

ANTIOXIDANT CAPACITY OF *Livistona saribus* FRUIT

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ABSTRACT

Livistona saribus shares similar family to *L. chinensis* which has been consumed by the Chinese and Japanese in maintaining general health. Therefore, the aim of this study is to assess *L. saribus*'s fruit antioxidant capacity which has not been studied before. The determination of antioxidant capacity was assessed *in vitro* and *in vivo*. Four (4) kinds of *L. saribus* extracts for both coat and seed; hexane, chloroform, methanol and water, were subjected to *in vitro* evaluation specifically, total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging, ferric reducing and ferrous chelation. *In vitro* results suggest that each fraction exhibits different antioxidant mechanism, since the antioxidants results were varied according to type of extracts and fruit parts (coat and seed). Tandem liquid chromatography and mass spectrometry (LC-MS/MS) assessment had identified that water seed fraction contains 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid and oxooctadecanoic acid, which are phenolic acid and stearic acid respectively that are purportedly contribute to its antioxidant action. Meanwhile, *in vivo* analysis utilising water seed fraction on Sprague Dawley (SD) rats suggests that 300 mg/kg body weight extract supplementation and oxidative stress induced, Group 4 heightened SOD activity and catalase activity that led to protection against lipid peroxidation. This preliminary analysis of *L. saribus* antioxidant capacity paved the way to new discoveries of potent antioxidant in health supplementation industry. The possible antioxidant mechanism of action of *L. saribus* extract includes its ability to scavenge reactive oxygen species or/and enhance endogenous antioxidants levels. At present study we have found that *L. saribus* has the ability to enhance the levels of an important intracellular antioxidant.

ABSTRAK

Livistona saribus berkongsi keluarga yang serupa dengan spesies *L. chinensis*, yang telah lama digunakan oleh orang Cina dan Jepun dalam menjaga kesihatan. Tujuan kajian ini dijalankan ialah untuk menguji kadar antioksidan buah *L. saribus* *in vitro* dan *in vivo*, yang mana belum pernah ada kajian yang dibuat sebelum ini. Empat (4) jenis ekstrak buah *L. saribus* (kulit dan biji); ekstrak hexane, chloroform, methanol dan air digunakan untuk analisis antioksidan *in vitro* seperti berikut; Kandungan Keseluruhan Phenolic (TPC), Kandungan Keseluruhan Flavonoid (TFC), Ujian Radikal DPPH, Ujian FRAP dan Ujian Ferrous Chelating. Analisis *in vitro* menunjukkan bahawa setiap ekstrak mempunyai mekanisme antioksidan yang berbeza mengikut jenis ekstrak dan bahagian buah *L. saribus* (sama ada bahagian kulit dan biji). Ujian *tandem liquid chromatography* dan *mass spectrometry* (LC-MS/MS) menunjukkan ekstrak air – biji mengandungi asid 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic and asid oxooctadecanoic, yang merupakan asid phenolic dan asid stearic, yang juga dikenal pasti menyumbang kepada ciri-ciri antioksidan. Sementara itu, analisis *in vivo* ekstrak air – biji kepada Tikus Sprague Dawley (SD) menunjukkan, dos 300 mg/kg berat badan berserta tekanan oksida yang diinduksi, Kumpulan 4 meningkatkan kadar aktiviti SOD dan catalase, menyumbang kepada perlindungan daripada peroksida lipid. Ujian awal kadar antioksidan *L. saribus* ini menyumbang kepada penemuan antioksidan baru dalam industri makanan kesihatan tambahan. Kebarangkalian mekanisme antioksidan *L. saribus* termasuklah kebolehan ia untuk menghapus sisa oksigen reaktif dan/atau meningkatkan antioksidan dalaman (*endogenous*). Penemuan kajian ini, *L. saribus* mempunyai kebolehan meningkatkan kadar antioksidan intrasel.

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LIST OF SYMBOLS AND ABBREVIATIONS

μg	microgram
μl	microliter
Abs	absorbance
cm	centimeter
EC ₅₀	effective concentration at 50% activity
EDTA	ethylene-diamine-tetraacetic acid
FeCl ₂	ferrous chloride
g	gram
GAE	gallic acid equivalent
H ₂ SO ₄	sulphuric acid
HOAc	acetic acid
HPLC	high performance liquid chromatography
IC ₅₀	inhibitory concentration at 50% activity
KI	potassium iodide
L	liter
LD ₅₀	lethality dose to kill 50% population
m	meter
M	molarity

mg	milligram
min	minute
ml	milliliter
Na ₂ HPO ₄	sodium phosphate monobasic
nm	nanometer
°C	degree celcius
OD	optical density
R _f	relative mobility
rpm	revolutions per minute
TLC	thin layer chromatography
w/v	weight / volume

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CHAPTER 1

INTRODUCTION

In human, oxidative stress is implicated with the development of illnesses like cancer, cardiovascular diseases (CVD), neurodegenerative diseases and atherosclerosis. The reason being is that excess reactive oxygen species (ROS) that is generated via aerobic metabolism failed to be neutralized by physiologic antioxidant mechanisms. Consequently, the free radicals disrupt the building blocks of cell, such as DNA, RNA and protein that in turn, dismantle basic cellular function.

Studies have demonstrated that the consumption of fruits, vegetables that contain antioxidants such as Vitamin C and E can assist in preventing and minimizing the impact of free radical action towards our body. This protective role against oxidative stress conferred by numerous kinds of antioxidant supplementation is investigated to understand its mechanistic action and benefits. Therefore, the main objective of this study is to explore *Livistona saribus* to be used in preventing and treating the effects of oxidative stress.

L. saribus fruit was selected as the study plant for antioxidant capacity because no study has been done in charting its constituents nor its nutritional benefit. The decision was on the basis that *L. chinensis*, a different species in similar family exhibit potent antioxidant potential. In addition to that, *L. chinensis* has been consumed by the Chinese and Japanese for generations in maintaining general well-being. Based on that premise, it was hypothesized that *L. saribus* may have up to par or better nutritional benefits than *L. chinensis*. Accordingly, this study aims to explore preliminary antioxidant profile of *L. saribus* with intention to understand its antioxidant properties, which may possibly proceed for human consumption after significant verification is done.

However, this project aims to measure the antioxidant capacity of *L. saribus* fruit extracts only, without comparing its capacity vis-à-vis the fruit extracts of *L. chinensis*. Also, this study aims to explore the antioxidant capacity between the different types of extracts, but not the antioxidant capacity of specific phytochemical compounds present in *L. saribus* fruit extracts.

Research Objectives:

- i. To identify chemical compounds in *L. saribus* fruit by Thin Layer Chromatography (TLC) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)
- ii. To determine the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of *L. saribus* fruit
- iii. To determine the antioxidant capacity of *L. saribus* fruit *in vitro* using DPPH Radical Scavenging Assay, Ferric Reducing Antioxidant Power (FRAP), and Ferrous Chelation Activity (FCA) and *in vivo* by measuring serum antioxidant enzymes: superoxide dismutase (SOD), and catalase and its lipid peroxidation in response to oxidative stress induction.

