ANTIOXIDATING EFFECT OF *Ampelocissus* sp. (ISI NYARU) ON STREPTOZOTOCIN-INDUCED DIABETIC RATS

ELLY ZULAIKHA ZAMRI

FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2020

ANTIOXIDANTING EFFECT OF *Ampelocissus* sp. (ISI NYARU) ON STREPTOZOTOCIN-INDUCED DIABETIC RATS

ELLY ZULAIKHA ZAMRI

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2020

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **ELLY ZULAIKHA BINTI ZAMRI**

Matric No: **SGF140010**

Name of Degree: **MASTER OF BIOTECHNOLOGY**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

ANTIOXIDATING EFFECT OF Ampelocissus sp. (ISI NYARU) ON STREPTOZOTOCIN - INDUCED DIABETIC RATS

Field of Study: **PLANT BIOTECHNOLOGY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this work;
(2) This work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do ought I reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation

ANTIOXIDATING EFFECT OF *Ampelocissus* sp. (ISI NYARU) ON STREPTOZOTOCIN-INDUCED DIABETIC RATS

ABSTRACT

The *Ampelocissus* sp. locally known as Isi Nyaru has been traditionally used by native Malaysian for medical purpose. The tuber was extracted with hexane, petroleum-ether, chloroform, methanol and water solvents. The seperation and detection of bioactive compounds were done using Thin Layer Chromatography (TLC) and Gas Chromatography Mass Spectrometry (GCMS) methods. The TLC results showed the presence of phenolic acids, flavonoids and terpenoids. While in GCMS has revealed the presence of hydrocarbon groups such as alkene, ester and ether, amino acid groups, dicarboxylic acid and cyclic aromatic compound. The total phenol content and total flavonoid content were highest in methanolic extract at 748.50 ± 0.01 mg (TAE)/g and 27.08 ± 0.04 mg (QE)/g respectively. The extracts were tested for antioxidant activities by DPPH radical scavenging activity, ferrous ion chelating activity (FCA) assays and ferric reducing antioxidant power (FRAP) assays. The methanol and water extracts rich in phenol and flavonoid based on Thin Layer Chromatography (TLC) result, exhibit the strongest antioxidant activities to DPPH, FCA and FRAP. According to IC_{50} reading of DPPH and FCA, methanol extract indicated 1.27 µg/mL and 1.72 µg/mL respectively while water extract indicated 1.22 µg/mL and 1.62 µg/mL respectively. The FRAP value for water extract is 11.73 mmol/g and 11.58 mmol/g for methanol extract. The Streptozotocin (STZ) diabetic-induced rats have been treated by 4000 mg/kg of methanol extract in acute oral toxicity test with no toxicity effects observed in rats. Methanol extract has been chosen for the anti-diabetic treatment of diabetic rats based on its free toxicity effects and has the largest yield production. The blood glucose

concentration of methanolic-treated diabetic rats showed the significant reduction which 17.4% (800 mg/kg methanol extract) and 12.4% (800 mg/kg methanol extract), while 21% reduction in glibenclamide-treated diabetic rats. There are slightly increased of body weight in diabetic rats treated with 800 mg/kg and 400 mg/kg methanol extract and glibenclamide which are 1.7%, 2.6% and 3.3% respectively. The activity of antioxidant enzymes which are catalase (Cat) and glutathione peroxidase (GPx) in diabetic rats showed a positive result when treated with extracts and standard. The results concluded that the antioxidant activity of methanol extract was primarily due to the high amount of phenolic and flavonoid content.

Keywords: *Ampelocissus* sp. (Isi Nyaru), streptozotocin (STZ) diabetic-induced rats, antidiabetic, polyphenolics, antioxidant enzymes

KESAN *Ampelocissus* sp. (ISI NYARU) TERHADAP TEKANAN OKSIDATIF TIKUS TERARUH DIABETIS OLEH STREPTOZOTOCIN

ABSTRAK

Ampelocissus sp. atau lebih dikenali sebagai Isi Nyaru telah lama digunakan oleh masyarakat tempatan sebagai bahan yang mampu merawat komplikasi penyakit. Sampel ubi dari *Ampelocissus* sp. (Isi Nyaru) telah diekstrak dengan pelarut hexane, petroleum-ether, klorofom, metanol dan air. Pengasingan dan pengesanan sebatian bioaktif telah dilakukan dengan menggunakan kaedah Thin Layer Chromatography (TLC) dan Gas Chromatography bergabung dengan Massa Spectrometri (GCMS) . Keputusan TLC menunjukkan kehadiran fenolik asid, flavonoid dan terpenoid. Manakala GCMS menunjukkan kehadiran sebatian kumpulan hidrokarbon seperti alkene, ester and ether, kumpulan asid amino, asid dicarboxylic and komponen gelung aromatik. Jumlah kandungan fenol dan flavonoid paling banyak dalam ekstrak metanol iaitu 748.50 ± 0.01 mg (TAE)/g dan 27.08 ± 0.04 mg (QE)/g. Pengasaian termasuk DPPH, FCA dan FRAP serta menguji kandungan polifenolik bagi setiap ekstrak. Metanol ekstrak menunjukkan IC_{50} 1.27 µg/mL bagi DPPH dan 1.72 µg/mL bagi FCA. Manakala IC_{50} bagi ekstrak air adalah 1.22 µg/mL bagi DPPH dan 1.62 µg/mL bagi FCA. Nilai FRAP tertinggi adalah dari ekstrak air (11.73 mmol/g) diikuti dengan metanol ekstrak (11.58 mmol/g). Aktiviti anti-diabetik seterusnya dilanjutkan secara *in vivo* menggunakan tikus teraruh diabetis oleh STZ. Tikus yang teraruh diabetis telah dirawat mengggunakan ekstrak metanol pada dos 4000 mg/kg bagi ujian OGTT dan tiada kesan toksik direkod pada tikus yang diuji. Ekstrak metanol telah dipilih bagi ujian selanjutnya berdasarkan bebas toksik dan penghasilan kuantiti ektrak yang tertinggi. Kepekatan glukosa darah tikus yang

teraruh diabetis yang dirawat oleh 800 mg/kg ekstrak metanol, 400 mg/k ekstrak metanol dan glibenclamide menurun sebanyak 17.4%, 12.4% dan 21. Berat tikus juga mempunyai sedikit peningkatan pada kumpulan yang sama iaitu sebanyak 1.7%, 2.6% dan 3.3%. Selain itu, aktiviti enzim antioksidan iaitu CAT dan GPx pada tikus teraruh diabetis yang terawat menunjukkan keputusan yang positif berbanding dengan tikus kawalan. Dapat disimpulkan bahawa penyumbang utama aktiviti antioksidan ekstrak metanol disebabkan oleh kandungan fenolik dan flavonoid yang tinggi.

Katakunci: *Ampelocissus* sp. (Isi Nyaru), tikus teraruh diabetis oleh STZ, anti-diabetik, polifenolik, enzim anti-oksida

ACKNOWLEDGEMENTS

In the name of Allah SWT, the Most Gracious and the Most Merciful. All praise is to Allah. First and foremost, I offer my sincere gratitude to my supervisor Associate Professor Dr. Jamaludin bin Mohamad for his continuous support, an exemplary guidance, monitoring, patience and constant encouragement throughout my Master research. The blessing, help & knowledge given by him time to time shall carry me a long way in the journey of life which I am about to embark.

Special thanks to my beloved mother and father, Hajah Nor Arini binti Abd Majid and Haji Zamri bin Hamzah for their endless love and support. Although I have been through a lot to complete the dissertation and almost give up at the end, they keep encouraged me to finish what I have started and help me to recover from financial difficulties. A big thanks to my lovely husband, Mohamad Azeem bin Mohamad Azman who have been very supportive throughout the journey. The greatest gift from Allah despite the ups and downs moments is my precious daughter, Annur Alyssa. She is a strongest backbone and enlighten my spirit to complete this journey.

My deepest appreciation also goes to my classmates, Syakirah Nurizzati, Rosniyati and Ilya Farhana for the helps and guidance in order to continue my writing till the end. A deeply grateful to all staff from Institute Science Biology, Faculty of Science, University of Malaya and all staff from LAB for their valuable assistance.

Last but not least, thanks to all my family members and friends for the endless moral support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
°C	:	Degree celcius
µl	:	Microlitre
µM	:	Micromolar
µg	:	Microgram
U	:	Unit
L	:	Litre
ml	:	Millilitre
mm	:	Milimeter
cm	:	Centimeter
nm	:	Nanometer
M	:	Molar
mM	:	milimolar
g	:	gram
mg	:	miligram
n	:	number
Rf	:	Retention factor
UV	:	Ultra violet
A	:	Absorbance
min	:	Minute
ROS	:	Reactive oxygen species
RNS	:	Reactive nitrogen species
SOD	:	Superoxide dismutase
Cat	:	Catalase
GPx	:	Glutathione peroxidase

GSH	:	Glutathione
NOSs	:	Nitric oxide synthases
eNOS	:	Endothelial nitric oxide synthase
NO	:	Nitric oxide
LDL	:	Low density lipoprotein
HDL	:	High density lipoprotein
PKC	:	Protein kinase C
MnSOD	:	Manganese superoxide dismutase
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NAD	:	Nicotinamide adenine dinucleotide
HETE	:	Hydroxyeicosatetraenoic
DNA	:	Deoxyribonucleic acid
COX-enzyme	:	Cyclooxygenase
HMG-CoA	:	3-hydroxy-3-methylglutaryl-CoA
GLUT4	:	Glucose transporter type 4
Cu/Zn- SOD	:	Copper/Zinc superoxide dismutase
O_2	:	Oxygen
H_2O_2	:	Hydrogen peroxide
Fe	:	Ferrous
$FeSO_4$	:	Ferrous sulfate
HES-DFO	:	Hydroxyethyl starch deferoxamine
Nfr2	:	Nuclear factor-erythroid-2
SAME	:	S-adenosyl methionine
T1DM	:	Type 1 diabetes mellitus
T2DM	:	Type 2 diabetes mellitus
VSMC	:	Vascular smooth muscle cells
AGE	:	Advanced Glycation end-product
L-NAME	:	N(ω)-nitro-L-arginine methyl ester

TZDs	:	Thiazolidinediones
TBARS	:	Thiobarbituric acid reactants
MDA	:	Malondialdehyde
BHT	:	Butylated hydroxytoluene
NaCl	:	Sodium chloride
TMP	:	Tetramethoxypropane
TAE	:	Tannic acid extract
QE	:	Quarcetin extract
TPTZ	:	2,4,6-Tri(2-pyridyl)-s-triazine
TLC	:	Thin layer chromatography
GCMS	:	Gas chromatography-mass spectrometry
1TPC	:	Total phenolic content
TFC	:	Total flavonoid content

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Diabetes mellitus is a major factor that contribute to cardiovascular disease (Pyörälä *et al.,* 1987). While microvascular complications of diabetes include nephropathy and retinopathy, macrovascular complications resulting in aetherosclerotic cardiovascular disease such as coronary artery disease, cerebrovascular disease and pheripheral vascular disease are the leading cause of death in the diabetic population. Besides knowing the chronic disease that cause by the diabetes, people should know how diabetes happens. It is very important for us to take precautions and prevent diabetes at the early age.

Type 1 and type 2 diabetes normally co-occur in the same families, suggesting common genetic susceptibility. Hence, the individual with the diabetic history family background should be extra careful. In most cases, diabetic is caused by a loss of the physical or functional β-cell mass, mainly due to an autoimmune process, type 1 etiological process, or need for insulin due to insulin resistance, type 2 process (Alberti *et al.*, 1998). Both of these major diabetes types are believed to have different stages of disease, ranging from non-insulin-requiring to insulin-requiring for control of survival. By referring the classification served by the World Health Organization, it is quite possible that both processes would operate in a single patient and contribute to the phenotype of the patient. Also, factor other than autoimmunity can lead to a defective insulin response to glucose. Both major diabetes are considered multifactorial disease with several predisposing heredity and environmental factors, some of which could be common to both types.

The Diabetes Control and Complications Trials (DCCT) showed that tight control of blood glucose is effective in reducing clinical complications significantly, but even optimal control of blood glucose could not avoid complication suggesting that alternative treatment strategies are needed. Since there is many studies have shown that oxidative stress, mediated mainly by hyperglycemia-induced generation free radicals, contributes to the development and progression of diabetes and related contributions, it became clear that treatment with antioxidants might be an effective strategy for reducing oxidative stress as well as diabetic complications (Johansen *et al.,* 2005).

In general, oxidative stress is generally described as an excess formation or inadequate removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Johansen *et al.,* 2005; Maritim *et al.,* 2003) . Diabetes has several sources of oxidative stress in diabetes including non-enzymatic, enzymatic and mitochondrial pathways. First attempt of human body is activate its natural defence against oxidative stress automatically. Reactive species can be eliminated by a number of enzymatic and non-enzymatic antioxidants mechanism. Three primary enzymatic antioxidants include superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GPx) while non-enzymatic antioxidants are vitamin A, C and E as well as a few more another molecules such are coenzymes and cofactors.

In the late 19th and early 20th centuries, chemists studied antioxidants, a loosely defined group of compounds characterised by their ability to be oxidised instead of other compounds present. At first, their uses ranged from food storage to the vulcanisation of rubber, the biologists only later realised the importance of antioxidants in health with the 1960s publications of vitamins and flavonoids (Tinel & Tschopp, 2004). In the 1970s, Cameron and Pauling, discovered that ascorbic acid (vitamin C) is

a potential human cancer protective agent (Cameron & Pauling, 1976). With many well-known researchers actively studying antioxidants as protecting agents (Willett & MacMahon, 1984), explanations of the impacts of antioxidants on cancer susceptibility and general health expanded rapidly in subsequent decades with research into mechanisms, molecular targets, and molecular interactions (Glade, 1999). In recent years, many conferences and reviews testify to the growing interest in the roles of the body's antioxidants system working together in human cells against toxic reactive oxygen species, their relationship with several pathophysiologic processes and their potential therapeutic implications (MatÉs *et al.,* 1999).

In spite of the use of insulin as a diabetic therapy for patients, antioxidants may also be considered as the alternative way for the treatment based on prior research. Small trials showed positive impacts on cardiovascular with the treatment of Vitamin E. The treatment of Type 1 diabetes patients with double-blind, placebo-controlled, vitamin E supplementation (1000 IU/day) for three months significantly enhanced endothelium-dependent vasorelaxation (Skyrme-Jones *et al.,* 2000). In another research, diabetic rats ware treated with with 0.5% of α-lipoic acid as a dietary supplement or with hydroxyethyl starch deferoxamine (HES-DFO) by weekly intravenous injections at a dose of 75 mg/kg. The treatments demonstrated the improvement of diabetes-induced decrease in endometrial blood flow, acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of sciartic nerve (Coppey *et al.,* 2001).

To discover novel type of antioxidants, some research needs to be done by using the existing source that never been explored before. The extract of *Ampilocisscus* sp. have some special properties as they are being used as traditional health supplement by *Orang Asli*. The scientific name of this *Isi nyaru* species is *Nothocissus spicifera* as has

been confirmed by taxonomist, Prof. Dr. Ong Hean Chooi from Institute Science Biology, University of Malaya.

In the modern medical era nowadays, traditional medicine has been forgotten from time to time, which they may help us to increase the medicinal value especially in treating diabetic disease. Thus, this experiment is carried out to determine the efficacy of different *Ampilocicuss* sp. extracts on diabetic treatment.

1.2 Objectives

i. To screen the identify of organic components of *Ampilocissus* sp. extracts.

ii. To determine the in vitro antioxidating activities of *Ampilocissus* sp. extracts.

iii. To evaluate antioxidant effect of *Ampilocissus* sp. Extracts on biochemical properties of streptozotocin-induced diabetic rats.

CHAPTER 2: LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetic is a chronic metabolic disorder with a rapidly growing epidemic (Golbidi *et al.,* 2011) that emphasizes the significance of continued research and the need for ne twchniques to both prevent and treat this pandemic. While obesity and physical inactivity are known to be significant risk factors for Type 2 diabetes mellitus (T2DM), recent evidence suggests that oxidative stress can contribute to the pathogenesis of TD2M by enhancing insulin resistance or impairing insulin secretion (Montonen *et al.*, 2004).

While diabetes management has focused largely on control of hyperglycemia, the disease's increasing burden is primarily associated with its vascular complications. This is reflected by a 4-fold increase in the incidence of coronary artery disease, a 10-fold increase in peripheral vascular disease, and a 3- to 4-fold higher mortality rate with as much as 75% of diabetics ultimately dying from vascular disease (Basha *et al.,* 2012). Consequently, the question of whether antioxidants could have a beneficial effect on reducing the risk of this condition, especially cardiovascular disease, has been intensively investigated, but the results remain inconclusive. If antioxidants play a protective role in the pathophysiology of diabetes and cardiovascular disease, it is importance to understand the physiological status of antioxidant concentrations among individuals at high risk , such as people with metabolic syndrome (Ford *et al.,* 2003).

2.2 Diabetes and Endothelium

Endothelial cells line the inner lumen of the entire vasculature and act as an interface between circulating blood and vascular smooth muscle cells (VSMC). Apart from being

the key participant during the process of angiogenesis, these dynamic structures can actively regulate basal vascular tone and vascular reactivity in physiological and pathological conditions. The balance between the vasodilation and vasoconstriction is maintained by the endothelium, and the disruption of this balance leads to endothelial dysfunction and causes damage to the arterial wall. Endothelial cell-derived factors also are critical mediators of VSMC growth and inflammation. Endothelial dysfunction in a basal state or after activation may be a critical and initiating factor in the development of diabetic micro- and macrovascular disease (Basha *et al.,* 2012).

The metabolic disturbances of diabetes, like hyperlipidemia, hyperinsulinemia, and hyperglycemia, act as "triggers" eventually causing endothelial dysfunction through influence of different "mediator" molecules. Several lines of evidence point to the fact that oxidative stress caused by these metabolic changes plays a key role in endothelial dysfunction (Calles-Escandon, 2001).

An ideal antioxidant should be readily absorbed by body and should prevent or quench free radical formation or chelate redox metals that increased the oxidative stress level (Rahman, 2007). The endogenous antioxidant defense system, which includes endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione (GSH), proteins, and low-molecular-weight scavengers, like uric acid, coenzyme Q, and lipoic acid have vital roles to minimize the level of free radical (Halliwell, 2011).

2.3 Diabetes and Oxidative Stress

A number of complications arise as a consequence that result from diabetes. Chronic hyperglycemia may increase the tissue-damaging effects. Since endothelial cells are

unable to limit glucose transport as well as other cells do, they are more vulnerable to the toxic effects of hyperglycemia (Bajaj *et al.*, 2012).

Oxidative stress results from an imbalance between radical-generating and radical-scavenging system such as increased free radical production or reduced activity of antioxidant defences or both. Some studies have shown that diabetes mellitus is accompanied by increased formation of free radicals and decreased antioxidant capacity, leading to oxidative damage of cell components (Bashan *et al.,* 2009).

