (Sigma-Aldrich), Sodium nitroferricyanide, Curcumin.

Superoxide radical scavenging assay: Sodium phosphate monobasic (NaH_2PO_4), Sodium phosphate dibasic (Na_2HPO_4), Nitro blue tetrazolium (NBT),Phenazine methosulfate (PMS), Nicotamide adenine dinucleotide (NADH), and Gallic acid.

3.3.6 Enzymatic and Non-enzymatic anti hyperglycaemic assays

Glycogen phosphorylase enzyme inhibition assay:

Glycogen phosphorylase α enzyme from rabbit muscle, Glycogen from rabbit liver,α-D glucose-1-phosphate, HEPES [4-(2-Hydroxyethyl) piperazine-1 –ethanesulfonic acid, N- (2-Hydroxyethyl) piperazine –N´-(2-ethansulfonic acid)], Magnesium chloride ($MgCl_2$), EGTA [Ethylene glycol-bis(2-aminoethylether)-N.N.N´,N´-tetraacetic acid), Ammonium molybdate, Malachite green, Potassium chloride, Caffeine.

Alpha-glucosidase enzyme inhibitory assay:

α-glucosidase enzyme of s.cerevisiae purchased from Sigma Aldrich, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), Sodium carbonate, and Acarbose.

Alpha amylase enzyme inhibitory assay:

α-amylase enzyme from porcine pancreas and soluble starch purchased from Sigma Aldrich, 3,5 Dinitrosalicylic acid (DNSA), Sodium hydroxide, Sodium potassium tartrate and Acarbose.

Non-enzymatic hemoglobin glycosylation assay:

Glucose, bovine hemoglobin (Fluka,Germany), Sodium azide, and Gallic acid.

3.4 Preparation for plant extraction

1 kg of *A. sinensis* leaves was collected and then cut into small pieces left for dried at 40°C in oven after shade dried for 1 week. The dried leaves are then finely grinded using blender into fine powder which weighs 280 gram. Grinded fine powder is then used for extraction purpose for biological assays.

3.5 Plant extraction and fractionation

50 gm of *A. sinensis* leaves powder was weighed and macerated in 10% methanol and left for overnight. The methanolic leaves extract was then filtered with Whatmann filter paper (No 1) and sequentially extracted via fractionation with solvents by increasing the polarity, *n*-hexane, dicholoromethane, *n*-butanol, chloroform and ethyl acetate. The final fraction of leave extract was collected and used as methanol aqueous fraction. Each fraction were then filtered and evaporated under vacuum rotatory evaporator at 40°C following procedure as described by Amzad *et al.* (2014).

The dried fractions were stored in refrigerator at 2-8°C. The dried fractions are then used for the biological assay determination of antioxidant and anti hyperglycaemic activities.

3.6. Separation of phytochemical bioactive compounds using Thin Layer Chromatography (TLC)

Aluminum plates (TLC Aluminum Silica Gel $60F_{254}$ sheets) size 20 x 20 cm was prepared. The plant extract will be loaded as a single line on the TLC plate and the chromatography was developed using chloroform and 10% methanol in chloroform as mobile solvent. The dried TLC plate is then view under UV-light and then spray with Phenol, Vanillin, Dragendorff's reagent to detect the presence of phenol, terpenoids and alkaloids compounds respectively.

Preparation of spraying reagents:

i. Phenol reagent

Phenol reagent was prepared using Folin-Coliteu reagent by the ration of 1:10 with distilled water. The solution was mixed well and kept in aluminium wrapped bottle. The preparation for the reagent was done in a dark room.

ii. Vanillin reagent

This spraying reagent is used for the detection of terpenoids compound.

About 1 ml of concentrated sulphuric acid (H_2SO_4) was added to 1 g of vanillin powder. The mixture was stirred and mixed with 100 ml of ethanol. The stock vanillin solution is kept in aluminium wrapped bottle. The dried TLC plates of samples were sprayed with the solution and were heated in hot plate at 110 °C for 2 -5 minutes. The appearance of purple or blue bands showed the presences of terpenoid compounds.

iii. Dragendroff reagent

Solution A – 0.85 g of bismuth nitrate was dissolved in 10 ml glacial acetic acid and 40 ml of distilled water.

Solution B- 8 g of potassium iodide was dissolved in 30 ml of distilled water.

The reagent was prepared by mixing 30 ml of solution A and 30 ml of solution and kept as stock reagent.

Spray reagent – Mixture of 50 ml stock solution with 100 ml glacial acetic acid and 500 ml of distilled water.

3.7 LCMS Analysis using *A.sinensis* plant extracts

The most active extract in antioxidant and anti hyperglycaemic activity were selected for the analysis of LCMS. The phytochemical constituents of the plant extract were determined with known standard references using ionization mode of positive and negative. The column that been used for analysis is Phenomenex Aqua C18-50 mm × 2.0 mm × 5 uM; Buffer: Water and Acetonitrile. The plant sample extract (1.0mg/ml) was prepared and were diluted with HPLC grade methanol. The samples were then further filtered using 0.2 uM nylon filter prior to avoid residue during analyses. The compounds found was based on using the standard samples.

3.8 Determination of total phenolic content (TPC)

Preparation of chemical reagents

i. Folin-Ciocalteu solution

Folin was prepared by diluting the solution with 10 fold of distilled water. The reagent need to be prepare freshly and in dark room.

ii.7.5% of sodium carbonate

7.5 g of sodium carbonate was measured and dissolved onto 100mL of distilled water. The solution need to be prepared freshly.

Preparation for standard:

About 10 mg/ml of Gallic acid was prepared as a stock standard and dissolved in methanol. Concentration of 0.05 – 1.6 mg/ml was prepared by using serial dilution.

Procedure for assay:

The total phenolic content of plant extract was measured by the Folin-Ciocalteu method. An aliquot of 20 µl of plant extract was mixed with prepared 100 µl of Folin- Ciocalteu reagent (Diluted 10-fold with distilled water , 1: 9) in a 96 well microplate. The mixture are incubated for 5 minutes and then further added with 75 µl of sodium carbonate (75 g/l).The mixture was left with incubation period of 2 hours in darkness at room temperature. The absorbance reading was measured after incubation period at 740 nm with microplate reader (Tecan Sunrise, Austria).The gallic acid (0.05 mg/ml – 1.6 mg/ml) was used as standard for the calibration of linear regression and distilled water was used as the blank. The total phenolic content was estimated as mg gallic acid equivalent to mg GAE/g dry weight extract.

3.9 Determination of total flavonoid content (TFC)

Preparation of chemical reagents:

i. Preparation of 5% of sodium nitrite solution :

5 g of sodium nitrite were measured and dissolved into 100 ml of distilled water.

ii. Preparation of 10% of aluminium chloride solution :

10 g of aluminium chloride was measured and dissolved into 100 ml of distilled water.

iii .Preparation of 1M sodium hydroxide solution :

2 g of sodium hydroxide pellets were measured and dissolved in 50 ml of distilled water.

Preparation for standard:

10 mg/ml of Quercetin was prepared as a stock standard. A concentration set of (0.05 mg/ml-1.6 mg/ml) were prepared using the stock by the dilution factor. The standard was dissolved in methanol for the assay.

Procedure for assay:

The total flavonoid content of the plant extract was measured with modified method and the assay was conducted using 96 microplate reader. Briefly, 50 µl of sample extract were added along with 70 µl of distilled water and 15 µl of 5 % sodium nitrite solution. The mixture was mixed and incubated for 5 minutes at room temperature. Then,15 µl of prepared 10% aluminium chloride solution was added into the mixture and kept for 6 minutes of incubation. It is the further added with 100 µl of 1 M sodium hydroxide solution into the mixture. The absorbance reading was immediately measured at 510 nm using microplate reader.

Calculation of total flavonoid contents :

The total flavonoid contents were determined by using a standard curve with Quercetin (0.05 – 1.6 mg/ml) as the standard. Total flavonoid content is expressed as mg QE/ g dry weight extract.

3.10 *In vitro* Antioxidant assay

3.10.1 DPPH radical scavenging activity

The reducing ability of antioxidant was measured by scavenging the DPPH radical. On accepting the hydrogen from a corresponding antioxidant as a donor would loses the characteristics of deep purple colour and the mechanism is well described in Figure 3.1.

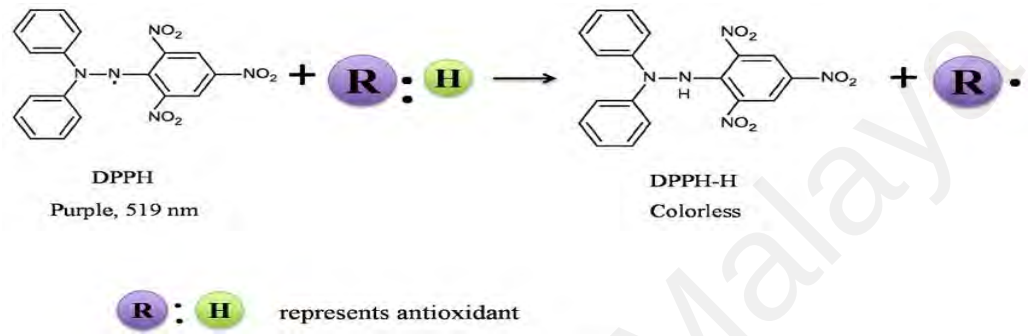

Figure 3.1: Mechanism action of DPPH scavenging activity (Ningjian & Kitts, 2014).

Preparation of chemical reagents:

i. 50 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The reagent of DPPH was prepared with 100 ml of 60 µM as stock solution. 0.002 g of DPPH was dissolved in 100 ml of methanol. The prepared stock reagent was wrapped with aluminium foil and kept in room temperature.

Preparation of standard:

0.01 g of ascorbic acid were measured and dissolved in methanol. Various concentrations of extract sample and standard (0.05- 1.6 mg/ ml) were prepared for test.

Procedure of assay:

40 μL of sample extracts of different concentrations (0.05–1.6 mg/mL) mixed with 200 μL of 50 μM DPPH solution in methanol. The mixture is then immediately shaken and

incubated for 15 minutes in the dark at room temperature. The decrease in absorbance is measured at 517 nm with a micro plate reader consisting of 96 wells.

Ascorbic acid (0.05-1.6 mg/ml) was used as a standard and the control was methanol. The percentages of the inhibition activity of the extracts are calculated as per the standard formula:

$$\text{DPPH radical scavaging activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100$$

3.10.2 Ferric Reducing Power (FRAP) assay

The ferric reducing power activity of the plant extract was determined using the method described by Muller *et al.* (2010) with some modification.

Principle of assay:

The reducing ability of antioxidant was measured from the conversion ferric ions into ferrous complex. It utilizes a ferric ion complex with tripyridyltriazine (TPTZ), where Fe (III) ions reduced to Fe (II) ions by antioxidants forming an intense blue color.

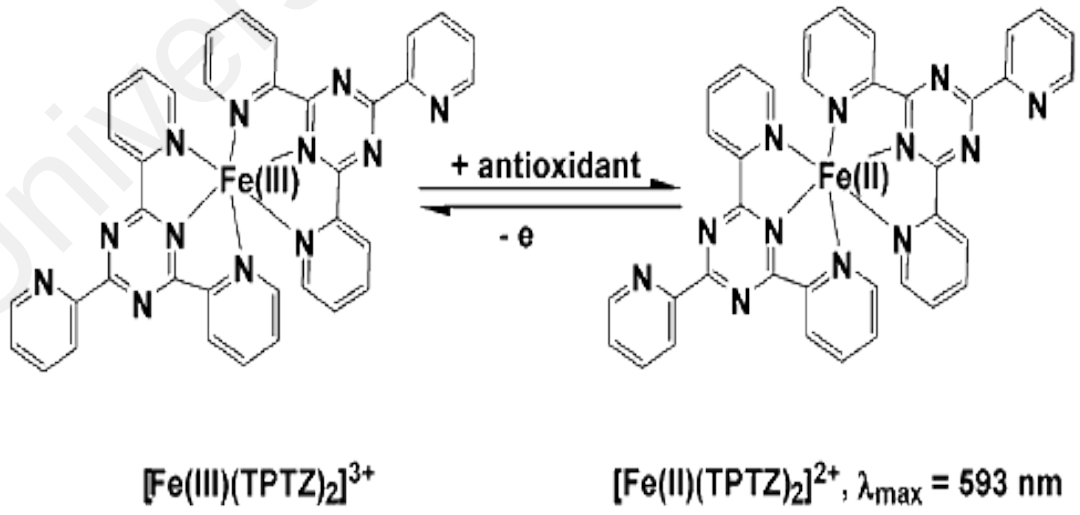

Figure 3.2: Mechanism action of ferric reducing antioxidant power (Iris *et al.,* 1996)

Preparation of chemical reagents:

i. 40 mM of concentrated hydrochloric acid (HCl) solution

About 1.46 ml of concentrated HCl was measured into reagent bottle. It was then further added distilled water to 1 litre of the solution. The solution was stored at room temperature.

ii. 0.3 M Acetate buffer (pH 3.6) solution

Briefly, 3.1 g of sodium acetate.tetrahedrate were measured and dissolved in 16ml of glacial acetic acid. The buffer solution was mixed thoroughly and distilled water was further added to make up the solution to 1 litre. The buffer solution was checked for its pH reading and adjusted to 3.6. The prepared buffer was stored at 4°C.

iii. 10 mM TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) solution

Briefly, 0.031 g of TPTZ was measured and dissolved in 10 ml of 40 mM HCl solution. The dissolved solution was stored at room temperature and wrapped with aluminium foil.

iii. 20 mM of Ferric chloride ($FeCl_3$) solution

Briefly, 0.054 g of $FeCl_3.6H_2O$ was measured and dissolved in 10 ml of distilled water. The solution was prepared freshly.

Preparation for standard:

0.01 g (10 mg/ml) of ferrous sulphate ($FeSO_4$) was measured and dissolved Methanol. Various concentration (0.05 mg/ml – 1.6 mg/ml) were prepared using serial dilution method.

Procedure for the assay:

The working solution of FRAP reagent were prepared freshly by mixing 0.3 M of acetate buffer (pH 3.6), 10 mM of 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) in 40 mM of HCl and 20 mM of Ferric chloride ($FeCl_3$) in the ratio of 10:1:1 per time of use. The prepared FRAP reagent were incubated at 37°C of water bath before use for assay. For the assay, 20 μL of extracts in methanol were mixed with 200 μL of FRAP reagent in 96- well microplate. After 8 minute of incubation time, the formation of the TPTZ-Fe^{2+} complex in the presence of antioxidant compounds in the extract is measured at 595 nm with a microplate reader (Tecan Sunrise, Austria). Ethanol was used as blank and ferrous sulfate ($FeSO_4$) solution (0.05-1.6 mg/ml) was used for standard calibration curve. The FRAP value was calculated according to the linear regression between standard solutions and absorbance at 595 nm. One unit of FRAP was defined as the reduction of 1 mole of Fe(III) to Fe (II). The results was estimated as mmol Fe2+/g of dry extract from duplicated tests.

$$\text{Ferrous chelating activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100\%$$

3.10.3 Metal chelating assay

The ferrous ion chelating activity (FCA) of the sample extracts is determined according to the procedure by measuring the formation of the Fe2+-ferrozine complex based on the method as describe by Decker *et al.* (1990).

Preparation of chemical reagents:

i. 2 mM Ferric (II) Chloride ($FeCl_2$) solution

0.0198 g of ferric (II) chloride ($FeCl_2$) was measured and dissolved in 50 ml of distilled water. The prepared reagent is wrapped with aluminium foil and stored in room temperature.

ii. 5 mM Ferrozine solution

0.0024 g of Ferrozine was measured and dissolved in 10 ml of distilled water. The prepared reagent is wrapped with aluminium foil and stored in room temperature.

Preparation of standard for assay:

10 mg/ml of EDTA-Na_2 was prepared and dissolved in distilled water. Sets of concentration (0.05 - 1.6 mg/ml) were prepared by serial dilution. The standard was freshly prepared.

Procedure of assay:

The sample extract of 100 μL is taken at different concentrations (0.05–1.6 mg/mL) and mixed with 120 μL distilled water and 10μL FeCl2 (2 mM) in a 96-wellmicroplate. Ferrozine (5 mM, 20 μL) need to be added to the mixture to initiate the reaction. The reaction mixture will be incubated at room temperature for 20 min and absorbance at 562 nm is measured along with EDTA-Na_2 (0.05–1.6 mg/ml) as a standard metal chelator. Methanol (100 μl) was used as a control; blank was without ferrozine (20 μl) of distilled water instead of ferrozine. All the tests were performed in duplicate, and the result are expressed as μg/ml and using formula given below to obtain chelating activity.

$$\text{Chelating activity } (\%) = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100$$

3.10.4 Nitric oxide radical scavenging activity (NORSA*)*

The nitric oxide radical scavenging assay (NORSA) has the basic principle where, it is briefly accessed the extract for inhibition ability on the production of nitrite ions. The generation of nitric oxide takes place from the reaction of sodium nitroprisside with oxygen. The nitric oxide radical converts to sulphanilamide acid which then further paired with naphthyl ethylenediamine (Panda *et al.,* 2011).

The pairing mechanism takes place by forming pink color intensity which can be measured at 546 nm. The reducing ability of antioxidant was measured by the reduced conversion of nitrate to nitrite using Griess reagent.

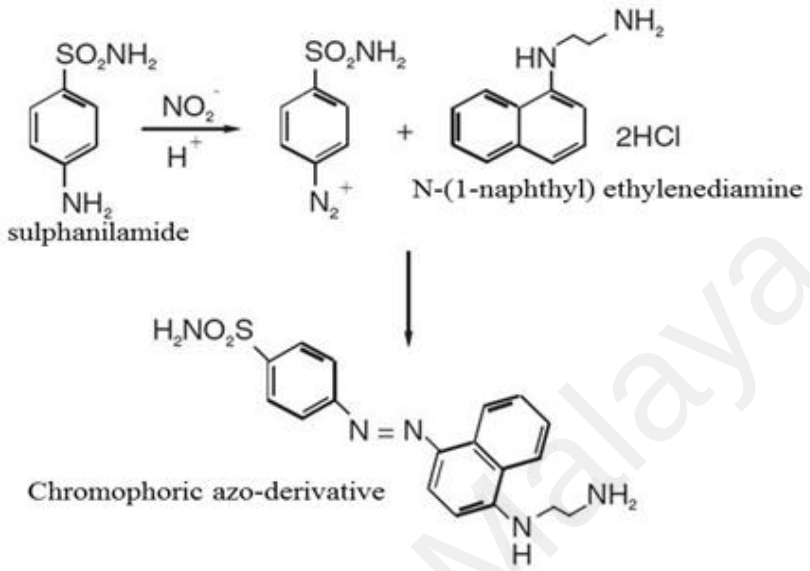

Figure 3.3 : Mechanism of nitric oxide radical scavenging activity (Kang *et al.,* 2006)

Preparation of chemical reagents:

i. 20 mM phosphate buffer saline (PBS)

Stock solutions of PBS (50 mM) were prepared and mixed with the following procedure:

Stock solution A – 1 M potassium dihydrogen phosphate (KH_2PO_4) dissolved in distilled water.

Stock solution B – 1 M dipotassium hydrogen phosphate (K_2HPO_4) dissolved in distilled water.

Stock solution C – 5 M sodium chloride (NaCl) dissolved in distilled water.

The solution were prepared by mixing 7.6 ml of stock A, 42.4 ml of stock B, 30ml of stock C and further diluted to one litre with distilled water. The desired pH (7.4) was adjusted and buffer was kept in flask prior to prepare 20 mM phosphate buffer saline solution. In order to prepare 100 ml of 20 mM phosphate buffer solution with pH 7.4, approximately 40mL of stock buffer solution was mixed with 60mL of distilled water. The pH reading are checked and adjusted using pH reading calibrator

ii. Griess reagent

Griess reagent (SIGMA-Aldrich) were used from ready stock solution and wrapped with aluminium foil as it is light sensitive.

iii. 10 mM sodium nitroferricyanide

0.0357 g of sodium nitroferricyanide was measured and dissolved in 12 ml of phosphate buffer-saline (PBS).

Preparation for standard:

10 mg/ml of curcumin was prepared and dissolved in methanol. Sets of concentration (0.05 - 1.6 mg/ml) were prepared by serial dilution. The standard is freshly prepared and stored in room temperature.

Procedure for assay:

This activity measured the formation of the nitrite ions in the reaction mixture that can be detected by using Griess reagent. 50 µl of each fraction sample (0.05-1.6 mg/ml) was added together with 50 µl sodium nitroferricyanide (10mM) in phosphate buffer saline (20 mM, pH 7.4). The mixture was then incubated for 2 hours and 30 minute at room temperature. After incubation, 125 µl of Griess reagent was added and left to be incubated again about 10 minutes.