CHAPTER 2

LITERATURE REVIEW

2.1 Studied Plant – *Livistona saribus*

Livistona saribus, or known as the taraw palm is a species of palm tree that is widely found in Southeast Asia particularly in Malaysia, Thailand, Jawa, and the Philippines. Its habitat is in well-drained ecology like rainforests or swamp forests. This palm can reach 40 meter in height and 40 to 65 cm stem diameter. Each leaf sheath sized from 25 to 30 cm, breaking up in mesh of coarse fibres; with each petiole from 1.5 to 2 meter long, armed with sharp brown spines, largest on proximal portion up to 60 mm long. Inflorescence branched to 4 orders, 1.5 to 2.5 meter long. Its flowers exist in clusters of 3 to 5, 1.5 to 2 mm long. The fruit is globose, slightly ellipsoid ranging from 1.5 to 2.5 cm diameter, glossy blue to blackish coat. The size of the seed contributes largely to the size of each fruit. The fruit is approximately 2 cm in diameter and the seed is about 0.5-0.7 cm diameter. The key features that differentiate *L. saribus* than other *Livistona* species are it has large canopy palms to 40m tall; lamina costapalmate; mid-leaf segments to 200 cm long; and fruit blue to purple at maturity (Dowe, 2009).

To date, there is no research conducted to identify and to evaluate the chemical compounds and the nutritional content of *L. saribus* fruit. However, *L. chinensis* fruit, one of the many *Livistona* species, possess medicinal properties such as anticancer and antibacterial (Kaur & Singh, 2008), which has long been benefited by the Chinese community. Besides, *L. chinensis* has demonstrated antioxidative capacity of scavenging free radicals stronger than Vitamin C (Yao et al., 2012). Based on the positive outlook of *L. chinensis*, it was hypothesized that *L. saribus* fruit exhibits potent antioxidant potential as both species shares

the same genus. Therefore, this project aims to embark on analyzing the antioxidant potential of *L. saribus* *in vitro* and *in vivo*.



Figure 2.1.1: *L. saribus* trees in Taman Botani, FRIM



Figure 2.1.2: *L. saribus* branches armed with sharp thorns.



Figure 2.1.3: Fallen *L. saribus* fruits

2.2 Oxidative Stress and Diseases

Numerous studies have demonstrated that oxidative stress is one of the primary roots of chronic diseases and aging in human. The oxidative stress takes effect when the body fails to neutralize excess free radicals generated by biochemical processes from cells and organelles. This occurrence results in the disruption of function or damage at cellular level including proteins (Baraibar et al., 2011), lipids (Murdolo et al., 2013) and DNA (Dizdaroglu et al., 2002; Wright et al., 2014). Free radical is in fact needed for redox homeostasis, such as the regulations of signal cascades or gene expression, pathogen killing and senescence (Dröge, 2002). However, at high concentration, free radicals are implicated with many pathogenesis like cancer (Zhang et al., 2013), neurodegenerative diseases (Schrag et al., 2013), diabetes (Rains & Jain, 2011), Parkinson's disease (Hauser & Hastings, 2013), cardiovascular diseases (Stephens et al., 2008), and psychiatric disorders (Ng et al., 2008) and aging (El Assar et al., 2013). Figure 2.2.1 summarizes the targets of free radicals and its effects on site.

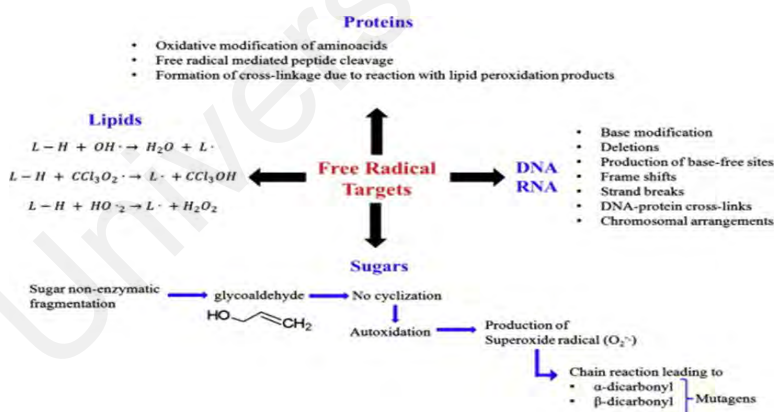


Figure 2.42-1: Targets of free radicals. Adapted from (Carocho & Ferreira, 2013)

2.3 Free Radicals and Oxidative Stress

Free radicals are unstable atoms, molecules or ions due to unpaired electrons and it is highly reactive towards other molecules. It is derived from three elements: oxygen, nitrogen and sulfur, hence creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS). In human physiologic system, ROS is significantly generated from the mitochondrial oxidative metabolism (Kowaltowski, [et al. de Souza Pinto, Castillo, & Vereesi](#), 2009). Although some may argue that the mitochondria is not the primary site of ROS production (Brown & Borutaite, 2012). The mitochondrial electron transport chain (ETC) is the site for energy production in the form of ATP via the action of oxidative phosphorylation. During the process, a number of electrons “leak” to oxygen prematurely, causing the formation of oxygen free radical superoxide. Superoxide is generated from both Complex I and III of the ETC, when it is in anionic form (O_2^-), it has already strongly charged to readily cross the inner mitochondrial membrane (Lushchak, 2014; Valko et al., 2007). It is estimated that 1-2% of oxygen consumed from respiration leaks as superoxide radicals, in which during normal respiration, intramitochondrial superoxide concentration can get to $10^{-11} - 10^{-12}$ M (Dhar & St. Clair, 2012). Therefore, aerobic organisms are constantly exposed to ROS as by-products of energy production in the mitochondria. The free radicals of ROS are the superoxide anion (O_2^-), hydroperoxyl radical ($HO_2\cdot$), hydroxyl radical ($\cdot OH$), nitric oxide (NO), and other species like hydrogen peroxide H_2O_2 , singlet oxygen (1O_2), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) (Carocho & Ferreira, 2013). Figure 2.3.1 shows the depiction of the formation of ROS.

The formation of ROS is portrayed as follows:

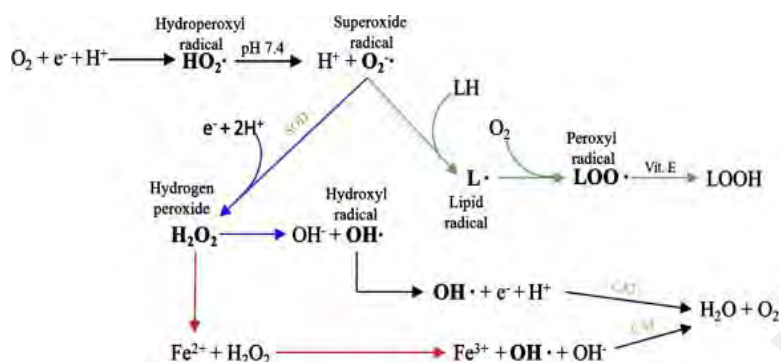


Figure 2.53-1 Overview of the reactions leading to the formation of ROS. Green arrows represent lipid peroxidation. Blue arrows represent the Haber–Weiss reactions and the red arrows represent the Fenton reactions. The bold letters represent radicals or molecules. The bold letters represent radicals or molecules with the same behavior (H_2O_2). SOD refers to the enzyme superoxide dismutase and CAT refers to the enzyme catalase. Adapted from (Carocho & Ferreira, 2013).