2.3.1 Increased Free Radical Production

There are multiple sources of reactive oxygen species (ROS) production in diabetes including those of mitochondrial and non-mitochondrial pathway. ROS accelerates the four important molecular mechanisms involved in hyperglycemia-induced oxidative tissue damage. These four pathways reactivation of protein kinase C (PKC), increased hexosamine pathway flux, increased advanced glycation end-product (AGE), and increased polyol pathay flux (Bashan *et al.,* 2009).

i. Mitochondrial sources

The mitochondrial respiratory chain is a non-enzymatic source of ROS. Hyperglecemia-induced generation of free radicals at the mitochondrial level is thought to be one of the major driver of the intense cycle of oxidative stress in diabetes (Brownlee, 2001) . He is briefly stated that increased extracellular glucose leads to an abundance of electron donors generated during Kreb's cycle, so driving the inner mitochondrial membrane potential upward, a state that is associated with mitochondrial dysfunction and increased ROS production. The augmented generation of pyruvate via accelerated glycolysis under hyperglycemic condition is thought to flood the mitochondria and thus generates

ROS formation at the level of complex II in the respiratory chain. Reactive oxygen species stimulates oxidation of LDL is not recognized by the LDL receptor and is subsequently taken up by scavenger receptors in macrophages to form foam cells and so lead to atherosclerotic plaques (Boullier *et al.,* 2001). The production of ROS is reduced by using either an uncoupler of oxidative phosphorylation or by the over expression of either uncoupling protein-1 or MnSOD, such that normalizing the levels of mitochondrial ROS with any of these agents will prevent glucose-induced activation of protein kinase C, formation of advanced glycation-end products, sorbitol accumulation, and NF-κB activation. These findings support the feasibility of targeting the triggering role of mitochondrial superoxide production in hyperglycemia-induced tissue damage (Nishikawa *et al.,* 2000).

ii. Non-mitochondrial sources

Non-mitochondrial sources of ROS include NAD(P)H oxidase, xanthine oxidase, uncoupled eNOS, lipoxygenase,cyclooxygenase, cytochrome P450 enzymes and other hemoprotein (Yung *et al.,* 2006). Common stimulators of vascular NAD(P)H are angiotensin II, thrombin, platelet-derived growth factor, and tumor necrosis factor-α. Inhibition of NADPH oxidase-dependent production of ROS in diabetes by a variety of PKC inhibitors suggests a regulatory role of PKC in hyperglycemia-induced NADPH oxidase activity. In keeping with this, PKC inhibitors decrease the expression of NADPH oxidase in high glucose-treated endothelial cells (Golbidi *et al.,* 2011).

The collective reference of xanthine oxidase and xanthine dehydrogenase is xanthine oxidoreductase. While both these enzymes catalyse the transformation of hypoxanthine toxanthine and then to uric acid, xanthine oxidase reduces

oxygen as an electron acceptor while xanthine dehydrogenase can reduce either oxygen or NAD+. Hydroxyl radicals, hydrogen peroxide, and superoxide are by products of xanthine oxidase. Although there is some uncertainty over the presence of xanthine oxidase in normal endothelial cells, it has been recognized as a course of oxidative stress in the pathogenesis of atherosclerosis, ischemia-reperfusion, and diabetes mellitus (Desco *et al.,* 2002).

Nitric oxide is produced by inducible and constuitive nitric oxide synthases (NOSs), enzyme systems that incorporate oxygen into L-arginine. If NOS lacks its substrate L-arginine or one of its co-factors ("uncoupled" NOS), NOS produces superoxide instead of nitric oxide. The excessive ROS generation is known to impair endothelial nitric oxide synthase (eNOS) activity and NO production, thereby affecting endothelium-dependent vasolidation (Desco *et al.,* 2002).

Lipoxygenase products, especially 12(S)-HETE and 15(S)-HETE, are involved in the pathogenesis, of several diseases including diabetes where they have proatherogenic effects and mediate the actions of growth factors and pro-inflammatory cytokines. High levels of glucose induce endothelium-derived vasoconstrictor prostanoids, indicating a role for cyclooxygenase-2 in diabetic vascuopathies. Addidtional proof to support a role for oxidative stress in the induction of COX expression is that glycemic control normalizes the expression of COX enzymes (Golbidi *et al.,* 2011).

Environmental factors such as cigarette smoke can increase the concentrations of free radicals in the body (Devasagayam *et al.,* 2004).

2.3.2 Decreased Antioxidant Defences

Cells have evolved various enzymatic and non-enzymatic antioxidant pathways, which work together synergistically to prevent the body from free-radical-induced damage (Bajaj *et al.,* 2012a).

There are several evidences to suggest that antioxidant defences may be lower in diabetes. These include reports of reduced plasma or serum total antioxidant status or free radical scavenging activity and increase plasma oxidisability in type 2 diabetic, together with reduced levels of specific antioxidants such as ascorbic acid and vitamin E. In addition, the activities of the antioxidant enzymes catalase, superoxide dismutase and gluthathione peroxidase have been described as reduced in diabetics. A diminution in the endothelial synthesis of NO has also been suggested in type 2 diabetics, which apart from detracting from vascular antioxidant defence, would of course compound any defect in the anti-atherogenic signalling role of NO (Laight *et al.,* 2000).

The content of oxidized fatty acids is increased and the anti-inflammatory and antioxidant activities of High Density Lipoproteins (HDLs) are impaired in T2DM (Morgantini *et al.,* 2011).

2.4 Antioxidant Therapy and Diabetes

Intracellular free radical formation inhibtion would provide a therapeutic approach to avoid oxidative stress and associated problems of the diabetic vascular. Antioxidants may perform at specific levels, inhibiting ROS formation or scavenge free radicals or increase the antioxidants defense enzyme capabilities. Supplementation with antioxidants of factors essential to nitric oxide (NO) production may potentially improve endothelial dysfunction in T2DM by re-coupling eNOS and mitochondrial

function, as well as decreasing vascular NAD(P)H oxidase activity (Hamilton *et al.,* 2007). In the event of macrovascular and microvascular problems, however, antioxidant treatment with blood pressure control, dyslipidemia management and ideal glucose control is beneficial (Jakus, 2000).

Antioxidant drug therapy can generally be divided into the use of antioxidant enzyme and substrates, biogenic elements, combined drugs, synthetic antioxidants, and drug with antioxidant activity. There are also a vast number of natural mechanisms of cellular defense as the naturally existing antioxidantelements that neutralizes free radical damage. The enzymatic antioxidant systems such as copper, zinc, manganese superoxide dismutase, glutathione reductase, glutathione peroxidase, and catalase may remove the ROS directly or sequentially, preventing their excessive accumulation and consequent adverse effects. Non-enzymatic antioxidant system consist of scavenging molecules that are endogenously produced such as glutathione, ubichinol, and uric acid or derivatives of the diet such as vitamins C and E, carotenoids, lipoic acid and selenium (Silva *et al.,* 2010). Exercise training results in an upregulation of antioxidant defence mechanisms in various tissues, presumably due to increased levels of oxidative stress that occurs during exercise (Golbidi *et al.,* 2011).

Well-established antioxidants derived from the diet are vitamins C, E, A and carotenoids, which have been studied intensively. In general, exogenous antioxidants can compensate for the lower plasma antioxidant levels often observed in T2DM and in prediabetic individuals, whether their diabetes is primarily genetic in origin or due to obesity and a sedentary lifestyle (Ruhe *et al.,* 2001). Vitamin C (ascorbic acid) and vitamin E (tocopherol) have well-described antioxidant properties. Vegetables and fruits have in their natural composition. Over the past decade, evidence has been accumulated

that plant polyphenols are an important class of defence antioxidants. These compounds are widespread virtually in all plant foods, often at high levels, and include phenols, phenolic acids, and flavonoids (Pietta, 2000).

In a prospective of cohort study, vitamin C intake was found to be significantly lower among incident cases of T2DM (Feskens *et al.,* 1995). In three prospective observational studies, serum α-tocopherol levels were associate with lower risk of type T1DM or T2DM. In another prospective study cohort of more than 4000 non-diabetic subjects over 23 years, vitamin E intake was significantly associated with a reduced risk of T2DM (Montonen *et al.,* 2004).

However, despite observational studies suggesting an association between antioxidant vitamin intake and reduced cardiovascular risk, this has not been borne out in interventional trials. Studies of the effect of ascorbic acid and tocopherol n endothelial dysfunction in T2DM have yielded mixed results (Hamilton *et al.,* 2007).

Coenzyme Q or ubiquinone may decrease oxidative stress not only by quenching reactive oxidant species but also by 'recoupling' mitochondrial oxidative phosphorylation, thereby reducing superoxide production. Alpha-lipoic acid, a critical cofactor for mitochondrial dehydrogenase reactions, is another compound with free radical-scavenging activity (Hamilton *et al.,* 2007). Lipoic acid was found to increase glucose transport in muscle cells in culture by stimulating translocation of GLUT4 from internal pools to the plasma membrane. In cultured adipocytes, treatment with lipoic acid protected the insulin receptor from oxidative damage, maintaining its functional integrity. A placebo-controlled explorative study of patients with T2DM indicated that oral administration of lipoic acid significantly increased insulin-mediated glucose

uptake, presumably by modulating insulin sensitivity (Jacob *et al.,* 1999). In addition to the antioxidants mentioned, a number of commonly used drugs, have been reported to have antioxidant activity, in addition to their primary pharmacological property. For example, gemfibrozil, a lipid-lowering fibrate, was previously reported to have antioxidant actions. Anti-hyperlipidemic statins are thought to exert antioxidant effect. In addition, it has been demonstrated that at least a part of the beneficial vascular effects of thiazolidinediones are linked with their antioxidant properties (Karasu, 2010).

2.5 Antioxidant Enzymes

Oxidants are desribed as substances that remove electrons from other compounds that are capable of producing free radicals that can cause oxidative cascades. Oxidants include oxygen, hydrogen peroxide, nitric oxide and their free radicals.

However, antioxidants are described as substances that inhibit the oxidation of other compounds. As has been mention before, all cells in the body contain powerful antioxidant enzymes. The three major classes of antioxidant enzymes are the superoxide dismutase, catalase and glutathione peroxidase. In addition, there are a lot of specialized antioxidant enzymes as well as vitamins, minerals, thiols, polyphenols and other compound reacting with detoxifying oxidants (Valko *et al.,* 2007)

2.5.1 Superoxide Dismutase

Superoxide dismutase (SOD) catalyzes the simultaneous oxidation and reduction of the superoxide radical to generate oxygen and hydrogen peroxide. It is an important enzyme that detoxifies the highly superoxide radical to create hydrogen peroxide. This antioxidant enzyme is found in entire body. In the human body, there are two in the mitochondria forms of SOD: Cu/Zn-SOD found in the cytosol and extracelluar space,

and Mn-SOD found in the mitochondria. Left unchecked, the superoxide radical is capable of producing countless other reactive species including peroxynitrite, lipid peroxyl and alkoxyl radicals which can cause destructive havoc throughout the body. The enzyme's main functions are to neutralize the superoxide radical and protect cellular proteins, lipids and DNA from ROS-induced destruction and maintain healthy cells, tissues, organs and bodies. There is a lso a need for optimal level of this enzyme to encourage healthy aging. Environmental radiation from radon, cosmic radiation-induced oxidation. Antioxidant activity of SOD helps to protect the healthy individual from everyday natural radiation exposure (Baynes *et al.,* 1996).

Supplemental SOD is available from bovine liver as well as vegetarian form from *Cucumis melo* (Ji, 1993).

2.5.2 Catalase

Hydrogen peroxide is catalyzed by catalase (Cat) enzyme to produce water and oxygen. Catalase often decribed as the "perfect catalyst", has an incredibly raised the rate of response that can decompose millions of hydrogen peroxide molecules every second. Interestingly, it was also reported that the enzyme acts as peroxidase and is known to catalyse the transformation of short chain alcohols to their corresponding aldehydes. The enzyme's tetrametric structure includes an iron at each active site. This structure is quite stable making the enzyme more resistant to pH, heat and proteolysis than many other enzymes (Allen *et al.,* 1983).

In peroxisomes, red blood cells and extracellular spaces, catalase is dicovered. Catalase levels in human bodys are highest in the liver and red blood cells and relatively low in heart and brain. This antioxidant enzyme serves numerous functions in

the human body including modullating inflammation, mutagenesis, and apoptosis. Catalase protects pancreatic β-cells and nerve cells as well as the oxygen-carrying capacity of red blood cells. Hydrogen peroxide radicals can be major source of cell injury and cell death. Catalase's ability to neutralize these damaging ROS inhibits apoptosis and helps regulate cell proliferation. Research has also found that catalase activity in tumor cells is often low (Allen *et al.,* 1983).

The fermentation of Aspergillus *niger* is the main source of supplemental catalase. (Ji, 1993).

2.5.3 Peroxidases

This diverse group of enzymes participates both in the immune defense against invading microorganisms and in neutralizing free radicals that initiate cellular damage. Like catalase, these enzymes catalyse the neutralization of hydrogen peroxide and help protect the body from ROS-induced tissue damage. Within this group of enzymes glutathione peroxidase is one of the most studied in mammalian systems (Kang *et al,*. 2008).

Glutathione peroxidase is used as an electron donor to reduce ROS by utilizing glutathione. Glutathione itself is not an enzyme, but simply a substrate for the glutathione peroxidase in antioxdiant response. The action of a second enzyme, glutathione reductase, regenerates reduced glutathione disulphide in human body. Glutathione also serves the body as an antioxidant by donating electrons to maintain the activity of other antioxidant compounds such as vitamin C and vitamin E. Like catalase, glutathione peroxidase is a tetrameric protein but with selenium at the catalytic site.

High levels of glutathione peroxidase have been shown to protect against oxidative stress in both in vitro cellular and in vivo animal studies (Rotruck *et al.,* 1973).

A standardized glutathione peroxidase is not currently available as a dietary supplement. Lactoperoxidase is another peroxidase found in the milk and saliva of mammals. This enzyme functions in the body as part of the innate immune system. The enzyme catalyzes hydrogen peroxide through a reaction that both neutralizes the ROS potential and creates antimicrobial compounds such as hypothiocyanate. Supplemental lactoperoxidase is currently available from cow's milk (Ji, 1993).

2.6 Natural Defense against Oxidative Stress and Antioxidants

A number of enzymatic and non-enzymatic antioxidant mechanisms can suppress reactive species. As mentioned above, SOD is one of the antioxidant enzymes which immediately converts $\cdot O_2^-$ to H_2O_2, which is the detoxified to water either by catalase in the lysosomes or by glutathione peroxidase during in the mitochondria. Another significant enzyme, glutathione reductase, which regenerates glutathione that is used as a hydrogen donor by glutathione peroxidase during the H2O2 removal. Maritim *et al.,* (2003) have reviewed in detail that diabetes has multiple effects on the protein levels and activity of these enzymes, which further augment oxidative stress by causing a suppressed defence response. For example, in the heart, which is an important target in diabetes and prone to diabetic cardiomyopathy leading to chronic heart failure, SOD and GPx expression as well as activity are decreased whereas catalase is increased in experimental models, of diabetes. In patient with chronic heart failure, all three enzymes are decreased in the smooth muscle (Linke *et al.,* 2005) and exercise training can upregulate the expression the expression and activity and activity of antioxidant enzymes. Increased isoprostane levels in diabetic patients with chronic heart failure are

correlated with antioxidant status and disease severity (Polidori *et al.,* 2004). Thus, modulation of these enzymes in target organs prone to diabetic complications such as heart and kidney may prove beneficial in the prevention and management of heart failure and kidney failure (Johansen *et al.,* 2005).

2.7 Antioxidant Enzyme Supplementation

Antioxidant enzymes are component of all living cells and natural component of the foods we eat. These enzymes, unfortunately, are often heat labile and do not survive the process of cooking. In 2007, a group of researchers reported on the effect of boiling, microwaving and baking various fruits and vegetables on the activity of SOD, catalase and glutathione peroxidase. These researchers revealed that the concentrations of antioxidant enzyme in nearly all fruits and vegetables tested (except tomato) were considerably diminished by heat treatment. This loss of antioxidant enzyme activity reduces the antioxidant potential of these foods (Vouldoukis *et al.,* 2004).

Dietary antioxidant enzymes may act to spare or renew other nutritional antioxidants. Their nature enzyme catalysts allow them to counteract oxidative reactions repeatedly as food passes through the digestive tract. Evidence suggests that at least some SOD may survive the digestive process and be absorbed for direct systemic supplementation. Research has also found that combination with gliadin may further enhance the gastric survival and SOD absorption. One research team studied the effect of vegetarian SOD on oxidative cell damage in healthy volunteers when exposed to hyperbaric oxygen and found that supplementation protected against DNA damage. Antioxidants enzyme supplements may be useful to support the general antioxidant system of the body as well as providing general immune support and helping to maintain healthy skin, muscles and connective tissues (Vouldoukis *et al.,* 2004).

Alternatively, different nutrients may increase the body's production or activity of antioxidant enzymes. It has been shown that dietary consumption of polyphenols increases antioxidant enzyme expression including SOD, catalase, and glutathione peroxidase. It is believed that these phytochemicals induce expression of antioxidant enzymes through the nuclear factor-erythroid-2-realted factor 2 pathway (Nfr2). Furthermore, some scientists thought that it is indirect antioxidant effect, rather than the antioxidant effect, rather than the antioxidant potential of dietary polyphenols that is most essential to their in vivo protective action. Phytochemicals that have been shown to induce antioxidant enzyme activity include resveratrol, green tea catechins, curcumin, lycopene, strawberry anthocyanins, oleuropein, and sulforophane. Research indicates that both endogenous and supplemental melatonin enhance the level and activity of antioxidant enzymes including catalase, glutathione peroxidase and Mn-SOD though the mechanism is currently unknown. Supplementation of the tripeptide glutathione or its component amino acids – glycine, glutamine and especially cysteine – may also promote the action of glutathione peroxidase, particularly if systemic glutathione levels are depleted. Supplementation of S-adenosylmethionine (SAMe), whey protein, α-lipoic acid and SOD work synergistically to eliminate the toxic potential of ROS (Wojcik *et al.*, 2010).

Antioxidant enzymes are essential component of the natural antioxidant defence system of the body. It is important to maintain sufficient levels of these enzymes in order to maintain excellent health and to replenish depletion caused by aging, strenuous exercise and environmental circumstances. The intake of antioxidant enzymes or nutrients that enhance their expression or activity can be a key component of a diet to -maintain health and well-being (Clarkson *et al.,* 2000).

2.8 Antioxidant Study from Animal Models

In experimental diabetic models, a variety of in vivo research have been conducted u sing antioxidants. Some observable biomarkers are used to measure the impacts of antioxidants on oxidative stress These markers usually include the enzymatic activities of catalase, SOD, GPx and glutathione reductase, as well as thiobarbituric acid reactants (TBARS) levels, an indirect measurement of free-radical production that has been shown to be consistently increased in diabetes. Normalization of the activity levels of any of these markers, and ultimately, the balance of free-radical production/removal would be an effective method to reduce ROS-induced damage. Many animal studies have been conducted with this objective and shown that changes in oxidative stress indicators caused by diabetes may be reversed when treated with different compounds (Johansen *et al.,* 2005).

Mekiňová *et al.* (1995) demonstrated that supplementation of streptozotocin (STZ) diabetic rats with vitamins C, E and beta-carotene for 8 weeks produced a significant reduction of TBARS level, glutathione reductase and GPx, GPx, an increase in Cu-SOD and no change in catalase activity in kidneys. Treatment with vitamins C and E was also shown to decrease urinary albumin excretion, glomerular basement membrane thickness and kidney weight in STZ diabetic rats. In the same study, vitamins C and E significantly lowered malondialdehyde (TBARS) levels and GPx activity while increasing catalase and SOD activities when compared to unsupplemented diabetic animals (Kornatowska *et al.,* 2004). A study by Cinar *et al.* (2001) demonstrated that supplementation with vitamin E significantly lowered liver and lung TBARS levels nd improved impaired endothelium-dependent vasorelaxation in STZ diabetic rat aorta.

A-lipoic acid, which is involved in mitochondrial dehydrogenase reactions, has gained a considerable amount of attention as an antioxidant. Studies have demonstrated that intraperitoneal administration of α-lipoic acid to STZ diabetic Wistar rats normalizes TBARS levels in plasma, retina, liver, and pancreas (Obrosova, Fathallah, & Greene, 2000). In the same study, Obrosova et.al observed a reduction of GSH activity in the diabetic retina and that supplementation with α-lipoic acid produced no change. However, another study demonstrated an increase in aorta-GSH-Px in STZ diabetic rats that was normalized by treatment with α-lipoic acid. Additionally, increased maximum contractile responses in diabetic aortic rings were ameliorated with α-lipoic acid treatment (Kocak *et al.,* 2000).

SOD activity is undoubtedly important to the regulation of oxidative status in diabetes. However, there is variation as to the status of this enzyme in the diabetic state. Some studies have reported decreased SOD activity (Kornatowska *et al.,* 2004) while others have shown increases or no change in enzyme (Mekiňová et al., 1995). A-lipoic acid has been observed to normalized diabetes-induced decreases of SOD in rat heart and retina one study demonstrated that treatment of STZ diabetic rats with α-lipoic acid reverse SOD-induced vasorelaxation, potentially due to the elimination of excess superoxide or hydrogen peroxide and the recovery of basal NO (Kocak *et al.,* 2000). A recent study by Brands *et al.* (2004) investigated the effect of oxidative stress in the development of hypertension in diabetes using the SOD mimetic tempol in a Type 1 model of diabetes where NOS is pharmacologically inhibited with a NOS inhibitor, L-NAME. In this model, hyperglycaemia causes hypertension implicating an important role for NO. Results of this study showed that if $\cdot O_2^-$ is eliminated by tempol early in the disease process, the hypertension and decrease in glomerular filtration precipitated by diabetes are prevented.