Absorbance reading was measured at 546 nm and all test were performed in duplicate. Curcumin (0.05 – 1.6mg) was used as standard and the percentage of inhibition of nitric oxide was then calculated using formula:

$$\text{NORSA inhibition (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100$$

3.10.5 Superoxide radical scavenging activity

Principle of assay: The scavenging activity was measured by reduction of nitro blue tetrazolium (NBT) in conversion to formazan.

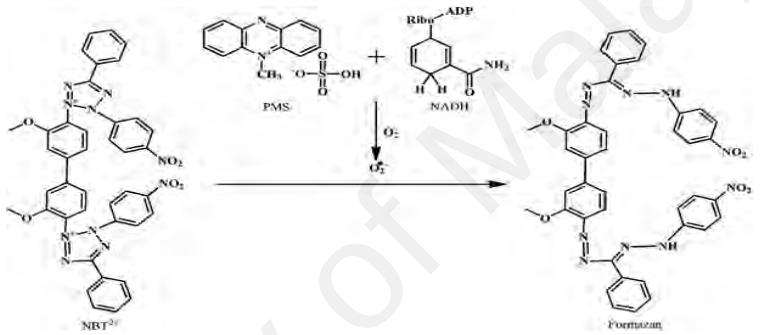

Figure 3.4 : Mechanism of superoxide radical utilize in conversion of NBT to formazan (Satish & Dilipkumar, 2005).

Preparation of chemical reagents:

i. 50 mM phosphate buffer (pH 7.4) solution

A stock of (0.1 M) phosphate buffer was prepared prior to be further diluted and prepare 0.05 M of PB for the assay.

Stock A – Preparation of 1.0 M sodium phosphate monobasic (NaH_2PO_4) :

To prepare 100 ml of 1.0 PB stock solution, approximately 11.9 g of sodium phosphate monobasic (MW =119.98 g/mol) was measured and dissolved in distilled water. The 100 ml of stock A was kept in flask in prior to prepare 0.05M PB solution.

Stock B – Preparation of 1.0 M sodium phosphate dibasic (Na_2HPO_4) :

To prepare 100 ml of 1.0 M PB stock solution, approximately 14.1 g of sodium phosphate dibasic (MW=141.96 g/mol) was measured and dissolved in distilled water. The 100 ml of stock B was kept in flask prior to prepare 0.05 M PB solution.

In order to prepare 1000 ml of PB solution (pH 6.8), 26.5 ml of stock A was mixed with 23.5 ml of stock B. The mixture was further diluted to 1000 ml with distilled water. Reading of pH meter was checked and adjusted prior to requirement of the assay. Therefore, to prepare 500 ml of 50 mM PB solution, 25 ml from the stock solution was measured and mixed 475 ml of distilled water. The prepared 50 mM PB was kept in flask and stored in 4°C.

ii. Preparation of 50 µM nitro blue tetrazolium (NBT)

1 mg of NBT was measured and dissolved in 24.5 ml phosphate buffer (PB) solution. The prepared solution are kept in flask and wrapped with aluminium foil as it is light sensitive.

iii. Preparation of 15 µM phenazine methosulfate (PMS)

0.8 mg of PMS was measured and dissolved in 176 ml of PB solution. The prepared solution are kept in flask and wrapped with aluminium foil as it is light sensitive.

iv. Preparation of 73 µM nicotinamide adenine dinucleotide (NADH)

11.1 g of NADH was measured and dissolved in 20 ml of PB solution. The prepared solution are kept in flask and wrapped with aluminium foil as it is light sensitive.

Preparation of extract and standard concentration:

The plant extracts were dissolved in phosphate buffer and serially diluted to make set of concentration (0.05 – 1.6 mg/ml). Meanwhile, 0.01 g (10 mg/ml) of Gallic acid was

measured and dissolved in phosphate buffer solution. Sets of concentration (0.05 - 1.6 mg/ml) were prepared by serial dilution. The standard is freshly prepared.

Procedure of assay :

The reduction of NBT was measured in the superoxide radical scavenging assay. The non-enzymatic phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide (NADH) do generate superoxide radicals also do reduce nitro blue tetrazolium (NBT) to formazan which produces purple color changes.

Briefly, 100 µl of NBT (50 µM in 50 mM phosphate buffer), 100 µl of NADH (73 µM in 50 mM phosphate buffer), 20 µl of extract (dissolves in phosphate buffer) and 20 µl of PMS (15 µM in 50 mM phosphate buffer) were all added together. Phosphate buffer was used as control; and gallic acid was used as standard. The mixture was then kept for incubation at room temperature for 15 minutes and absorbance reading was taken at 530 nm. The entire test was performed in duplicate. The inhibition of superoxide was calculated using the formula:

$$\text{Superoxide radical scavenging activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100\%$$

3.11. *In vitro* anti hyperglycaemic assay

3.11.1 Glycogen phosphorylase enzyme inhibitory assay

The inhibition of glycogen phosphorylase was measured by inhibiting the release of phosphate from glucose-1-phosphate for the synthesis of glycogen (Loughlin *et al.,* 2008).

Preparation of chemical reagents:

i. Preparation of 0.1 M sodium phosphate buffer (PB) solution :

Stock solutions of PB (0.2 M) of 100 ml was prepared and mixed with the following procedure.

Stock A – 0.2 M of sodium phosphate monobasic (NaH_2PO_4)

To prepare 12 ml of 0.2 M stock solution, 0.331 g of sodium phosphate monobasic was dissolved in 12 ml distilled water. The stock solution was kept in flask prior to prepare 0.1 M sodium phosphate buffer.

Stock B - 0.2 M of sodium phosphate dibasic (Na_2HPO_4)

To prepare 42 ml of 0.2 M stock solution, 1.495 g of sodium phosphate dibasic are dissolved in 42 ml distilled water. The stock solution was kept in flask prior to prepare 0.1 M sodium phosphate buffer.In order to prepare 100 ml of sodium phosphate buffer (pH7.4), approximately 9.5 ml of stock A and 40.5 ml of stock B was mixed. It was further diluted to 100 ml with distilled water. The buffer solution was mixed well and stored in 4°C in refrigerator.

ii. Preparation of HEPES buffer solution (pH 7.2):

0.1 M of HEPES was prepared as stock solution according to the method as described by Dawson *et al*. (1986). In order to prepare 100 ml stock solution, approximately 2.3831 g of HEPES (MW=238.31 g/mole) was measured and dissolved in 100 ml of HPLC grade water. The pH reading was measured and adjusted to pH 7.2. The prepared buffer solution was kept in flask and stored prior to prepare 50 mM of HEPES buffer for the assay.

iii. Preparation of 1 M of hydrochloric acid (HCl) :

To prepare 1 litre of stock solution, approximately 82.78 ml of HCl

(MW= 36.46 g/mole) was measured and mixed with 917.22 ml of distilled water.

iv. Preparation of 1M of sodium hydroxide (NaOH) solution :

To prepare 100 ml of solution, 0.4 g of NaOH (MW= 40 g/mole) was measured and dissolved well in 100 ml HPLC water grade. The solution was kept for magnetic stirring process until it is full dissolved and stored in 4°C.

v. Preparation of Glycogen phosphorylase enzyme stock solution

Stock solution:

A stock of 45 units (5 mg) glycogen phosphorylase enzyme was measured and dissolved in 5 ml of 50 mM HEPES buffer.The assay requires 4.5 unit/ml of enzyme. Therefore, the stock solution was diluted into 2-fold. To prepare 10 ml enzyme, approximately 5 ml of stock enzyme was diluted with 5 ml of 50 mM HEPES buffer. The reading of pH was adjusted to pH 7.2. The enzyme solution was kept in flask and stored in 4°C.

vi. Preparation of 50 mM HEPES buffer containing (100 mM KCl, 2.5 mM EGTA,2.5 mM $MgCl_2$, 0.25 mM glucose-1-phosphate, and 1 mg/ml glycogen):

To prepare 10 ml of the solution, following chemicals are measured; 0.75 g of KCl, 0.095 g of EGTA, 0.0510 g of $MgCl_2$, 0.0084 g of glucose-1-phosphate, and 0.1 g of glycogen. Those measured chemicals were dissolved in 100 ml of 50 mM HEPES buffer with pH of 7.2. The solution was kept in flask and stored in 4°C.

vii. Preparation 1M HCl containing 10mg/mL of ammonium molybdate 0.38mg/mL of malachite green :

To prepare 100 ml of the solution, approximately 1 gram of ammonium molybdate and 0.038 g of malachite green were measured and dissolved in 100 ml of 1 M HCl. The solution were mixed well an kept in a flask wrapped with aluminnium foil as light sensitive.The solution was stable as it"s kept in 4°C.

Preparation for extract and standard:

Plant extract was dissolved with 5% of DMSO in distilled water. Serial dilutions of concentration (0.05– 1.6 mg/ml) were prepared. 0.01 g (10 mg/ml) of caffeine was measured and dissolved distilled water. Serial dilutions of concentration (0.05 – 1.6 mg/ml) were prepared.

Procedure of assay :

The glycogen phosphorlyase α activity of *A. sinensis* was evaluated against GPα in the direction of glycogen synthesis with the release of phosphate from glucose-1-phosphate. The sample and standard (20 µl) was mixed with 40µl of 50mM HEPES (pH 7.2) which containing 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$, 0.25 mM glucose-1-phosphate and 1 mg/ml of glycogen in a microplate reader. Then, the reaction of mixture was initiated by adding 50 µl of enzyme (GPα) in 50mM HEPES (pH7.2) buffer and left for incubation at 22°C for 30 minutes. The mixture was then subsequently incubated with 150 µl of 1M HCl solution which contained 10mg/ml ammonium molybdate and 0.38 mg/ml of malachite green for 5 minutes. The absorbance reading was measured at wavelength of 620 nm for the phosphate. Caffeine (0.05– 1.6 mg/ml) were used as standard and HEPES Buffer as control (Loughlin *et al.,* 2009).The inhibition percentage of glycogen phosphorlyase activity was then calculated and the test was performed in duplicate. The inhibitory activity was calculated using the formula:

$$\text{Inhibitory activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100\%$$

3.11.2 Alpha glucosidase inhibitory assay

The inhibitory of alpha glucosidase was measured by determining the reduction of disaccharide hydrolysis into absorbable monosaccharide as shown in the Figure 3.5. The assay was carried out using the modified procedure of McCue & Shetty (2004). The effect of the plant fractions on alpha glucosidase activity was determined using α-glucosidase from *Saccharomyces cerevisiae.*

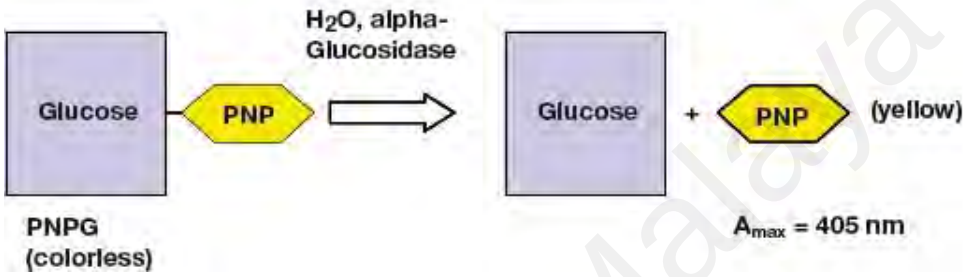

Figure 3.5: Mechanism action of alpha glucosidase enzyme and PNPG (Tania *et al.,* 2017).

Preparation of chemical reagents:

i. Preparation of α- glucosidase enzyme (0.1 U/ml)

The α-glucosidase enzyme used was obtained *Saccharomyces cerevisiae* (Sigma-Aldrich). The working solution containing 0.1 U/ml of enzyme was prepared and diluted using 0.01 M phosphate buffer (pH 7.4). The prepared enzyme are kept in flask and stored in 4°C.

ii. Preparation of 5 mM nitrophenyl-α-D-glucopyranoside (PNPG)

The *p*-nitrophenyl-α-D-glucopyranoside (PNPG) was used as substrate for the assay. To prepare 10 ml of 5 mM PNPG (MW = 315.24 g/mole), approximately 0.0157 g of PNPG were measured. The measured chemical was dissolved in 0.01 M phosphate buffer (pH 7.4). The prepared solution was kept in flask and stored in 4°C.

iii. Preparation of 0.2 M sodium carbonate (Na_2CO_3)

To prepare 10ml of 0.2 M sodium carbonate (MW= 105.99g/mole), 0.212g of the chemical were measured and dissolved in 0.1 phosphate buffer (pH 6.8).

Preparation of plant extracts and standard:

Plant extracts were dissolved in 5% of DMSO in distilled water and 0.01 M of phosphate buffer. Sets of concentration (0.05 -1.6 mg/ml) were prepared for the assay. The plant extract was observed for the inhibition of the enzyme with comparison of acarbose as standard. 0.01 g (10 mg/ml) of acarbose was measured and dissolved 0.01 M phosphate buffer (pH 6.8). Serial dilutions of concentration (0.05 – 1.6 mg/ml) were prepared.

Procedure for assay :

The inhibitory α-glucosidase activity was determined using spectrometrically in microplate reader based on *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as substrate. Briefly, 50 µl of sample of enzyme solution (0.1 U/ml) α-glucosidase in 0.01 M Phosphate buffer saline (pH 7.4) and 50 µl of the test extracts in 0.5 % DMSO of 0.01 M phosphate buffer were mixed. The mixture was then kept for pre-incubation at 37°C for 15 minutes for initiating the reaction. After pre-incubation, 50 µl of PNPG solution were added (5 mM PNPG in 0.01 M phosphate buffer) and incubated for 10 minutes. After incubation, 80 µl of 0.2 M Na_2CO_3 in 0.1 M phosphate buffer was added to the wells to stop the reaction. The amount of PNPG was quantified at 405 nm. The phosphate buffer was used as control for the assay. The entire test was performed in duplicate and in dark room. The inhibitory of alpha amylase was calculated using the formula:

$$\text{Inhibitory activity (\%)} = A_{control} - A_{sample/standard} / A_{control} \times 100\%$$

3.11.3. Alpha amylase inhibitory assay

The inhibitory activity was measured on the reduction of alpha bond polysaccharide hydrolysis to glucose. The assay were carried out with slight modifications of method (Kazeem *et al.,* 2013).

Preparation of chemical reagents:

i. Preparation of 50 µg/ml amylase enzyme

The alpha amylase enzyme from porcine pancreas (Sigma Aldrich) was prepared freshly. 2.5 g of alpha amylase was measured and dissolved in 50 ml of 100 mM phosphate buffer saline (pH 6.9). The enzyme solution was stored in -20°C to maintain the stability of enzyme for the assay.

ii. Preparation of 1% starch in 100 mM PBS

1 g of soluble starch from potato (Sigma Aldrich) was measured and dissolved in 100 ml of 100 mM of PBS. The starch solution was prepared freshly prior to assay.

iii. Preparation of Dinitrosalicylic Acid (DNSA) solution

The DNSA solution was prepared in dark room at room temperature. 3 g of lyophilized of 3,5 dinitrosalicylic acid (DNS) was measured and added into 300 ml of distilled water. The solution was kept on a hot plate with aluminium foil covered flask to allow it to be fully stirred. After that, 4.8 g of sodium hydroxide (NaOH) was added into the solution and was let it to be dissolved. And finally 36 g of sodium potassium tartrate was measured and added into the solution. The solution was kept for continuous stirring and left at room temperature.

Preparation of plant fractions and standard :

The plant fractions sample was dissolved in phosphate buffer saline (7.4) and a set of concentration (0.05 - 1.6 mg/ml) was prepared. Acarbose was used as positive control and standard for the alpha amylase inhibitory assay, which was in the form of lyophilized. Therefore, for a stock solution, 10 mg/ml of acarbose was prepared. The stock solution was further been diluted in PBS (pH 7.4) and sets of concentration (0.05 - 1.6 mg/ml) was prepared.

Procedure of assay :

The inhibitory alpha-amylase activity was determined with slight modifications. 250 µl of each fraction and acarbose at different concentration (0.05 -1.6mg/ml) was added with 500 µl of porcine pancreatic amylase (2 U/ml) in phosphate buffer (100 mM, pH6.8) at 37°C for about 20 minutes. Then, 250 µl of 1% starch was dissolved in 100 mM phosphate buffer (pH 6.8) was further added to the mixture and incubated at 37°C for 1 hour. After the incubation, dinitrosalicylate color reagent (DNS) reagent was added to each tube with the volume of 1 ml and kept for boiling at 80°C for 15 minutes. The samples were then kept for cooling and absorbance of the resulting mixture was taken at 540 nm. The inhibitory activity of alpha-amylase was calculated and expressed as percentage of control without the inhibitors. The entire test was performed in duplicate.

The inhibitory of alpha amylase was calculated using the formula:

$$\text{Alpha - amylase inhibitory activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100\%$$

3.11.4 Non-enzymatic hemoglobin glycosylation assay

The reduction of haemoglobin glycosylation was measured by decrease in the formation of glucose-hemoglobin complex, increase of free haemoglobin. The decrease of absorbance over 72 hours indicates inhibition of haemoglobin glycosylation (Megha *et al.,* 2013).

Preparation of chemical reagents:

i. Preparation of 2% glucose

2 g of Glucose was measured and dissolved in 100mL of prepared 0.01 M phosphate buffer saline (pH 7.4). The solution was prepared freshly and was kept in flask.

ii. Preparation of 0.06 % of bovine haemoglobin

0.06 g of bovine hemoglobin was measured and dissolved in 100 ml of prepared 0.01 M phosphate buffer saline (pH 7.4). The solution was prepared freshly and was kept in flask.

iii. Preparation of 0.02% of sodium azide

0.02 g of sodium azide was measured and and dissolved in 100 ml of prepared 0.01 M phosphate buffer saline (pH 7.4). The solution was prepared freshly and was kept in flask.

Preparation plant fractions:

Plant fractions were dissolved in 5% of DMSO in distilled water and 0.01 M of phosphate buffer (pH 7.4). Sets of concentration (0.05 -1.6 mg/ml) were prepared for the assay.

Preparation of standard :

The plant fractions was evaluated for the inhibition of the enzyme with comparison of gallic acid as standard. 0.01 g (10 mg/ml) of gallic acid was measured and dissolved in 0.01 M phosphate buffer (pH 7.4). A serial dilution of concentration (0.05 – 1.6 mg/ml) was prepared.

Procedure for assay :

The anti hyperglycaemic activity of *Aquilaria sinensis* were investigated by estimation degree of non-enzymatic hemoglobin glycosylation. Briefly, Glucose (2%), hemoglobin from bovine (0.06%) and sodium azide (0.02%) were prepared in 0.01 M phosphate buffer, pH 7.4. About 1 ml of each of the prepared solution were then mixed with 1 ml of each concentration of plant sample into the test tubes. The gallic acid (0.05-1.6 mg/ml) were used as standard and phosphate buffer as control. The mixture was incubated in dark at room temperature for 72 hours. The degree of glycosylation of haemoglobin was measured at 520 nm for 24 hours, 48 hours and 72 hours incubation. The absorbance of haemoglobin glycosylation was measured at 520 nm and the inhibition was calculated as described by Suganya *et al.* (2014). All the tests were performed in duplicate. The inhibitory activity was calculated using the formula:

$$\text{Inhibitory activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100\%$$

3.12. Statistical Analysis

All data were presented as the mean ± SEM. The means of the data were subjected to statistical analysis using GraphPad Prism 7.0. The IC_{50} values were calculated by the formula Y = 100*A1/(X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t tests. $p < 0.05$ was considered significant.

CHAPTER 4

RESULTS

4.1 Yield extraction of *Aquilaria sinensis* leaves extracts

Dried and powdered leaves of *A. sinensis* were used in this study to carry out extraction and further analysis. The methanolic leaf extract of *A. sinensis* was extracted via solvent fractionation with *n*-hexane, dichloromethane, *n*-butanol, chloroform, ethyl acetate and 10 % ratio of methanol aqueous .The yield was varied with the respective solvent.

Yield of extractable solutes shown in the Table 4.1. The results indicated the highest yield obtained from 10 % methanol aqueous fraction (11.51 g) which gave dark brown powder. It is then followed by *n*-butanol fraction (1.52 g) which gave dark orange powder, ethyl acetate fraction (1.10 g) which gives brown powder, chloroform fraction (1.02 g) which gave dark green powder, and dichloromethane (0.48 g) which gave greyish powder. However, *n*-hexane fraction gives the lowest yield of only (0.14g) which gives thin layer of greyish powder.