However, the ROS is not entirely a “bad molecule” to exist in the body, since studies have demonstrated that the ROS regulates the redox (reduction/oxidation) mechanism in cell signaling pathways such as proliferation, metabolism, differentiation, apoptosis, iron homeostasis, DNA-damage response and antioxidant and anti-inflammatory response (Circu & Aw, 2010; Halliwell, 2000; Ray, et al., 2012). Thus, the study of ROS is not limited to its damaging effects but to its beneficial functions as well.

2.4 Antioxidants and Oxidative Stress

Dietary polyphenolics are regarded as potent antioxidants in disease prevention (Rice-Evans et al., 1997). With numerous evidence associating oxidative stress induced by free radicals with the onset of various diseases and accelerated aging, the role of antioxidant to scavenge free radicals has captured many attention. Antioxidant is defined as “any substance that delays, prevents or removes oxidative damage” (Gutteridge & Halliwell, 2010). Therefore, the

characteristic of an antioxidant is to counteract free radicals damaging effect and to reduce excess free radicals in the body. Our body has the capability to scavenge ROS endogenously via the action of superoxide dismutase, catalase and glutathione peroxidase and glutathione. Also, antioxidant rich diet (eg. polyphenolic compound) from diet or commercial antioxidant supplements like glutathione, Vitamin C and E have been implicated with reduced risk of developing chronic diseases like cardiovascular diseases, cancer and diabetes (Harris, et al., 2014; Rendón-Ramírez et al., 2014; Rink et al., 2013; Wootton-Beard & Ryan, 2011). However, the findings concerning the efficacy of Vitamin C and E supplements and the incidence of chronic diseases have been inconsistent (Bjelakovic, Nikolova, Simonetti, & Gluud, 2004; Dietrich et al., 2009).

There are two types of antioxidants: endogenous and exogenous. Endogenous class is the inherent antioxidant in the body while exogenous antioxidants are the ones need to be taken to help pre-existing antioxidative mechanism to neutralize free radicals. Figure 2.4.1 summarizes the classes of antioxidant compounds.

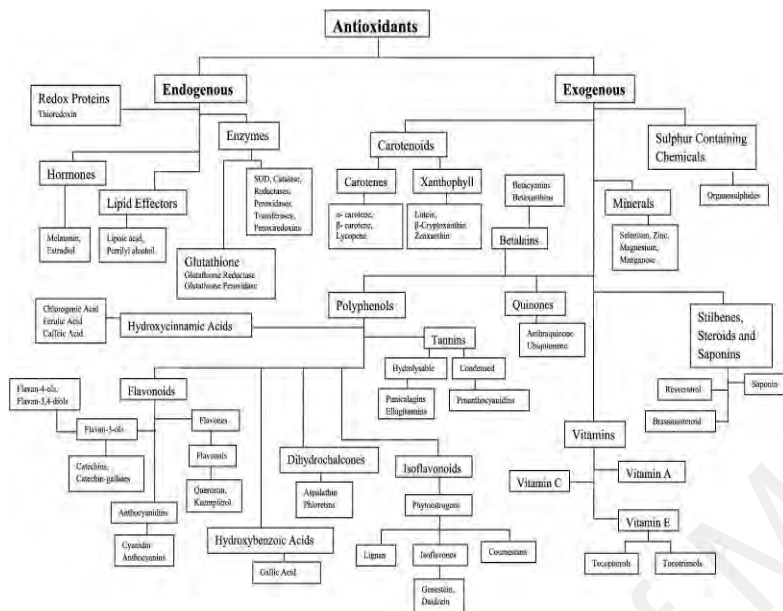


Figure 2.64.1: Classes of antioxidant compounds. Adapted from: (Wootton-Beard & Ryan, 2011).

From a mechanistic point of view, antioxidants can be classified into preventing, scavenging and repair and *de novo* antioxidants (Niki, 2010). The preventing antioxidants serve as a first line defense through the inhibition of the formation of reactive oxygen and nitrogen species, for example by reducing superoxide and lipid hydroperoxide radicals to water and lipid peroxide respectively and by removing metal ions such as iron and copper. The scavenging antioxidants work by eliminating reactive species before it attacks biological molecules as a second line defense; for instance, superoxide dismutase (SOD) converts superoxide to hydrogen peroxide and carotenoids scavenge singlet oxygen. Many phenolic compounds and aromatic amines act as scavenging antioxidants. While various enzymes work in third line defense by repairing damages, and reconstructing the lost function (Niki, 2010). For example, the reparative mechanism of non-bulky DNA damage due to oxidative action is countered by the base

excision repair (BER) system at the faulty DNA base that involves glycosylase, endonuclease, polymerase, and ligase action to correct the oxidative DNA damage (Berquist & Wilson Iii, 2012).

2.5 Phytochemicals - Antioxidant Compounds

Phenolics are characterized as having at least one aromatic ring with one or more hydroxyl groups attached. Phenolic compounds are the secondary metabolites in plants that have little or no role in photosynthesis, respiration or growth and development but which accumulate at high concentration (Crozier [et al.](#), [Jaganath](#), & [Clifford](#), 2009). Dietary polyphenolic-rich foods have been extensively studied due to its' benefits on human health like anticarcinogenic properties (Anand et al., 2008; Hollman, 2014). It is important to note that there are various bioactivities of polyphenols in human, such as their metabolites, the effects on the modulation of enzymes and the interactions with other macromolecules (Jakobek, 2015).

Some of the phenolic groups are phenolic acids, acetophenones, phenylacetic acids, hydroxycinnamic acids, coumarins, naphthoquinones, xanthenes, stilbenes, and flavonoids. Flavonoids are the largest of phenolic group and are found throughout the plant kingdom, and they are specifically found on the epidermis of leaves and the skin of fruits (Crozier et al., 2009). Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings connected by a three carbon bridge C₆-C₃-C₆. The main subgroups of flavonoids are (1) flavonols, (2) flavones, (3) anthocyanidins, (4) flavanones, (5) and isoflavones, while those that are comparatively minor in components in diet are (7) dihydroflavonols, (8) flavan-3,4-diols, (9) coumarins, (10) chalcones, (11) dihydrochalcones, (12) and aurones (Crozier et al., 2009).