In another research, the extract of dried leaves *V. amygdalina* were used to treat diabetic induced rats. The reduction in body weight in the diabetic groups was regained following administration of the extract of *V. amygdalina*. The extract is considered safe and had little or no effect on blood glucose, MDA and GSH levels of the normal rabbits. Extract significantly reduced glucose and MDA concentrations but increased GSH levels in the diabetic rabbits. Similarly, the extract had no effect on the activities of SOD, CAT and GPx in normal rabbits, however in diabetic rabbits, the enzymes activities increased dose-dependently. This finding provides basis for the use of *V. amygdalina* as potential antidiabetic antioxidant agent and may be useful for its hypoglycemic property (Owolabi *et al.,* 2011).

In summary, there are differences in response to antioxidants in experimental diabetes in the prevention of cardiovascular complications. Studies in experimental models provide a foundation for the clinical studies but results should be interpreted cautiously since the experimental models of diabetes, duration and type of antioxidant treatment and markers oxidative stress investigated in these studies exhibit a wide range.

2.9 Antioxidant Study from Clinical Trials

Although studies with antioxidants in experimental models as well as observational studies strongly suggest that antioxidants should confer beneficial effects in reducing cardiovascular complications in diabetes, clinical evidence for the use of antioxidants is not solid. It should be emphasized that clinical trials with antioxidants in diabetes are limited and majority of these trials focused on the use of vitamin E and C and lately αlipoic acid (Johansen *et al.*, 2005).

Small trials with vitamin E demonstrated beneficial cardiovascular effects. In a double-blind, placebo-controlled, randomized study, vitamin E supplementation (1000 IU/day) for three months in patients with T1DM (n = 41) significantly improved endothelium-dependent vasorelaxation (Skyrme-Jones *et al.,* 2000).

In another study, Beckman et al. reported that administration of vitamin E (800 IU/day) and C (1000 mg/day) combination for six months had a positive effect on endothelium-dependent vasorelaxation in T1DM patients (n = 26) but had no effect in T2DM (n = 23). Gaede et al. (2001) reported that vitamin E (680 mg/day) and C (1250 mg/day) combination significantly improved renal function in T2DM.

Other clinical trials on a larger scale include the Heart Outcomes Prevention Evaluation (HOPE) trial (Yusuf, Dagenais, Pogue, Bosch, & Sleight, 2000), Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease (SPACE) trial (Boaz et al., 2000), the Steno trial (Gæde et al., 2003), the Primary Prevention Project (PPP) trial (Sacco et al., 2003) and the Study to Evaluate Carotid Ultrasound Changes in Patients Treated With Ramipril and Vitamin (SECURE) trial (E. M. Lonn et al., 2001).

The HOPE trial enrolled patients 55 years of age or older who were at high risk for cardiovascular disease and recruited significant number of patients with diabetes (Yusuf *et al.,* 2000) . Results with vitamin E and Ramipril were evaluated separately as compared to respective placebo groups. The primary endpoint was a composite of myocardial infarction, stroke and death from cardiovascular causes. The trial was stopped for ethical reasons after 4.5 years follow-up by the recommendations of and independent data and safety monitoring board based on the beneficial effects of

Ramipril on cardiovascular events in the concurrent treatment group and lack of effect in the vitamin E treatment group. Results of the study demonstrated that there was no significant difference in the primary outcome between vitamin E and placebo groups (Yusuf et al., 2000). Analyses of the secondary endpoints of the study, which included total mortality, hospitalizations for heart failure and unstable angina, revascularization and nephropathy revealed vitamin E supplementation for 4.5 years failed to provide any benefit in cardiovascular outcomes or nephropathy (Lonn *et al.,* 2002). It was also reported that there were no significant adverse events associated with vitamin E. The HOPE trial was the largest trial conducted thus far for the use of antioxidants in diabetes.

The SECURE trial was designed as a sub-study of the HOPE trial to evaluate the effects of long-term treatment with Ramipril and vitamin E on atherosclerosis progression in high-risk patients. While Ramipril slowed down atherosclerosis changes, vitamin E had no effect as compared to placebo group.

The SPACE and PPP trials were designated to evaluate the effect of high dose and low dose of vitamin E respectively towards diabetic patients. The primary end point was a composite of cardiovascular death, stroke or myocardial infarction. The SPACE trial showed 46% decrease in the primary end point events in the vitamin E group and this was mainly due to a 70% reduction in total myocardial infarction (Boaz *et al.,* 2000). The PPP trial was stopped prematurely by the recommendations of an independent data and safety monitoring board based on the consistent beneficial effects of aspirin as compared to placebo group. However, there was no significant effect of vitamin E treatment either in diabetic or nondiabetic subjects.

Studies with α-lipoic acid are approved for the treatment of diabetic neuropathy and results are more promising than those obtained with vitamin E. In the Alpha-Lipoic Acid in Diabetic Neurpathy (ALADIN) I, II and III studies, infusion of α-lipoic acid significantly improved patient symptoms (Ziegler et *al.,* 1995) including nerve function (Reljanovic *et al.,* 1999).

The DEKAN (Deutsche kardiale autonome neuropathie) study evaluated the effect of 800 mg α-lipoic acid or placebo in diabetic patients with cardiac autonomic neuropathy for 4 months and showed that rate variability, an indicator of cardiac autonomic neuropathy, significantly improved with α-lipoic acid treatment (Ziegler *et al.,* 1997).

In summary, there are restricted clinical trials in diabetic patients with conventional antioxidants. Vitamin E did not provide a significant benefits on cardiovascular results. However, as in diabetic neuropathy studies, when the research population was restricted to diabetic patients alone, α-lipoic acid has been shown to be efficient. This antioxidant may be a viable option in trials focusing on cardiovascular outcomes in diabetes.

In addition to the many antioxidants examined above, a number of commonly used drugs have shown promising antioxidant activity in addition to their primary pharmacological activity. These drugs include thiazolidinediones (TZDs), HMG-CoA reductase inhibitor (statins), and inhibitors of the renin-angitensin system (Johansen *et al.,* 2005).

In conclusion, in relation to their main pharmacology activities, most of the agents that are a major component of pharmacotherapy in diabetes have been shown to have

antioxidant characteristics. These antioxidant properties make the case for use of these drugs even more compelling. Particularly in light of the lacklustre results seen in clinical trials with antioxidant supplementation, health care providers should redouble their efforts to ensure adequate usage of the demonstrably effective agents summarized above (Johansen *et al.,* 2005).

2.10 Studied Plant - *Ampelocissus* sp.

Ampelocissus is a genus of *Vitaceae* having hundreds of species found variously in tropical Africa, Asia, Central America and Oceania. Simple descriptions of this species, they are climbers, woody or herblike and hermaphrodrotic or polygamo-diaecious. They have unbranched tendrils or sometimes bifurcate.

Until now, extensive studies on this species have been conducted around the globe are leading to certain diseases. Previous investigation of the Philippine *Ampelocissus* sp. roots for cancer cell growth inhibitory components led to the isolation of a new acetogenin characterized as 22-epicalamistrin. While two other constituents proved to be known acetogenin uvaribonin and chalcone. All these components indicate substantial inhibitory activity of cancer cell development agaisnt a panel of human cancer cell lines (Pettit *et al.,* 2008).

Furthermore, the antibacterial, antifungal and antioxidant activities of *Ampelocissus grantii* (Baker) Planch, an African medicinal plant was analyzed to create the scientific basis of the use of the plant in folk medicine to fight bacteria and it uses in the therapy of certain cancers (Zongo *et al.,* 2009). Ethnobotanical analyses have shown that the plant is used alone or blended with other medicinal plants to treat shigellosis and fever including malaria (Adjanohoun *et al.,* 1980), urinary schistosomiasis (Bah *et*

al., 2006). It is also used to cure old wound (Inngjerdingen *et al.,* 2004), to treat cancer (Muhammad *et al.,* 2005), diarrhoea (Etuk *et al.,* 2009), trypanosomiais, rheuniatism and muscular pain (Bizimana *et al.,* 2006).

In vitro and *in vivo* studies of trypanocidal activity has been demonstrated (Bizimana *et al.,* 2006). An antimicrobial activitymay be considered if the diameter of inhibition zone observed is 9 mm or more around the paper disc (Kitzberger et al., 2007). The diameter of inhibition zone obtained by gram-positive bacteria generally larger than those obtained with gram-negative bacteria. This is in phase with several prior research which suggested that gram-positive bacteria are more sensitive to plant extracts and their components than gram-negative bacteria (Kelmanson *et al.,* 2000). Plant extracts with antimicrobial activities are usually active on multi-drug resistant human pathogens (Oskay *et al.,* 2010).

CHAPTER 3: MATERIALS AND METHOD

3.1 Chemicals and Reagents

Chemicals	Manufacturer
Hexane	Pharmaco-Aaper
Petroleum-ether	Pharmaco-Aaper
Chloroform	Pharmaco-Aaper
Methanol HPLC-grade-99%	Lab Alley
Mure water HPLC-grade-99%	Lab Alley
Saline	Lab Alley
n-heptane	Lab Alley
Ethanol	Lab Alley
Vanilin	Sigma-Aldrich
Dragendorff	Sigma-Aldrich
Aniseldehyde	Sigma-Aldrich
Folin-ciocalteu reagent	Sigma-Aldrich
Sodium carbonate solution	Sigma-Aldrich
Quarcetin	Sigma-Aldrich
DPPH	Sigma-Aldrich
FeCl2	Sigma-Aldrich
FeCl3	Sigma-Aldrich
FeSO4	Sigma-Aldrich
Ferrozine	Sigma-Aldrich
TPTZ	Sigma-Aldrich
HCl	Sigma-Aldrich
Streptozotocin	Sigma-Aldrich

Aluminium chloride solution	Thermo Fisher Scientific
Sodium hydroxide solution	Thermo Fisher Scientific
Phosphate buffer	Thermo Fisher Scientific
Pottasium bichromate	Thermo Fisher Scientific
Glutathione Peroxidase Kit	Merck Millipore

3.2 Plant Materials

Figure 3.1 : Tuber of *Nothociccus spicifera* (Isi Nyaru)

The fresh tuber part of *Ampelocissus* sp. *(*Isi Nyaru) were collected in Endau–Rompin, Pahang forest. The plant material were properly cut into small pieces and dried in oven at 60℃ until it became well dried for grinding. After drying, the plant material was grounded well into fine powder.

3.3 Extraction of Plant Chemical Compound

The tuber was dried in oven and ground to fine powder with mechanical grinder. 100 g of sample powder was macerated in 400 ml of hexane, petroleum-ether, chloroform, methanol and water for 24 hours at 40 ℃ and properly covered with aluminum foil and labelled. After 8 hours of extraction, each extract was filtered through Whatmann's filter paper no. 1 separately. The filtrate then was concentrated by vacuum-rotary evaporation at 45 C and freeze dried to obtain the yield. The crude extract was stored in an impervious amber bottle until used.

3.4 Separation and Detection of Phytochemical Compounds

3.4.1 Qualitative Analysis by Thin Layer Chromatography

The extracts were checked by Thin Layer Chromatography (TLC) on analytical plates over silica gel. TLC was carried out to isolate the principle components that were present in most effective extracts of plant. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system capable of showing better resolution (Biradar *et al.,* 2013).

Method

The above prepared plant extracts were applied on pre-coated TLC plates by using capillary tubes and developed in a TLC chamber using suitable mobile phase. The developed TLC plates were air dried and observed under ultra violet light UV at both 254 nm and 366 nm. They were later sprayed with different spraying reagents which are vanilin, dragendorff, folin and aniseldehyde. The plate sprayed with vanillin need to be placed in hot air oven for 1 min for the development of color in separated bands. The movement of the analyze was expressed by its retention factor (Rf). Values were calculated for different sample (Biradar *et al.,* 2013) .

$$\text{Rf} = \frac{Distance\ travel\ by\ solute}{Distance\ travel\ by\ solvent} \quad (3.1)$$

3.4.2 Identification of Phytochemical Compounds with Gass Chromatography Mass Spectrometry (GC-MS)

Reagents

6% methanolic KOH (w/v), n-heptane, BFSTA (N,O-bis(trimethylsilyl)trifluoroacetamide silylation reagent. It reacts with a range of polar organic compounds replacing active hydrogens with a – Si(CH_3) 3 (trimethylsilyl) group, TCMS (trimethylchlorosilane). It is a silylation catalyst increases the reactivity of other silylation reagents (Kamthan *et al.,* 2012).

Equipment

Freeze dryer suitable for lyophilisation, freezer (-80℃) for sample storage, vortexer, silanol free glass vials, GC-MS instrument, Rtx5MS- 30m column (0.25-mm ID and 0.25µmdf) (Kamthan *et al.,* 2012).

Method

Alkaline hydrolysis

500 µL of 6% methanolic KOH (w/v) was added to the dried sample and incubated at 85℃ for 30 minute to 1 hour. Then, 250 µL of dH_2O and 750 µL of n-heptane were added and well-vortexed. It is allowed to stand for sometimes till the layers get separated. The upper phase of the mixture was transferred to a fresh vial. The above step is repeated twice. Heptane was allowed to evaporate completely within 16 to 24 hours (Kamthan *et al.,* 2012).

Derivatization

Prior to injection into GC-MS, 100 µL of derivatization reagent (80µL BFSTA + 20µL TMCS) were added to the dried residue and incubated at 65℃ for 1 hour. The most critical point is to avoid any water of moisture during derivatization especially silylating step is highly vulnerable (Kamthan *et al.,* 2012).

GC-MS analysis

For GC-MS analysis, 1µL of sample was injected in split mode in the instrument. A Rtx5MS -30m column with 0.25 mm ID and 0.25 µm df is used. The standardized parameters for GC-MS are as follows:

Injection temperature : 300℃

Interface temperature : 300℃

Ion source should be adjusted to 250℃

Carrier gas : Helium (flow rate of 1 $mL\,min^{-1}$)

The analysis was performed by using 1 min of isothermal heating at 100℃ followed by heating at 300 ℃ for 20 minute as its temperature program. Mass spectra were recorded as 2 $scan\,sec^{-1}$ with a scanning range of 40 to 850 m/z. Quantify each component based on peak areas and normalization based on internal standard (Kamthan *et al.,* 2012).

3.5 Determination of Total Phenolic Contents

Total phenol content (TPC) was measured by the Folin-Ciocalteu method (Müller *et al.,* 2010). Briefly, 20 µL of sample extracts was mixed with 100 µL of Folin-Ciocalteu reagent (diluted 10-fold with distilled water in a 96-well microplate, incubated for 5 min, and 75 µL sodium carbonate solution (75 g/L) was added. After incubation period of 2 hours in darkness at room temperature, the absorbance was measured at 740 nm with a

microplate reader (Tecan Sunrise, Austria). Tannic acid (100 – 1000 µL) was applied as standard for calibration and construction of a linear regression line and water was used as blank. The total phenolic content was estimated as mg tannic acid equivalent (mg TAE/g) of dry extract.

3.6 Determination of Total Flavanoid Contents

Total flavonoid contents (TFC) were measured according to the method (Sasipriya *et al.,* 2012) with some modification. Briefly, 50 µL extracts were added with 70 µL of distilled water and 15 µL of 5% sodium nitrite solution in a 96-well microplate. The solution was well mixed and incubated for 5 min at room temperature. Then, 15 µL of 10% aluminium chloride solution was added into the mixture. After 6 min of incubation, 100 µL of 1 M sodium hydroxide solution was added, and the absorbance was measured at 510 nm with a microplate reader (Tecan Sunrise, Austria). The total flavonoid contents were estimated from quercetin (200 – 1000 µM) standard curve, and the results were expressed as mg quercetin equivalent (mg QE)/g of dry extract.

3.7 Antioxidant Activity Assays

3.7.1 2,2-diphenyl-1-picryhydrazyl (DPPH) Method

The free radical scavenging activity of extracts was measured in terms of hydrogen donating ability using DPPH radical as decribed by the method (Mărghitaş *et al.,* 2009) with a slight modification. Briefly, 40 µL of sample extracts of different concentration (0.002 – 2 mg/mL) were mixed with 200 µL of 50 µM DPPH solution in ethanol. The mixture was immediately shaken and incubated for 15 min in the dark at room temperature. The decrease in absorbance was measured at 517 nm with a microplate reader (Tecan Sunrise, Austria). Ascorbic acid (0.002 – 2 mg/mL) was used as a

standard and the blank was ethanol. The percentage of inhibition activity of the extracts was calculated according to the following equation:

DPPH radical scavenging activity (%) = $\frac{A\ control - A\ sample\ or\ standard}{A\ control}$ x 100 (3.2)

A = Absorbance

The concentration of extracts required to scavenge 50% (IC_{50}) of DPPH radical was estimated from the graph plotted against the percentage inhibition and compared with the standard. All the tests were performed in triplicate, and the results were expressed as mg/mL.

3.7.2 Metal Chelating Activity

The ferrous ion chelating activity (FCA) of the extracts was determined according to the procedure (Srivastava, Raut, Wagh, Puntambekar, & Kulkarni, 2012) by measuring the formation of Fe^{2+}-ferrozine complex based on the methods described by (Decker & Welch, 1990). 100 µL extracts at different concentration (0.002 – 2 mg/mL) were mixed with 120 µL distilled water and 10 µL 2mM of FeCl2 in a 96-well microplate. 20 µL of 5 mM ferrozine was added to the mixture to initiate the reaction. The reaction mixture was incubated at room temperature for 20 min and absorbance at 562 nm was measured along with EDTA-Na_2 (0.002 – 2 mg/mL) as a standard metal chelator. 100 µL of ethanol was used as a control; blank was without ferrozine (20 µL of distilled water instead of ferrozine). The percent Fe^{2+}-ferrozine complex formation was calculated according to the following formula:

Ferrous ion chelating activity (%) = $\frac{A\ control - A\ sample\ or\ standard}{A\ control}$ x 100 (3.3)

A = Absorbance

The concentration of extracts required to chelate 50% of the Fe^{2+} ion (IC_{50}) was calculated from the graph against the percentage of inhibition. All the tests were performed in triplicate and the results were expressed as mg/mL.

3.7.3 Ferric Reducing Antioxidant Power (FRAP)

The FRAP activities of extracts were measured as previously described method (Müller *et al.,* 2010). 20 µL of extracts in ethanol were mixed with 200 µL of daily prepared FRAP reagent, which contained 5 mL of 10 mM TPTZ in 40 mM HCl, 5 mL of 20 mM $FeCl_3$, and 50 mL 0f 0.3 M acetate buffer (pH 3.6) in 96-well microplate. After 8 min of incubation time, the formation of the TPTZ-Fe^{2+} complex in the presence of antioxidant compounds in the extract was measured at 595 nm with the microplate reader (Tecan Sunrise, Austria). Ethanol was used as blank. $FeSO_4$ solution (0.2 mM – 1 mM) was used for standard calibration curved. The FRAP value was evaluated according to the linear regression between standard solutions and absorbance at 595 nm and the results were estimated as mmol -Fe^{2+}/g of dry extract from triplicated tests.

3.8 Animal Study

Sprague Dawley (SD) rats weighing 160 g – 200 g were purchased from Laboratory Animal Research Unit, University National of Malaysia (LARU, UKM). The rats were housed in open cages under standard laboratory conditions with tap water and standard rat pallet diet, in 12 h/12 h light/dark cycle at temperature of 22 . 1 C with a relative humidity of 65±5%. The animals were allowed to acclimate for two weeks. The study was conducted in accordance with the principles outlined in the guide for "Animal Use Protocol" prepared by the Animal Experimental Unit, Faculty of Medicine, University of Malaya (Ethic no. 2015-181006/IBS/R/EZZ).