Table 4.1: Percentage yield of each fraction from 100 g of *Aquilaria sinensis leaves*

Fractions	**Percentage of Yield (g/100g dry weight)**
n-hexane	0.14 %
Chloroform	1.02 %
Ethyl Acetate	1.1 %
n-butanol	1.52 %
Dichloromethane	0.48 %
10% Methanol Aqueous	11.51 %

4.2 Detection of phytochemical bioactive compounds using Thin Layer Chromatography (TLC)

The presence of phytochemical bioactive compound in each plant fractions; *n*-hexane, dicholormethane, *n*- butanol, chloroform, ethyl acetate and methanol aqueous fractions were detected using the spraying agents.

Table 4.2 : Presence of number of spots as phytochemical compound in fractions.

Fractions	Alkaloid	Terpernoids	Phenolic
***n*-hexane**	2 spots	4 spots	1 spot
chloroform	4 spots	8 spots	12 spots
ethyl acetate	2 spots	7 spots	3 spots
***n*-butanol**	3 spots	6 spots	4 spots
dichloromethane	3 spots	2 spots	4 spots
10 % methanol aqueous	2 spots	1 spot	1 spot

The numbers resembles positive spots after using the spraying agents.

From the TLC analysis, the plant fractions have shown indication of numbers of bands formed as presence of secondary metabolite compounds. The *n*-hexane and methanol aqeuous fractions t have shown presence of alkaloid compound. The chloroform and dichloromethane fraction has exhibited more phenolic compound indication compared to the rest of fractions. Meanwhile, the ethyl acetate and *n*-butanol fraction have shown more presence of terpenoids compounds.

Determination of Retention factor (R_f) value

The R_f value of each fractions was obtained by using 10 % methanol in chloroform / chloroform (1 :2) as a mobile phase. The migration distance of each fraction and the separation distance were measured.

Table 4.3 : Travel distance of the solvent in each fraction

Fractions	Distance travel of the solvent- (10 % methanol in chloroform)	Distance travel of the solvent (Chloroform)
n-hexane	6.5 cm	6.5 cm
Chloroform	6.4 cm	6.5 cm
Ethyl acetate	6.6 cm	6.5 cm
n-butanol	6.5 cm	6.5 cm
dichloromethane	6.5 cm	6.4 cm
Methanol aqueous	6.5 cm	6.6 cm

$$R_f \text{ value} = \frac{\text{Travel distance of compound}}{\text{Travel distance of solvent}}$$

It was observed that the less polar compound is observed weakly by the silica gel and thus moved faster. Whereas, the molar polar compound is absorbed well by the silica gel and moves slowly. The compounds with difference of polarity are separated and viewed under the UV.The type of compound was further detected using the spraying reagents dragendroff, vanillin and phenol. The concentration of the compound affects the color intensity.

Table 4.4 : Thin Layer Chromatography of *A. sinensis* leaves *n*- hexane extract using mobile phase of 10% methanol in chloroform.

Label compound	R_f Value	Color under Visible light	Color under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
H1	0.38	-ve	-ve	-ve	Orange (+)	-ve	Alkaloid
H2	0.78	-ve	-ve	-ve	-ve	Blue (+)	Terpenoids
H3	0.93	-ve	Light blue (+)	-ve	-ve	Dark blue (++)	Terpenoids
H4	0.97	-ve	-ve	-ve	Orange (++)	-ve	Alkaloid
H5	0.98	-ve	Yellow (++)	Pale Yellow (++)	-ve	-ve	Phenolic

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band observed (-ve)

Table 4.5: Thin Layer Chromatography of *Aquilaria sinensis* leaves *n*-hexane extract using solvent chloroform

Label compound	R_f Value	Color under Visible light	Color under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
H5	0.89	-ve	-ve	-ve	-ve	Light blue (+)	Terpenoids
H6	0.93	-ve	-ve	-ve	-ve	Blue (+)	Terpenoids

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band observed (-ve)

Table 4.6: Thin Layer Chromatography of *Aquilaria sinensis* leaves chloroform extract using solvent 10 % methanol in chloroform

Label compound	R_f Value	Color under Visible light	Color under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
C1	0.10	-ve	Light blue (+)	-ve	-ve	Pale Green (+)	Phenolic
C2	0.30	-ve	Light blue (+)	Dark Blue (++)	-ve	-ve	Phenolic
C3	0.43	-ve	Bright Blue (+++)	-ve	-ve	Dark blue (++)	Phenolic
C4	0.54	-ve	Bright blue (+++)	-ve	-ve	Blue (++)	Terpenoids
C5	0.54	-ve	-ve	-ve	-ve	Light green (+)	Terpenoids
C6	0.54	-ve	Light Blue (+)	-ve	-ve	Dark blue (++)	Phenolic
C7	0.59	-ve	Yellow (++)	-ve	Orange (++)	-ve	Alkaloid

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band observed (-ve)

Table 4.7 : Thin Layer Chromatography of *Aquilaria sinensis* leaves chloroform extract using solvent 10% methanol in chloroform

Label compound	R_f Value	Color under Visible light	Color under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
C8	0.7	-ve	Red (++)	-ve	Orange (+)	-ve	Alkaloid
C9	0.75	-ve	Red (++)	-ve	Light orange (+)	-ve	Alkaloid
C10	0.78	Visible	Bright green (+)	-ve	-ve	Dark blue (++)	Phenolic
C11	0.79	-ve	Dark blue (++)	-ve	-ve	Purple (++)	Terpenoids
C12	0.85	-ve	Bright Red (+++)	-ve	Orange (+)	-ve	Alkaloids
C13	0.85	-ve	Bright Blue (+++)	-ve	-ve	Green (+++)	Terpenoids
C14	0.89	Visible	Light blue (+)	-ve	-ve	Green (+)	Phenolic
C15	0.93	-ve	Dark Blue (++)	-ve	-ve	Dark Blue (+++)	Terpenoids
C16	0.98	-ve	Dark blue (+)	-ve	-ve	Blue (++)	Phenolic

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band observed (-ve)

Table 4.8 : Thin Layer Chromatography of *Aquilaria sinensis* leaves chloroform extract using solvent chloroform

Label compound	R_f Value	Color under Visible light	Color under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
C17	0.03	-ve	Bright green (++)	-ve	-ve	Purple (++)	Terpenoids
C18	0.17	-ve	Bright blue (++)	-ve	-ve	Green (+++)	Terpenoids
C19	0.46	-ve	Dark blue (+)	-ve	-ve	Dark blue (+++)	Terpenoids

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band observed (-ve)

Table 4.9 : Thin Layer Chromatography of *Aquilaria sinensis* leaves ethyl acetate extract using solvent 10% methanol in chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
E1	0.03	-ve	Dark red	-ve	-ve	Red (+++)	Terpenoids
E2	0.04	-ve	Dark red	Blue (++)	-ve	-ve	Phenolic
E3	0.05	-ve	Light blue	-ve	-ve	Light blue (+)	Terpenoids
E4	0.09	-ve	Dark blue	-ve	-ve	Red (++)	Terpenoids
E5	0.22	-ve	Light blue	-ve	-ve	Light blue (+)	Terpenoids
E6	0.99	-ve	Bright red (+++)	-ve	Orange (++)	-ve	Alkaloid

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band observed (-ve)

Table 4.10 : Thin Layer Chromatography of *Aquilaria sinensis* leaves ethyl acetate extract using solvent chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
E7	0.03	-ve	Dark Red	-ve	-ve	Dark brown (+++)	Terpenoids
E8	0.06	-ve	Red	Green (++)	-ve	-ve	Phenolic
E9	0.11	-ve	Red	-ve	-ve	Blue (+)	Terpenoids
E10	0.11	-ve	Red	-ve	-ve	Blue (+)	Terpenoids
E11	0.91	-ve	Bright blue (+++)	-ve	Light orange (+)	-ve	Alkaloid

Indication of color intensity of spots:	Strong (+++)	medium (++)	weak (+)	No color/band observed (-ve)

Table 4.11 : Thin Layer Chromatography of *Aquilaria sinensis* leaves *n*-butanol extract using solvent 10% methanol in chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
B1	0.04	-ve	Blue	Green Yellowish (++)	-ve	-ve	Phenolic
B2	0.07	-ve	Purple	Blue (+++)	-ve	-ve	Phenolic
B3	0.07	-ve	-ve	-ve	-ve	Pink (++)	Terpenoids
B4	0.07	-ve	-ve	-ve	-ve	Dark red (+++)	Terpenoids
B5	0.46	-ve	-ve	-ve	Orange (++)	-ve	Alkaloid
B6	0.76	-ve	-ve	-ve	-ve	Blue (++)	Terpenoids
B7	0.95	-ve -ve	Red	-ve	Orange (++)	-ve	Alkaloid

Indication of color intensity of spots: Strong (+++) Medium (++) weak (+) No color/band observed (-ve)

Table 4.12: Thin Layer Chromatography of *Aquilaria sinensis* leaves *n*-butanol extract using solvent chloroform.

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
B8	0.03	-ve	Red	Dark Yellow (++)	-ve	-ve	Phenolic
B9	0.04	-ve	-ve	-ve	-ve	Dark red (+++)	Terpenoids
B10	0.06	-ve	Blue	Green yellow (++)	-ve	-ve	Phenolic
B11	0.85	-ve	Red	-ve	Orange (++)	-ve	Alkaloid

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band (-ve)

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Table 4.13 : Thin Layer Chromatography of *Aquilaria sinensis* leaves dichloromethane extract using solvent 10% methanol in chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
DC1	0.04	-ve	Blue (++)	Green Yellowish (++)	-ve	-ve	Phenolic
DC2	0.07	-ve	Purple (++)	-ve	-ve	Blue (+++)	Phenolic
DC3	0.24	-ve	-ve	-ve	Orange (+)	-ve	Alkaloid
DC4	0.98	-ve	Red (+)	-ve	Orange (+)	-ve	Alkaloid

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band (-ve)

Table 4.14 : Thin Layer Chromatography of *Aquilaria sinensis* leaves dichloromethane extract using solvent chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
DC5	0.03	-ve	Red (++)	Dark Yellow (+++)	-ve	-ve	Phenolic
DC7	0.06	ve	Blue (++)	Green yellow (++)	-ve	-ve	Phenolic
DC8	0.77	-ve	Light Blue (+)	-ve	Orange (+)	-ve	Alkaloid
DC9	0.80	-ve	Light Blue (+)	-ve	-ve	Dark blue (++)	Tepernoids
DC10	0.94	-ve	Light Blue (+)	-ve	-ve	Light blue (+)	Terpenoids

Indication of color intensity of spots: Strong (+++) medium (++) weak (+) No color/band (-ve)

Table 4.15 : Thin Layer Chromatography of *Aquilaria sinensis* leaves 10% methanol aqueous extract using solvent 10% methanol in chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
MA1	0.11	-ve	Red	Blue (++)	-ve	-ve	Phenolic
MA2	0.94	-ve	-ve	-ve	-ve	Blue (++)	Tepernoids
MA3	0.98	-ve	-ve	-ve	Dark orange (+++)	-ve	Alkaloid

Indication of color intensity of spots: Strong (+++) Medium (++) weak (+) No color/band observed (-ve)

Table 4.16 : Thin Layer Chromatography of *Aquilaria sinensis* leaves 10 % methanol aqueous extract using solvent chloroform

Label compound	R_f Value	Colors under Visible light	Colors under UV light	Observation of spraying reagents			Remarks
				Phenols	Drangendroff	Vanillin	
MA4	0.22	-ve	-ve	-ve	Orange (++)	-ve	Alkaloid

Indication of color intensity of spots:	Strong (+++)	Medium (++)	weak (+)	No color/band observed (-ve)

4. 3 LCMS analysis

4.3.1 LCMS analysis of methanol aqueous fraction

The analysis of 10% methanol aqueous fraction using LCMS showed the presences of several compound.

Table 4.17 : LCMS analysis of methanol aqueous of *A. sinenis*

Compound	Name of compound	RT	Mass	Formulae
1	Hypoxanthine	3.234	136.0389	$C_5 H_4 N_4 O$
2	Norswertianolin	3.33	422.0836	$C_{19} H_{18} O_{11}$
3	Acetyl-maltose	3.335	384.1282	$C_{14} H_{24} O_{12}$
4	Isosorbide-2-glucuronide	3.437	322.0909	$C_{12} H_{18} O_{10}$
5	Mangiferin	3.617	422.0857	$C_{19} H_{18} O_{11}$
6	2,4',6,6'-Tetranitro-2',4-azoxytoluene	7.824	406.0499	$C_{14} H_{10} N_6 O_9$
7	Orotidine	7.933	288.0594	$C_{10} H_{12} N_2 O_8$
8	Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside	8.085	594.1584	$C_{27} H_{30} O_{15}$
9	Sulfometuron	8.254	350.0681	$C_{14} H_{14} N_4 O_5 S$
10	Sulfometuron methyl	8.748	364.0826	$C_{15} H_{16} N_4 O_5 S$
11	Met Trp Asp	9.084	450.156	$C_{20} H_{26} N_4 O_6 S$