Phytochemicals have long been used worldwide for thousands of years to treat and prevent ailments, thus, the relation between phytochemicals and traditional medicine or phytomedicine is integral as it is at the heart of conventional health care system (Phillipson, 2001). “Phyto” is derived from a Greek word that means plant; hence, phytochemicals are naturally occurring chemical compounds in plants. World Health Organization (WHO) defines traditional medicine as “...health practices, approaches, knowledge, and beliefs incorporating plant, animal and mineral based medicine, spiritual therapies, manual techniques, and exercises, applied singularly or in combination to treat, diagnose and prevent illness or maintain well-being.” (Cordell, 2011).

There are many phytochemicals that have been identified to possessing beneficial properties to humans such as phenolic, flavonoids, alkaloids, quassinoids etc. One of the main concerns of traditional medicines is that, it is not fully practiced in modern health care setting is due to the lack of consistency of chemical compounds present in a certain dose. This is particularly important in healthcare policy to ensure safety and efficacy of medicine prescribed (Cordell, 2011). The current trend in natural product research is in the direction of drug discovery to enhance existing findings and to discover new drugs from nature. This trend could be seen in China as an example, in which they are extensively validating data on Chinese herbs, also commercializing the herbs of traditional Chinese medicine worldwide (Cordell, 2011; Gurib-Fakim, 2006) This worldwide movement of understanding traditional medicine using current technologies can be extremely beneficial to the fate modern healthcare as it can enhance and bridge the original link between phytochemicals and traditional medicine.

2.6 Endogenous Antioxidants

For this project, the antioxidant capacity of polyphenolic compounds from *L. saribus* fruit were analyzed *in vitro* and *in vivo* using the rat model by analyzing its effects on antioxidant enzymes, superoxide dismutase (SOD) and catalase. Also, the hypothesized protective role of *L. saribus* fruit extract was assessed in its capacity to limit lipid peroxidation.

Polyunsaturated acids (PUFA) are targets for oxidants, once it is initiated, the chain of radical attack on the fatty acids chain causes lipid peroxidation. Consequently, this accumulation of lipid peroxidation such as malondialdehyde (MDA) provide the most common biochemical marker for oxidative stress (Seljeskog, 2006). Increased thiobarbituric-acid reactive substances (TBARS) in a system due to oxidative stress is implicated with diseases like DNA damage (Marchetti, 2015), and behavioral diseases (Tsai, 2015; Chakraborty, 2009).

Aerobic organisms have developed efficient defense system against oxidative insult, be it enzyme or non-enzyme antioxidants. The superoxide dismutase (SOD) family is specific in catalyzing the dismutation of superoxide anion radical byproduct, from oxidative metabolism from the mitochondria, also from extracellular sources such as ionizing radiation (Miao & St. Clair, 2009; Zelko [et al.](#), [Mariani,](#) & [Folz](#), 2002). There are 3 different SOD isoforms that have been characterized in mammals: copper-zinc superoxide dismutase (Cu/ZnSOD) encoded as gene *sod1* gene, manganese superoxide dismutase (MnSOD) encoded as *sod2* gene and extracellular superoxide dismutase (ECSOD) encoded as *sod3* gene (Miao & St. Clair, 2009). The control and compartmentalization of SODs in expression and activity levels contribute to the level of SOD and its consequent localized ROS level (Miao & St. Clair 2009). Genetic comparisons indicate that *sod1* and *sod3* genes are similar at certain levels of amino

acid homology, but *sod2* does not share considerable amino acid homology with either *sod1* or *sod3* (Miao & St. Clair 2009). Among the members of SOD family, MnSOD is shown to be the only enzyme that is important for aerobic environment survival living in physiologic system, also its association with diseases such as cancer development and progression, pulmonary and cardiovascular dysfunction, and neurodegenerative diseases (Becuwe [et al.](#), [Ennen, Klotz, Barbieux, & Grandemange](#), 2014; Dhar & St. Clair, 2012; Miao & St. Clair, 2009). The study revealed the higher the level of MnSOD, the greater the accumulation of H₂O₂; hence, the observation shows MnSOD contributes to the flux of H₂O₂ in cells and therefore MnSOD influences the production of H₂O₂ in cells and therefor their biological state (Buettner [et al.](#), [Ng, Wang, Rodgers, & Schafer](#), 2006).

The catalase enzyme on the other hand catalyzes hydrogen peroxide into water and oxygen. Hydrogen peroxide is a toxic by-product of metabolic processes; therefore, it must be converted into less harmful substances to prevent damage to cells and tissues. Catalase is expressed in many organisms especially in the liver, kidney and erythrocytes that serves as protective role in oxidative stress. The enzyme is mainly located in the peroxisomes, but it is also functionally present in the cytoplasm, in the mitochondria of rat cardiomyocytes and in the cytoplasmic membrane of human cancer cells (Glorieux, 2015).

2.7 Free Radicals, Oxidative Stress and Pathological Illness

Free radicals are defined as any molecules or atoms with one or unpaired electrons. These unpaired electrons are unstable and highly reactive. The role of free radicals such as superoxide radical and hydroxyl has been pin pointed as the initiator of several diseases including cancer (Cowey & Hardy, 2006), diabetes (Furukawa et al., 2012), cardiovascular disease (Stocker & Keaney, 2004), hypercholesterolemia (Ohara et al., 1993) and hypertension (Nakazono et al., 1991). Neurodegenerative diseases such as Alzheimer and Parkinsons are also linked to the damage caused by ROS (Blake, 1987).

At cellular level, free radicals of oxygen atom known as Reactive oxygen species (ROS), which are very unstable molecules, have immense oxidative effects on cellular constituents and functions (e.g lipid, protein and DNA). Reactive oxygen species (ROS) including hydroxyl radicals, superoxide anions and hydrogen peroxide which are produced by activated granulocytes, play an important role in many biochemical processes such as intracellular message in the cell differentiation, apoptosis, immunity and defence microorganisms (Inoguchi et al., 2000). Meanwhile in an obese person, ROS main targets are the polyunsaturated fatty acids in cell membranes causing lipid peroxidation and malondialdehyde (MDA) which can lead to damage of cell structures and function fat cells and tissues (Keane, 2003). Antioxidant treatment can stop radical scavenge and reduce the risk of pathological condition Human body can counteract with ROS by exogenous and endogenous antioxidants. Endogenous antioxidants are known as antioxidant enzymes because they are produced by the enzyme mechanism in our body (Sies, 1997). Several endogenous antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione (GSH) and catalase (CAT). These different types of endogenous antioxidants act by various mechanisms at cellular radical scavenging system. Superoxide dismutase (SOD) scavenges by speeding up its dismutase, catalase (CAT) a heme enzyme scavenges

hydrogen peroxide, and glutathione peroxidase (GPX) scavenge peroxide and other peroxide compounds (Blake et al, 1987).

The amount of antioxidants in our body must be enough to overwhelm the amount of ROS to sustain normal balance of healthy physiological system and function (Richardson, 1993). However, when ROS generation is more than the generation of antioxidant capacity, a new inequilibrium of ROS, which then leads to a condition known as oxidative stress (Sies, 1997). Oxidative stress contributes to many pathological conditions.