3.8.1 Oral Acute Toxicity

The oral acute toxicity (OAT) of selected extract from *Ampelocissus* sp. was evaluated in healthy SD rats including both sexes 3 males and 3 females according to the guidelines of the Organization for Economic Cooperation and Development (Kitano, 1992). The rats were fasted overnight (16 h) and orally fed with high dose of extract which is 5000 mg/kg. This particular dose used is decided by following the highest dose stated in OECD for animal study for toxicity test (Barlow *et al.,* 2002). Rats were observed continuously for 3 hours and then at 6 hours intervals for 24 hours and finally after every 24 hours up to 14 days for any physical signs of toxicity such as food consumption, urination, writhing, response to touch and decreased respiratory rate or for any lethality.

3.8.2 Oral Glucose Tolerance Test (OGTT) in Non-diabetic Rats

The OGTT were performed in normal male SD fasted rats. Animals were deprived of food for 16 hours before and during the experiment but were allowed free access to water. The rats were divided into four groups. 400 and 800 mg/kg of methanol extract were administrated orally to two groups (Group III and IV) while 5 mg of glibenclamide to each rats in Group II (n = 3) 30 minute before glucose load (3 g/kg). First group considered as controls, received distilled water (10 ml/kg) instead of the extract, glibenclamide and glucose. Blood samples were taken before and after the administration of the extract and blood glucose level was determined by using a glucometer, Multi-sure, in all animals at 0, 30, 60, 90, and 120 minute (Nyunaï *et al.*, 2009).

3.8.3 Induction of Diabetes in Rats

After acclimatization period, diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ) (60 mg/kg body weight) in citrate buffer (pH 4.5). Diabetes was evident within 10 days after administration of STZ. The rats with blood glucose level above 130 mg/dL were considered diabetic and were involved in the study.

3.8.4 Extract Administration and Body Observation

6 normal rats and 24 diabetic rats were fasted for 12 hours and divided equally into 5 groups. Treatment with the extract started on 11th day of STZ in treatment and then was continued daily for 28 days. The extract was given orally as a single dose in the morning. Group I (normal control and Group II (diabetic control) received distilled water, Group III and Group V received standard drug Glibenclamide (5 mg/kg) and low dose extract (400 mg/kg) respectively, while Group IV received high dose of extract (800 mg/kg). The change in body weight was observed during the treatment period. 800 mg/kg and 400 mg/kg were used to observe the efficacy and safety between high dose and low dose on reducing oxidative stress in diabetic rats (McDonnell *et al.,* 2017)

3.8.5 Determination of Blood Glucose

Blood glucose level was determined by Multi-sure glucometer. The tail of the rat was pricked by lancet and a little of blood dripped on glucometer strip. Level of blood glucose was stated as mg/dL.

3.9 Determination of glutathione peroxidase (GPx) by using Glutathione Peroxidase Assay Kit Merck Millipore (Cat. No. 353919).

Background or Non-enzymatic Wells: Add 120 µl diluted Assay Buffer and 50 µL Co-Substrate Mixture to 3 wells. Positive Control Wells (bovine erythrocyte glutathione peroxidase): Add 100 µl diluted Assay Buffer, 50 µL of Co-Substrate Mixture, and 20 µL diluted Glutathione Peroxidase (Control) to 3 wells. Sample Wells: Add 100 µL diluted Assay Buffer, 50 µL of Co-Substrate Mixture, and 20 µL sample to 3 wells. To obtain reproducible results, the amount of glutathione peroxidase added to the well should cause an absorbance decrease between 0.02 and 0.135/min. When necessary, samples should be diluted with diluted Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. Note: The amount of sample added to the well should always be 20 µl. To determine if an additional sample control should be performed see the Interferences section. Initiate the reactions by adding 20 µL Cumene Hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the Cumene Hydroperoxide as quickly as possible. Carefully shake the plate for a few seconds to mix. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points. Note: The initial absorbance of the sample wells should not be above 1.2 or below 0.5.

Calculating the results by determination of the reaction rate

a. Determine the change in absorbance (ΔA_{340}) per minute by:

i. Plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown below using bovine erythrocyte glutathione peroxidase) -or-

ii. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min} = \frac{A340\ (\text{Time } 2) - A340\ (\text{Time } 1)}{Time\ 2\ (\text{min}) - Time\ 1\ (\text{min})} \quad (3.4)$$

b. Determine the rate of ΔA_{340}/min. for the background or non-enzymatic wells and subtract this rate from that of the sample wells.

c. Use the following formula to calculate the Glutathione peroxidase activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373 µM-1*. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP+ per minute at 25°C. *The actual extinction coefficient for NADPH at 340 nm is 0.00622 µM-1cm-1. This value has been adjusted for the path length of the solution in the well (0.6 cm).

3.10 Determination of Catalase (Cat) Activity

The catalase activity was determined based on the disappearance of added hydrogen peroxide in the presence of enzyme source (Sinha, 1972). To measure catalase activity, 50 µL of serum sample or standard and 50 µL of 0.2 M H_2O_2 solution, 50 µL of 0.01 M phosphate buffer and 100 µL 5% potassium bichromate were added to each well of 96-microplate well. The mixture was incubated for 30 min at 37℃. After cooling, the absorbance of the solution was read at 570 nm. The intensity of the absorbance was linearly related to the H_2O_2 concentration. The catalase activity was stated as µmol H_2O_2 /min/mL.

3.11 Statistical Analysis

Results were analyzed by SPSS 22.0 version. Results were expressed as the mean ± standard error (SEM) for the three independent experiments. The significance analysis has been performed by using ANOVA, followed by Duncan's multiple comparison test.

CHAPTER 4: RESULTS

4.1 Extraction Yields

In this study, to separate the chemical compounds according to polarity, non-polar to highly polar solvents were chosen. 100 g of Isi Nyaru powder was extracted with hexane in the first step, the followed petroleum-ether, chloroform, methanol and water. The extraction was performed by incubating the mixture solution in a water bath at 40ºC for 24 hours. These extracts were then evaporated under pressure and stored in -20ºC for further test. The yields of extraction have varied depending on extraction solvent used as shown in Table 4.1 below.

Table 4.1: Samples of *Ampelocissu*s sp. Crude extracts at five different solvent systems.

Solvents	Yield Extracts (g)	Color	Form
Hexane	2.61	Light yellow	Sticky
Petroleum-ether	2.07	Dark yellow	Sticky
Chloroform	3.40	Green	Sticky
Methanol	38.76	Dark brown	Solid
Water	14.21	Light brown	Powder

4.2 Separation of phytochemical bioactive compounds

4.2.1 Qualitative analysis by Thin Layer Chromatography (TLC)

Table 4.2: TLC analysis of tuber *Ampelocissus* sp. crude extract.

Extract	Band	R_f Value	Color		Reagent				Remarks
			Visi-ble	UV light	Vanilin	Dragen-dorff	Folin	Anisel-dehyde	
Hexane	1	0.963	-	P 2+	B 3+	-	B 4+	P 4+	Phenol, Flavonoid & Terpenoid
	2	0.913	-	P 2+	B 2+	-	B 4+	P 3+	
	3	0.850	-	P 2+	P 4+	-	B 4+	P 3+	
Petroleum-ether	1	0.971	-	P 2+	P 2+	-	B 3+	P 2+	Phenol, Flavonoid & Terpenoid
	2	0.914	-	-	B 3+	-		P 2+	
	3	0.743	-	P 2+	P 1+	-	B 1+	P 2+	
	4	0.657	-	-	P 1+	-	B 3+	P 3+	
	5	0.543	-	P 1+	B 4+	-	B 1+	P 1+	
	6	0.514	-	-	P 1+	-	B 4+	P 3+	

Extract	Band	R_f Value	Color		Reagent				Remarks
			Visi-ble	UV light	Vanilin	Dragen -dorff	Folin	Anisel-dehyde	
	7	0.357	-	P 1+	P 4+	-	B 1+	P 1+	
	8	0.271	-	P 1+	P 1+	-	B 1+	P 1+	
	9	0.229	-	P 1+	P 1+	-	B 1+	P 1+	
	10	0.157	-	-	P 1+	-	B 1+	P 1+	
Chloroform	1	0.971	-	-		-		P 2+	Phenol, Flavonoid & Terpenoid
	2	0.929	-	-	B 1+	-		P 2+	
	3	0.843	-	-	B 3+	-	B 1+	P2+	
	4	0.637	-	P 1+	P 1+	-	B 1+	P 2+	
	5	0.571	-	-	B 4+	-	B 3+	P 3+	
	7	0.400	-	P 2+	-	-	B 2+	P 2+	
	8	0.329	-	-	-	-		B 1+	
	9	0.286	-	P 2+	P 2+	-	B 1+	P 2+	
	10	0.257	-	-	P 2+	-	B 2+	B 3+	

Extract	Band	R_f Value	Color		Reagent				Remarks
			Visi-ble	UV light	Vanilin	Dragen -dorff	Folin	Anisel-dehyde	
	11	0.214	-	P 2+	P 2+	-	B 1+	B 3+	
	12	0.157	-	-	P 2+	-	B 2+	B 3+	
	13	0.129	-	P 1+	P 2+	-	B 1+	P 1+	
	14	0.100	-	-	-	-		B 2+	
	15	0.043	-	P 1+	-	-	B 1+	P 1+	
Methanol	1	0.971	-	P 2+	R 2+	-	-	R 2+	Phenol &
	2	0.186	-	P 2+	R 2+	-	-	R 2+	Flavonoid
Water	1	0.900	-	P2+	R 2+	-	-	R 2+	Phenol &
	2	0.214	-	P 2+	R 2+	-	-	R 2+	Flavonoid

Indications: 4+ Very strong 3+ Strong 2+ Weak 1+ Very weak

Colour:

- No colour B Blue

P Purple R Red

The present study carried out in Isi Nyaru showed the presence of medicinal active constituents. The phytochemical active compounds were qualitatively analysed by Thin Layer Chromatography and the results are presented in Table 4.2. TLC analysis has revealed that these compounds were phenol, flavonoids and terpenoid due to colour appeared after TLC plates were sprayed with vanillin, dragendorrf, folin and aniseldehyde reagents.

In this phytochemical screening, phenol and flavonoid were present in all solvent extracts. Terpenoid were found in hexane, petroleum-ether and chloroform extracts while not in methanol and water extracts.

4.2.2 Gas Chromatography Mass Spectrometry (GCMS)

Initial investigation showed the presence of phenolic and flavonoid compounds in methanol crude extract of tuber *Ampelocissus* sp. Further test was done using Gas Chromatography Mass Spectrometry (GCMS). The GCMS spectrum revealed the presence of polyphenols components with different retention times.

The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different ratios. These mass spectra were identified from Mass Bank the high resolution mass spectral database as presented in Table 4.3 and Table 4.4

The compounds found in *Ampelocissus* sp. methanol extract are alkene, ester and ether compounds. While there is a lot of amino group compound found in water extract. Amino acid is an important elements in body system.

Table 4.3: Compounds identified in the water extract of Isi Nyaru in GCMS.

Retention time (RT)	Compound Name	Molecular formula	Molecular weight (g/mol)	Peak area (%)	Compound Nature
25.914	Diethanolamine	$C_4H_{11}NO_2$	105.14	0.360	Ethanolamines
26.615	Semicarbazide	CH_5N_3O	75.071	1.797	Monocarboxylic acid amide
27.103	2-methylbutylamine	$C_5H_{13}N$	87.166	1.545	Monoalkylamines
27.312	Maleic acid	$C_4H_4O_4$	116.072	2.534	Dicarboxylic acid
27.804	1,2-dihydropyrimidine	$C_4H_6N_2$	82.106	1.085	Cyclic aromatic compound
33.357	3-aminopropionitrile	$C_3H_6N_2$	70.095	0.261	β- amino group
36.913	N-butylurea	$C_5H_{12}N_2O$	116.164	0.300	Amide
44.079	2-aminopropanoic acid	$C_3H_7NO_2$	89.094	2.053	Amino acid
45.279	4-aminobutyric acid	$C_4H_9NO_2$	103.121	0.252	Amino acid

Table 4.4: Compounds identified in the methanol extract of Isi Nyaru in GCMS.

Retention time (RT)	Compound Name	Molecular formula	Molecular weight (g/mol)	Peak area (%)	Compound Nature
8.150	1,5-Hexadiene, 3,3,4,4-tetrafluoro	$C_6H_6F_4$	154.11	0.604	Alkene
11.981	1-Buten-3-yne	C_4H_4	25.07	0.114	Alkene
20.436	Propanedioic acid, bis(trimethylsilyl) ester	$C_{10}H_{22}O_4Si_2$	248.425	0.342	Ester
23.108	L-(+)-Tartaric acid, bis(trimethylsilyl) ether, bis(trimethylsilyl) ester	$C_{16}H_{38}O_6Si_4$	438.81	1.452	Ester
27.086	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime			27.404	
27.251	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime			18.661	
27.372	d-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)	$C_{22}H_{55}NO_6Si_5$	570.10	0.318	Ether
27.486	d-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1E)			19.616	
27.768	d-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)			3.280	
28.539	Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester	$C_{19}H_{38}O_5Si_4$	458.84	5.137	Ester

4.3 Polyphenolic Contents of Isi Nyaru

Tannic acid and quercetin were used as a standard reference in order to determine the total phenolic content and total flavonoid content of crude extracts of *Ampelocissus* sp. respectively. The standard curve of tannic acid and quercetin were prepared at six different concentrations.

The various contents of polyphenol were detected among the fractions, and the results are presented in Table 4.5. The results showed that, depending on the types of solvent used, the quantities of Total Phenol Contents (TPC) and Total Flavonoid Contents (TFC) were varied.

Methanol crude extract revealed the highest total phenolic content with 748.5 mg (TAE)/g followed by water, petroleum-ether, chloroform and hexane crude extracts with 737.31 mg (TAE)/g, 154.21 mg (TAE)/g, 17.07 mg (TAE)/g and 2.07 mg (TAE)/g respectively.

The highest amount of total flavonoid content was detected in methanol crude extract and followed by water extract with 27.08 mg (QE)/g and 17.04 mg (QE)/g. The medium yield was observed in chloroform while the lowest yields were indicated by petroleum-ether and hexane with 4.26 mg (QE)/g, 2.44 mg (QE)/g and 0.88 mg (QE)/g respectively.

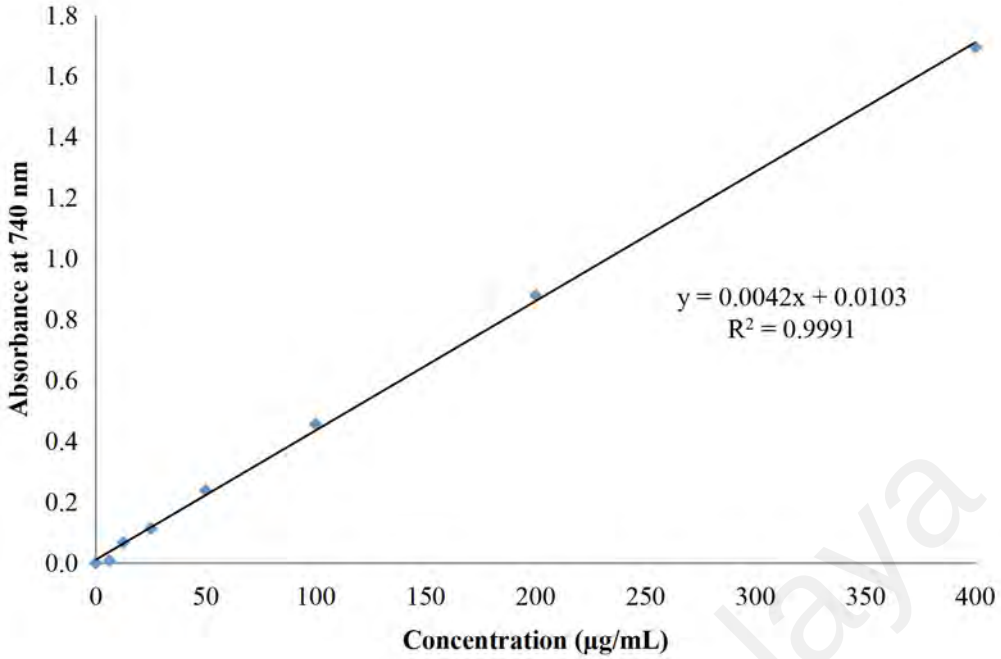

Figure 4.1: Standard curve of Tannic acid in Total Phenol Content.

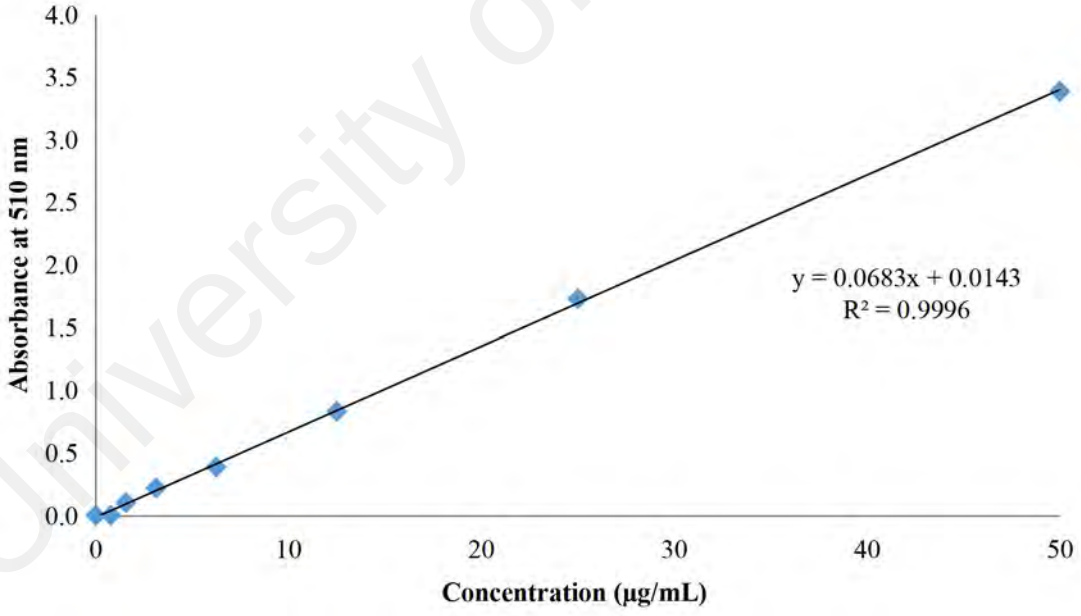

Figure 4.2: Standard curve of Quercetin in Total Flavonoid Content

Table 4.5: TPC and TFC of *Ampelocissus* sp. extracts. Each value is represented as mean ± SE (n = 4). The means with different lower case letters (a,b,c and d) in the same column are significantly different at P < 0.05 (ANOVA, followed by Duncan's multiple comparison test).

Extract (2 mg/mL)	Phenolic Content (mg (TAE)/g)	Flavonoid Content (mg (QE)/g)
Hexane	$2.07 \pm 0.05_a$	$0.88 \pm 0.02_a$
Petroleum-ether	$154.21 \pm 0.08_c$	$2.44 \pm 0.08_a$
Chloroform	$17.07 \pm 0.01_b$	$4.26 \pm 0.03_b$
Methanol	$748.50 \pm 0.01_d$	$27.08 \pm 0.04_d$
Water	$737.31 \pm 0.07_d$	$17.04 \pm 0.10_c$

4.4 Antioxidant Activity Assay

In this study the antioxidant of *Ampelocissus* sp. extracts was evaluated by using three different assays.

4.4.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

To determine DPPH radical scavenging activity is based on the formation of the DPPH-H non-radical form in the presence of hydrogen donating antioxidants in the extracts that can be detected at 517 nm. Since ascorbic acid is a potent antioxidant, it was used as a standard.

Table 4.6: Percentage inhibition of DPPH radical by standard reference of ascorbic acid. Percentage of inhibition are expressed as mean ± SE, (n=4). (SPSS Statistic Data Editor 22.0 Version).

Concentration of Ascorbic acid (µg/mL)	Percentage of DPPH inhibition (%)
2	72.90 ± 0.01
20	88.83 ± 0.03
200	98.82 ± 0.01
2000	99.41 ± 0.02

The percentage of DPPH inhibition was increased with increasing concentration of ascorbic acid. The concentration of ascorbic acid is ten times of dilution volume which are 2 µg/mL, 20 µg/mL, 200 µg/mL and 2000 µg/mL. The percentage of DPPH inhibition were 72.90 %, 88.83 %, 98.82 µg/mL and 99.41 % respectively.