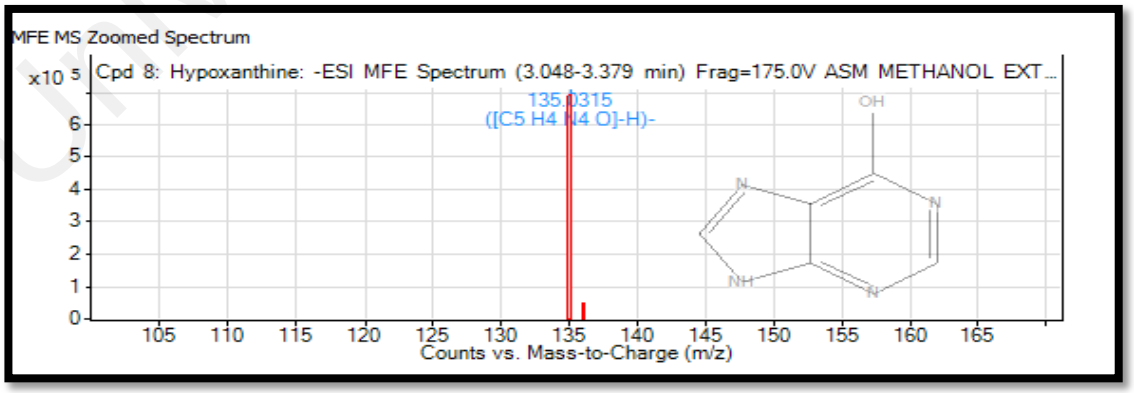


Figure 4.1: MS spectra of Hypoxanthine

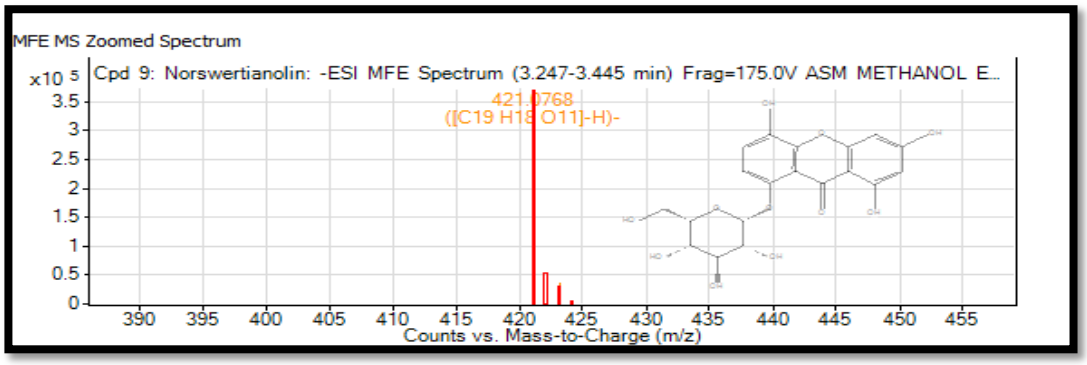


Figure 4.2 : MS spectra of Norswertianolin

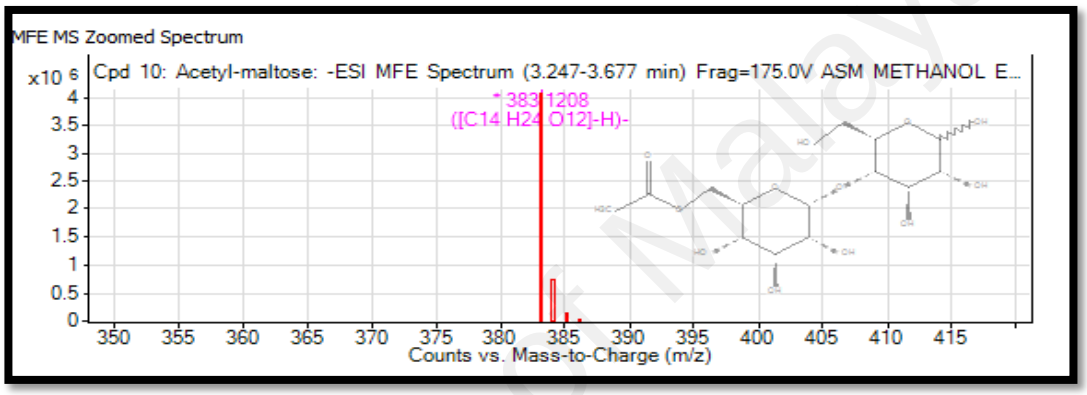


Figure 4.3: MS spectra of Acetyl-maltose

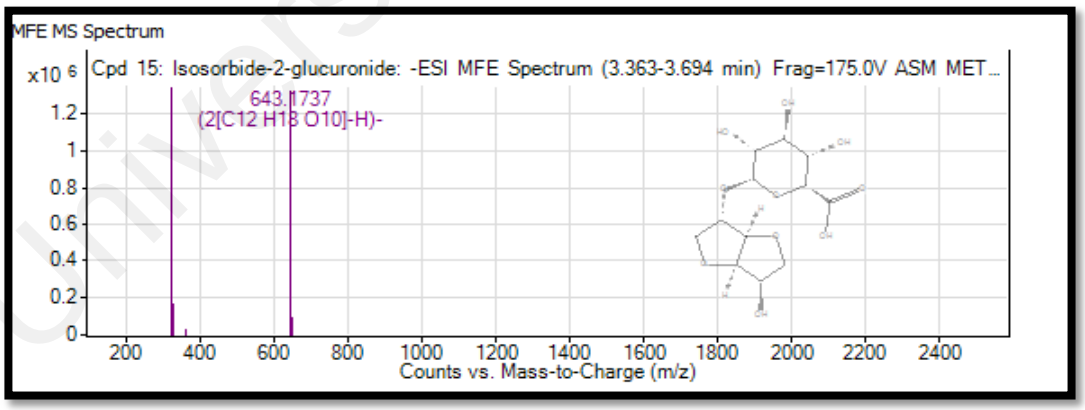


Figure 4.4: MS spectra of Isosorbide-2-glucuronide

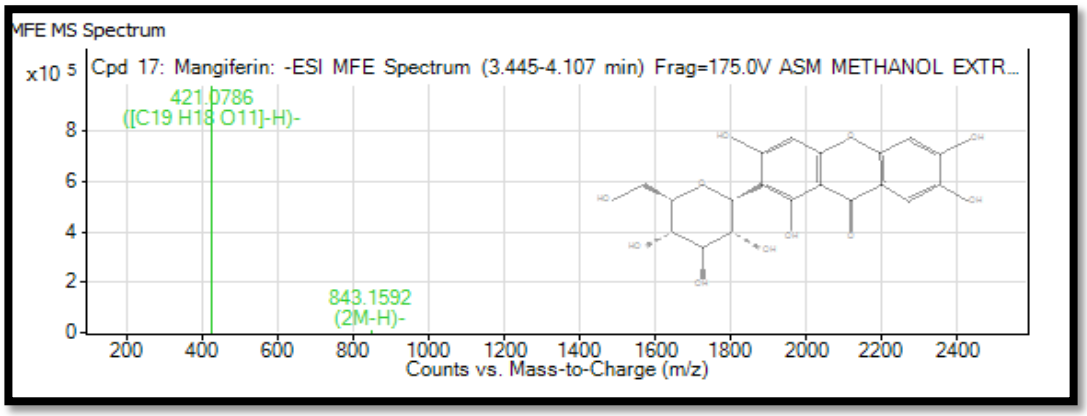


Figure 4.5: MS spectra of Mangiferin

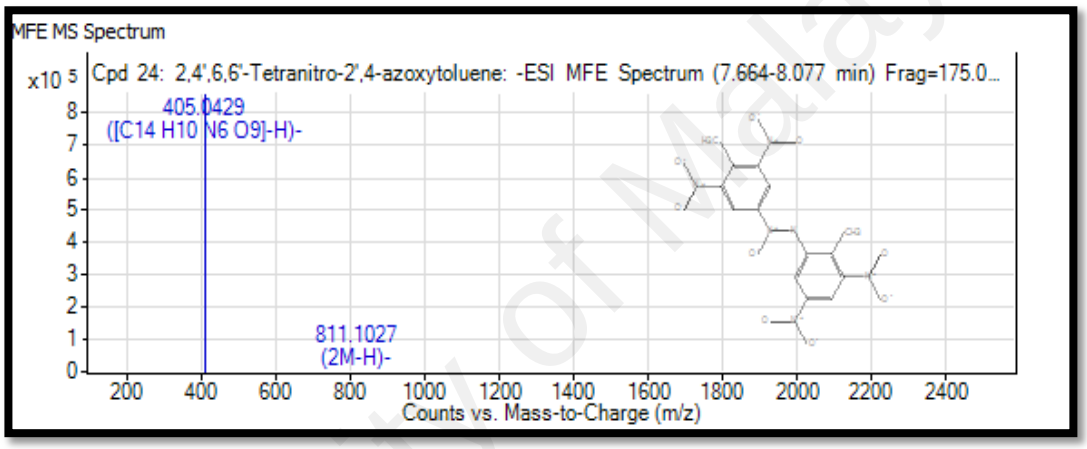


Figure 4.6 : MS spectra of 2,4',6,6'-Tetranitro-2',4-azoxytoluene

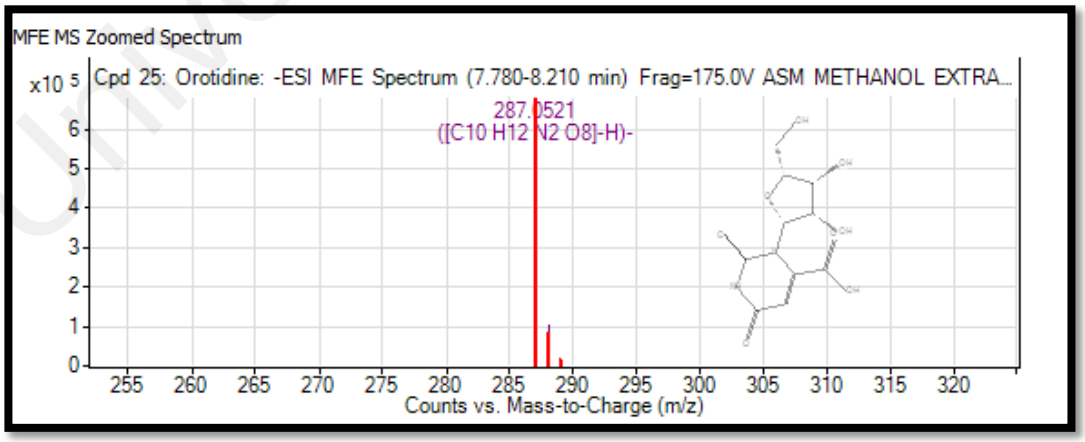


Figure 4.7 : MS spectra of Orotidine

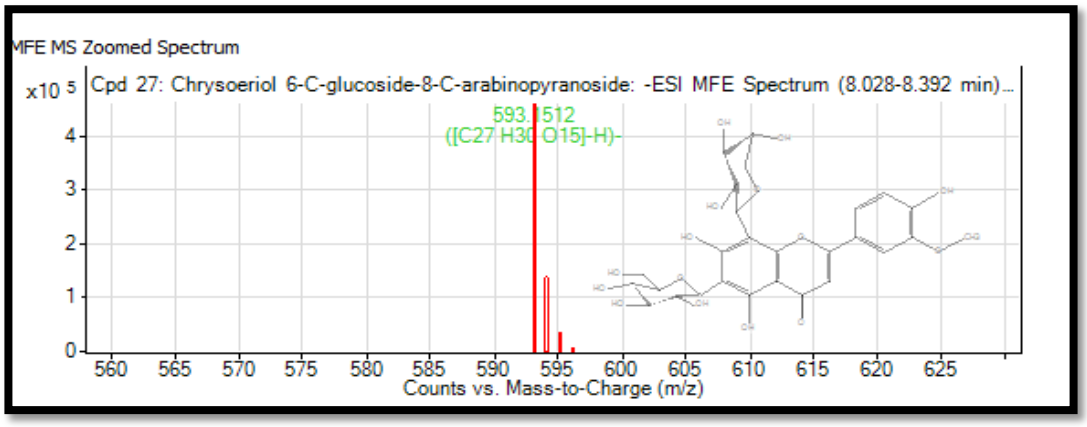


Figure 4.8 : MS spectra of Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside

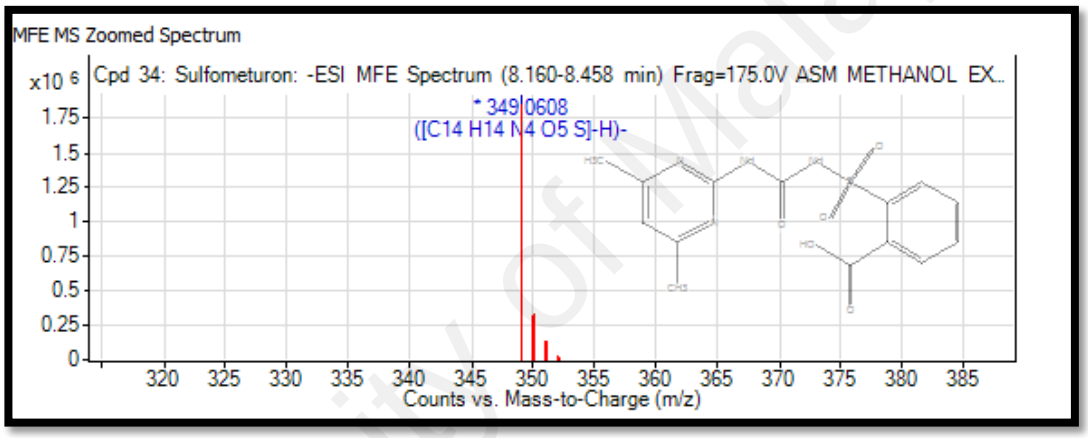


Figure 4.9 : MS spectra of Sulfometuron

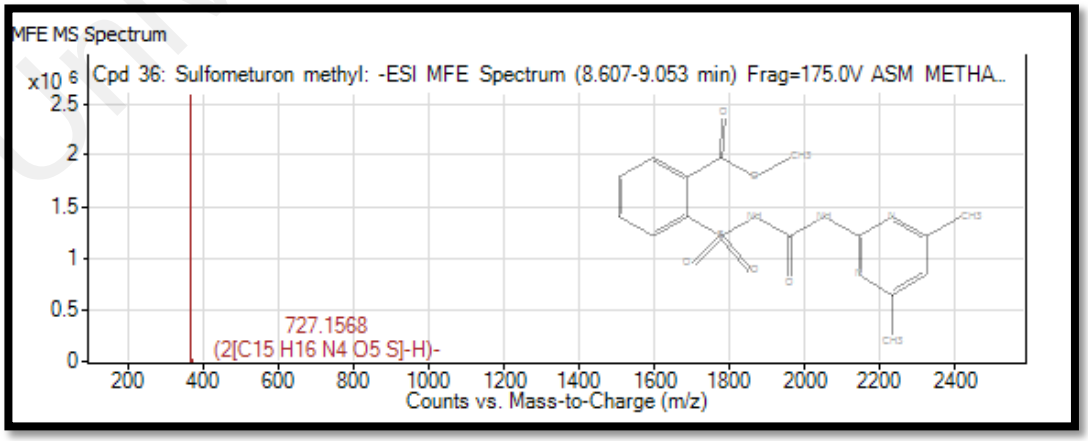


Figure 4.10 : MS spectra of Sulfometuron methyl

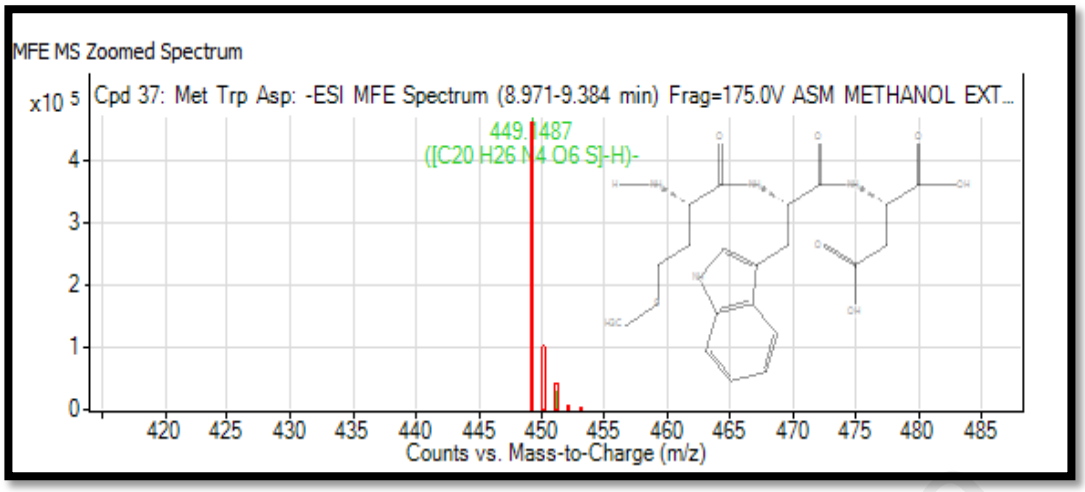


Figure 4.11: MS spectra of Met Trp Asp

4.3.2 LCMS analysis of ethyl acetate fraction

Table 4.18 shows LCMS analysis of ethyl acetate fraction which detected the presences of compounds.

Table 4.18: LCMS analysis of ethyl acetate of *A. sinenis*

Compound	Name of compound	RT	Mass	Formulae
Compound 1	Tripeteroside	0.92	422.085	$C_{19}H_{18}O_{11}$
Compound 2	D-Proline	0.937	115.0634	$C_5H_9N0_2$
Compound 3	Gln-Gln-Trp	3.834	460. 2073	$C_{21}H_{28}N_6O_6$
Compound 4	Sulpho NONOate	4.494	141.9708	$H_2N_2O_5S$

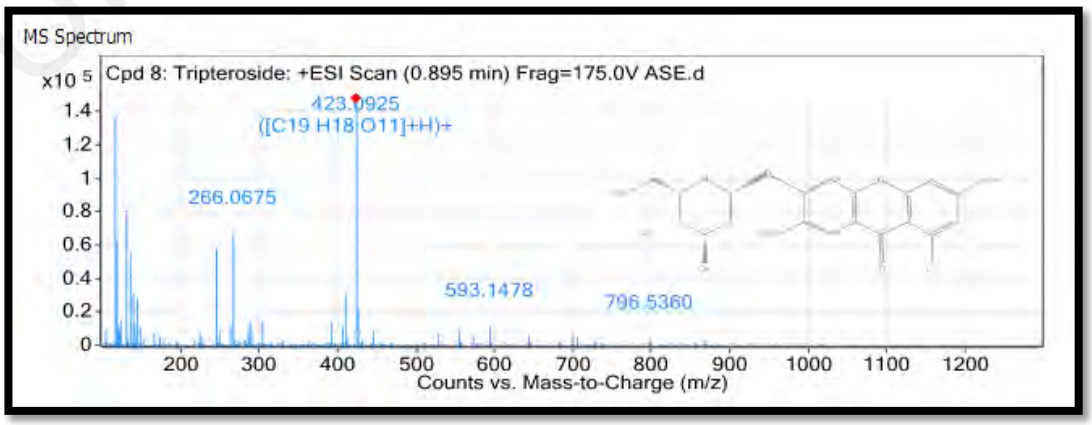


Figure 4.12 : MS spectra of Tripeteroside

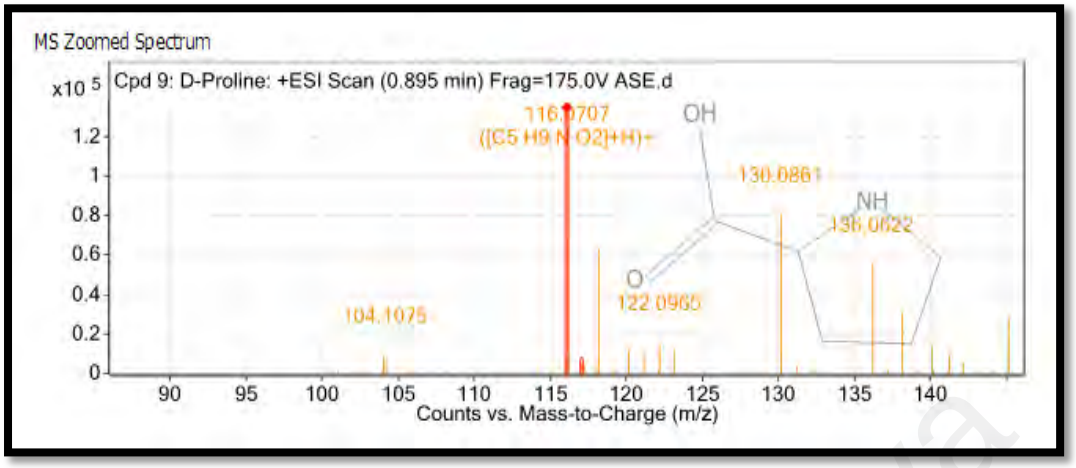


Figure 4.13 : MS spectra of D-Proline

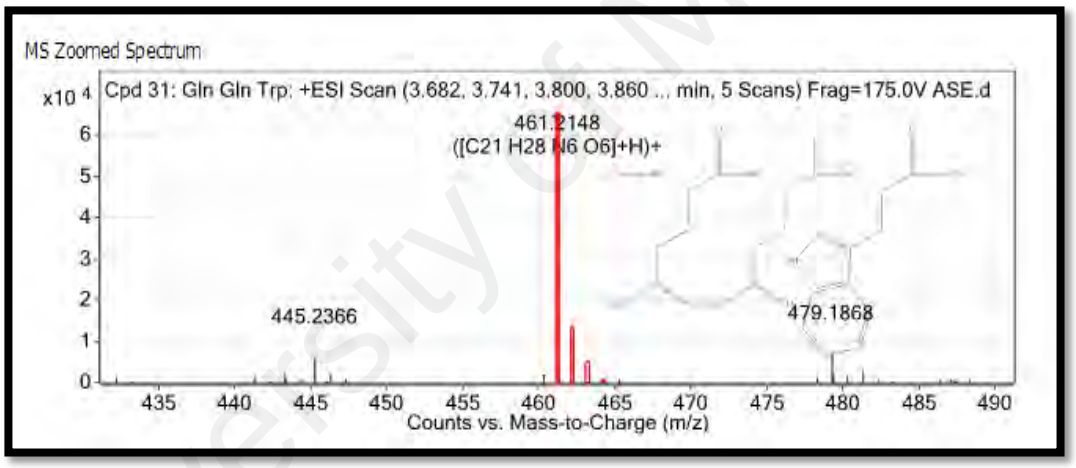


Figure 4.14 : MS spectra of Gln-Gln-Trp

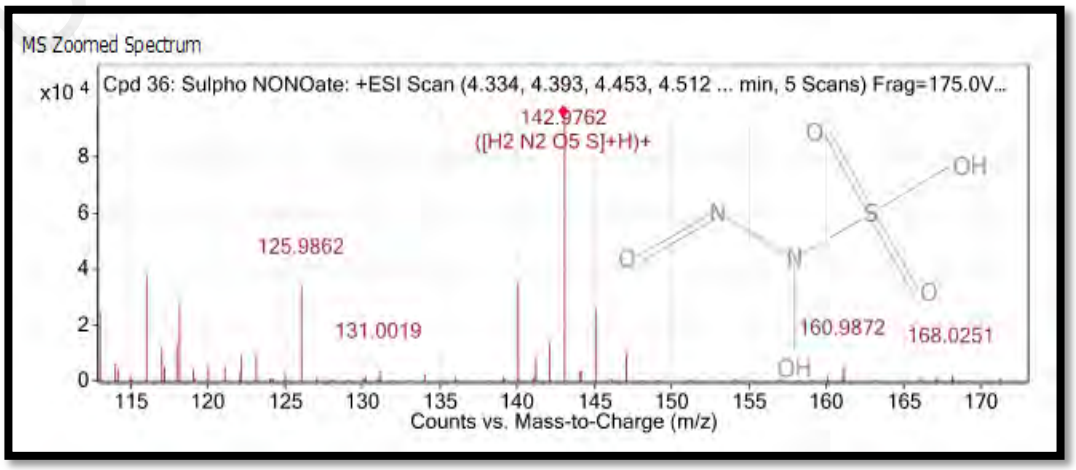


Figure 4.15: MS spectra of Sulpho NONOate

4.3.3 LCMS analysis of *n*-butanol fractions

Table 4.19: shows LCMS analysis of *n*-butanol fraction in *A. sinensis.*

Compound	Name of compound	RT	Mass	Formulae
Compound 1	C16 Sphinganine	0.824	273.2673	C_{16} H_{35} N O_2
Compound 2	Adenine	0.913	135.0552	C_5 H_5 N_5
Compound 3	Tripteroside	0.93	422.0858	C_{19} H_{18} O_{11}
Compound 4	Isopimpinellin	0.972	246.0531	C_{13} H_{10} O_5
Compound 5	Trp Leu Val	1.031	416.2424	C_{22} H_{32} N_4 O_4
Compound 6	2,3-dinor Thromboxane B1	1.048	344.2203	C_{18} H_{32} O_6
Compound 7	Granisetron	1.091	312.194	C_{18} H_{24} N_4 O
Compound 8	Lys Cys His	1.226	386.1732	C_{15} H_{26} N_6 O_4 S
Compound 9	Oleoyl Ethyl Amide	1.358	309.3036	C_{20} H_{39} N O
Compound 10	Stearamide	1.802	283.2878	C_{18} H_{37} N O
Compound 11	Tyr Asn His	8.667	432.1757	C_{19} H_{24} N_6 O_6