The current environment and pollutions exposed us to both exogenous and endogenous source of oxidations. Exogenous source of oxidants are ultraviolet radiation foreign chemicals and environmental pollutants. Endogenous source of oxidants includes mitochondrial respiration, metal ions and enzyme such as NADPH/myeloperoxidase system of phagocytes, lipoxygenase and xanthine oxidase (Halliwell, 2000)

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2.8 Free Radicals and Hyperglycemia and Diabetes

Excessive amount or over production of ROS from external or internal such as mitochondrial transport chain and excessive stimulation of NADPH is dangerous in physiological system (Cadenas & Sies, 1998). One pathological condition that arise from continuous exposure of ROS is diabetes. Diabetes mellitus is a clinical syndrome associated with chronic elevation of glucose or hyperglycemia. Pathological conditions and life threatening complications includes hypertension, retinopathy, nephropathy and other cardio vascular diseases may arise from diabetes mellitus. Type 2 diabetes is the most common form of diabetes almost 60% and only about 10% of patients suffering from type 1 diabetes (Niedowicz & Daleke, 2005). Pancreas is where the site of insulin enzyme production is vulnerable to ROS and insulin resistance is a major contributor and progression of diabetes (Weyer et al., 2001). Figure 2.8.1 depicts the mechanism of pancreatic-cell death in type 1 and type 2 diabetes.

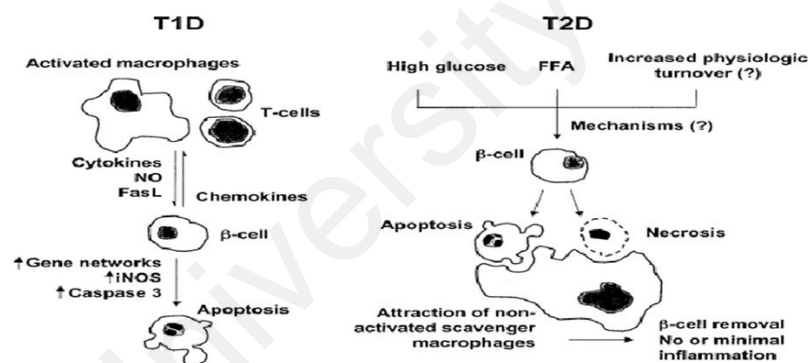


Figure 2.78.1 Mechanisms of Pancreatic -Cell Death in Type 1 and Type. (Adapted from Miriam *et al.*, 2005).

Increased oxidative stress has been the major factor for pathological condition of hyperglycemia or elevated sugar level. And at the same time hyperglycemia can also trigger the production of secondary oxidative stress due to presence of ROS. The process of hyperglycemia involves various mechanisms including oxidative phosphorylation, glucose autooxidation, NADPH, oxidase, lipoxygenase, cytochrome P450 monooxygenases and nitric oxide synthase (NOS). A cascade of processes known as NADH oxidative phosphorylation (Casper et al., 2005) and mitochondrial β -oxidation (Rosca et al., 2005) lead to excess accumulation of intracellular triglyceride in muscle and liver and eventually lead to insulin resistance. Figure 2.8.2 shows the vascular complications in diabetes mellitus.

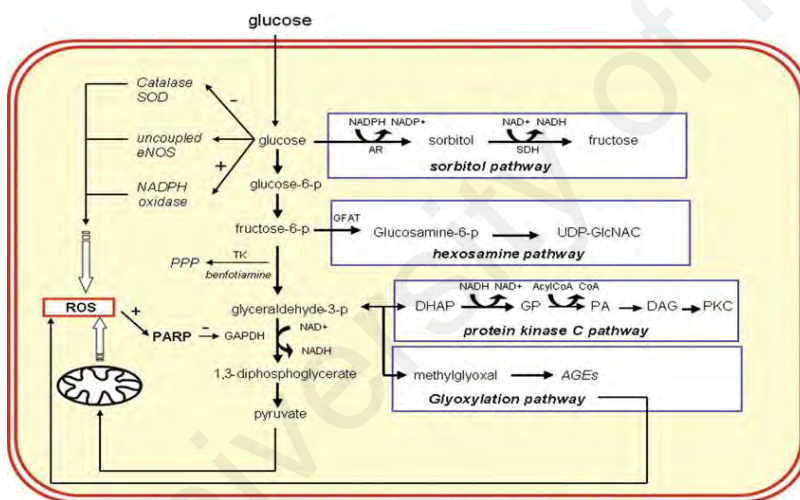


Figure 2.8.2 Vascular complications in diabetes mellitus: the role of endothelial dysfunction Clinical Science. Casper et al., 2005.

A study by Evans et al (2003) has suggested that a prolonged exposure to high glucose, fatty acids may result to β -cell dysfunction. The lack of endogenous antioxidant enzymes of catalase, glutathione peroxidase and superoxide dismutase causes the β -cells to vulnerable to ROS attack (Grankvist et al., 1981). This deficiency of endogenous antioxidants will further more aggravated by the damage of mitochondrial β -cell due to oxidative stress characterized by low insulin secretion. In addition, the low capability of β -cells to metabolize hydrogen peroxide through catalase and glutathione made it even worse. An adaptation of this condition was done in vitro where cultured β -cells under oxidative stress by the presence of H_2O_2 increases production of cyclin dependent-kinase (CDK) inhibitor and decreases insulin mRNA, cytosolic ATP and calcium flux in cytosol (Kaneto et al., 1999).

Expression and function of mitochondrial β -cell protein plays a pivotal role for insulin production. It is said that elevated glucose or hyperglycemia can contribute to expression of specific protein known as Uncoupling protein-2 (*UCP2*). An increase *UCP2* activity leads to the formation of superoxide radicals (Krauss et al, 2003). Another factor that can contribute to β -cell dysfunction is an interruption with pancreas duodenum homeobox-1 (*PDX-1*). The gene of *PDX-1* regulates insulin that involves glucose regulation. An interruption of ROS and oxidative stress to *PDX-1* will cause glucose toxicity (Robertson et al., 2003). An evidence reinforced that a prolonged exposure of ROS and oxidative stress would disintegrate the mRNA and *PDX-1* and critically imposes glucose toxicity (Robertson *et al*, 1992). Diabetes especially diabetes type 2 warranted a greater clinical care and treatment because β -cells become an easy target of ROS and oxidative stress.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

The *L. saribus* fallen fruits were collected from Forest Research Institute of Malaysia (FRIM), Kuala Lumpur. Voucher specimen number: KLU 49448, UM Herbarium. The sample fruit was then authenticated by plant taxonomist, Prof Dr Ong Hean Chooi from the Institute of Biological Sciences, University of Malaya. The fruits were dried in an oven at 40°C for a week, in which the seed and the coat were separated by hand. Then, the coat and seed were grinded into fine powder for the purpose of extraction and stored in air tight containers at room temperature until use.