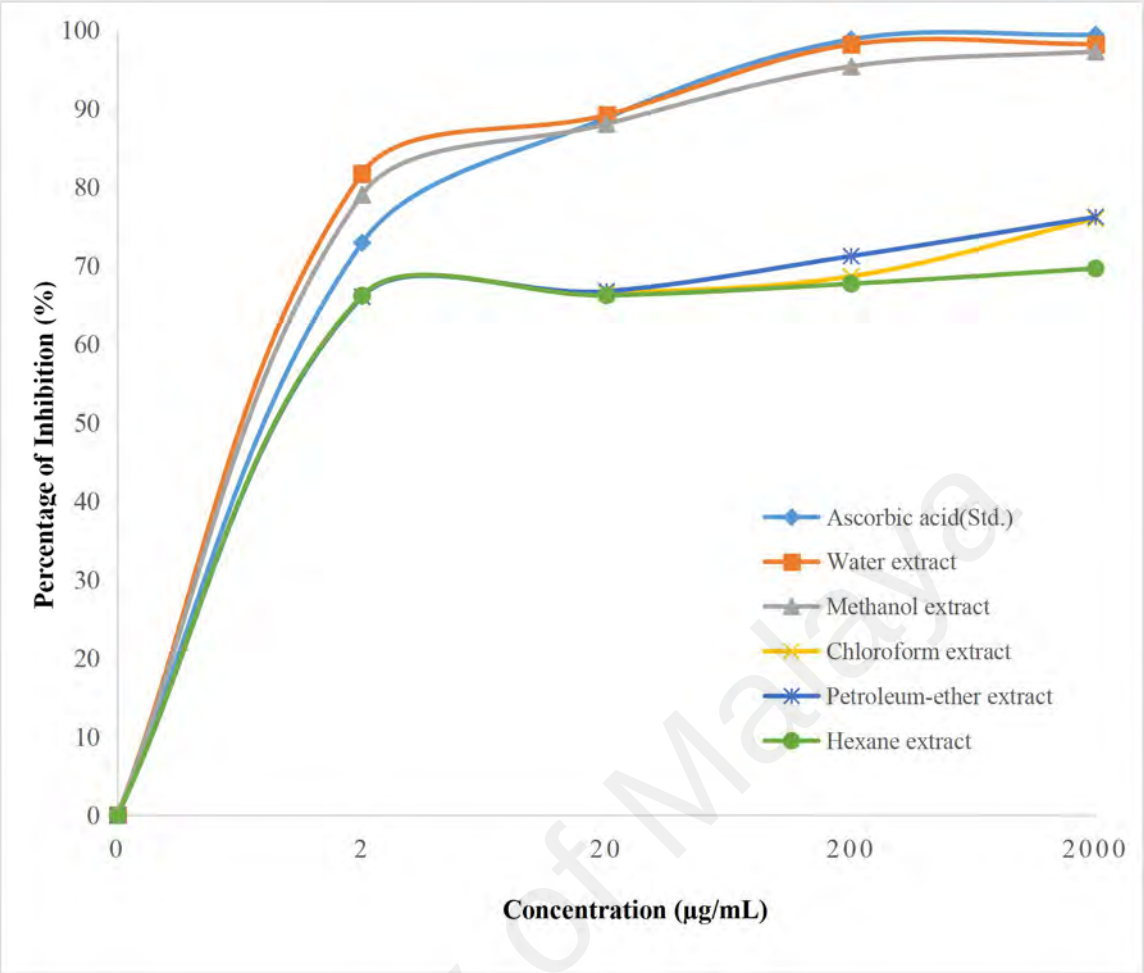

Figure 4.3: Scavenging activity percentage of DPPH of hexane, petroleum-ether, chloroform, methanol and water aqueous extracts of *Ampelocissus* sp.

IC_{50} has been determined from the graph to ensure the efficiency of the extracts to scavenge free radical at 50% inhibition. In comparison with ascorbic acid, water and methanol extracts possess higher percentage of inhibition than ascorbic acid while hexane, petroleum-ether and chloroform have lower percentage of inhibition than ascorbic acid. Based on the percentage of scavenging activity, ascorbic acid has IC_{50} = 1.37 µg/mL which slightly higher than water extract IC_{50} = 1.22 µg/mL and methanol extract IC_{50} = 1.27 µg/mL. While chloroform, petroleum-ether and hexane share the same value of IC_{50} which is 1.51 µg/mL.

4.4.2 Metal Chelating Activity

The assay performed is to measure chelating ability of ferrous ion was based on the chelation of this ion with ferrozine to form ferrous-ferrozine complex which can be detected at 562 nm. Five concentrations of $EDTANa_2$ were prepared.

Table 4.7 Percentage metal chelating by standard reference of $EDTANa_2$. Percentage of inhibition are expressed as mean ± SE, (n=4). (SPSS Statistic Data Editor 22.0 Version).

Concentration of $EDTANa_2$ (µg/mL)	Percentage of ferrozine- Fe^{2+} complex (%)
2	99.71 ± 0.02
20	98.78 ± 0.07
200	86.67 ± 0.08
2000	70.78 ± 0.02

The concentration of $EDTANa_2$ is ten times of dilution volume which are 2 µg/mL, 20 µg/mL, 200 µg/mL and 2000 µg/mL. The percentage of inhibition Ferrozine - Fe^{2+} complex formation by EDTA percentage were 99.71%, 98.78%, 86.67% and 70.78% respectively.

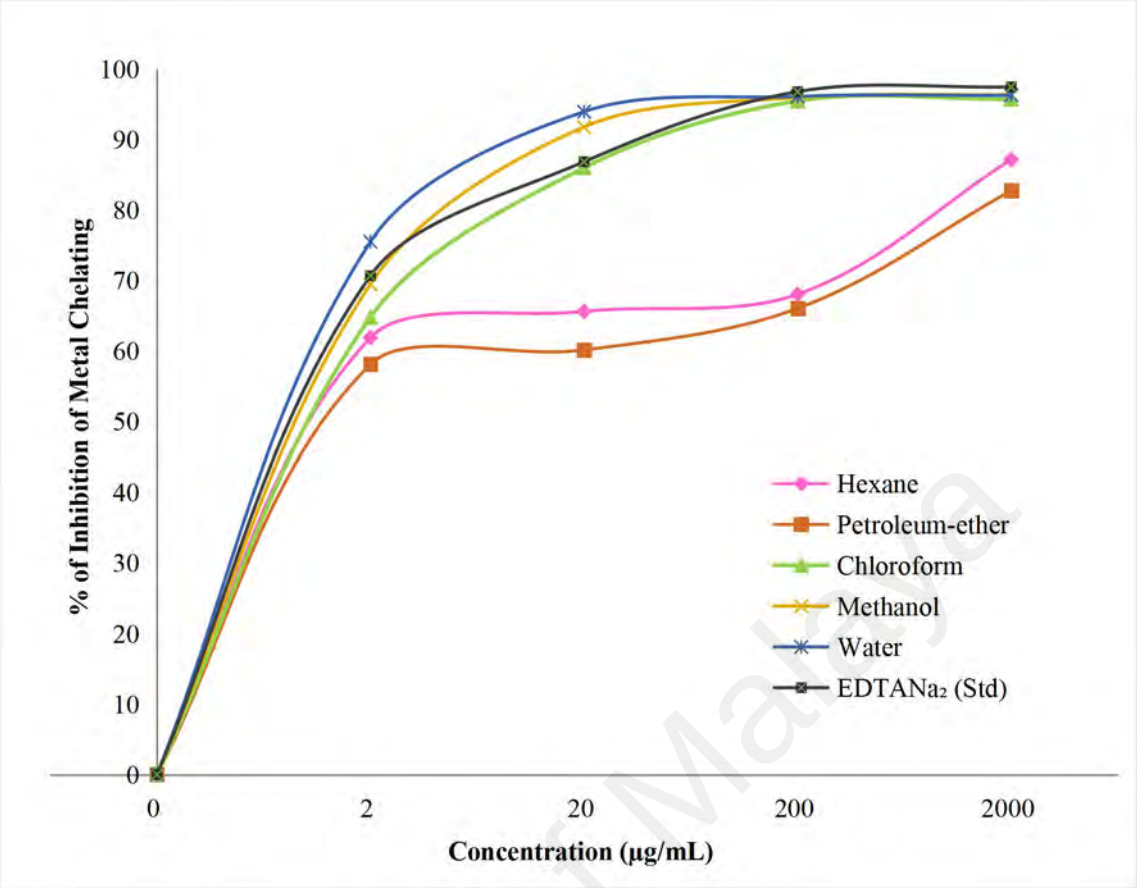

Figure 4.4: Metal chelating activity of hexane, petroleum-ether, chloroform, methanol and water aqueous extracts of *Ampelocissus* sp.

Figure 4.4 shows the percentage of inhibition Ferrozine - Fe^{2+} complex formation by hexane, petroleum-ether, chloroform, methanol and water aqueous extracts. In comparison with $EDTANa_2$ IC_{50} = 1.41 µg/mL, methanol extract has the highest IC_{50} = 1.72 µg/mL, followed by water extract IC_{50} = 1.62 µg/mL, chloroform extract IC_{50} = 1.54, petroleum-ether IC_{50} = 1.44 µg/mL and hexane IC_{50} = 1.32 µg/mL.

4.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP value of extracts was measured based on the reaction between antioxidant potentials and Fe^{3+}-TPTZ complex to produce blue color Fe^{2+}-TPTZ form in the extracts. FRAP values of all extracts were evaluated according to the linear regression from FRAP standard curve in Figure 4.5.

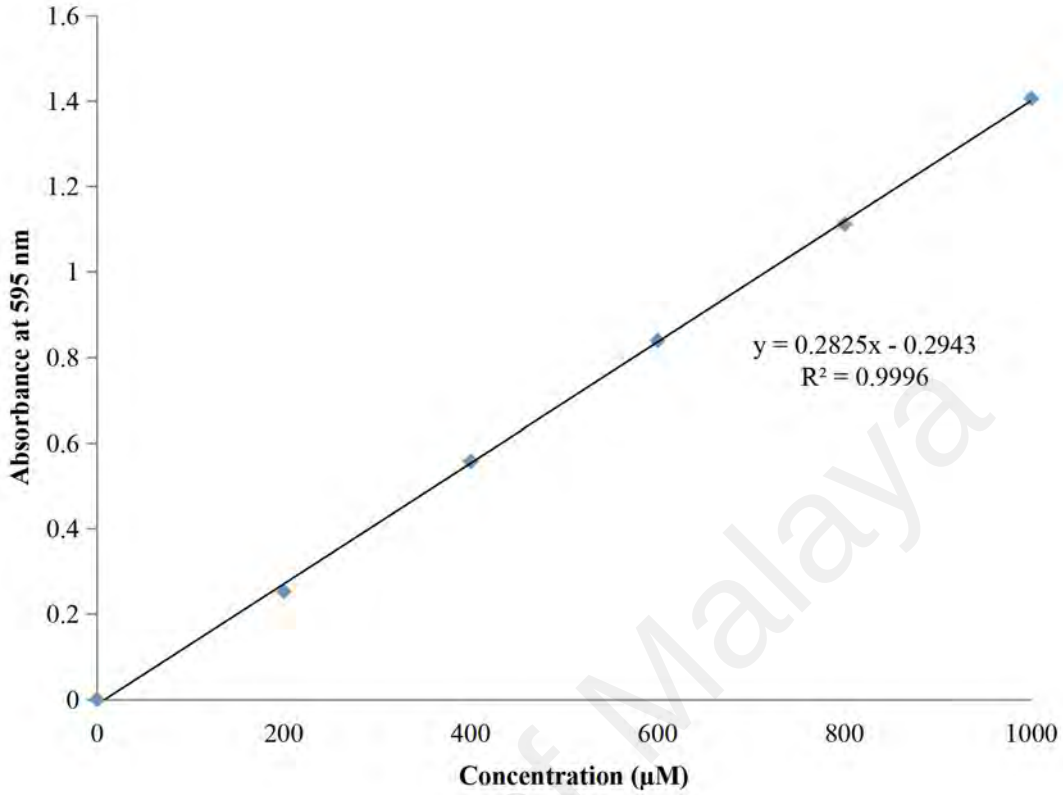

Figure 4.5: Standard curve of ferrous sulphate in FRAP.

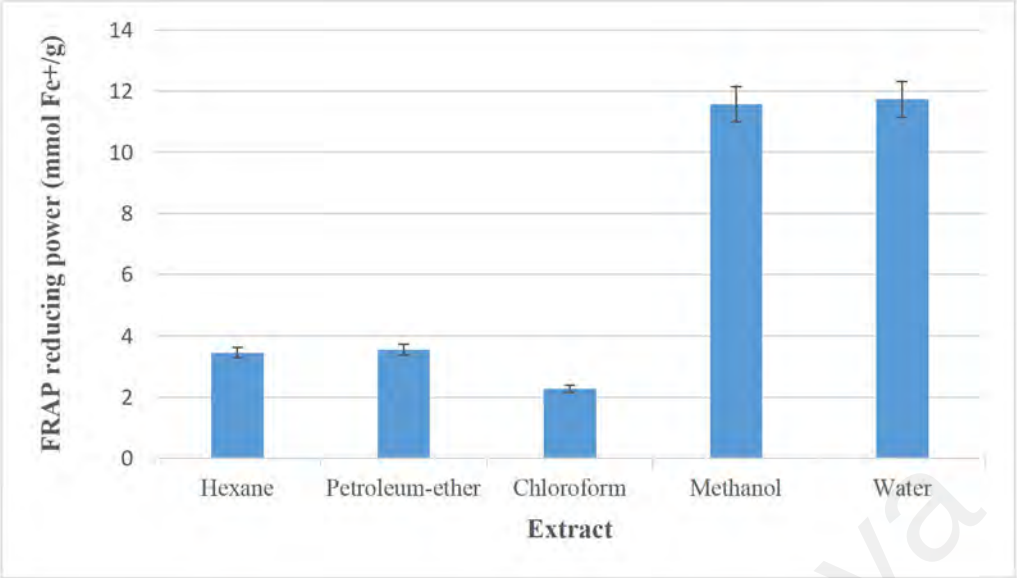

Figure 4.6: Ferric reducing antioxidant power assay.

The highest FRAP value was detected in water extract (11.73 mmol Fe^{2+}/g of dry extracts) and slightly lower for methanol extract (11.58 mmol Fe^{2+}/g of dry extracts). The reducing ability of chloroform (2.27 mmol Fe^{2+}/g of dry extracts) is the least efficient compared to hexane extract (3.56 mmol Fe^{2+}/g of dry extracts) and petroleum-ether extract (3.56 mmol Fe^{2+}/g of dry extracts).

4.5 Animal Study

4.5.1 Acute Oral Toxicity Test

Three male and three female of six weeks Sprague-dawley rats was administrated with methanol crude extract of *Ampelocissus* sp. via intrapatoneal at dose of 4000 mg/kg. Table 4.6 showed that methanol crude extract did not show any lethality or any toxic reaction in 14 days.

Table 4.8: Number of exposed and survived after administrated with methanol crude extract of *Ampelocissus* sp.

Number of SD rats exposed	Number of survived			Toxic reactions
	24h	72h	14 days	
6	6	6	6	-

4.5.2 Oral Glucose Tolerance Test (OGTT)

OGTT was performed to investigate the ability of SD rats to use glucose which is the body's main source of energy. 3 mg/kg glucose was administrated by oral gavage to normal control and glucose-induced hyperglycemic rats. The reductions of blood glucose levels were compared with control group.

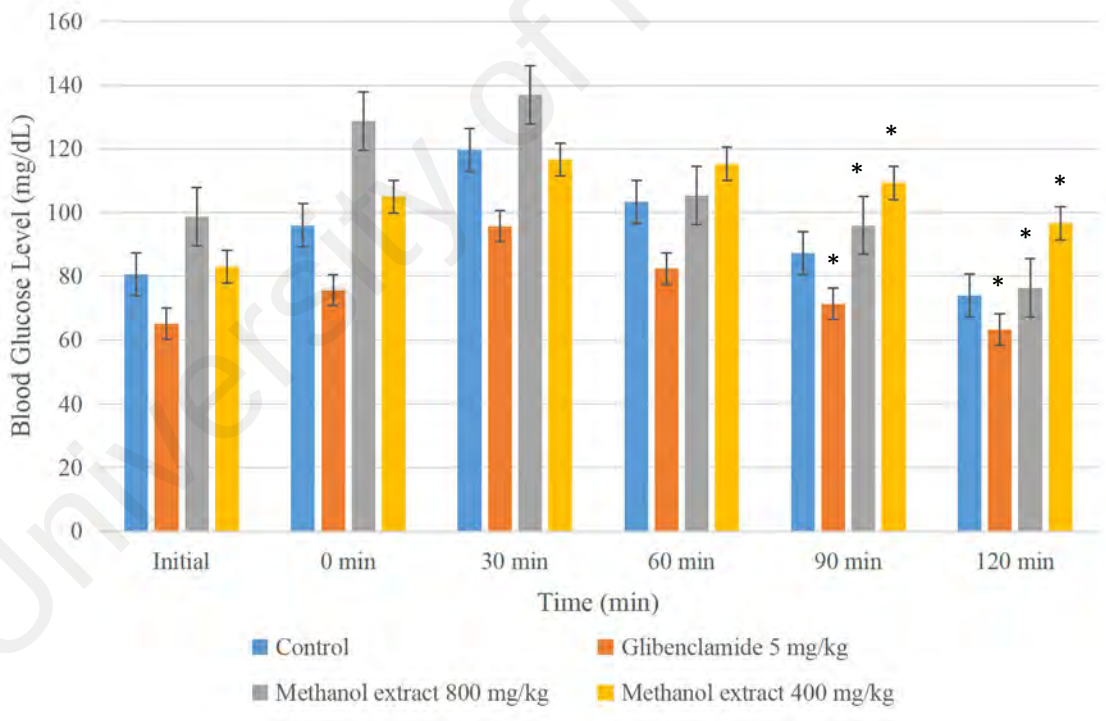

Figure 4.7: Blood glucose level of SD rats after administrated with *Ampelocissus* sp. methanol extract and glibenclamide. The sign (*) means significantly different from the control value of diabetic control group at $p < 0.05$.(ANOVA, followed by Duncan's multiple comparison test).

Figure 4.7 showed an increased in blood glucose level for all groups from 0 minutes until 30 minutes and start to decreased at minute of 60. Methanol extract of 800 m/kg showed a significant falls as well as glibenclamide as compared to control group. In addidtion, there is no significant reduction of blood glucose level in rats treated with 400 mg/kg methanolic extract.

4.5.3 Effect of Methanolic Extract of *Ampelocissus* sp. on Blood Glucose Level

The hyperglycaemic effects of methanolic *Ampelocissus* sp. Extracts on fasting blood glucose levels of diabetic induced rats were showed in Table 4.7. There was a significantly decreased in blood glucose level in diabetic group treated with glibenclamide and methanolic extract as compared to normal group.

Table 4.9 Effect of methanolic extracts of *Ampelocissus* sp. in fasting blood glucose level of diabetic rats. The sign (*) means significantly different from the control value of normal group at $p < 0.05$. (ANOVA, followed by Duncan's multiple comparison test).

Groups	Blood Glucose Level (mg/dL)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	109 ± 3	95 ± 3	104 ± 2	91 ± 5	107 ± 4
Diabetic control	205 ± 65	195 ± 46	208 ± 47	204 ± 54	296 ± 76*
Glibenclamide 5 mg/kg	258 ± 90	246 ± 101	108 ± 11	98 ± 11	90 ± 5
Methanol extract 400 mg/kg	375 ± 65	374 ± 75	276 ± 77	238 ± 72	194 ± 51*
Methanol extract 800 mg/kg	367 ± 95	345 ± 89	290 ± 82	223 ± 57	131 ± 15*

From the table above, there is no significant changes of blood glucose level in normal rats. SD rats treated with low dose (400 mg/kg) and high dose (800 mg/kg) of methanol extract showed a significant decreased of blood glucose level about 12.4 % and 17.4 % respectively. There is also a significant falls between those extracts and glibenclamide which indicate the highest reduction of blood glucose level which is 21%.