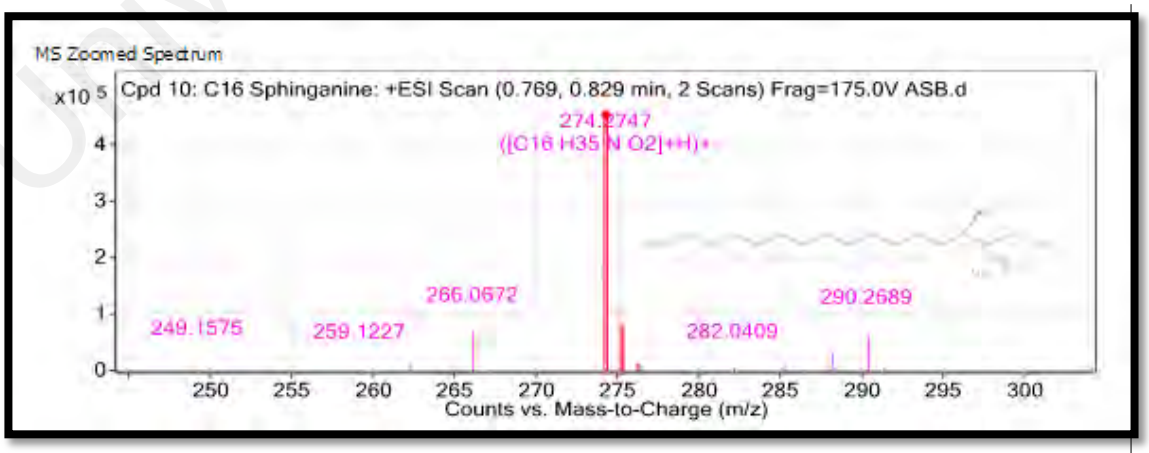


Figure 4.16 : MS spectra of C16 Sphinganine

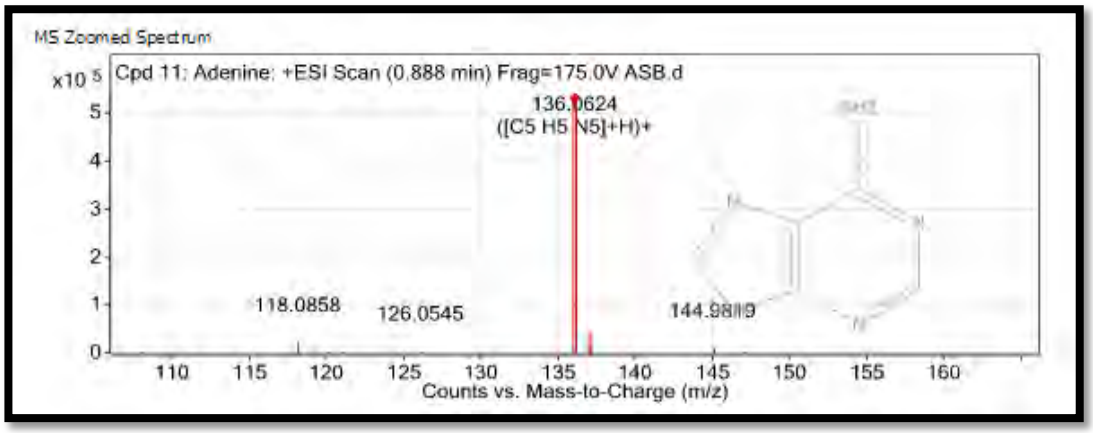


Figure 4.17 : MS spectra of Adenine

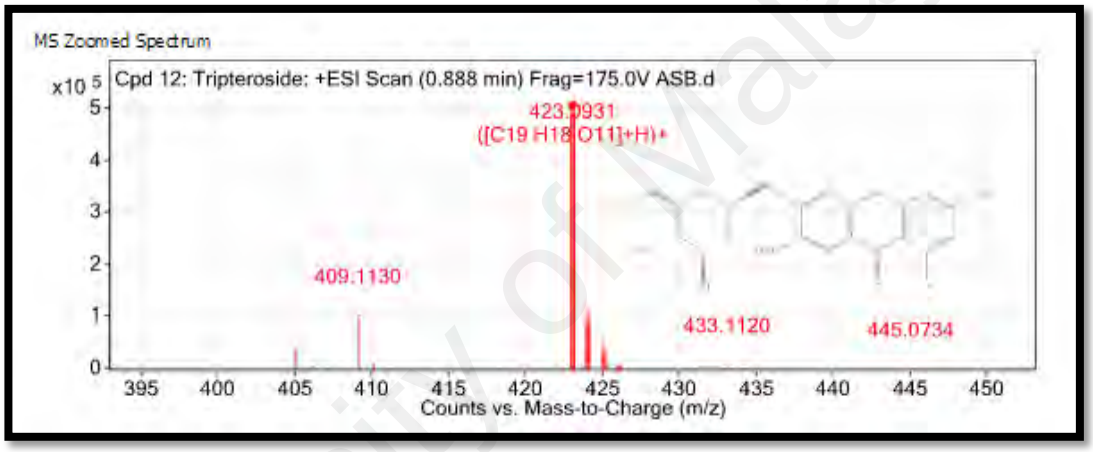


Figure 4.18 : MS spectra of Tripteroside

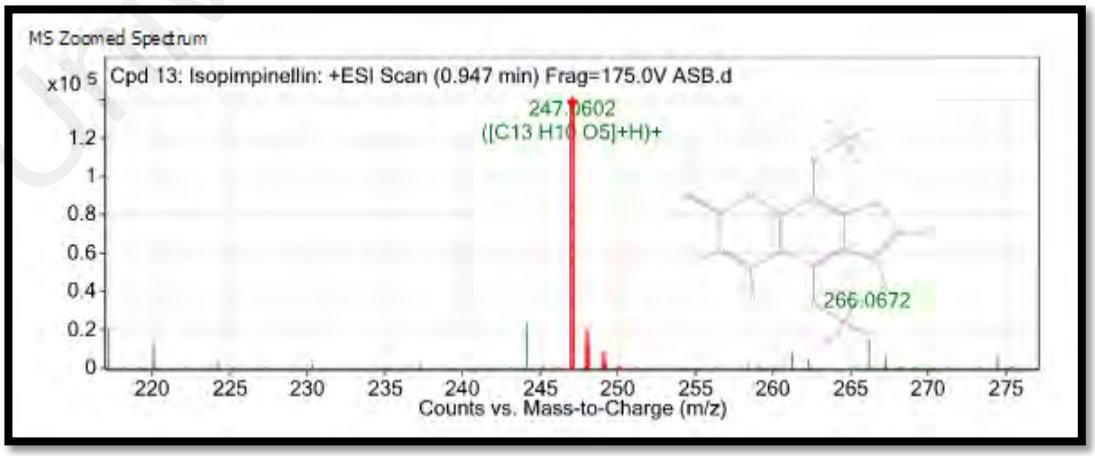


Figure 4.19 : MS spectra of Isopimpinellin

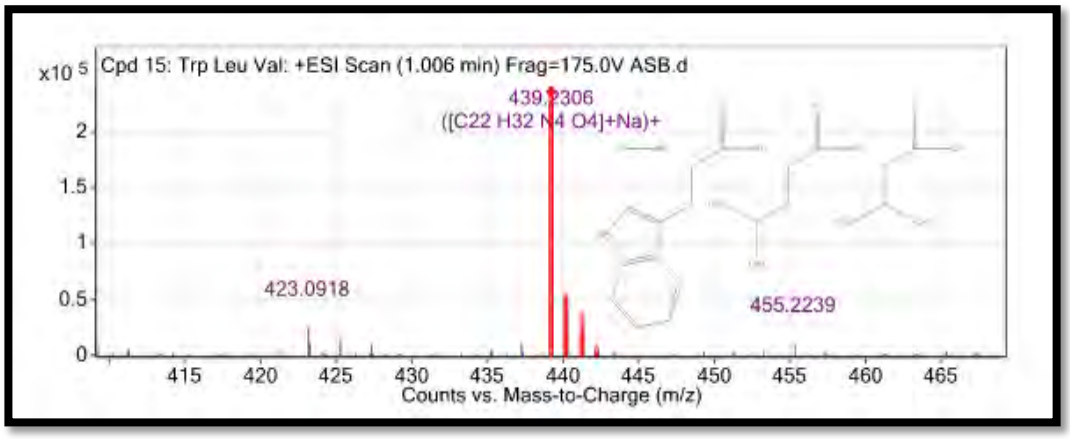


Figure 4.20: MS spectra of Trp Leu Val

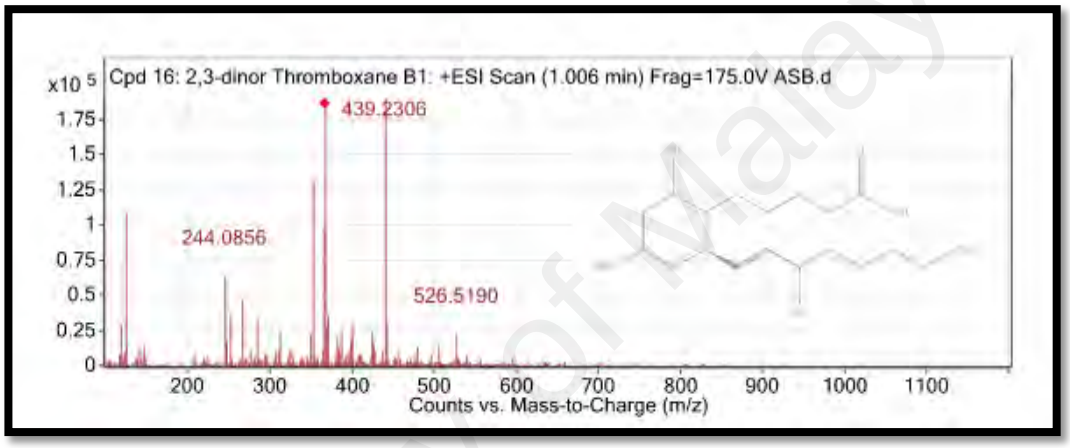


Figure 4.21: MS spectra of 2,3-dinor Thromboxane B1

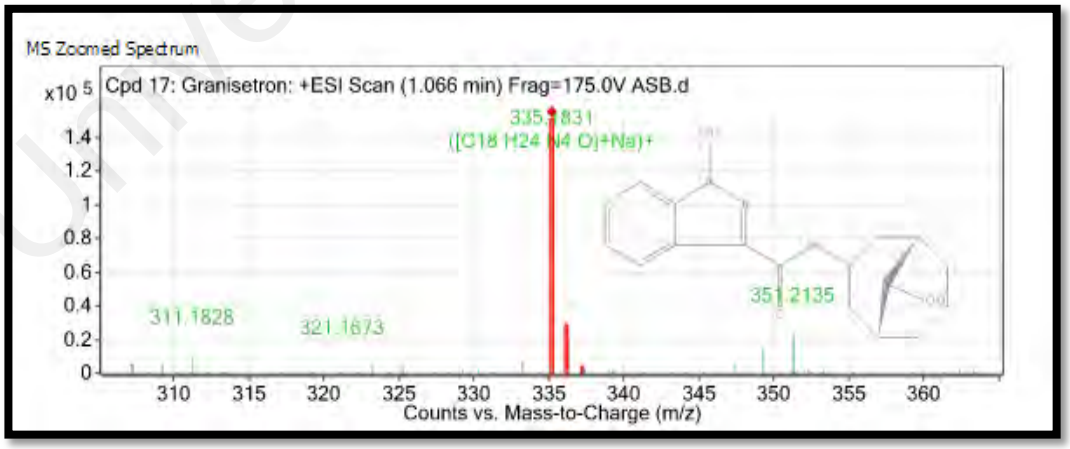


Figure 4.22: MS spectra of Granisetron

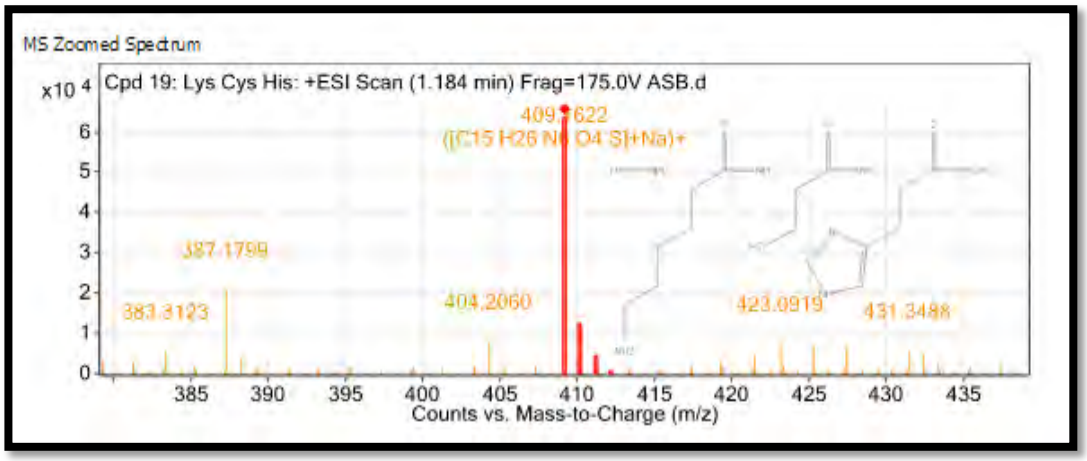


Figure 4.23: MS spectra profile of Lys Cys His

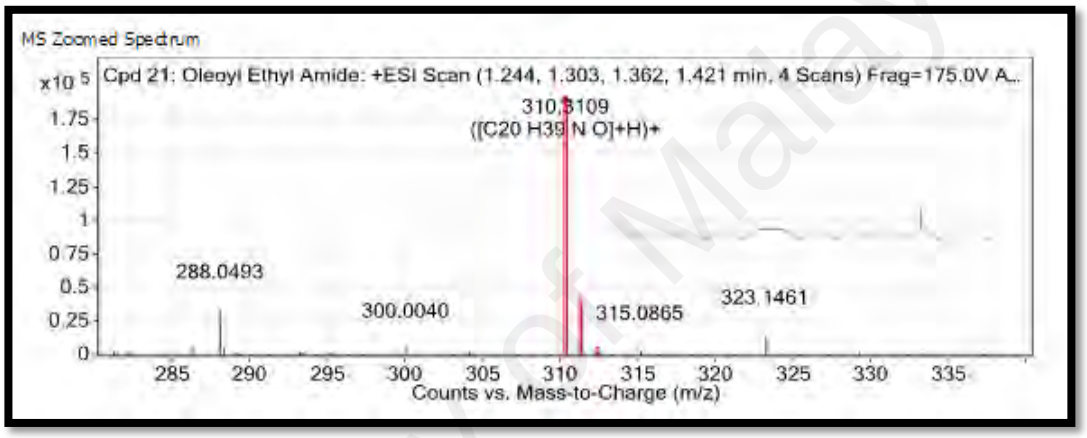


Figure 4.24: MS spectra of Oleoyl Ethyl Amide

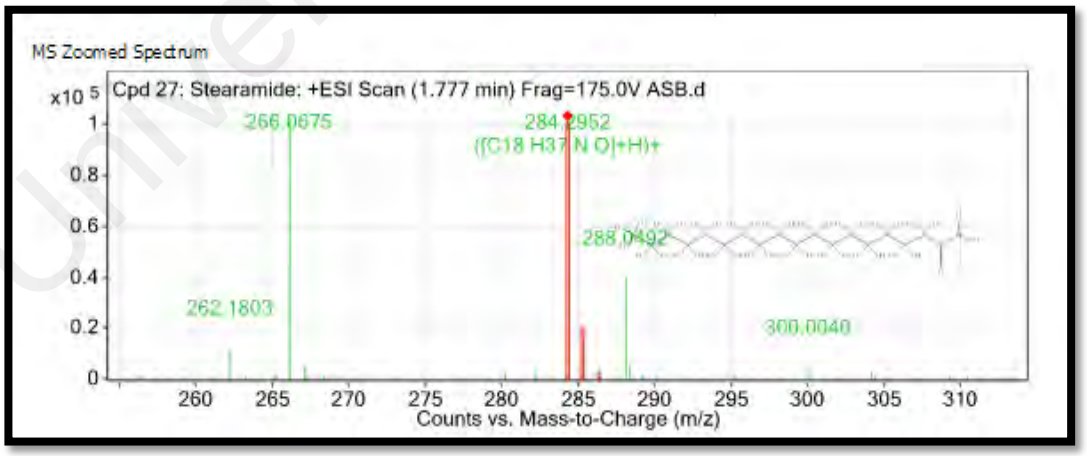


Figure 4.25: MS spectra of Stearamide

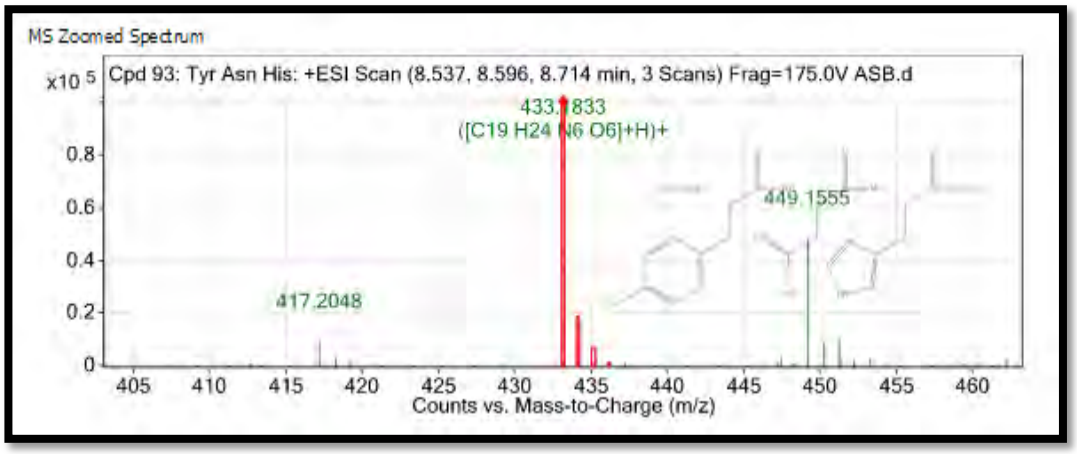


Figure 4.26: MS spectra of Tyr Asn His

4.4 Determination of total phenolic content (TPC)

The total phenolic content of the *A. sinensis* fractions, were determined by Folin-Ciocalteu's method with standard curve (Figure 4.27) of gallic acid as reference (Y = 0.5392x -0.4509 and R^2 =0.9714).

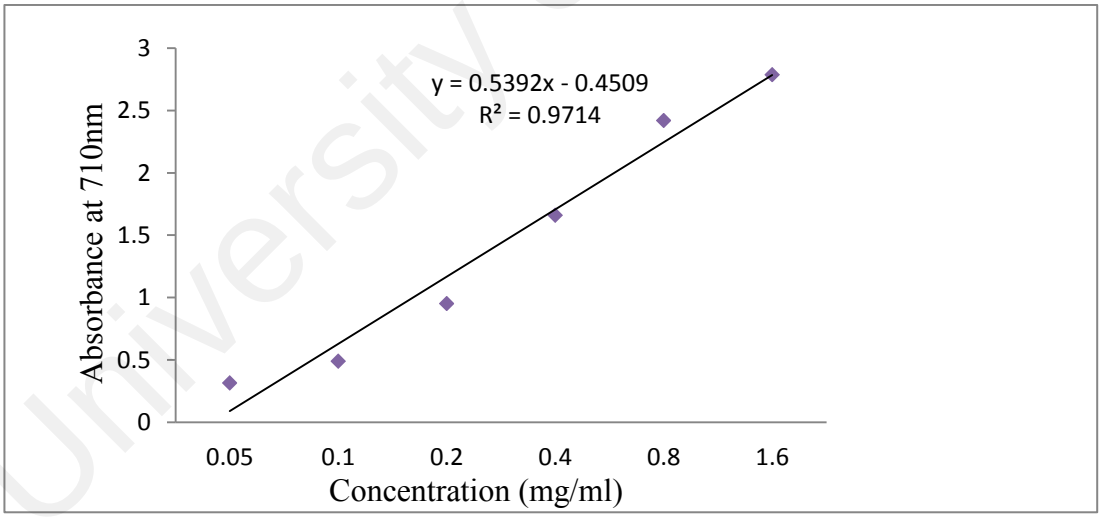


Figure 4.27 : The standard curve of gallic acid

Table 4.20 : The TPC of *A. sinensis* fractions.

Fractions	TPC (mg GAE/g dry weight) ± SEM
***n*-hexane**	0.68 ± 0.01
Chloroform	0.84 ± 0.03
Ethyl Acetate	1.69 ± 0.02
***n*-butanol**	1.45 ± 0.04
Dichloromethane	0.74 ± 0.05
10 % Metanol Aquoeus	0.63 ± 0.02

Each values of fraction indicates as mean ± SEM

The results in Table 4.20 showed that ethyl acetate fraction have the highest total phenolic content at 1.69 ± 0.02 mg GAE/g dry weight followed by, butanol fraction at 1.45 ± 0.04 mg GAE/g dry weight, chloroform fraction at 0.84 mg GAE/g dry weight and dicholoromethane fraction at 0.74 ± 0.05 mg GAE/g dry weight. The hexane and methanol aqueous fraction have lesser phenolic content at 0.68 mg GAE/g dry weight and 0.63 ± 0.02 mg GAE/g dry weight, respectively.

The results suggested that TPC of fractions tends to increase with increasing polarity of the solvents used for the extraction medium.

4.5 Determination of total flavonoid content (TFC)

The total flavonoid content of the *A. sinensis* fractions were determined from the standard curve of quercetin.

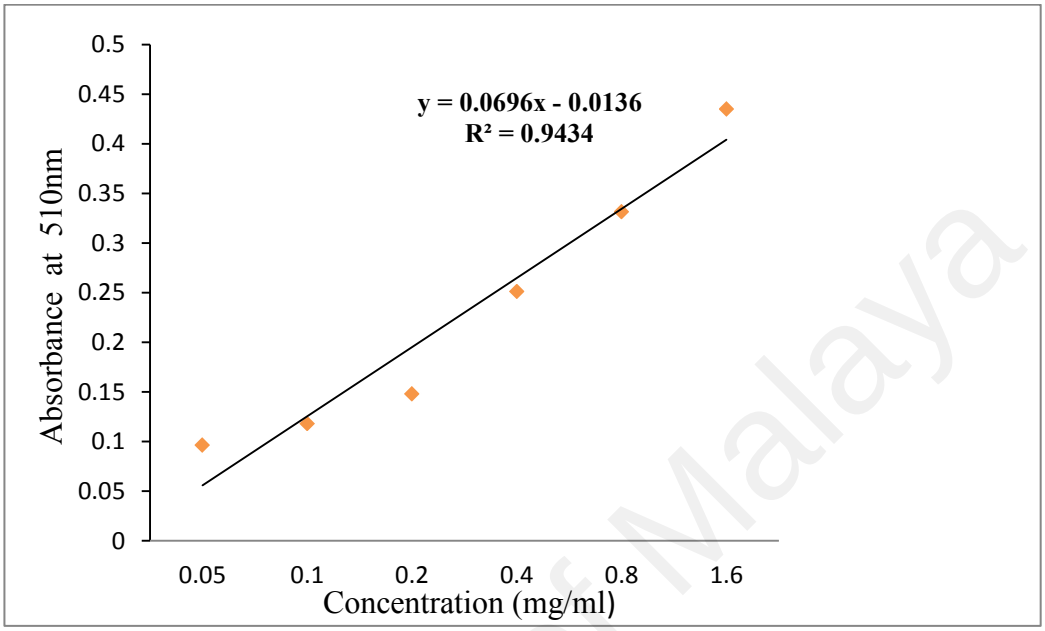


Figure 4.28 : The standard curve of quercetin

The results in Table 4.21 showed that butanol fraction have the highest total flavonoid content at 3.59 ± 0.07 mg QE/g dry weight and followed by ethyl acetate fraction at 2.40 ± 0.04 mg QE/g dry weight. The chloroform and methanol aqueous fraction have moderate flavonoid content at 0.77 ± 0.04 mg QE/g dry weight and 0.70 ± 0.01 mg QE/g dry weight, respectively. However, the lowest flavonoid content obtained in dichloromethane fraction at 0.63 ± 0.05 mg QE/g dry weight and hexane fraction at 0.61 ± 0.01 mg QE/g dry weight. This could be an indication that lesser flavonoid content of *A. sinensis* was found in non-polar solvents and higher content in polar solvents.