3.2 Preparation of Sample Extracts

The powdered fruits of *L. saribus* (coat and seed) were subjected to soxhlet extraction using *n*-hexane, chloroform and methanol consecutively for 48 hours for each fraction. The crude extracts were concentrated into powder or essence using rotary evaporator (Rotavapor R-210, Switzerland) at temperature below 40 °C.

Water extraction was done by immersing the samples (coat and seed) in distilled water at 1:10 ratio for 5 days below 50 °C. The water extracts were then filtered and dried using freeze dryer.

All crude extracts were stored in air tight glass tubes at 4 °C. The *n*-hexane, chloroform, and methanol extracts were dissolved in 5% DMSO for analysis. While water extracts were dissolved in 100% distilled water.

3.3 Separation and Identification of *L.saribus* Chemical Compounds with TLC

3.3.1 Visible Light

TLC chromatograms of each sample was observed under visible light. The R_f values observed bands were calculated.

3.3.2 Ultra Violet Light

TLC chromatograms were observed under UV light to mark for fluorescent bands to calculate the R_f values.

3.3.3 Dragendorff Reagent

Dragendorff reagent was prepared by mixing 5 ml of Solution A (1.7g basic bismuth nitrate in 100 ml H₂O-OHAc (80:20) with 5 ml Solution B (40 g powdered potassium iodide in 100 ml distilled water), 20 ml acetic acid and 70 ml distilled water. Dragendorff reagent was sprayed on the TLC plates. Then, the R_f values were calculated. Dragendorff reagent is used for the detection of alkaloids.

3.3.4 Vanillin Reagent

The reagent was prepared by mixing 5% ethanol sulfuric acid with 1% ethanol vanillin. Vanillin reagent was sprayed on the TLC plates. Then, the R_f values were calculated. Vanillin reagent is used for the detection of terpenoids.

3.3.5 Anisaldehyde Reagent

Anisaldehyde reagent was prepared by mixing 10ml glacial acetic acid with 85ml methanol and 5 ml concentrated sulphuric acid. After the TLC plates were sprayed with anisaldehyde reagent, the plates were heated at 100 °C for 5-10 minutes prior to viewing and calculating the R_f values. Anisaldehyde reagent is used for the detection of phenolic compounds.

3.4 Analysis of Compounds with LC-MS/MS

All quantitative analyses were performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) from AB Sciex 3200Q Trap with Perkin Elmer FX15 uHPLC system. 20 µl sample (*L. saribus* water extract) was injected into the LC system equipped with column Agilent Zorbax C18 (50 mm x 4.6 mm x 5 µM column). Sample elution was carried out at 15 minutes rapid screening using gradient elution comprising Buffer A (water with 0.1% formic acid and 5 mM ammonium formate) and Buffer B (acetonitrile with 0.1% formic acid and 5 mM ammonium formate). Elution was performed using the following gradient: 10% A and 90% B from 0.01 minute to 8 minutes, hold for 2 minutes and back to 10%B in 0.1 minute and reequilibrated for 5 minutes. Peaks were analyzed by mass spectrometer at negative ion mode. Identification of phenolic compounds was performed under full scan mode in the range of 100-1200 m/z.

3.5 Determination of Total Phenol and Total Flavonoid Content

3.5.1 Total Phenolic Content (TPC) by Folin-Ciocalteu Method

The procedure for the measurement of phenolic content was evaluated with alterations to the gallic acid standard concentrations (Medina, 2011). The gallic acid standard calibration at (25 - 200 µg/mL) was used to construct the standard reference. In this procedure, 0.9 mL distilled water was transferred to test tubes, followed by 100 µL of gallic acid standards, extracts (1 mg/mL), and distilled water for blank, and mixed. Next, 100 µL of Folin-Ciocalteu reagent (10 times diluted) was added, mixed and allowed to rest at room temperature for 5 minutes. Then, 1 mL 7% Na₂CO₃ sodium carbonate was added, followed by the addition of 400 µL distilled water. The mixture was allowed to react for 90 minutes at room temperature and dark condition and 200 µL aliquots were transferred to 96 well plate. The absorbance was measured using Hi-Tech

UVM340 at 725 nm. The results were expressed by mg gallic acid equivalent (mg GAE) / g of dry extracts from triplicate tests.

3.5.2 Total Flavonoid Content (TFC)

The measurement of flavonoid content was assessed according to the aluminum chloride method (Zhang et al., 2011). The quercetin standard calibration at (200 – 1000 µg/ml) was used to construct the standard reference. 0.5ml of each sample (1 mg/mL) and 300 µl 5% sodium nitrite (Na₂NO₂) were mixed in a test tube and left at room temperature for 5 minutes. Then, 300 µl 10% aluminum chloride (AlCl₃), 2ml 1M sodium hydroxide (NaOH) and 1.9 ml distilled water were added and mixed. The absorbance was measured at 510 nm by using microplate reader (Tecan Sunrise, Austria). The results were expressed by mg quercetin equivalent (mg QE) /g of dry extracts from triplicate tests.

3.6 In vitro Antioxidant Assays

3.6.1 Free Radical DPPH Scavenging Assay

The measurement of the activity of DPPH was evaluated based on the method (Ablat et al., 2014) with slight modifications in the volume and the type of standard used. The ascorbic acid standard calibration at (5 - 320 µg/ml) was used to construct the standard reference. 20 µL of extracts or standard was added to a microtiter plate, followed by 150 µL of 50 µM DPPH dissolved in methanol. The mixture was then incubated for 30 minutes in room temperature and dark condition. The decrease in absorbance was measured 517 nm microplate reader (Tecan Sunrise, Austria). The inhibition percentage by the extracts were measured according to the formula

$$\text{DPPH scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample or standard}}}{A_{\text{control}}} \times 100\%$$

The concentration of extracts required to scavenge 50% of DPPH radical (IC₅₀) was estimated from the graph plotted between concentration and percentage inhibition in

comparison to the standard. All tests was conducted in triplicate and the IC₅₀ values were expressed in µg/mL.

3.6.2 Ferric Reducing Antioxidant Power (FRAP)

The FRAP activities was measured in reference to the method (Ablat et al., 2014). 20µL sample (5 – 320 µg/mL) was added into a microplate, followed by 200 µL of FRAP reagent. The FRAP reagent consisted of 5 mL 10 mM TPTZ in 40 mM HCl, 5 mL 20 mM FeCl₃ and 0.3M acetate buffer (pH 3.6). After 10 minutes of incubation time, the TPTZ Fe²⁺ complex formation was measured at 595nm with a microplate reader (Tecan Sunrise, Austria). Ferrous sulfate (200 – 1000 µM) was used as the standard calibration to measure FRAP activity. Methanol was used as blank. The FRAP activity was calculated based on the linear regression between standard solutions against absorbance at 595nm from triplicate tests. The results were expressed as mmol Fe²⁺ /g of dry extract.