4.5.4 Effect of Methanolic Extract of *Ampelocissus* sp. on Body Weights

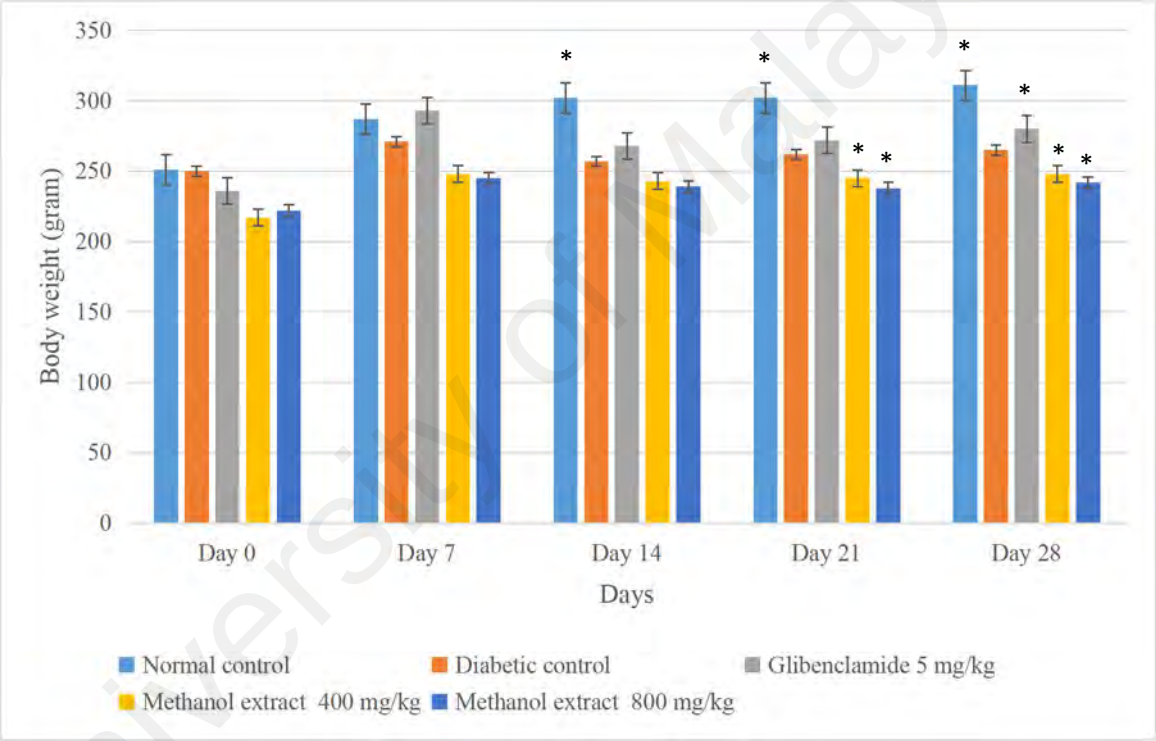

Figure 4.8: Effect in body weight after administrated with methanolic extract and glibenckamide as a standard drug. The sign (*) indicated values significantly different from diabetic control group at $p < 0.05$. (ANOVA, followed by Duncan's multiple comparison test).

Decreased in body weight were observed during the treatment of diabetic rats with methanol extract of *Ampelocissus* sp. and glibenclamide as standard drug during the first two weeks of this experiment. Results showed there is a slightly increased of weight in STZ-induced rats treated with 400 mg/kg and 800 mg/kg methanol extract rats which only 1.7 %, 2.6 % respectively. While the rats treated

with glibenclamide indicate 3.3 % increased of body weight. There is no significant increase of body weight in diabetic control rats.

4.5.5 Effect of Methanolic Extract of *Ampelocissus* sp. on Glutathione peroxidase Activity

The GPX activity in serum 4.10. Serum GPx activity in the untreated diabetic rats was significantly less than control which are 77.43 and 100.35 nmol/min/mL respectively. Serum GPX activity of the methanol extracts-treated diabetic rats was significantly different from that of the untreated diabetic rats. They are 87.61 nmol/min/mL for 400 mg/kg methanol extract and 93.34 nmol/min/mL for 800 mg/kg methanol extract. The GPX activities in the serum of the methanol extracts-treated diabetic rats were relatively high than untreated diabetic control. But there is slightly significantly lower GPx activity in glibencamide-treated rats than methanol extracts-treated rats and normal rats which only 65.97 nmol/min/mL.

Table 4.10: GPx assay of serum in rats treated by methanol extract and glibenclamide (n=2).

Groups	Glutathione peroxidase activity (nmol/min/mL)
Normal control	100.35 ± 15.14
Diabetic control	77.43 ± 11.53
Glibenclamide 5 mg/kg	65.97 ± 6.02
Methanol extract 400 mg/kg	87.61 ± 3.75
Methanol extract 800 mg/kg	93.34 ± 14.68

4.5.6 Effect of Methanolic Extract of *Ampelocissus* sp. on Catalase Activity

Serum CAT activities are shown in Table 4.11. Serum CAT activity in the untreated diabetic rats was significantly higher than the control which are 52.38 μM/min/mL and 42.89 μM/min/mL respectively. Serum CAT activity of methanol extracts-treated diabetic rats do not have any significant different as compared to normal untreated rats but signifiantly lower that diabetic untreated rats. They are 41.15 μM/min/mL for 400 mg/kg methanol extract and 41.70 μM/min/mL for 800 mg/kg methanol extract. The serum glibenclamide-treated diabetic rats showed no significant different between normal and methanol extracts-treated rats of CAT activity.

Table 4.11: Catalase activity assay of serum in rats treated by methanol extract and glibenclamide (n=2).

Groups	Catalase activity (μM/min/mL)
Normal control	42.89 ± 0.02
Diabetic control	52.38 ± 0.08
Glibenclamide 5 mg/kg	41.83 ± 0.07
Methanol extract 400 mg/kg	41.15 ± 0.05
Methanol extract 800 mg/kg	41.70 ± 0.03

CHAPTER 5: DISCUSSION

Samples of *Ampelocissus* sp. were prepared by dried it under room temperature. The dried samples were then grinded to become powder. An extraction is a processes that are widely used to obtain crude extract contain bioactive compounds on plants samples (Chirinos et al., 2007). Scientists have studied and analyzed the impact of different types of solvents, such as methanol, hexane, and ethyl alcohol, for the purpose of antioxidant extraction from various plants parts, such as leaves and seeds. In order to extract different phenolic compounds from plants with a high degree of accuracy, various solvents of differing polarities must be used. Moreover, scientists have discovered that highly polar solvents, such as methanol, have a high effectiveness as antioxidants (Wong & Kitts, 2006). In this study, a serial of extensive extraction method was used involving the extraction with non-polar solvent which is hexane to more polar solvent which is water to ensure a wide polarity range of bioactive compounds could be extracted. For aqueous extraction, it was done by soaking in aqueous bath at 40 ℃ for 8 hours. The extracts were then filtered through Whatmann's filter paper. The filtrate obtain was concentrated by rotary evaporation at 45 ℃ and freeze dried to obtain the yield as recorded in Table 4.1. During the extraction, temperature is one of the most vital factors to be controlled as different bioactive compound has different stability that may affected by temperature. To protect these bioactive compounds from thermal decomposition, the temperature was controlled and maintained at optimum temperature which is 40 to 45 ℃. There are several others factors can influence the extraction efficiency, including extraction method, solvent type and concentration, particle size of plant materials, extraction time and temperature, solvent to solid ratio and extraction pH (Chirinos *et al.,* 2007).

Seperation and detection of chemical compounds were carried out using thin layer chromatography (TLC). TLC is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, aluminium, or cellulose on a flat, inert substrate. It has the advantage of faster runs, better seperations, and the choice between different adsorbents. TLC takes advantage of the different affinity of the analyte with the mobile and stationary phase to achieve the seperation of complex mixtures of organic molecules (Kumar *et al.,* 2013).

In this study, thin layer chromatography uses a thin glass plate coated with either silica gel as the solid phase. The mobile phase is a solvent chosen according to the properties of the components in the mixture. The principle of TLC is the distribution of a compound between a solid fixed phase applied to a glass or plastic plate and a liquid mobile phase, which is moving over the solid phase. A small amount of a compound or mixture is applied to a starting point just above the bottom of TLC plate. The plate is then developed in the developing chamber that has a shallow pool of solvent just below the level at which the sample was applied. The solvent is drawn up through the particles on the plate through the capillary action, and as the solvent moves over the mixture each compound will either remain with the solid phase or dissolve in the solvent and move up the plate. Whether the compound moves up the plate or stays behind depend on the physical properties of that individual compound and thus depend on its molecular structure, especially functional groups. The solubility rule “Like Dissolves Like” is followed. The more similar the physical properties of the compound to the mobile phase, the longer it will stay in the mobile phase. The mobile phase will carry the most soluble

compounds the furthest up the TLC plate. The compounds that are less soluble in the mobile phase and have a higher affinity to the particles on the TLC plate will stay behind (Singhal *et al.,* 2009).

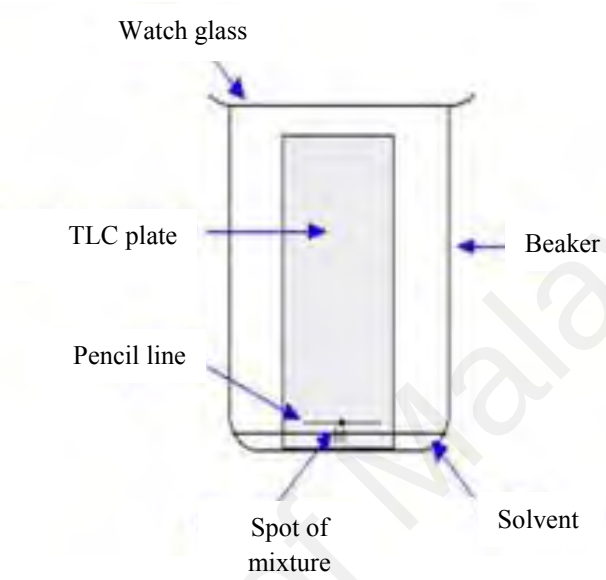

Figure 5.1. Chromatogram

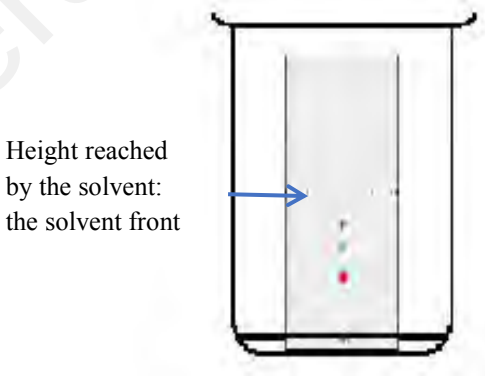

Figure 5.2. Developing tank

There is a several number of solvent system used for each extract to obtain the good seperation of bioactive compound. Solvent system for hexane crude extract is 50 methanol: 50 chloroform, 80 hexane: 20 acetone for chloroform and

petroleum-ether, 20 methanol: 80 chloroform and 50 methanol: 50 chloroform for methanol and water crude extract respectively.

In Table 4.2, each compound possessed its own relative R_f value. It means that the values are reported relative to a standard, or it means that you compare the R_f values of compounds run on the same plate at the same time. The larger an R_f of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger R_f is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. Conversely, if the structures of the compounds in a mixture is known,it is easy to predict that a compound of low polarity will have a larger R_f value than a polar compound run on the same plate.

Some compounds are sufficiently coloured, for example natural and synthetic dyes, and nitrophenols, to give an absorption in the visible part of the electromagnetic spectrum. However, it is the remaining part of the visible radiation that is reflected and is seen by either the naked eye or light-detecting equipment.As recorded in Table 4.2 there is colorless bioactive compound spot as they are not visible by naked eyes. Colourless separated components on the TLC/high performance (HP) TLC layer may absorb electromagnetic radiation at shorter wavelengths than the visible spectrum. These are detected in the UV range, normally at 400 to 200 nm. Such compounds are detected using Chromatography: Thin layer (Planar) / Spray Reagents 907 UV-sensitive detectors, e.g. photomultipliers. Often exposure to UV light at short-wave radiation (254 nm) or long-wave radiation (366 nm) is all that is necessary for absorbing or fluorescing substances to be observed (Kumar *et al.,* 2013). Four types of reagent has been used in this experiment. They

are vanillin, dragendorff, folin and aniseldehyde. Each reagent has specific group of compound detection in TLC. As has been recorded in Table 4.2 hexane, petroleum-ether and chloroform crude extract contained phenol, flavonoid and terpenoid while methanol and water crude extract have phenol and flavonoid. These compounds were grouped by their colors appearance after the reagents were sprayed.

Anisaldehyde - sulphuric acid is a universal reagent for natural products that makes color differentiation possible. It is a reagent for anitoxidants, steroids, prostaglandins, carbohydrates, phenols, glycosides, sapogenins, essential oil components or terpenes, antibiotics and mycotoxins (Smith & Petterson, 1984). According to the book by Ivor Smith published in 1960, the initial reaction of vanillin would be electrophilic aromatic substitution of a phenol and give out yellow spots on TLC plate while Folin-Ciocalteu reagent was used to detect phenolic functional group of capsaicinoids yielding the blue color. A simple analytical platform based on thin-layer chromatography coupled with paper-based analytical device for determination of total capsaicinoids in chilli samples (Dawan *et al.,* 2017). Alkaloid detecting reagents are solutions of the salts of heavy metals such as Dragendorff"s (potassium iodide-bismuth nitrate). The mechanism of action is proposed to occur via coupling of the reagent's heavy metal atom in the reagent with the nitrogen in the alkaloid to form ion pairs. The ion pairs form an insoluble precipitate. Using Dragendorff's reagent, a reaction occurs between $BiI4^-$ and the alkaloid. In the ppt complex, $BiI4^-$ could determine by flam atomic absorption spectrometry. The color could vary from orange-red, yellow-orange, red-black, pink-purple depending on the species or genus (Coe *et al.*, 1996).

Gas chromatography-mass spectrometry was done to investigate and characterize the chemical composition of the different crude extracts from the tuber

of *Ampelocissus* sp. water and methanol extracts were chose for further investigation.

Qualitative determination of the different biologically active compounds from water and methanol crude extracts of *Ampelocisu*s sp. using gas chromatography–mass spectrometry revealed different types of high and low molecular weight chemical entities with varying quantities present in each of the extracts as presented in Table 4.3 and Table 4.4. These chemical compounds are considered biologically and pharmacologically important. Furthermore, the two different extracts possess unique physicochemical characteristics which may be attributed to the compounds naturally present in significant quantities in the tuber of *Ampelocissus* sp**.** The compounds found in *Ampelocissus* sp. methanol extract are alkene, ester and ether compounds. While there is a lot of amino group compound found in water extract. Amino acid is an important elements in body system. In comparison between chromatography–mass spectrometry and thin layer chromatogeraphy, it is the analysis method of choice for smaller and volatile molecules such as benzenes, alcohols and aromatics, simple proteins and simple molecules such as steroids, fatty acids, and hormones (Zeng *et al.,* 2017). It can also be applied towards the study of liquid, gaseous and solid samples. While thin layer chromatography is to seperate non-volatile mixture (Logoyda, 2018).

Various studies have shown that many plants are rich source of antioxidants. For instance, vitamins A, C, E, and phenolic compounds such as flavonoids, tannins, and lignins, found in plants, all act as antioxidants (Suffredini *et al.,* 2004). Thus, the total phenolic content (TPC) and total flavonoid content (TFC) in *Ampelocissus* sp. tuber is determined by using tannic acid and quarcetin as standards respectively. As

recorded in Table 4.5, it was elucidated that the water and methanol extracts showed higher phenolic content than the hexane, petroleum-ether and chloroform extracts. Water and methanol extract also exhibited a high content of flavonoid and phenol .The total polyphenolic content with regards to different solvents used for extraction was as follows: methanol > water > petroleum-ether > chloroform > hexane. Meanwhile the total flavonoid content: methanol > water > chlororform> petroleum-ether > hexane.

A good solvent is characterized by its optimal extraction and its capacity in conserving the stability of the chemical structure of desired compounds (Harborne, 1984). Therefore the type of extraction solvent and its polarity may have a significant impact on the level of extracted polyphenols. The polarities of the polyphenols range from polar to non-polar, optimum extraction of polyphenols is usually obtained in the polar solvent which have a better efficiency of solvation as a result of interactions (hydrogen bonds) between the polar sites of the antioxidant compounds and the solvent than nonpolar one (Winter *et al.,* 1962). Therefore, water and an aqueous mixture of methanol are frequently used for recovering polyphenols. Hexane gave a low level of antioxidant compounds because of their lower efficiency of solvation. The acetone molecules are known as proton acceptors only while methanol and water, are also proton donors (Boeing *et al.*, 2014).

Antioxidants assays activities were conducted by three different method which are 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, ferric reducing power assay and metal chelating assay. The data obtain showed small range of standard error (SE). To describe variation, SE was chose instead of standard deviation (SD) because SE gives the smallest of the error bars.

DPPH method was developed by Blois (1958) with the viewpoint to determine the antioxidant activity in a like manner by using a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33). The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine (Contreras-Guzman & Strong 1982).

DPPH is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, like most other free radicals. The delocalisation also gives rise to the deep violet colour, with an absorption in ethanol solution at around 520 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet colour (Kadare & Singh, 2011).

In this experiment, ascorbic acid is used as standard to compare with other samples. Based on the result showed in Figure 4.4, water and methanol crude extract does not have much different percentage of inhibition as compared to ascorbic acid but the IC_{50} of both extract (water extract IC_{50} = 1.22 µg/mL; methanol extract IC50 = 1.27 µg/mL) were slighly lower than IC_{50} of ascorbic acid (IC_{50} = 1.37 µg/mL). The percentage of inhibition of hexane, petroleum-ether and hexane lower than ascorbic acid and they are sharing the same IC_{50} which is 1.51 µg/mL which is the highest among others. The high scavenging activity of water and methanol extracts may due to the presence of polyphenols as found in TLC as well as in polyphenolic content assay.

The chelating of Fe^{2+} by extracts was estimated by the method of Müller *et al.*, (2010). Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (AboulEnein *et al.,* 2003). The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. Water extract is the most active extract interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. IC_{50} of the extract for chelating activity was 1.62 µg/ml which is higher than the positive standard EDTA (IC_{50} = 1.41 µg/mL). Methanol extract also detected to have higher percentage of inhibiton followed by standard $EDTANa_2$, chloroform (IC_{50} = 1.54 µg/mL), hexane (IC_{50} = 1.32 µg/mL) and petroleum-ether (IC_{50} = 1.44 µg/mL).

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ). The free radical chain breaking takes place through donating a hydrogen atom. At low pH of about 3.6, reduction of Fe^{3+}-TPTZ complex to blue colored Fe^{2+}-TPTZ takes place, which has absorbance at 593 nm. FRAP values were varied between extracts. The highest FRAP value was detected in water extract (11.73 mmol Fe^{2+}/g of dry extracts) and slightly lower for methanol extract (11.58 mmol Fe^{2+}/g of dry extracts). The reducing ability of chloroform (2.27 mmol Fe^{2+}/g of dry extracts) is the least efficient compared to hexane extract (3.56

mmolFe^{2+}/g of dry extracts) and petroleum-ether extract (3.56 mmol Fe^{2+}/g of dry extracts). The results obtained are highly reproducible and related linearly with the molar concentration of the antioxidants present. This is in accordance with the results reported by Benzie *et al,.* (1999) and Jeong *et al.,* (2004).

The acute toxicity test is the first step on toxicological analysis of medicinal plants (Deciga-Campos *et al.,* 2007) this test was observed in non-diabetic rats administrated with *Ampelocissus* sp. methanol extract. Non-toxicity test was experimental at dose 4000 mg/kg body weight during 14 days study period. Table 4.8 showed the results of the acute toxicity test of methanol extract. No toxic sign such as death, distress, fearfulness, urination or defecation, pain response and spontaneous activities were observed. This result revealed that *Ampelocissus* sp. methanol extract did not caused any acute toxicity and the LC_{50} values was more than 4000 mg/kg as no death were found during experiment.