Table 4.21 : The TFC of each fraction in the highest concentration.

Fractions	TFC (mg/QE/g dry weight) ± SEM
n-hexane	0.61 ± 0.01
Chloroform	0.77 ± 0.04
Ethyl Acetate	2.40 ± 0.04
n-butanol	3.59 ± 0.07
Dichloromethane	0.63 ± 0.05
10 % Metanol Aquoeus	0.70 ± 0.01

Each data of fraction was express as TFC ± SEM

4.5 *In vitro* antioxidant assay

4.5.1 DPPH radical scavenging activity

The determination of DPPH radical scavenging activity was based on the formation of the DPPH-H non -radical form in the presence of hydrogen donating antioxidants in the fractions and was detected at 517 nm. The DPPH radical scavenging activity of *A. sinensis* fractions were tested at different concentrations ranging from 0.05 – 1.6 mg/ml against the ascorbic acid as a standard. The highest DPPH scavenging activity was observed in the ethyl acetate and butanol fractions at 83.24% and 83.81% inhibition, respectively. However, hexane and dichloromethane fractions exhibited the lowest DPPH inhibition at 41% and 29.26 %, respectively. The rank of highest antioxidant activity starts with ethyl acetate > *n*-butanol> methanol aqueous > chloroform> *n*-hexane and dicholormethane.

Table 4.22: IC_{50} of DPPH inhibition of *Aquilaria sinensis* fractions

Fractions	IC_{50} (mg/ml)
Hexane	> 1.6 ± 0.08
Chloroform	0.52 ± 0.03
Ethyl acetate	0.10 ± 0.02
Butanol	0.12 ± 0.02
Dichloromethane	> 1.6 ± 0.10
Methanol aqueous	0.18 ± 0.03
Ascorbic acid (Standand)	0.08 ± 0.03

The results above are expressed as mean ± SEM of duplicate determination.

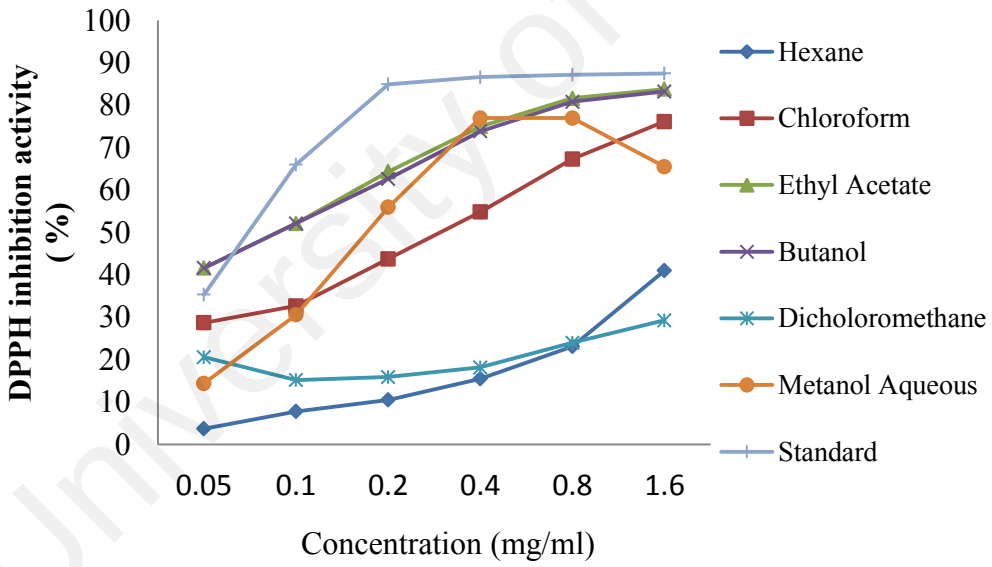


Figure 4.29 : The inhibition percentage of DPPH of *A. sinensis*

Results from Table 4.22, showed the scavenging abilities of fractions were expressed as IC_{50} values. The inhibitory activity of *A. sinensis* were concentration-dependent and lower IC_{50} value indicates higher antioxidant activity in the fraction.

The ethyl acetate and *n*-butanol fraction were capable of exhibiting high inhibition of DPPH with lowest IC_{50} value of 0.10 ± 0.02 and 0.12 ± 0.02 mg/ml. Meanwhile, 10 % methanol aqueous and chloroform fractions showed moderate IC_{50} value of 0.18 ± 0.03 mg/ml and 0.52 ± 0.03 mg/ml , respectively. Highest IC_{50} value and lowest inhibitory activity was exhibited by the hexane and dicholoromethane with IC_{50} value more than 1.6 mg/ml. The degree of discoloration from purple to yellow was observed in the assay as it suggested the activity of antioxidant .The color changes due to the hydrogen transfer by the antioxidant and reduced form of DPPH radical. The results suggested that the presences of phenolic and flavonoid compounds in *A. sinensis* fractions might contribute in scavenging DPPH free radicals.

4.6.2 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power of the *A. sinensis* fractions,was determined from standard curve of ferrous sulphate (Figure 4.30). The FRAP value of each fraction was measured based on the reaction of antioxidant potential of the plant fraction and ferric reducing power of TPTZ complex with the formation blue color in the fractions. The fraction that contained highest amount of the total flavonoid and phenolic compound showed the highest reducing power activity. The result suggested that, phenolic compound are most efficient in reducing power in *A. sinensis* fractions.

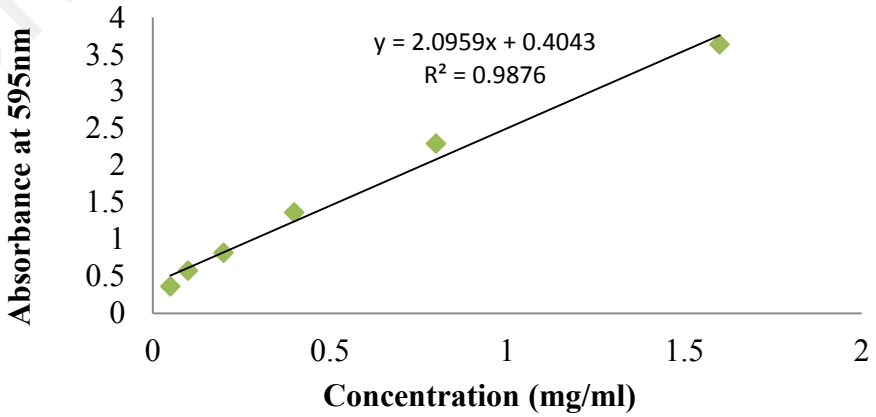


Figure 4.30 : The standard curve of ferrous sulphate

Table 4.23: Ferric reducing antioxidant power value of the *A. sinensis* fractions

Fractions	FRAP(mmol/ g dry weight fractions)
n-hexane	ND
Chloroform	0.096 ± 0.003
Ethyl Acetate	0.368 ± 0.069
n-butanol	0.299 ± 0.025
Dichloromethane	0.127 ± 0.002
10% Methanol Aqueous	ND

Each value indicates as FRAP ± SEM & ND represents the value of undetected.

Higher FRAP value was observed in the fraction of ethyl acetate at 0.368 mmol/g dry weight and followed by *n*-butanol with 0.299 mmol/g dry weight. A moderate reducing ability was detected in dichloromethane and chloroform fraction at 0.127 mmol/ g dry weight and 0.096 mmol/ g dry weight, respectively. However, hexane and 10% methanol fractions exhibited the poorest reducing ability and negative sign indicates that the fractions low inhibitory effect in FRAP assay. The ferric reducing capability (R^2 =0.987) results again correlated with the DPPH inhibition assay as well total phenolic content (R^2 = 0.9714) and total flavonoid content (R^2 =0.9434).

Color changes from deep blue violet to colorless was observed in the assay, and this indicates reduction of ferric ion took place by the antioxidant. The color intensity showed the potency of the antioxidant in each fractions. These results suggested that presences of phenolic compound serve a good antioxidant by ability to reduce ferric ion. The highest rank of ferric reducing abilities starts with chloroform > *n*-butanol > ethyl acetate.

4.6.3 Metal chelating assay

The assay is to measure chelating ability of ferrous ion which is based on the ion chelation with ferrozine to form ferrous-ferrozine complex and can be detected at 562 nm. The chelation of metal ion assay was carried out to access the chelation capacity of the *A. sinensis* fractions. The fractions were measured at the different concentrations from 0.05- 1.6 mg/ml and the results were shown in Figure 4.31 and Table 4.24.

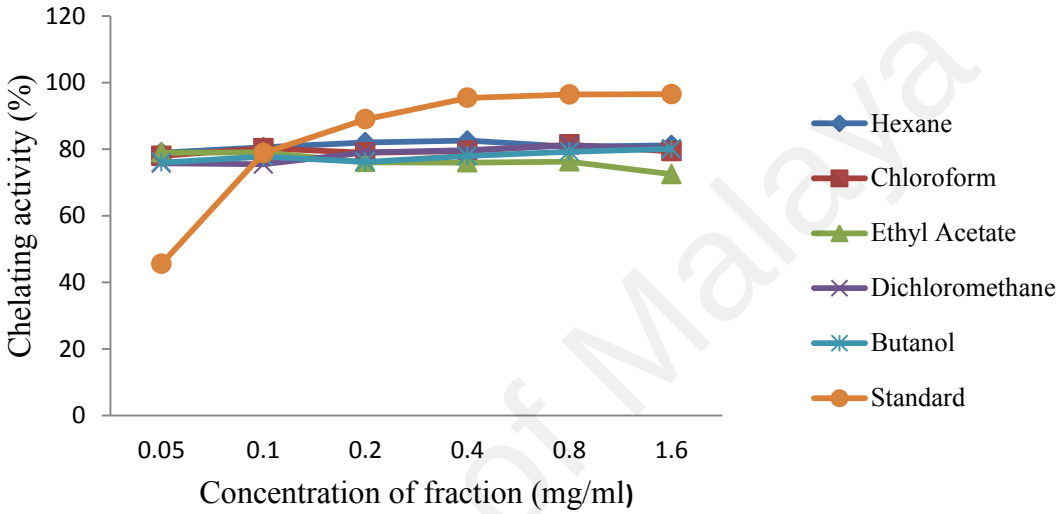


Figure 4.31: Metal chelating activities of *A. sinensis* fractions

Table 4.24: IC_{50} of metal chelating activity using *A.sinensis*

Fractions	**(IC_{50}) mg/ml ± SEM**
n-hexane	< 0.05 ± 0.008
Chloroform	< 0.05 ± 0.009
Ethyl acetate	< 0.05 ± 0.010
Dichloromethane	< 0.05 ± 0.006
n-butanol	< 0.05 ± 0.011
EDTA(Standard)	0.060 ± 0.012

Results are expressed as (IC_{50}) ± SEM.

Based on the results, fractions of *A. sinenis* leaves showed remarkable chelation power with IC_{50} value of <0.05 mg/ml. Chelation metal was also analysed with comparison of standard reference, EDTA which have exhibited significant capacity to chelate ferrous ion at 96.56% and IC_{50} of 0.060 ± 0.012 mg/ml. The fractions showed lower chelating activity when compared to EDTA or standard used in the assay. However, the highest chelating activity found in the hexane fraction at 81.2% with IC_{50} of < 0.05± 0.008 and followed by butanol dichloromethane, and chloroform fractions at 80.1% , 80%, and 79 % respectively. A moderate chelating activity was observed in the ethyl acetate fraction at 72%. The active *A. sinensis* fractions had successfully interfered the formation of ferrous and ferrozine complex indicates as potential metal chelator. The results suggested that the presences of phenolic compounds in the plant fractions, contributed to the efficiency of chelating metal ions.

4.6.4 Nitric oxide radical scavenging activity (NORSA)

The nitric oxide radical scavenging activity was determined using different concentration of each fraction and the results are shown in Figure 4.32 and Table 4.25

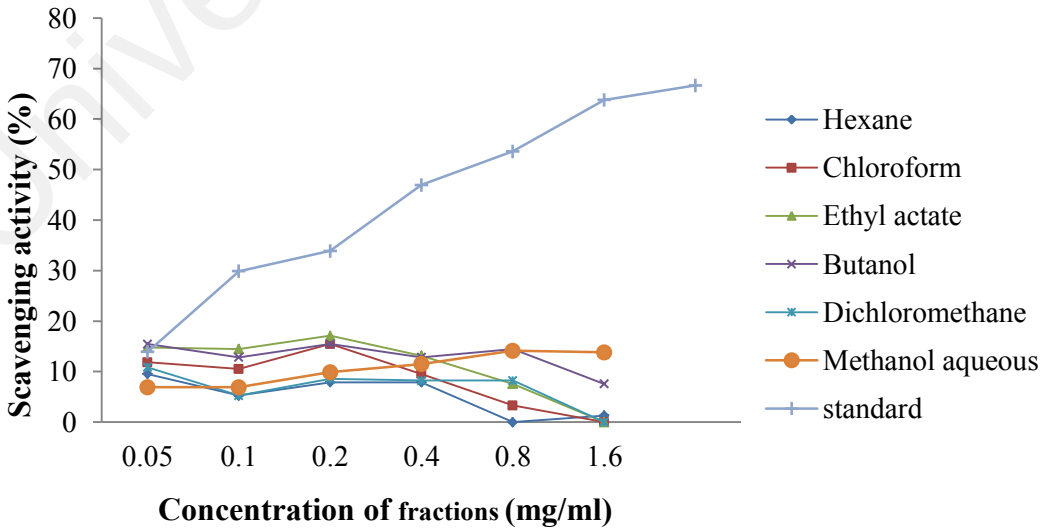


Figure 4.32 : Nitric oxide radical scavenging activity using the *A. sinensis* fractions

Table 4.25: The IC_{50} of *A. sinensis* fractions for NORSA activity.

Fraction	IC_{50} ± SEM (mg/ml)
n-Hexane	>1.6 ± 0.002
Chloroform	>1.6 ± 0.010
Ethyl acetate	>1.6 ± 0.005
n-Butanol	>1.6 ± 0.001
Dichloromethane	>1.6 ± 0.002
10 % Methanol Aqueous	>1.6 ± 0.001
Curcumin(Standard)	0.885 ± 0.012

Results are expressed in IC_{50} ± SEM

Based on the result obtained, it was shown that methanol aqueous fraction exhibited highest nitric oxide radical scavenging at 14.1% with IC_{50} value of more than 1.6 ± 0.001 mg/ml, but not effectively scavenged above 50 %. All of the fractions showed a poor scavenging capabilities of nitric oxide radical compared to the standard, curcumin that showed the highest inhibition at 66.67 % with IC_{50} value of 0.885 ± 0.012 mg/ml. However, the highest scavenging fraction suggested that the compound presenting in the methanol aqeuous could be the main contributor to scavenge nitric oxide radicals. The phenolic rich ethyl acetate fraction has shown moderate scavenging NO using *A. sinensis* leaves compared to the rest of fractions.

4.6.5 Superoxide radical scavenging activity

In the cellular damage and reaction, superoxide radical is typically be formed first and magnifies the effects more as it contribute in producing other oxidizing agents and cell damaging free radicals. In this study of superoxide radical scavenging using *A. sinensis* fractions was evaluated and the ability of scavenging radical using PMS, NBT and NADH.

Table 4.26: The IC_{50} of *Aquilaria sinensis* for superoxide radicals activity.

Fractions	IC_{50} ± SEM
n-Hexane	> 1.6 ± 0.015
Chloroform	> 1.6 ± 0.006
Ethyl acetate	0.172 ± 0.005
n-Butanol	1.520 ± 0.010
Dichloromethane	> 1.6 ± 0.007
10% Methanol Aqeous	0.180 ± 0.008
Standard (Gallic acid)	< 1.6 ± 0.002

Results expressed as IC_{50} ± SEM

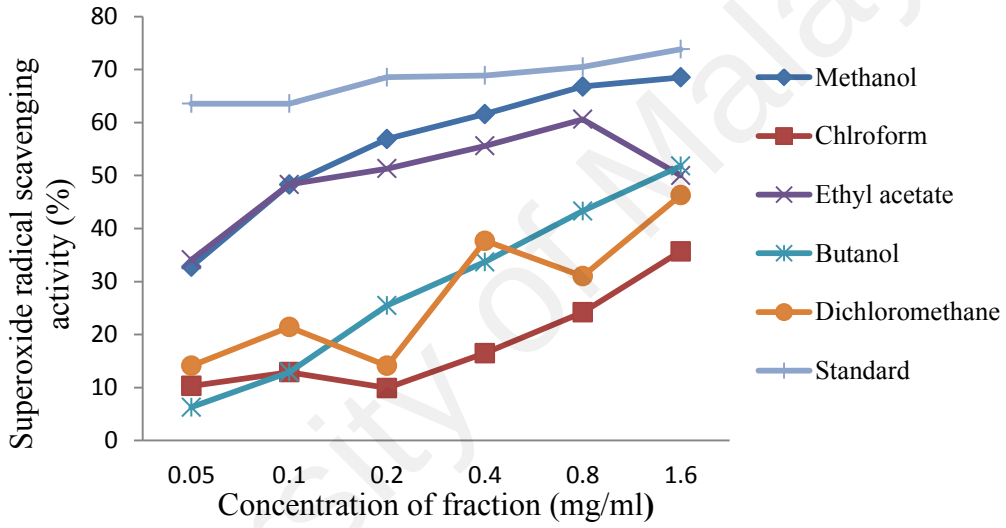


Figure 4.33 : The inhibitory activity of superoxide radical of *A. sinensis* fractions.

As shown in the Figure 4.33 and Table 4.26, each of fraction for scavenging activity ranging from 0.05 to 1.6 mg/ml are dose dependent. The highest extend of scavenging was methanol aqueous fraction at 68.5 % inhibition with IC_{50} value of 0.180 ± 0.008 mg/ml. It is the followed by ethyl acetate fraction at 50 % inhibition with IC_{50} value of 0.172 ± 0.005 and butanol fraction at 51% inhibition with IC_{50} value of 1.520 ± 0.010 mg/ml. The scavenging activities of hexane fraction, chloroform fraction, and dichloromethane fraction have shown poor inhibitory activity and IC_{50} value of higher than 1.6 mg/ml.

4.7. *In vitro* anti hyperglycaemic assay

4.7.1 Glycogen phosphorylase enzyme inhibitory assay

The assay was used to analyses the fractions for the GPa enzyme inhibition, which inhibit the release of phosphate from glucose-1-phosphate for the synthesis of glycogen. The fractions and caffeine as standard was assayed at the concentration ranging from 0.4 to 1.6 mg/ml.

Table 4.27: The IC_{50} of *Aquilaria sinensis* for glycogen phosphorylase inhibition.

Fractions	IC_{50}± SEM (mg/ml)
n-Hexane	>1.6 ± 0.07
Chloroform	ND
Ethyl acetate	>1.6 ± 0.09
n-Butanol	ND
Dichloromethane	>1.6 ± 0.10
10% Metanol aqeuous	>1.6 ± 0.05
Caffeine (Standard)	>1.6 ± 0.06

Results expressed as IC_{50}± SEM (mg/ml) and ND represents the value of undetected.