3.6.3 Ferrous Chelation Assay

The ferrous chelation activity was measured according to the method described (Ablat et al., 2014). EDTA (5 – 320 µg/ml) was used as the standard calibration to measure the chelating activity. In brief, 10 µL FeCl₂ (2mM), 100µL extracts or standard (5 – 320 µg/mL), 120 µL distilled water and 20 µL Ferrozine (5mM) were pipetted into each well in a microtiter plate. The mixture was then incubated for 20 minutes at room temperature and the final absorbance was read at 562 nm. Methanol was used as blank without ferrozine (20 µL of distilled water instead of ferrozine). The percent inhibition of Fe²⁺- ferrozine complex according to this formula:

$$\text{Ferrous Ion Chelation Activity (\%)} = \frac{A_{\text{control}} - A_{\text{standard or sample}}}{A_{\text{control}}}$$

The concentration of extracts to chelate 50% of Fe²⁺ ion (IC₅₀) was calculated from the graph of extract concentrations against percent inhibition. The results are from triplicate tests and were expressed as µg/mL.

3.7 Experimental Rat Studies

3.7.1 Experimental Animals

Male Sprague Dawley (SD) rats approximately 6 to 8 week old were used in this study (n=6 each group). The rats were housed in cages (4.5 to 4.8m²) under standard laboratory conditions (temperature, 19 °C, relative humidity 50%) in compliance with the requirements of Institutional Animal Care and Use Committee (IACUC) of the University of Malaya. Animal Ethical No: ISB/4/03/2015/AAS (R). Light-dark cycle 12 hours light and 12 hours dark will be employed. Access to rat pellet and water are provided *ad libitum*.

3.7.2 Oral Acute Toxicity Test

The male (n = 3) and female (n = 3) rats were acclimatized for two weeks. Then, the rats were fasted overnight for 16 hours and initially be orally fed with 300 mg/kg body weight of seed water extract to determine the acute toxicity according to the guidelines of the 423 OECD guideline for Acute Oral Toxicity. The control groups received distilled water and standard rat pellet only. The rats were observed continuously 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, gasping, response to touch, and decreased respiratory rate or for any lethality.

3.8 Animal Experimental Design

The rats were divided into 7 groups of 6 rats each as follow. All groups were subjected to two (2)-week treatment.

Group 1: Rats received distilled water and standard rat pellet *ad libitum*.

Group 2: Rats received Quercetin (50 mg/kg) on alternate days (day 1, day 3, day 5...)

Group 3: Rats received sodium tungstate (50 mg/kg) alone on alternate days.

Group 4: Rats received sodium tungstate (50 mg/kg) and high dose extract (300 mg/kg) on alternate days.

Group 5: Rats received sodium tungstate (50 mg/kg) and low dose extract (30 mg/kg) on alternate days.

Group 6: Rats received sodium tungstate (50 mg/kg) and Quercetin (50 mg/kg) on alternate days.

Group 7: Rats received with extract alone (300 mg/kg) on alternate days.

Twenty-four hours after the sodium tungstate challenge followed by fasting, the rats were anesthetized with (Ketamine 50 mg/kg and Xylazine 5 mg/kg) before obtaining the blood and euthanized (done by drug overdose) to collect the livers and kidneys. Blood was collected by cardiac puncture about 1 to 2 ml for analysis.

3.9 Determination of Serum Antioxidant Activity

3.9.1 Serum Superoxide Dismutase (SOD) Activity

The capability of SOD to inhibit the autoxidation of epinephrine to adrenochrome is the basis for this test (Misra & Fridovich, 1972). The brown color formation of adrenochrome was measured at 480 nm using spectrophotometer. 100 μ L of plasma or blank sample was mixed with 2800 μ L of sodium carbonate and 100 μ L of epinephrine. Distilled water was used as blank. After the epinephrine solution was added, the absorbance of test solutions was measured at 480 nm at 30 $^{\circ}$ C.

Inhibition (%) was determined by this formula:

$$\frac{\Delta \text{Absorbance of blank each minute} - \Delta \text{Absorbance of plasma each minute}}{\Delta \text{Absorbance of blank sample each minute}} \times 100\%$$

One unit / mL SOD was determined by: % inhibition x dilution factor

3.9.2 Serum Catalase (CAT) Activity

The catalase activity was determined by colorimetric method for the loss of added hydrogen peroxide in the presence of enzyme catalase that reduced bichromate to chromic acetate (Sinha, 1972). One mL plasma was added to 2 mL of chromogen (5% potassium bichromate) and mixture solution was boiled in boiling water for 10 minutes. After cooling, the absorbance of the solution was measured at 570 nm. Absorbance versus H₂O₂ was linearly correlated. The catalase activity was expressed as μmol H₂O₂/mL.

3.9.3 Serum Glutathione Peroxidase (GPx) Activity

The glutathione peroxidase activity was evaluated by using the Calbiochem® Glutathione Peroxidase Assay Kit (Merck, Germany). The background wells consisted of 120 μl 1X assay buffer and 50 μl co-substrate mixture. The positive control wells contained 100 μl 1X assay buffer, 50 μl co-substrate mixture and 20 μl diluted glutathione peroxidase. Meanwhile, the sample wells consisted of 100 μl 1X assay buffer, 50 μl co-substrate mixture and 20 μl rat serum. The co-substrate mixture is comprised of lyophilized powder of NADPH, glutathione and glutathione peroxidase. All tests were done in triplicates and the decrease in absorbance at 340 nm was monitored using a plate reader.

The GPx activity formula:

$$\frac{\Delta A_{340}/\text{min}}{0.00373\mu\text{M}^{-1}} \times \frac{0.19\text{ml}}{0.02\text{ml}} \times \text{sample dilution} = \frac{\text{nmol}}{\text{min}}/\text{ml}$$

3.9.4 Lipid Peroxidation Analysis (TBARS)

The estimation of malondialdehyde (MDA) production as the by-product of lipid peroxidation using thiobarbituric acid reagent is the basis of this assay (Jentzsch et al.,

1996). The standard solutions were prepared using 200 μl of MDA standard solution was used instead of plasma. MDA stock solutions were prepared by hydrolysis of 50 μl TEP (10 mM) in 10 ml 0.01M hydrochloric acid for 10 minutes at room temperature. MDA stock solution was diluted with distilled water to 5.0, 2.5, 1.25, 0.625, 0.3125 and 0.156 μM . 200 μl plasma or standard MDA were mixed with 25 μl BHT in ethanol (3 mM) and 200 μl orthophosphoric acid (0.2 M) in 1.5ml Eppendorf micro test tubes and vortexed for 10s. 25 μl TBA reagent dissolved in sodium hydroxide (0.11 M) were added and vortexed again. The reaction mixture was incubated at 90 $^{\circ}\text{C}$ for 45 min in a water bath. The tubes were then cooled at room temperature. Next, 500 μl *n*-butanol was added to extract TBARS. 50 μl saturated sodium chloride solution was added to the mixture to facilitate separation. The test tubes were then centrifuged at 12000 rpm for 1 min. Two-hundred fifty microliters of the upper phase butanol were transferred in a 96-well plate microtiter plate. Absorption was read at both 535 nm and 572 nm to correct for baseline absorption in a multititer plate reader. MDA equivalents (TBARS) were calculated using the difference in absorption at the two wavelengths and quantification was made with the aid of calibration curve.