The oral glucose tolerance test (OGTT) is a widely used to evaluate apparent insulin release and insulin resistance in various clinical setting (Stumvoll *et al.,* 2000). For Oral Glucose Tolerance Test, the blood samples were analyzed for glucose content at 0, 30, 60, and 120 minutes, respectively. The single dosed of methanol extract, dose at 800 mg/kg produced a significant hypoglycemic effect in normal rats. Glibenclamide-treated rats also showed a greater significant falls of blood glucose level compared to 400 m/kg methanol extract. This results revealed that methanol extracts may stimulate insulin secretion as well as glibenclamide does.

Refer to American Diabetes Association (2010), type 1 diabetes mellitus (T1DM) is a chronic disease that results from an autoimmune destruction of β-cells of the pancreas. Therefore, insulin deficiency and hyperglycemia are the main outcomes of

T1DM. Recently, drug formulation from natural herbs, for treatment of diabetes mellitus drugs and other diseases, attracted the attention of many researchers (Sheweita *et al.,* 2002). Streptozotocin-induced rats developed clinical features and signs, which are similar to those found in T1DM (Park *et al.,* 2013). This study have been conducted to investigate the antidiabetic effects of methanolic extract of *Ampelocissus* sp. through determination of blood glucose level, body weight and antioxidant enzymes in STZ-induced diabetic rats.

There is 4.5% increased of blood glucose level in normal rats but it was still under a normal line of glucose reading. Low dose (400 mg/kg) and high dose (800 mg/kg) of methanol extract showed a significant different of blood glucose level by decreasing about 12.4 % and 17.4 % respectively. There is also a significant different between those extracts and glibenclamide which indicate the highest reduction of blood glucose level which is 21%. The hypoglycemic activity of methanol extract might be the result of an improved insulin level. This suppression of hyperglycemia may result from the inhibition of α-glucosidase and -amylase enzymes reported by Sarma *et al.,* (2016). There is a large standard error observed for blood glucose level in rats treated by glibenclamide for day 7 because there is a notable differences in the glucose reading level in each rat.

Tissue proteins are known to contribute to the body weight (Rajkumar et al., 1991). Decreases in body weight have been associated with diabetes mellitus due to excessive breakdown of tissue protein and could be due to catabolism of fats and protein (Rajagopal & Sasikala, 2008). The breakdown of adipocytes and muscles tissues in STZ-induced rats are due to the frequent urination and over conversion of glycogen to glucose (Lin *et al.,* 2006). In this study the decreased in body weight

were observed during the treatment of diabetic rats with methanol extract of *Ampelocissus* sp. and glibenclamide as standard drug at the early stage of experiment. Results showed there slightly increased of weight in STZ-induced rats treated with 400 mg/kg and 800 mg/kg methanol extract as well as glibenclamide and diabetic control rats. There is also a significant increased of body weight in normal rats. This is due to no disruption of body system and enhancing of body function by treating with methanloic extract of *Ampelocissus* sp. These results revealed that methanol extracts may not have greater significance affect towards body weight of diabetic rats.

Chronic diseases such as diabetes and cardiovascular disease increase oxidants and decrease antioxidants in patients. Conditions that increase oxidants and reduce antioxidants also exacerbate these diseases. In diabetic patients, hyperglycemia causes glycation of macromolecules such as proteins, lipids, and nucleic acids. The glycation of macromolecules can alter their functions, increase lipid peroxidation, and reduce antioxidant enzyme activity, causing damage to the cells. Clinical and experimental studies have shown that disturbing the balance of the oxidant–antioxidant system can contribute to the pathogenesis of chronic diseases such as cancer, cardiovascular disease, diabetes, and many diabetic complications (Ahmadvand *et al.,* 2014).

Based on the results obtained in Table 4.10 and Table 4.11, this study showed that methanol extracts of *Ampelocissus* sp. can increase the reduced serum GPX and CAT in STZ-induced diabetic rats. There is much evidence that free radicals play a key role in the most pathogenic pathway of diabetic injuries (Stadler, 2013). Free radicals such as superoxide can increase lipid peroxidation, carcinogenesis,

inflammation, early aging, cardiovascular diseases, and tissue damage in diabetes due to reduction of function of antioxidant enzymes such as CAT and GPx (Ahmadvand *et al.,* 2014). Much evidence indicates that oxidative stress plays a key role in the pathogenesis of diabetes (Sarmento *et al.*, 2013).

These antioxidants and antioxidant enzymes protect the cells against oxidative stress-mediated injuries by converting the toxic free radicals to non-toxic products (Kim *et al.,* 2013; Tekiner-Gulbas *et al.,* 2013). Therefore the use of antioxidants as complementary therapies can be useful for the treatment of diseases related to oxidative stress. GPx activities in untreated diabetic is slightly higher than glibenclamide-treated diabetic rats. Methanolic-extracts-treated diabetic rats exhibits a good GPx activities compared to untreated diabetic and glibenclamide-treated rats.

Methanol extracts of *Ampelocissus* sp. resulted in similar serum CAT activities of the treated animals similar to as those of the control group (normal) as well as diabetic rats treated with glibenclamide as standard. However, there is a significantly higher of CAT activity in untreated diabetic compared to treated diabetic rats. This may due to the body has to full force the body system to work harder in order to counter the positive feedback mechanism of CAT activity at the early phase of diabetic disease.

The antioxidant enzymes GPX and CAT are considered to be indicators of antioxidant status (Flores-Mateo *et al.*, 2009). Previous studies showed that antioxidants such as glibenclamide and metformin (Erejuwa *et al*., 2010), vitamins E and C, melatonin (El-Batch et al., 2011), zinc (Lima et al., 2011), vanadium and Trigonella, salidroside (Li *et al.*, 2011), flavonoids (Stefek, 2011), taurine , N-acetyl

cysteine, L-arginine (Granstam *et al.*, 2011), lycopene , succinic acid monoethyl ester , rutin Kamalakkannan, *et al*., 2006), aminoguanidine (Stoppa *et al*., 2006), and natural phenolic compounds (Ranilla *et al*., 2010) increased antioxidant enzymes and antioxidant status in diabetics.

These results agree that methanol extract can increase GPX and CAT activities. Therefore, methanol extracts as an antioxidant with beneficial effects on antioxidant enzymes might be useful in reducing the complications of various types of tissue damage seen in diabetics. Antioxidant therapy is one of the most important treatment strategies for the prevention and slowing of the progression of diabetic complications such as hyperglycemia, hyperlipidemia, hepatic damage, and nephropathy (Ahmadvand *et al.,* 2014).

CHAPTER 6: CONCLUSION

Sample of tuber of *Ampelocissus* sp. was extracted with aqueous hexane, petroleum-ether, chloroform, methanol and water solvents. The phytochemical bioactive compounds of those extracts were identified using Thin Layer Chromatography and showed the existence of phenols, flavonoids, saponins and terpenoids. The examination on the methanol and water aqueous extract of *Ampelocissus* sp. with gas chromatography mass spectrometer (GCMS) displayed the occurrence of 20 phytochemical compounds which are biologically important for body system. Methanol extracts of *Ampelocissus* sp. showed the highest content of phenols and flavonoids content followed by water extracts. For antioxidant test, a good percentage of activity was achieved in 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, metal chelating assay and ferric reducing power assay by methanol and water extracts against standards. Methanol extracts was chose for further investigation on animal test. Acute toxicity test proven that methanol extract of *Ampelocissus* sp. contain non-toxic subtances due to the absence of toxic sign such as death and pain response. The administrations of *Ampelocissus* sp. methanolic extracts during anti-diabetic study showed positive response in blood glucose level of oral glucose tolerance test (OGTT). The treatment of STZ-induced diabetic rats with 800 mg/kg methanol extracts and glibenclamide showed a close percentage of blood glucose reduction between them which are 17.4 % and 21 %. There is a slightly significant changes in body weight after 28 days of treatment. From this experiment, methanol extracts help increased and enhance the function of GPx and CAT antioxidant enzyme in diabetic rats. It can be concluded that the antioxidant activity of methanol extract was primarily due to the high amount of phenolic content.

REFERENCES

Aboul-Enein, A. M., El-Baz, F. K., El-Baroty, G. S., Youssef, A., & Abd El-Baky, H. H. (2003). Antioxidant activity of algal extracts on lipid peroxidation. *Journal of Medical Sciences, 3*(1), 87-98.

Adjanohoun, E. J., Assi, L. A., Floret, J., Guinko, S., Koumaré, M., Ahyi, A., & Raynal, J. (1980). Medecine traditionelle et pharmacopee. Contribution aux etudes ethnobotaniques et floristiques au Mali. *Paris.: Agence de Cooperation Culturelle et Technique*.

Ahmadvand, H., Dehnoo, M. G., Cheraghi, R., Rasoulian, B., Ezatpour, B., Azadpour, M., & Baharvand, K. (2014). Amelioration of altered serum, liver, and kidney antioxidant enzymes activities by sodium selenite in alloxan-induced diabetic rats. *Reports of Biochemistry & Molecular Biology, 3*(1), 14.

Alberti, K. G. M. M., & Zimmet, P. f. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabetic Medicine, 15*(7), 539-553.

Allen, R., Farmer, K., & Sohal, R. (1983). Effect of catalase inactivation on levels of inorganic peroxides, superoxide dismutase, glutathione, oxygen consumption and life span in adult houseflies (Musca domestica). *Biochemical Journal, 216*(2), 503-506.

Ametov, A. S., Barinov, A., Dyck, P. J., Hermann, R., Kozlova, N., Litchy, W. J., . . . O'Brien, P. C. (2003). The sensory symptoms of diabetic polyneuropathy are improved with α-lipoic acid. *Diabetes Care, 26*(3), 770-776.

Association, A. D. (2010). Diagnosis and classification of diabetes mellitus. *Diabetes Care, 33*(Supplement 1), S62-S69.

Bagi, Z., Koller, A., & Kaley, G. (2004). PPARγ activation, by reducing oxidative stress, increases NO bioavailability in coronary arterioles of mice with Type 2 diabetes. *American Journal of Physiology-Heart and Circulatory Physiology, 286*(2), H742-H748.

Bah, S., Diallo, D., Dembélé, S., & Paulsen, B. S. (2006). Ethnopharmacological survey of plants used for the treatment of schistosomiasis in Niono District, Mali. *Journal of Ethnopharmacology, 105*(3), 387-399.

Bajaj, S., & Khan, A. (2012a). Antioxidants and diabetes. *Indian Journal of Endocrinology and Metabolism, 16*(8), Article 267.

Bajaj, S., & Khan, A. (2012b). Antioxidants and diabetes. *Indian Journal of Endocrinology and Metabolism, 16*(Suppl 2), Article 267.

Barlow, S. M., Greig, J. B., Bridges, J. W., Carere, A., Carpy, A. J. M., Galli, C. L., ... & Madsen, C. (2002). Hazard identification by methods of animal-based toxicology. *Food and Chemical Toxicology*, *40*(2-3), 145-191.

Bashan, N., Kovsan, J., Kachko, I., Ovadia, H., & Rudich, A. (2009). Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. *Physiological Reviews, 89*(1), 27-71.

Baynes, J. W., & Thorpe, S. R. (1996). The role of oxidative stress in diabetic complications. *Current Opinion in Endocrinology, Diabetes and Obesity, 3*(4), 277-284.

Benzie, I. F., Chung, W., & Strain, J. (1999). "Antioxidant"(reducing) efficiency of ascorbate in plasma is not affected by concentration. *The Journal of Nutritional Biochemistry, 10*(3), 146-150.

Berry, C., Anderson, N., Kirk, A. J., Dominiczak, A. F., & McMurray, J. J. (2001). Renin angiotensin system inhibition is associated with reduced free radical concentrations in arteries of patients with coronary heart disease. *Heart, 86*(2), 217-220.

Biradar, S. R., & Rachetti, B. D. (2013). Extraction of some secondary metabolites & thin layer chromatography from different parts of *Centella asiatica L.*(URB). *American Journal of Life Sciences, 1*(6), 243-247.

Bizimana, N., Tietjen, U., Zessin, K.-H., Diallo, D., Djibril, C., Melzig, M. F., & Clausen, P.-H. (2006). Evaluation of medicinal plants from Mali for their in vitro and in vivo trypanocidal activity. *Journal of Ethnopharmacology, 103*(3), 350-356.

Boaz, M., Smetana, S., Weinstein, T., Matas, Z., Gafter, U., Iaina, A., . . . Fainaru, M. (2000). Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *The Lancet, 356*(9237), 1213-1218.

Boeing, J. S., Barizão, É. O., e Silva, B. C., Montanher, P. F., de Cinque Almeida, V., & Visentainer, J. V. (2014). Evaluation of solvent effect on the extraction of phenolic compounds and antioxidant capacities from the berries: application of principal component analysis. *Chemistry Central Journal, 8*(1), 48.

Boullier, A., Bird, D. A., Chang, M. K., Dennis, E. A., Friedman, P., Gillote-Taylor, K., . . . Shaw, P. (2001). Scavenger receptors, oxidized LDL, and atherosclerosis. *Annals of the New York Academy of Sciences, 947*(1), 214-223.

Brands, M. W., Bell, T. D., & Gibson, B. (2004). Nitric oxide may prevent hypertension early in diabetes by counteracting renal actions of superoxide. *Hypertension, 43*(1), 57-63.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature, 414*(6865), 813-820.

Calles-Escandon, J., & Cipolla, M. (2001). Diabetes and endothelial dysfunction: A clinical perspective. *Endocrine Reviews, 22*(1), 36-52.

Cameron, E., & Pauling, L. (1976). Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. *Proceedings of the National Academy of Sciences, 73*(10), 3685-3689.

Chirinos, R., Rogez, H., Campos, D., Pedreschi, R., & Larondelle, Y. (2007). Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers. *Separation and Purification Technology*, *55*(2), 217-225.

Çınar, M., Ülker, S., Alper, G., linnaz, u., & Evinc, A. (2001). Effect of dietary vitamin E supplementationon vascular reactivity of thoracic aorta in streptozotocin-diabetic rats. *Pharmacology, 62*(1), 56-64.

Clarkson, P. M., & Thompson, H. S. (2000). Antioxidants: what role do they play in physical activity and health? *The American Journal of Clinical Nutrition, 72*(2), 637-646.

Coe, F. G., & Anderson, G. J. (1996). Screening of medicinal plants used by the *Garifuna* of Eastern Nicaragua for bioactive compounds. *Journal of Ethnopharmacology, 53*(1), 29-50.

Contrerasguzman, E., & Strong, F. (1982). Determination of tocopherols (vitamin-E) by reduction of cupric ion. *Journal of the Association of Official Analytical Chemists*.

Coppey, L. J., Gellett, J. S., Davidson, E. P., Dunlap, J. A., Lund, D. D., & Yorek, M. A. (2001). Effect of antioxidant treatment of streptozotocin-induced diabetic rats on endoneurial blood flow, motor nerve conduction velocity, and vascular reactivity of epineurial arterioles of the sciatic nerve. *Diabetes, 50*(8), 1927-1937.

Da Silva, S. B., Costa, J., Pintado, M., Ferreira, D., & Sarmento, B. (2010). Antioxidants in the prevention and treatment of diabetic retinopathy–a review. *Journal of Diabetes and Metabolism, 1*, Article #111.

Dawan, P., Satarpai, T., Tuchinda, P., Shiowatana, J., & Siripinyanond, A. (2017). A simple analytical platform based on thin-layer chromatography coupled with paper-based analytical device for determination of total capsaicinoids in chilli samples. *Talanta, 162*, 460-465.

Deakin, S., Leviev, I., Guernier, S., & James, R. W. (2003). Simvastatin modulates expression of the PON1 gene and increases serum paraoxonase a role for sterol regulatory element–binding protein-2. *Arteriosclerosis, Thrombosis, and Vascular Biology, 23*(11), 2083-2089.

Déciga-Campos, M., Rivero-Cruz, I., Arriaga-Alba, M., Castaneda-Corral, G., Angeles-López, G. E., Navarrete, A., & Mata, R. (2007). Acute toxicity and

mutagenic activity of Mexican plants used in traditional medicine. *Journal of Ethnopharmacology, 110*(2), 334-342.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry, 38*(3), 674-677.

Desco, M.-C., Asensi, M., Márquez, R., Martínez-Valls, J., Vento, M., Pallardó, F. V., . . . Viña, J. (2002). Xanthine oxidase is involved in free radical production in type 1 diabetes protection by allopurinol. *Diabetes, 51*(4), 1118-1124.

Devasagayam, T., Tilak, J., Boloor, K., Sane, K. S., Ghaskadbi, S. S., & Lele, R. (2004). Free radicals and antioxidants in human health: current status and future prospects. *Journal of Association of Physicians of India, 52*(7), Article #4.

Dobrian, A. D., Davies, M. J., Schriver, S. D., Lauterio, T. J., & Prewitt, R. L. (2001). Oxidative stress in a rat model of obesity-induced hypertension. *Hypertension, 37*(2), 554-560.

El-Batch, M., Hassan, A. M., & Mahmoud, H. A. (2011). Taurine is more effective than melatonin on cytochrome P450 2E1 and some oxidative stress markers in streptozotocin-induced diabetic rats. *Journal of Agricultural and Food Chemistry, 59*(9), 4995-5000.

Erejuwa, O. O., Sulaiman, S. A., Wahab, M. S. A., Sirajudeen, K. N. S., Salleh, M. S. M., & Gurtu, S. (2010). Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin-induced diabetic rats. *International Journal of Molecular Sciences, 11*(5), 2056-2066.

Etuk, E., Ugwah, M., Ajagbonna, O., & Onyeyili, P. (2009). Ethnobotanical survey and preliminary evaluation of medicinal plants with antidiarrhoea properties in Sokoto state, Nigeria. *Journal of Medicinal Plants Research, 3*(10), 763-766.

Feskens, E. J., Virtanen, S. M., Räsänen, L., Tuomilehto, J., Stengård, J., Pekkanen, J., . . . Kromhout, D. (1995). Dietary factors determining diabetes and impaired glucose tolerance: a 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study. *Diabetes Care, 18*(8), 1104-1112.

Flores-Mateo, G., Carrillo-Santisteve, P., Elosua, R., Guallar, E., Marrugat, J., Bleys, J., & Covas, M.-I. (2009). Antioxidant enzyme activity and coronary heart disease: meta-analyses of observational studies. *American Journal of Epidemiology, 170*(2), 135-147.

Ford, E. S., Mokdad, A. H., Giles, W. H., & Brown, D. W. (2003). The metabolic syndrome and antioxidant concentrations findings from the Third National Health and Nutrition Examination Survey. *Diabetes, 52*(9), 2346-2352.

Fukui, T., Noma, T., Mizushige, K., Aki, Y., Kimura, S., & Abe, Y. (2000). Dietary troglitazone decreases oxidative stress in early stage type II diabetic rats. *Life Sciences, 66*(21), 2043-2049.

Gaede, P., Poulsen, H., Parving, H. H., & Pedersen, O. (2001). Double-blind, randomised study of the effect of combined treatment with vitamin C and E on albuminuria in Type 2 diabetic patients. *Diabetic Medicine, 18*(9), 756-760.

Gæde, P., Vedel, P., Larsen, N., Jensen, G. V., Parving, H.-H., & Pedersen, O. (2003). Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *New England Journal of Medicine, 348*(5), 383-393.

Glade, M. J. (1999). Food, nutrition, physical activity and the prevention of cancer: a global perspective. *American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research. Nutrition, 15*, 523-526.

Granstam, S.-O., & Granstam, E. (2011). Endothelin-induced changes in blood flow in STZ-diabetic and non-diabetic rats: relation to nitric oxide synthase and cyclooxygenase inhibition. *The Journal of Physiological Sciences, 61*(6), 497.

Haendeler, J., Hoffmann, J., Zeiher, A. M., & Dimmeler, S. (2004). Antioxidant effects of statins via s-nitrosylation and activation of thioredoxin in endothelial cells a novel vasculoprotective function of statins. *Circulation, 110*(7), 856-861.