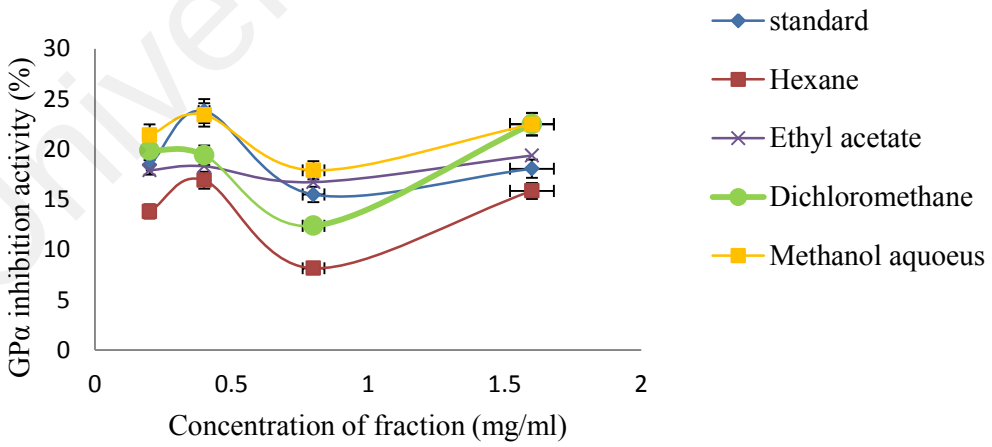


Figure 4.34 : Inhibitory activity of glycogen phosphorylase enzyme using *A.sinensis* fractions.

The results shown in Figure 4.34 and Table 4.27, represent the percentage inhibition of glycogen phosphorylase according to the each fractions that were used to evaluate the release of phosphate from glucose-1- phosphate. In this study, methanol aqueous fraction showed a good inhibition at 22.47 % with IC_{50} value of 1.6 ± 0.05 mg/ml, followed by dichloromethane at 22.5% with IC_{50} value of more than 1.6 ± 0.10 mg/ml, and ethyl acetate fraction at 19.37% with IC_{50} value of more than 1.6 ± 0.09 mg/ml.

The low inhibitory activity was observed in ethyl acetate fraction that contained high phenol contents which suggested that phenols was not appear effective in inhibition of glycogen phosphorylase in *A. sinensis* leaves fractions. Similarly, the other entire fraction showed poor inhibitory activity of glycogen phosphorylase. The phytochemical constituents in the *A. sinensis* fractions were ineffective to inhibit the carbohydrate hydrolysis enzyme.

4.7.2 Alpha glucosidase inhibitory assay

The alpha glucosidase inhibitor capable in retarding the digestion of carbohydrate and delays the process of absorption. This could reduce the post-prandial blood glucose of hyperglycemic patient to prevent absorption of carbohydrate after food intake. A high and good inhibition of alpha glucosidase reduces the high postprandial blood glucose level.In this study, the *A. sinensis* leave fractions were evaluated for the inhibitory of the alpha glucosidase enzyme and a satisfactory inhibitory activity was observed as shown in the Figure 4.35 and Table 4.28

Table 4.28: The IC_{50} of *A.sinensis* for alpha glucosidase inhibition activity.

Fractions	IC_{50}± SEM
n-Hexane	IC_{50} >1.6 ± 0.01
Chloroform	IC_{50} >1.6 ± 0.03
Ethyl acetate	IC_{50} < 1.6 ± 0.03
n-Butanol	ND
Dichloromethane	0.33 ± 0.06
10% Metanol aqeuous	ND
Standard	IC_{50} >1.6 ± 0.02

Results are expressed as IC_{50}± SEM

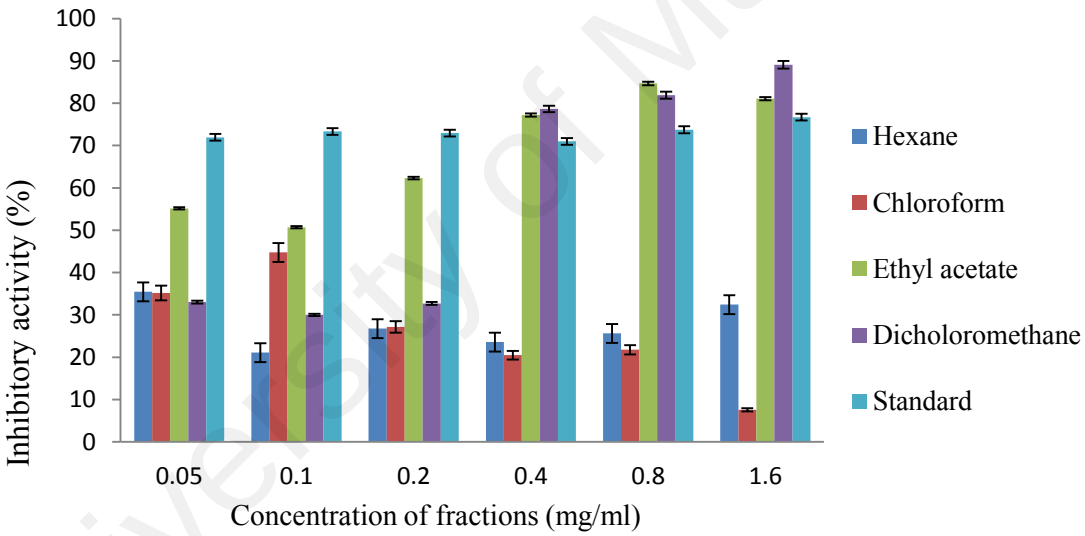


Figure 4.35: The inhibition of alpha glucosidase enzyme using *A. sinensis* fractions

The results shows,dichloromethane fraction demonstrated the highest inhibition of alpha glucosidase enzyme activity at 89.07% with IC_{50} value of 0.33 ± 0.060 mg/ml. The high inhibition was then followed by ethyl acetate at 81.07 % with IC_{50} value less than 1.6 ± 0.030 mg/ml. However, poor inhibitory was detected in *n*-hexane fraction with IC_{50} value more than 1.6 ± 0.010 mg/ml and in chloroform fraction with IC_{50} value more than 1.6 ± 0.030 mg/ml, respectively. However, *n*-butanol and 10%methanol aqueous fractions demonstrated undetected. The higher inhibitory activity might be contributed by presences of phenolic content in dichloromethane and ethyl acetate fraction.

4.7.3 Alpha amylase inhibitory assay

Alpha amylase is an enzyme which able to hydrolyse alpha bonds of alpha linked polysaccharide to yield high levels of glucose. However, the alpha amylase inhibitors bind to alpha bond of polysaccharide into monosaccharide. The *in vitro* alpha amylase inhibitory assay was evaluated with the fraction that showed good antioxidant activity.

The percentage of inhibition at 0.05,0.1,0.2,0.4,0.8,1.6 mg/ml concentration of fraction showed concentration dependent on reduction in percentage inhibition. Acarbose, the α-amylase inhibitor was used as a standard drug.

Table 4.29: The IC_{50} of *A. sinensis* for alpha amylase inhibition activity.

Fractions	IC_{50} (mg/ml) ± SEM
***n*- Hexane**	IC_{50} >1.6 ± 0.03
Chloroform	ND
Ethyl acetate	IC_{50} >1.6 ± 0.04
***n*- Butanol**	ND
Dichloromethane	ND
10 % Metanol aqeuous	IC_{50} >1.6 ± 0.05
Acarbose (Standard)	0.19 ± 0.012

Results are expressed as IC_{50} ± SEM (mg/ml) and ND as undetected

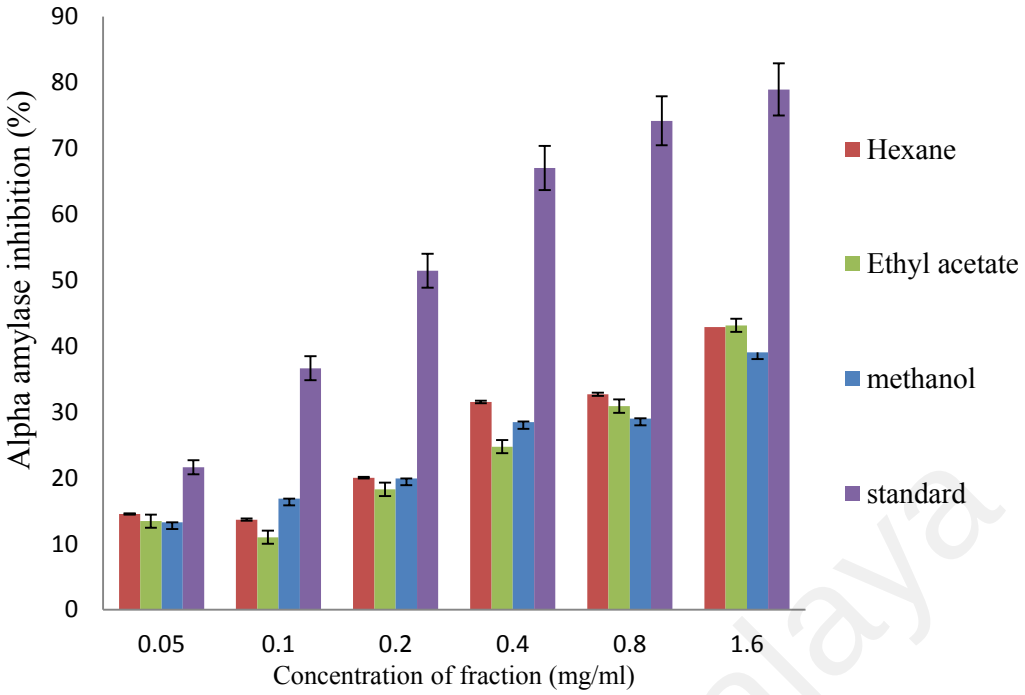


Figure 4.36: The inhibition of alpha-amylase enzyme by *A. sinensis* fractions

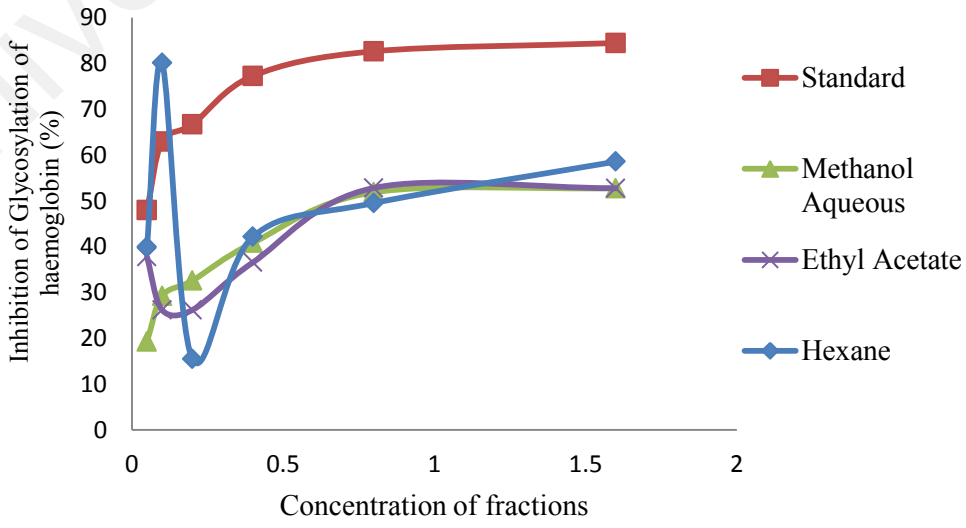
From the result obtained, the ethyl acetate fraction showed inhibition at 43.14 % with IC_{50} value less than 1.6 ± 0.04 mg/ml has demonstrated the strongest inhibition activity. The *n*-hexane fraction do showed high inhibition at 42.91% with of IC_{50} value less than 1.6 ± 0.03 mg/ml. The methanol aqueous fraction showed the lowest inhibition at 39% against the pancreatic α-amylase with IC_{50} value more than 1.6 ± 0.05 mg/ml. The concentration of for the inhibitor required more than 50% of inhibition (IC_{50}). The plant fractions were compared with acarbose as standard which showed a highest inhibitory activity at 78.96 % with IC_{50} value of 0.19 ± 0.012 mg/ml. However, none of the fractions were able to achieve the potential inhibitor requirement yet slightly lower than 50%.All the plant fractions were still appeared to inhibit the enzyme moderately as concentration dependent.

4.7.4 Non-enzymatic hemoglobin glycosylation assay

An increase in the glycosylation was observed on the incubation of 24, 48 and 72 hours especially in the ethyl acetate fraction. The plant fractions significantly inhibited the haemoglobin glycosylation which then increases free haemoglobin concentration. This indicates this plant exhibit a potent anti hyperglycaemic activity with dosage dependent for percentage of haemoglobin glycosylation inhibition. It can be concluded that the concentration of fraction increases with the free haemoglobin and decreases the formation of glucose- haemoglobin complex.

i. Incubation of 24 hours:

A good increase in the inhibition of haemoglobin glycosylation was observed along the measurement of free haemoglobin at 24 hour incubation. The *n*-hexane fraction exhibited the highest inhibition at 58.55% with IC_{50} value of 0.84 ± 0.01 mg/ml. Meanwhile, the ethyl acetate and methanol aqueous have exhibited with good inhibition at 52.74% with IC_{50} value of 0.74 ± 0.008 mg/ml and 52.76% with IC_{50} value of 0.72 ± 0.006 mg/ml, respectively. In comparison to the standard, gallic acid had exhibited strongest inhibition at 82.42 % with IC_{50} value more than 1.6 ± 0.004 mg/ml.



. **Figure 4.37 :** Inhibition of glycosylation of haemoglobin at 24 hours by *A. sinensis*

Table 4.30 : The IC_{50} of haemoglobin glycosylation of *A. sinensis* at 24 hours

Fractions	IC_{50}± SEM (mg/ml)
***n*-Hexane**	0.08 ± 0.01
Chloroform	ND
Ethyl acetate	0.74 ± 0.008
***n*-Butanol**	ND
Dichloromethane	ND
10 % Metanol aqeuous	0.72 ± 0.006
Gallic acid (Standard)	IC_{50} < 1.6 ± 0.004

Results are expressed as IC_{50}± SEM (mg/ml) and ND as undetected

ii. Incubation of 48 hours:

The inhibition of haemoglobin glycosylation was observed along the measurement of free haemoglobin at 48 hour incubation. The highest inhibition was observed from the methanol aqueous fraction at 45.58% with IC_{50} value more than 1.6 ± 0.04 mg/ml. However, a slight decrease in percentage of methanol aqueous extract inhibition was observed. Meanwhile, the ethyl acetate fraction exhibits inhibition at 41.76% with IC_{50} value more than 1.6 ± 0.04 mg/ml and hexane fraction at 43.82% with IC_{50} value more than 1.6 ± 0.07 mg/ml. A slight decrease in the inhibitory activities was observed in the assay for all the fractions except standard drug. The gallic acid had still exhibited strongest inhibition at 84.35% and IC_{50} value of 0.17± 0.02.

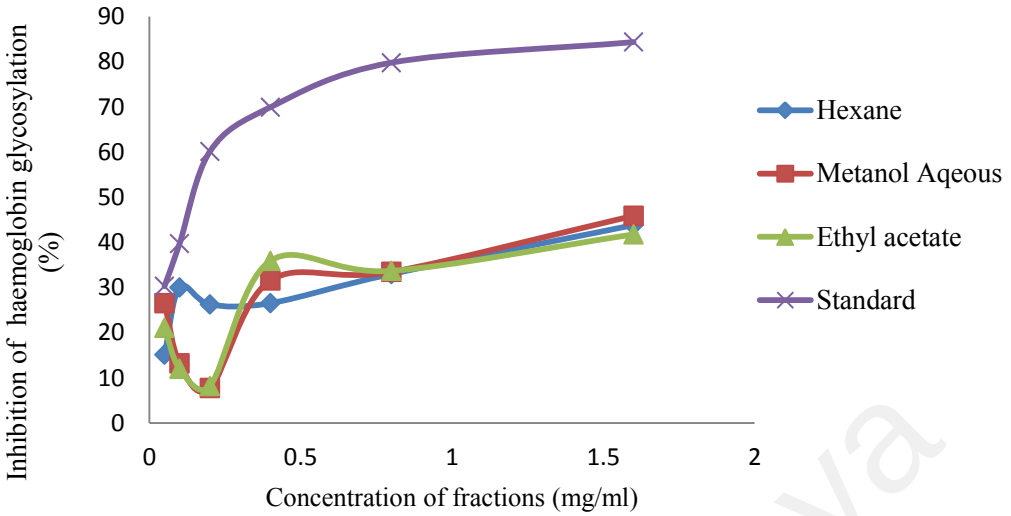


Figure 4.38: The inhibition of glycosylation of haemoglobin at 48 hours by *A. sinensis* fractions.

Table 4.31 : The IC_{50} of haemoglobin glycosylation of *A. sinensis* at 48 hours

Fractions	IC_{50} ± SEM (mg/ml)
***n*-Hexane**	IC_{50} >1.6 ± 0.07
Chloroform	ND
Ethyl acetate	IC_{50} >1.6 ± 0.04
***n*-Butanol**	ND
Dichloromethane	ND
10 % Metanol aqeuous	IC_{50} >1.6 ± 0.04
Gallic acid (Standard)	0.17 ± 0.02

Results are expressed as IC_{50}± SEM (mg/ml)

iii. Incubation of 72 hours:

The inhibition of haemoglobin glycosylation was observed along the measurement of free haemoglobin at 72 hour incubation. The highest inhibition was observed from the ethyl acetate fraction at 65.25% with IC_{50} value of 0.873 ± 0.12 mg/ml.Meanwhile, the methanol aqueous fraction exhibited inhibition at 63.39% with IC_{50} value of 0.77 ± 0.03 mg/ml and hexane at 63.87% with IC_{50} value of 0.91 ± 0.20 mg/ml .

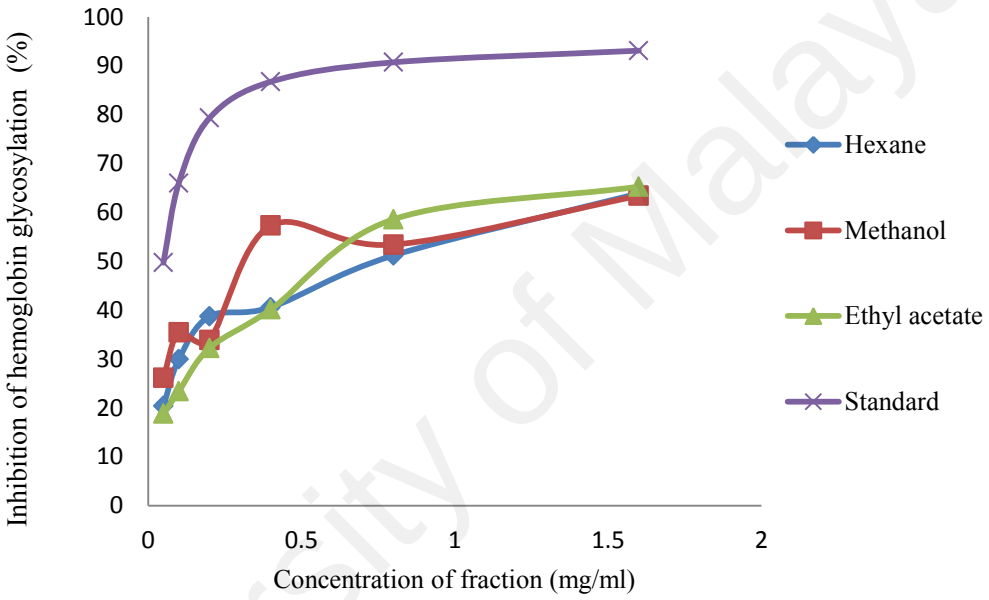


Figure 4.39: The inhibition of glycosylation of haemoglobin at 72 hours by *A. sinensis* fractions.

Table 4.32: The IC_{50} of haemoglobin glycosylation of *A. sinensis* fractions at 72 hours

Fractions	IC_{50} ± SEM (mg/ml)
***n*-Hexane**	0.91 ± 0.20
Chloroform	ND
Ethyl acetate	0.87± 0.12
***n*-Butanol**	ND
Dichloromethane	ND
Metanol aqeuous	0.77 ± 0.03
Standard	0.05 ± 0.003

Results are expressed as IC_{50} ± SEM (mg/ml).

A slight increase and improvised inhibitory activities was observed in the assay for all the fractions as compared from 48 hours incubation. In comparison to the standard, Gallic acid had still exhibited at 93.09% with IC_{50} value of 0.05 ± 0.003 mg/ml, as the most strongest inhibition. In this assay, the ethyl acetate fraction have shown a good progress on inhibiting the glycosylation of haemoglobin and have increases the free amount of haemoglobin. The ethyl acetate fraction indicates as the most potent glycosylation inhibitor and therefore can efficiently prevent from the formation of HbA_{1c}

CHAPTER 5

DISCUSSION

One of the applicable strategies for the management of diabetes is through the usage of natural antioxidative agents and inhibitors of glucose producing enzymes. It can be a therapeutic option with free of side effects for treating hyperglycemia condition associated with oxidative stress. This study investigated the bioactive phytochemical compound presented in *Aquilaria sinensis* leaves which able to act as anti-oxidative agents and possesses anti-diabetic properties.