Hamilton, S. J., Chew, G. T., & Watts, G. F. (2007). Therapeutic regulation of endothelial dysfunction in type 2 diabetes mellitus. *Diabetes and Vascular Disease Research, 4*(2), 89-102.

Harborne, J. B. (1984). Methods of plant analysis. In *Phytochemical Methods* (pp. 1-36). Springer, Dordrecht.

Inngjerdingen, K., Nergård, C. S., Diallo, D., Mounkoro, P. P., & Paulsen, B. S. (2004). An ethnopharmacological survey of plants used for wound healing in Dogonland, Mali, West Africa. *Journal of Ethnopharmacology, 92*(2), 233-244.

Jacob, S., Ruus, P., Hermann, R., Tritschler, H., Maerker, E., Renn, W., . . . Rett, K. (1999). Oral administration of RAC-α-lipoic acid modulates insulin sensitivity in patients with type-2 diabetes mellitus: a placebo-controlled pilot trial. *Free Radical Biology and Medicine, 27*(3), 309-314.

Jakus, V. (2000). The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. *Bratislavske Lekarske Listy, 101*(10), 541-551.

Jentzsch, A. M., Bachmann, H., Fürst, P., & Biesalski, H. K. (1996). Improved analysis of malondialdehyde in human body fluids. *Free Radical Biology and Medicine, 20*(2), 251-256.

Jeong, S. M., Kim, S. Y., Kim, D. R., Jo, S. C., Nam, K. C., Ahn, D. U., & Lee, S. C. (2004). Effect of heat treatment on the antioxidant activity of extracts from citrus peels. *Journal of Agricultural and Food Chemistry*, *52*(11), 3389-3393.

Ji, L. L. (1993). Antioxidant enzyme response to exercise and aging. *Medicine and Science in Sports and Exercise, 25*(2), 225-231.

Johansen, J. S., Harris, A. K., Rychly, D. J., & Ergul, A. (2005). Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. *Cardiovascular Diabetology, 4*(5), 1-11.

Kamalakkannan, N., & Prince, P. S. M. (2006). Rutin improves the antioxidant status in streptozotocin-induced diabetic rat tissues. *Molecular and cellular biochemistry, 293*(1-2), 211.

Kamthan, A., Kamthan, M., Chakraborty, N., Chakraborty, S., & Datta, A. (2012). A simple protocol for extraction, derivatization, and analysis of tomato leaf and fruit lipophilic metabolites using GC-MS. *Protocol Exchange*, *10*.

Kang, P., Dalvie, D., Smith, E., & Renner, M. (2008). Bioactivation of lumiracoxib by peroxidases and human liver microsomes: identification of multiple quinone imine intermediates and GSH adducts. *Chemical Research in Toxicology, 22*(1), 106-117.

Karasu, Ç. (2010). Glycoxidative stress and cardiovascular complications in experimentally-induced diabetes: effects of antioxidant treatment. *The Open Cardiovascular Medicine Journal, 4*(1).

Kedare, S. B., & Singh, R. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology, 48*(4), 412-422.

Kędziora-Kornatowska, K., Szram, S., Kornatowski, T., Szadujkis-Szadurski, L., Kędziora, J., & Bartosz, G. (2004). Effect of vitamin E and vitamin C supplementation on antioxidative state and renal glomerular basement membrane thickness in diabetic kidney. *Nephron Experimental Nephrology, 95*(4), e134-e143.

Kelmanson, J. E., Jäger, A. K., & van Staden, J. (2000). Zulu medicinal plants with antibacterial activity. *Journal of Ethnopharmacology, 69*(3), 241-246.

Kim, H. Y., Sin, S. M., Lee, S., Cho, K. M., & Cho, E. J. (2013). The butanol fraction of bitter melon (*Momordica charantia*) scavenges free radicals and attenuates oxidative stress. *Preventive Nutrition and Food Science, 18*(1), 18.

Kitano, M. (1992). Updating of OECD guidelines for the testing of chemicals. *Water Science and Technology, 25*(11), 465-472.

Kitzberger, C. S. G., Smânia, A., Pedrosa, R. C., & Ferreira, S. R. S. (2007). Antioxidant and antimicrobial activities of shiitake (*Lentinula edodes*)

extracts obtained by organic solvents and supercritical fluids. *Journal of Food Engineering, 80*(2), 631-638.

Kocak, G., Aktan, F., Canbolat, O., Ozoğul, C., Elbeğ, S., Yildizoglu-Ari, N., & Karasu, C. (2000). Alpha-lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocin-diabetes. *Diabetes, Nutrition & Metabolism, 13*(6), 308-318.

Kumar, S., Jyotirmayee, K., & Sarangi, M. (2013). Thin layer chromatography: a tool of biotechnology for isolation of bioactive compounds from medicinal plants. *International Journal of Pharmaceutical Sciences Review and Research*, *18*(1), 126-132.

Laight, D., Carrier, M., & Änggård, E. (2000). Antioxidants, diabetes and endothelial dysfunction. *Cardiovascular Research, 47*(3), 457-464.

Li, F., Tang, H., Xiao, F., Gong, J., Peng, Y., & Meng, X. (2011). Protective effect of salidroside from Rhodiolae Radix on diabetes-induced oxidative stress in mice. *Molecules, 16*(12), 9912-9924.

Lima, V. B. d. S., Sampaio, F. d. A., Bezerra, D. L. C., Moita Neto, J. M., & Marreiro, D. d. N. (2011). Parameters of glycemic control and their relationship with zinc concentrations in blood and with superoxide dismutase enzyme activity in type 2 diabetes patients. *Arquivos Brasileiros de Endocrinologia & Metabologia, 55*(9), 701-707

Lin, J. K., & Lin-Shiau, S. Y. (2006). Mechanisms of hypolipidemic and anti-obesity effects of tea and tea polyphenols. *Molecular Nutrition & Food Research, 50*(2), 211-217.

Linke, A., Adams, V., Schulze, P. C., Erbs, S., Gielen, S., Fiehn, E., . . . Hambrecht, R. (2005). Antioxidative effects of exercise training in patients with chronic heart failure increase in radical scavenger enzyme activity in skeletal muscle. *Circulation, 111*(14), 1763-1770.

Logoyda, L. (2018). Development of methods for the chromatographic identification of active pharmaceutical ingredient from group of angiotensin-converting enzyme inhibitors in pharmaceuticals. *International Journal of Green Pharmacy (IJGP)*, *11*(04).

Lonn, E., Yusuf, S., Hoogwerf, B., Pogue, J., Yi, Q., Zinman, B., . . . Gerstein, H. C. (2002). Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes results of the Hope Study and Micro-Hope Substudy. *Diabetes Care, 25*(11), 1919-1927.

Lonn, E. M., Yusuf, S., Dzavik, V., Doris, C. I., Yi, Q., Smith, S., . . . Teo, K. K. (2001). Effects of ramipril and vitamin E on atherosclerosis the study to evaluate carotid ultrasound changes in patients treated with ramipril and vitamin E (SECURE). *Circulation, 103*(7), 919-925.

Mărghitaş, L. A., Stanciu, O. G., Dezmirean, D. S., Bobiş, O., Popescu, O., Bogdanov, S., & Campos, M. G. (2009). In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. *Food Chemistry, 115*(3), 878-883.

Maritim, A., Sanders, a., & Watkins, r. J. (2003). Diabetes, oxidative stress, and antioxidants: a review. *Journal of Biochemical and Molecular Toxicology, 17*(1), 24-38.

MatÉs, J. M., Pérez-Gómez, C., & De Castro, I. N. (1999). Antioxidant enzymes and human diseases. *Clinical Biochemistry, 32*(8), 595-603.

McDonnell-Dowling, K., & P Kelly, J. (2017). The role of oxidative stress in methamphetamine-induced toxicity and sources of variation in the design of animal studies. *Current neuropharmacology*, *15*(2), 300-314.

Mekiňová, D., Chorvathova, V., Volkovova, K., Staruchova, M., Graňičová, E., Klvanova, J., & Ondreič, R. (1995). Effect of intake of exogenous vitamins C, E and β-carotene on the antioxidative status in kidneys of rats with streptozotocin-induced diabetes. *Food/Nahrung, 39*(4), 257-261.

Misra, H. P., & Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry, 247*(10), 3170-3175.

Montonen, J., Knekt, P., Järvinen, R., & Reunanen, A. (2004). Dietary antioxidant intake and risk of type 2 diabetes. *Diabetes Care, 27*(2), 362-366.

Morgantini, C., Natali, A., Boldrini, B., Imaizumi, S., Navab, M., Fogelman, A. M., . . . Reddy, S. T. (2011). Anti-inflammatory and antioxidant properties of HDLs are impaired in type 2 diabetes. *Diabetes, 60*(10), 2617-2623.

Muhammad, S., & Amusa, N. (2005). The important food crops and medicinal plants of north-western Nigeria. *Research Journal of Agriculture and Biological Sciences, 1*(3), 254-260.

Müller, L., Gnoyke, S., Popken, A. M., & Böhm, V. (2010). Antioxidant capacity and related parameters of different fruit formulations. *LWT-Food Science and Technology, 43*(6), 992-999.

Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S.-i., Matsumura, T., Kaneda, Y., . . . Hammes, H.-P. (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature, 404*(6779), 787-790.

Nyunaï, N., Njikam, N., Abdenneb, E., Mbafor, J., & Lamnaouer, D. (2009). Hypoglycaemic and antihyperglycaemic activity of Ageratum conyzoides L. in rats. *African Journal of Traditional, Complementary and Alternative Medicines, 6*(2).

Obrosova, I. G., Fathallah, L., & Greene, D. A. (2000). Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: effect of DL-α-lipoic acid. *European Journal of Pharmacology, 398*(1), 139-146.

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry, 95*(2), 351-358.

Oskay, M., Oskay, D., & Kalyoncu, F. (2010). Activity of some plant extracts against multi-drug resistant human pathogens. *Iranian Journal of Pharmaceutical Research*, 293-300.

Owolabi, M., Jaja, S., Olatunji, O., Oyekanmi, O., & Adepoju, S. (2011). Attenuation of oxidative damage in alloxan induced diabetic rabbits following administration of the extract of the leaves of *Vernonia amygdalina*. *Free Radicals and Antioxidants, 1*(3), 94-101.

Park, J.-H., Jung, J.-H., Yang, J.-Y., & Kim, H.-S. (2013). Olive leaf down-regulates the oxidative stress and immune dysregulation in streptozotocin-induced diabetic mice. *Nutrition Research, 33*(11), 942-951.

Pettit, G. R., Mukku, V. J., Cragg, G., Herald, D. L., Knight, J. C., Herald, C. L., & Chapuis, J.-C. (2008). Antineoplastic Agents. 558. Ampelocissus sp. Cancer Cell Growth Inhibitory Constituents (1). *Journal of natural products, 71*(1), 130-133.

Pietta, P.-G. (2000). Flavonoids as antioxidants. *Journal of natural products, 63*(7), 1035-1042.

Polidori, M. C., Praticó, D., Savino, K., Rokach, J., Stahl, W., & Mecocci, P. (2004). Increased F 2 isoprostane plasma levels in patients with congestive heart failure are correlated with antioxidant status and disease severity. *Journal of Cardiac Failure, 10*(4), 334-338.

Pyörälä, K., Laakso, M., & Uusitupa, M. (1987). Diabetes and atherosclerosis: an epidemiologic view. *Diabetes/Metabolism Reviews, 3*(2), 463-524.

Rajkumar, L., Srinivasan, N., Balasubramanian, K., & Govindarajulu, P. (1991). Increased degradation of dermal collagen in diabetic rats. *Indian Journal of Experimental Biology, 29*(11), 1081-1083.

Rajagopal, K., & Sasikala, K. (2008). Antihyperglycaemic and antihyperlipidaemic effects of *Nymphaea stellata* in alloxan-induced diabetic rats. *Singapore Medical Journal, 49*(2), 137.

Ranilla, L. G., Kwon, Y.-I., Apostolidis, E., & Shetty, K. (2010). Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Bioresource Technology, 101*(12), 4676-4689.

Reljanovic, M., Reichel, G., Rett, K., Lobisch, M., Schuette, K., Möller, W., . . . Mehnert, H. (1999). Treatment of diabetic polyneuropathy with the

antioxidant thioctic acid (α-lipoic acid): a two year multicenter randomized double-blind placebo-controlled trial (ALADIN II). *Free Radical Research, 31*(3), 171-179.

Rotruck, J., Pope, A., Ganther, H., Swanson, A., Hafeman, D. G., & Hoekstra, W. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science, 179*(4073), 588-590.

Ruhe, R. C., & McDonald, R. B. (2001). Use of antioxidant nutrients in the prevention and treatment of type 2 diabetes. *Journal of the American College of Nutrition, 20*(5), 363-369.

Sacco, M., Pellegrini, F., Roncaglioni, M. C., Avanzini, F., Tognoni, G., & Nicolucci, A. (2003). Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in Type 2 diabetic patients results of the primary prevention project (PPP) trial. *Diabetes Care, 26*(12), 3264-3272.

Sarma, R., Kumari, S., Elancheran, R., Deori, M., & Devi, R. (2016). Polyphenol rich extract of *Garcinia pedunculata* fruit attenuates the hyperlipidemia induced by high fat diet. *Frontiers in Pharmacology, 7*, 294.

Sarmento, R. A., Silva, F. M., Sbruzzi, G., Schaan, B. D. A., & Almeida, J. C. D. (2013). Antioxidant micronutrients and cardiovascular risk in patients with diabetes: a systematic review. *Arquivos Brasileiros de Cardiologia, 101*(3), 240-248.

Sasipriya, G., & Siddhuraju, P. (2012). Effect of different processing methods on antioxidant activity of underutilized legumes, *Entada scandens* seed kernel and *Canavalia gladiata* seeds. *Food and Chemical Toxicology, 50*(8), 2864-2872.

Sheweita, S., Newairy, A., Mansour, H., & Yousef, M. (2002). Effect of some hypoglycemic herbs on the activity of phase I and II drug-metabolizing enzymes in alloxan-induced diabetic rats. *Toxicology, 174*(2), 131-139.

Smith, W. T., & Patterson, J. M. (1984). Functional group analysis. *Analytical Chemistry, 56*(5), 63-68.

Singhal, S., Singhal, N., & Agarwal, S. (2009). Pharmaceutical analysis II. *Thin layer Chromatography, Pragati prakashan*, 98-111.

Sinha, A. K. (1972). Colorimetric assay of catalase. *Analytical Biochemistry, 47*(2), 389-394.

Skyrme-Jones, R. A. P., O'Brien, R. C., Berry, K. L., & Meredith, I. T. (2000). Vitamin E supplementation improves endothelial function in type I diabetes mellitus: a randomized, placebo-controlled study. *Journal of the American College of Cardiology, 36*(1), 94-102.

Srivastava, P., Raut, H. N., Wagh, R. S., Puntambekar, H. M., & Kulkarni, M. J. (2012). Purification and characterization of an antioxidant protein (~ 16kDa) from *Terminalia chebula* fruit. *Food Chemistry, 131*(1), 141-148.

Stadler, K. (2013). Oxidative stress in diabetes. In *Diabetes* (pp. 272-287). Springer, New York, NY.

Stefek, M. (2011). Natural flavonoids as potential multifunctional agents in prevention of diabetic cataract. *Interdisciplinary Toxicology, 4*(2), 69-77.

Stoppa, G. R., Cesquini, M., Roman, E. A., Ogo, S. H., & Torsoni, M. A. (2006). Aminoguanidine prevented impairment of blood antioxidant system in insulin-dependent diabetic rats. *Life Sciences, 78*(12), 1352-1361.

Suffredini, I., Sader, H. S., Gonçalves, A. G., Reis, A. O., Gales, A. C., Varella, A. D., & Younes, R. N. (2004). Screening of antibacterial extracts from plants native to the Brazilian Amazon Rain Forest and Atlantic Forest. *Brazilian Journal of Medical and Biological Research, 37*(3), 379-384.

Stumvoll, M., Mitrakou, A., Pimenta, W., Jenssen, T., Yki-Järvinen, H., Van Haeften, T., . . . Gerich, J. (2000). Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes Care, 23*(3), 295-301.

Tekiner-Gulbas, B., D Westwell, A., & Suzen, S. (2013). Oxidative stress in carcinogenesis: new synthetic compounds with dual effects upon free radicals and cancer. *Current Medicinal Chemistry, 20*(36), 4451-4459.

Tinel, A., & Tschopp, J. (2004). The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science, 304*(5672), 843-846.

Touyz, R. (2004). Reactive oxygen species and angiotensin II signaling in vascular cells: implications in cardiovascular disease. *Brazilian Journal of Medical and Biological Research, 37*(8), 1263-1273.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44-84.

Vouldoukis, I., Conti, M., Krauss, P., Kamate, C., Blazquez, S., Tefit, M., . . . Dugas, B. (2004). Supplementation with gliadin-combined plant superoxide dismutase extract promotes antioxidant defences and protects against oxidative stress. *Phytotherapy Research, 18*(12), 957-962.

Wanasundara, J. P., & Shahidi, F. (1994). Alkanol-ammonia-water/hexane extraction of flaxseed. *Food Chemistry, 49*(1), 39-44.

Willett, W. C., & MacMahon, B. (1984). Diet and cancer—an overview. *New England Journal of Medicine, 310*(11), 697-703.

Wilson, S. H., Simari, R. D., Best, P. J., Peterson, T. E., Lerman, L. O., Aviram, M., . . . Lerman, A. (2001). Simvastatin preserves coronary endothelial function in hypercholesterolemia in the absence of lipid lowering. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*(1), 122-128.

Winter, C. A. (1962). Risley EA, Nuss GW. Carrageenan-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine, 111*, 544-547.

Wojcik, M., Burzynska-Pedziwiatr, I., & Wozniak, L. (2010). A review of natural and synthetic antioxidants important for health and longevity. *Current Medicinal Chemistry, 17*(28), 3262-3288.

Wong, P. Y., & Kitts, D. D. (2006). Studies on the dual antioxidant and antibacterial properties of parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) extracts. *Food Chemistry*, *97*(3), 505-515.

Yung, L. M., Leung, F. P., Yao, X., Chen, Z.-Y., & Huang, Y. (2006). Reactive oxygen species in vascular wall. *Cardiovascular & Haematological Disorders-Drug Targets (Formerly Current Drug Targets-Cardiovascular & Hematological Disorders), 6*(1), 1-19.

Yusuf, S., Dagenais, G., Pogue, J., Bosch, J., & Sleight, P. (2000). Vitamin E supplementation and cardiovascular events in high-risk patients. The heart outcomes prevention evaluation study investigators. *The New England Journal of Medicine, 342*(3), 154-160.

Zeng, L., Zhou, Y., Fu, X., Mei, X., Cheng, S., Gui, J., ... & Yang, Z. (2017). Does oolong tea (Camellia sinensis) made from a combination of leaf and stem smell more aromatic than leaf-only tea? Contribution of the stem to oolong tea aroma. *Food chemistry*, *237*, 488-498.

Ziegler, D., Hanefeld, M., Ruhnau, K.-J., Hasche, H., Lobisch, M., Schütte, K., . . . Malessa, R. (1999). Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III Study). ALADIN III study group. Alpha-lipoic acid in diabetic neuropathy. *Diabetes Care, 22*(8), 1296-1301.

Ziegler, D., Hanefeld, M., Ruhnau, K., Mei, H., Lobisch, M., Schütte, K., . . . Group, A. S. (1995). Treatment of symptomatic diabetic peripheral neuropathy with the anti-oxidant α-lipoic acid. *Diabetologia, 38*(12), 1425-1433.

Ziegler, D., Schatz, H., Conrad, F., Gries, F. A., Ulrich, H., Reichel, G., & Group, D. S. (1997). Effects of treatment with the antioxidant α-lipoic acid on cardiac autonomic neuropathy in NIDDM patients: a 4-month randomized controlled multicenter trial (DEKAN Study). *Diabetes Care, 20*(3), 369-373.

Zongo, C., Akomo, E.-F. O., Savadogo, A., Obame, L. C., Koudou, J., & Traore, A. S. (2009). In vitro antibacterial properties of total alkaloids extract from *Mitragyna inermis* (Willd.) O. Kuntze, a west African traditional medicinal plant. *Asian Journal of Plant Sciences, 8*(2), 172-177.