The sequential extraction using solvents of increasing polarity were used to extract the bioactive compounds from the *A. sinensis*. A solvent with low polarity such as hexane, functions to extract compounds with low or non-polarity such as alkaloids, terpenoids aglycones and fatty acids (Ayaffor *et al.,* 1994). Besides that, polar solvents including methanol and ethyl acetate were used for the extraction of hydrophilic compounds. For the extraction of lipophilic compounds, solvents as dichloromethane, hexane and chloroform were used (Cosa *et al*., 2006). Using *n*-butanol solvent, fatty acid compound can be extracted from the leaves. Yield of each solvent was obtained as shown in Table 4.1. The differences of yield fractions might be due to availability of extractable compound in fractions and various chemical composition (Sultana *et al.,* 2009).

In the present study, the phytochemical screening using Thin Layer Chromatography (TLC) have revealed that *A. sinensis* leaves fraction significantly showed the presences of alkaloids, phenolics, and terpenoids in the sequential fractions.

Based on the previous studies by Bahrani *et al. (*2014), proved the existence of alkaloids, flavonoids, terpenoids and phenols in *A.sinensis* species leaves. The presences of secondary metabolites indicates the plant have potential antioxidant activity and could possess anti hyperglycaemic properties.

From the LCMS analysis, the aqueous leaves of *A.sinensis* has shown several presences of bioactive compound which includes mangiferin, hypoxanthine, norswertianolin, tripteroside and stearamide. Each of the compounds magnifies its own special medical properties and comes from different subclass of phytochemical compound. However, the most related antidiabetic compound, mangiferin that is known as anti hyperglycaemic compound by Ichiki *et al.* (1998)

Mangiferin is known as xanthanoid and phenolic compound, which is commonly found in mangoes. It is first xanthones to be investigated for pharmacological studies and biological activities. Based on previous research, mangiferin was reported to have anti-hyperglycemia properties, antioxidant, antitumor and antibacterial properties. Studies have shown that mangiferin are capable in reducing plasma blood glucose level in rodent model, significantly are capable in reducing glycosylated hemoglobin and increases the antioxidant activity (Periyar *et al.,* 2012). On the other hand, presences of hypoxanthine are capable of protecting the oxidant-induced cell injury by inhibiting the activation of protein. The natural hypoxantine owns the antioxidant properties and anti-infammatory properties which is favourable for diabetes complication (Fleischhacker *et al.,* 2010). Meanwhile, presence of Norswertianolin was analyzed from 10% methanol aqueous fraction, which has the functional as central nervous depressant and anti-tuberculostatic properties (Amritpal, 2008). The tripteroside compound possess as a strong inhibitor of platelet aggregation (Lin *et al.,* 1993). Studies have proved that, the dysfunction of platelet in diabetes mellitus often leads to cardiovascular complication.

In the study, presences of secondary metabolite, tripetoside compound capable of regulating the platelets and minimize the platelet aggregation response by oxidative stress in hyperglycemic patient (Nicholas *et al.,* 2011).

Plant phenolics are known as major class of bioactive constituents as it does have free radical scavenging activity. These constituents are capable to scavenge radicals due to the high reactivity of hydrogen and electron donor as well chelating activity. Hence, total phenolic content was evaluated using the plant fractions of *A. sinensis* and expressed as mg/GAE/g dry weight. The phenolic content are highly evaluated in the ethyl acetate fraction at 1.69 ± 0.02 mg GAE/g dry weight and *n*-butanol fraction was 1.45 ± 0.04 mg GAE/g dry weight.

In general, flavonoids are also known to be effective as free radical scavengers and possess antioxidant properties. Therefore, total flavonoid content was evaluated using the plant fractions. Apparently, TFC content was highly detected in ethyl acetate (2.40 ± 0.04 mg QE/g dry weight) and *n*-butanol (3.59 ± 0.07 mg QE/g dry weight). The results suggested that effectiveness of antioxidant could correlated with the high flavonoid content of the plant fractions

About five complementary assay was conducted to determine and evaluate the anti-oxidative profile of the plant fractions. The antioxidant assays includes the scavenging ability on free radicals and chelating abilities of metal.

The DPPH radical is the most common and intensive method to determine *in vitro* antioxidative activity of compounds in the plant fractions. It able to study the neutralizing effect of the compound presented in the crude fractions on the free radicals. Antioxidants do scavenge the DPPH radical by the ability of donating hydrogen. The DPPH assay holds on the principle that a hydrogen atom donor is an antioxidant.

Hence, DPPH is commonly used as substrate to investigate the radical scavenging abilities of antioxidative agents. The reduction of DPPH radical is highly dependent on the amount of antioxidant presents in the fractions.

In this study, the scavenging activity of *A. sinenis* plant fractions was compared with a standard, ascorbic acid which is naturally known as antioxidant. The concentrations are required to inhibit 50 % radical scavenging effect, which is known as $IC_{50.}$ However, all of the fractions exhibited scavenging activities in a concentration-dependent manner. The scavenging ability of the plant fractions demonstrated almost 2 fold lower than the standard (IC_{50}= 0.08 ±0.03 mg/ml). The results reveal that these fractions have significant DPPH radical scavenging activity and imply the abundances of antioxidative phytochemical presents in the leaves of *A. sinensis* especially in the ethyl acetate fraction which shows good scavenging ability with IC_{50} 0.10 ± 0.02 mg/ml. On the other hand, butanol fraction has showed a good scavenging abilities with a slight higher IC_{50} 0.12 ± 0.02 mg/ml compared to the standard and ethyl acetate fraction.Similar studies using *Aquilaria* species was conducted and methanol and ethyl acetate fraction had showed highest antioxidant activity (Hadi *et al.,* 2016). Ethyl acetate fraction may include phenolic or glycoside compounds, which associated with the antioxidant activity of the plant. This further suggested that the plant do have the ability to scavenge free radicals and remediate oxidative stress associated metabolic disease especially diabetes.

Based on the previous research, phytochemical compound that has capacity of reducing power are potential source of antioxidant activity. Therefore, the ferric reducing antioxidant power (FRAP) assay was also been carried out using the plant fractions. The basic principle behind the assay was to evaluate the ability of *A. sinensis* to reduce the ferric ion into ferrous ions. It is also known that only electron-donating antioxidant compounds are capable for reduction of the Fe^{3+} complex of tripyridyltriazine ($Fe(TPTZ)^{3+}$) to the intensely blue colored Fe^{2+} complex ($Fe(TPTZ)^{2+}$) . Based on the evaluation of the plant fraction, ethyl acetate (0.3681 mmol/g) do have the highest ability of reducing power activity and followed by dicholoromethane (0.127 mmol/g).

A moderate reducing power was detected in chloroform fraction (0.096 mmol/g) and butanol fractions (0.029 mmol/g).The ethyl acetate fraction, which has highest TPC, TFC has strong correlation with the reducing power capability. This could be due to the presences of the phenolic compound in the fractions.

The generation of reactive oxygen species also generated as by-products of the mitochondrial electron transport chain. However, the reduced metals may undergo the Fenton reaction and forms radicals that lead to oxidative stress. One of the important mechanisms of antioxidant is to have the abilities to chelate metals and catalyze Fenton reaction. Metal chelating capacity is significant as it able to reduce the excessive transition metal ions which able to contribute to lipid peroxidation if it‟s untreated (Aboulenein *et al.,* 2003). The chelation capacity was evaluated using the *Aquilaria sinensis* leaves fractions, which resulted most of the fractions exhibited as good chelators. The metal chelating activity of the plant fractions showed a great significance, as it can be potent chelator of the transition metal ions, which contributes to the oxidative stress in diabetic complications. Metal chelation therapy also well-known treatment for the patients with diabetic nephropathy. In the results of current study, suggested that the metal chelation ion of natural phytochemical constituents in ethyl acetate fraction of *A. sinensis* could be functional as therapeutic approach (Rasleen *et al.,* 2014).

Nitric oxide is a free radical with unpaired electron that mainly involved in the regulation of various physiological processes. The most common regulation includes the neurotransmission, antimicrobial and vascular homeostasis.

Nevertheless, the excessive production of nitrite ions could cause oxidative stress and chronic diseases. The natural source of antioxidants could be most prominent way of encountering the excessive production of nitrite ions. The scavengers of nitric oxide radical have the ability to disrupt the interaction of oxygen and reduce the generation of

nitrite ions (Miller, 1993). The potential scavengers do act as proton donor, and simultaneously decreases the absorbance, which is an effective for the measurement of nitric oxide inhibitor.

In this study, nitric oxide radical scavenging activity was accessed with the plant fractions of *A. sinensis* leaves and result was analyzed. Curcumin was used as standard which gives IC_{50} value of 0.89 ± 0.012 mg/ml. Based on the results, poor reduction of nitrite oxide radicals was observed in all of the fractions. The degree of nitric oxide inhibition was found to be ineffective by concentration of the plant fractions. This indicates that the fractions may not contain bioactive compound which has capability to inhibit the generation of nitric oxide radicals. It could also be suggested that properties of phenolic compound is the plant inactive in the scavenging of NO in *A. sinensis* leaves.

Superoxide radical is known as one of the strongest reactive oxygen species and generated when NADH oxidase takes up electron from NADH. However, the excess production of the radical often leads to oxidative stress and cellular damage. In order to determine the plant fractions are capable of neutralizing and scavenge the superoxide radical, *in vitro* superoxide radical scavenging assay were conducted. In the assay, the superoxide radicals are generated in PMS-NADH by oxidizing NADH and assayed by the measurement of reduced nitroblue tetrazolium (NBT).

The decrease of absorbance reading with the potential antioxidants indicates the consumption of superoxide anion in the assay. As shown in Table 4.26 and Figure 4.33, the IC_{50} value for the inhibition of superoxide radicals using the plant fractions and quercetin as standard. Methanol aqueous fraction IC_{50} value of 0.180 ± 0.008 mg/ml have exhibited highest scavenging activity among the rest of fractions and yet slightly lower than standard IC_{50} value lesser than 1.6 mg/ml, respectively.

However, the lowest scavenging ability of superoxide radical was evaluated in ethyl acetate fraction at 0.172 ± 0.005 mg/ml as it is detected as poor inhibitor for superoxide radicals. A decrease in absorbance was observed in 10 % methanol aqueous fraction, which indicates the consumption of superoxide radical and antioxidant activity of the plant. The effective inhibitor suggested to contain phenolic compound in the fraction to contribute the scavenging activity.

Based on the results obtained from the assays, the fractions which have good antioxidant activity was subjected to *in vitro* anti hyperglycaemic assay for further analysis.

The failure of insulin to suppress the output of hepatic glycogen phosphorylase, has been concerned in the management of diabetes. One of the best therapeutic approaches for the natural anti hyperglycaemic source is through inhibition of glucose phosphorylase enzyme which is highly elevated in diabetic patients. The hepatic glucogen phosphorylase enzyme do catalyzes and break down glycogen into glucose-1-phosphate. The catalyzation causes glycogenolysis and gluconeogenesis contribute to the elevated blood glucose level. The Inhibition of hepatic glycogen phosphorylase can able to stop the catalyzation and suppress the production of glucose.

Therefore, the plant fractions of *A. sinensis* leaves were screened for the inhibition of glycogen phosphorylase enzyme. The glycogen phosphorlyase α activity of *A. sinensis* was evaluated against GPα (Rabbit muscle-Sigma) in the direction of glycogen synthesis with the release of phosphate from glucose-1-phosphate.The plant fractions exhibited lesser inhibition activity compared to the standard, caffeine. The poor inhibitory activity was observed in ethyl acetate which is highly phenol content and suggested that phenols do not appear effective in inhibition of glycogen phosphorylase in *A. sinensis* leaves.

The alpha-amylase enzyme or known as intestinal digestive enzyme. It can be a good strategy to lower the postprandial blood glucose levels by inhibiting the alpha amylase enzyme. Although alpha amylase inhibitor does exist in the pharmaceutical practice to manage diabetes, it is still prone to gastrointestinal side effects.

Hence, considerations were given to explore natural source of alpha amylase inhibitor and free from undesirable side effects. Based on research findings, it have proved that the phenolic compounds are capable of inhibiting alpha amylase and maintaining postprandial hyperglycemia (Ani, 2008). In the current study, the plant fractions of *A. sinensis* leaves were investigated for the potential inhibitor of α-amylase enzyme. The results suggested that the pancreatic α-amylase successfully inhibited by the polar and non-polar components of the plant fractions. The standard drug, acarbose was shown to be potent inhibitor of α-amylase. Meanwhile, plant fractions have exhibited significant inhibition which shown as good potency inhibitor. An inhibition highly dependent on concentration was observed in the plant fractions and standard drug. The ethyl acetate fraction showed highest inhibition at 43.14% compared to rest of fractions. The finding suggested that inhibition of the enzyme could be due to the presences for phenolic compound in the plant fractions. Based on previous research on *in vitro* studies, the presences of flavonoids and polyphenols could attribute to the α-amylase inhibitory activity (Jung *et al.,* 2006).

Alpha glucosidase is a carbohydrate metabolism enzyme, which is capable of degrading complex carbohydrates to form monosaccharaides units, which is glucose. The degradation causes release of non-reducing sugar which is easily to be absorbed into the intestinal. The enzyme α-glucosidase in pancreatic and intestinal does carry out hydrolysis of disaccharide. High and frequent absorption of monosaccharide results into elevated level of postprandial blood glucose level especially in diabetic patient. Therefore, inhibitors of carbohydrate hydrolysis enzyme are in the need of controlling the blood glucose level. In this study, the plant fractions have significantly showed a

strong inhibition of α-glucosidase enzyme with IC_{50} values higher the standard drug, acarbose. This do indicate that the plant fractions are capable potent inhibitor than acarbose and most likely to exert a competitive type of inhibition (Kim *et al.,* 2005).

However, the highest inhibitory activity was detected in dichloromethane fraction at 89.07% and ethyl acetate fraction at 81.06%. Thus, the ethyl acetate and dichloromethane fractions contains a good inhibitor which is capable of binding to the active site and allosteric site of the α-amylase enzyme. It can further implicit that the fractions are capable for the reduction of complex carbohydrate hydrolysis and consequently lowers the postprandial blood glucose level. The inhibitors of α-glucosidase would be medium polarity chemical compound from the plant fractions. Based on the findings, the presence of phenolic, alkaloid and terpenoids as attributed by previous *in vitro* studies, it contributes to the inhibition activity (Andrade *et al.,* 2007).

Glycosylated hemoglobin is also known as glycohemoglobin or as hemoglobin A1C (HbA_{1C}). The glycosylated hemoglobin do help in reflecting long term average glucose level and indicator of diabetic control. The normal level of glycosylated hemoglobin is (<7%) which is rarely achieved by the hyperglycemic conditions. The formation of glycosylated hemoglobin takes place when the hemoglobin exposed and binds to plasma glucose. The amount of glycosylated hemoglobin is higher in hyperglycemic condition, due to poor control of plasma glucose level and leads to generation of reactive oxygen species (Adisa *et al.* 2004). However, natural sources as plants have tendency to increases free hemoglobin by inhibiting glycosylation and advanced glcosylation end products (AGEs).

In this study, plant fractions from *A. sinensis* leaves were screened for the inhibition of the non-enzymatic hemoglobin glycosylation. The plant fractions have successfully displayed a good inhibition of hemoglobin glycosylation over the period of 72 hours.

The percentage of inhibitory activity of the standard and plant fractions was based on concentration dependent.

The plant fractions significantly inhibited the haemoglobin glycosylation which then increases free hemoglobin concentration. The ethyl acetate fraction of *A. sinensis* exhibited the highest inhibition of glycosylation yet lower compared to the standard drug. However, it reached nearly 65.25 % over the period of 72 hours. It helped to indicate that the plant fractions are capable to decrease the hemoglobin-glucose complex formation and increases free hemoglobin. This results do suggested the inhibition could be attributed to presence of bioactive compound like alkaloids, flavonoids and phenolic compounds. According to Seddighe *et al.* (2002), flavonoids in plant capable of inhibiting glycosylation of hemoglobin.

This could deliberately suggest that presences of the bioactive compound, mangiferin, hypoxanthine, norsewalin and tripteroside could be the contributor of the plant extract to scavenge several types of radicals and as well by inhibiting the glucose producing enzymes, and hemoglobin glycosylation (Lian *et al.,* 2008). The presences bioactive compound in *A. sinensis* leaves, capable to regenerate the β-cells of pancreas, which could be highly potent anti hyperglycaemic drug for better therapeutic approach.

CHAPTER 6

CONCLUSIONS

Based on the compilation of experiment results, *Aquilaria sinensis* leaves contain various amount of phenolic and flavonoid compound, which contribute and influenced the antioxidant and anti hyperglycaemic properties.The rich total phenolic content in ethyl acetate fraction has exhibited good activity in DPPH, FRAP, Nitric oxide and superoxide radical scavenging activity assays. Furthermore, the ethyl acetate fraction showed good results in the anti hyperglycaemic assay especially in 72 hours of haemoglobin glycosylation. Good scavenging properties and enzyme inhibitory abilities might be due to the presences of Mangniferin and Tripteroside compound , which were analysed in LCMS. These findings suggested that *A. sinensis* could be a potential source of natural antioxidant having great importance as therapeutic agent and preventing oxidative stress related degenerative diseases mainly diabetes. As it can be a good therapeutic approach in managing diabetes efficiently. The plant can be also served as daily dietary of patient in terms of tea or drinks. In conclusion, the findings based on all the antioxidant assay and anti hyperglycaemic assay showed that *A. sinensis* have a high potential to be explored further to identify the anti-diabetic compound presented in the plant. The current study appeared to be the first to study on the additional antioxidant assay; superoxide radical scavenging activity, nitric oxide radical scavenging activity and anti hyperglycaemic assay of non-enzymatic hemoglobin glycosylation inhibitiory assay and alpha amylase inhibitory activity of *A. sinensis* leaves.

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APPENDIX

7.1 Thin Layer chromatography of cholorform xtract of *Aquilaria sinensis*

Detection under UV light- Bands emitted colors

Vannilin-H_2SO_4- Positive purple bands observed as terpenoid compound

Drangendroff‟ reagent- Positive orange bands observed as alkaloid compound

Phenol‟s reagent – Positive green bands observed as phenolic compound

7.2 .1 *In vitro* DPPH radical scavenging assay experimental data using *Aquilaria sinensis*

Extract	Mean	Standard Deviation	Inhibition activity
Hexane	30.49219	15.4217	41 %
Chloroform	50.56667	18.96545	76.14 %
Ethyl actate	66.43	16.92406	83.81 %
Butanol	65.71667	16.59329	83.24 %
Dichloromethane	20.52	5.361563	29.26 %
Methanol aqueous	53.405	25.71337	65.5 %
Standard	74.61667	20.9293	87.5%

7.2.2: FRAP assay experimental data using *Aquilaria sinensis*

Extract	Mean	Standard Deviation	Standard Error Mean
Hexane	0.2755	0.012021	0.0085
Chloroform	0.5725	0.003536	0.0025
Ethyl actate	0.728	0.098288	0.0695
Butanol	0.7275	0.035355	0.025
Dichloromethane	0.2815	0.002121	0.0015
Methanol aqueous	0.2315	0.00495	0.0035

7.2.3 : Metal chelating activity experimental data using *Aquilaria sinensis*

Extract	Mean	Standard Deviation	Chelating activity (%)
Hexane	81.045	1.259583	81.2%
Chloroform	79.6	1.157584	79.4%
Ethyl actate	76.5	2.427344	72.5%
Butanol	77.9	1.632176	80.15%
Dichloromethane	78.56667	2.362767	80.1%
EDTA	83.64333	19.87723	96.56%

7.2.4: Nitric oxide radical scavenging assay (NORSA) experimental data using *Aquilaria sinensis*

Extract	Mean	Standard Deviation	Standard Error Mean	Inhibition activity (%)
Hexane	0.143917	3.88123	1.584506	9.53
Chloroform	0.146333	5.729448	2.339037	15.46
Ethyl actate	0.137	6.341468	2.588893	17.11
Butanol	0.132083	2.957794	1.207514	15.46
Dichloromethane	0.141667	3.799833	1.551275	10.86
Methanol aqueous	0.136	3.206093	1.308882	14.14
Curcumin	0.39625	17.99371	7.345901	66.67

7.2.5: Superoxide radical scavenging activity experimental data

Extract	Mean	Standard Deviation	Inhibition activity (%)
Hexane	20.25	14.91291	21.2
Chloroform	18.25	10.04505	35.7
Ethyl actate	49.98333	8.956655	50
Butanol	28.915	17.51331	51.8
Dichloromethane	27.43333	13.1608	46.3
Methanol aqueous	55.81333	13.44105	68.5
Standard	68.15167	4.015378	73.84