CHAPTER 4

RESULTS

4.1 Phytochemical Detection by TLC on *Livistona saribus*

Table 4.1 Thin Layer Chromatography Analysis 10% Methanol in Chloroform for *L. saribus* Chloroform Coat Fraction

Band	Observation					Comment
	R _f		Detection using Reagent			
	Visible Light	UV Light	Vanillin	Anisaldehyde	Dragendorff	
B1	0.067	0.067	-	Green (+)	-	Phenolic
B2	-	0.100	-	Green (+)	Yellow (+)	Phenolic / Alkaloids /
B3	-	0.133	-	Green (+)	-	Phenolic
B4	0.167	0.167	-	-	Yellow (+)	Alkaloid
B5	0.217	0.217	-	-	Yellow (+)	Alkaloid
B6	-	0.267	-	Green (+)	-	Phenolic
B7	0.317	0.317	Purple (+++)	-	-	Terpenoid
B8	-	0.350	Purple (++)	-	-	Terpenoid
B9	0.367	0.367	-	-	Yellow (++)	Alkaloid
B10	-	0.400	-	Green (+)	Yellow (+)	Phenolic / Alkaloid

Table 4.1 Continued

Compound	Observation					Comment
	R _f		Detection using Reagent			
	Visible Light	UV Light	Vanillin	Anisaldehyde	Dragendorff	
B11	0.467	0.467	-	-	Yellow (++)	Alkaloid
B12	0.533	0.533	Purple (+)	-	-	Terpenoid
B13	-	0.567	-	Purple (++)	-	Phenolic /
B14	-	0.600	-	Green (+)	Yellow (+)	Phenolic / Alkaloid
B15	0.667	0.667	Purple (+)	-	-	Terpenoid
B16	0.716	0.716	-	Green (++)	Yellow (+)	Phenolic / Alkaloid
B17	-	0.750	-	Green (++)	Yellow (+)	Phenolic / Alkaloid
B18	0.833	0.833	-	Green (+++)	Yellow (+++)	Phenolic / Alkaloid

+ indicates mild intensity

++ indicates medium intensity

+++ indicates strong intensity

Table 4.2 Thin Layer Chromatography Analysis 10% methanol in Chloroform for *L. saribus* Chloroform Seed Fraction

Compound	Observation					Comment
	R _f		Detection using Reagent			
	Visible Light	UV Light	Vanillin	Anisaldehyde	Dragendorff	
B1	-	0.583	-	Green (+)	-	Phenolic
B2	0.667	0.667	Purple (+)	-	-	Terpenoid
B3	-	0.750	-	Green (+)	-	Phenolic
B4	0.767	0.767	-	Purple (+)	-	Phenolic
B5	-	0.833	-	Green (+)	Yellow (++)	Phenolic / Alkaloid
B6	-	0.900	Purple (+)	-	-	Terpenoid

+ indicates mild intensity

++ indicates medium intensity

+++ indicates strong intensity

Table 4.3 Thin Layer Chromatography Analysis 10% Methanol in Chloroform for *L. saribus* n-hexane Seed Fraction

Compound	Observation					Comment
	R _f		Detection using Reagent			
	Visible Light	UV Light	Vanillin	Anisaldehyde	Dragendorff	
B1	-	0.550	-	Green (+)	-	Phenolic
B2	-	0.683	-	Green (++)	-	Phenolic
B3	-	0.700	Purple (+)	-	-	Terpenoid
B4	-	0.733	-	Purple (+)	-	Phenolic
B5	-	0.833	-	Green (+)	Yellow (++)	Phenolic / Alkaloids
B6	-	0.900	Purple (+)	-	-	Terpenoid

+ indicates mild intensity

++ indicates medium intensity

+++ indicates strong intensity

4.2 LC-MS/MS Profile of *L. saribus* Seed Water Extract

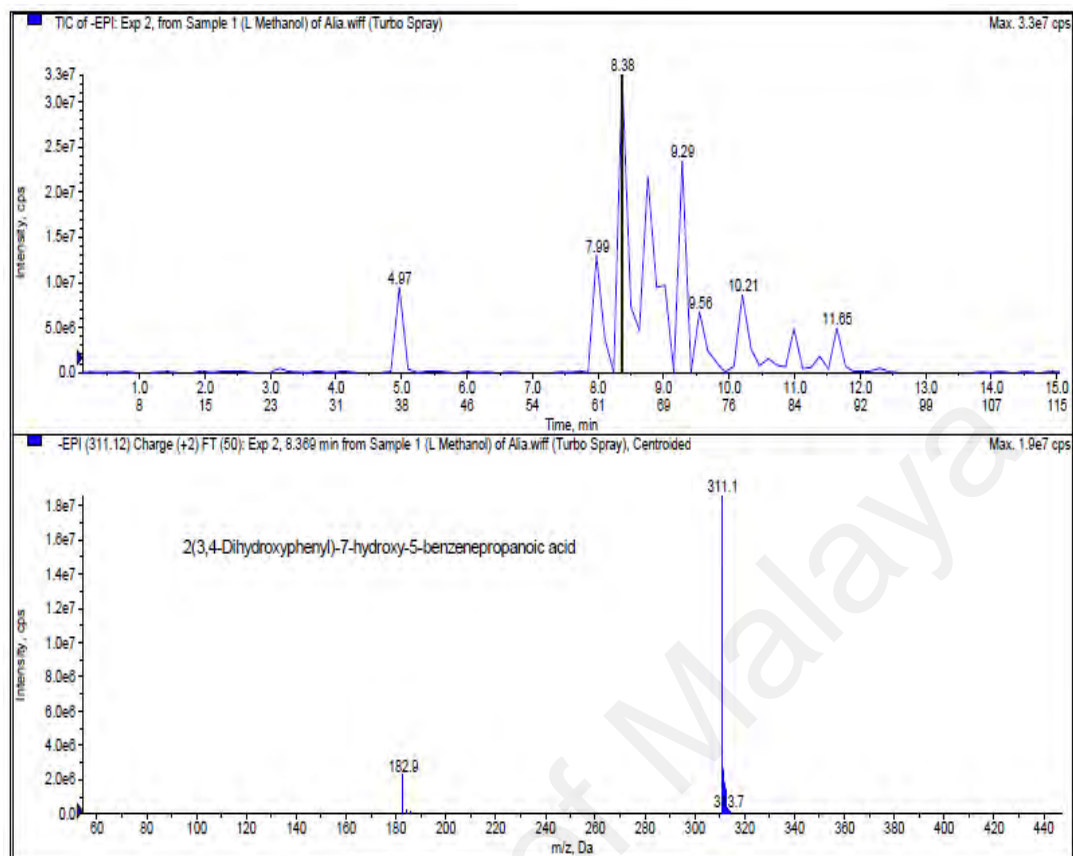


Figure 4.1 LCMS/MS of 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid from *L. saribus* seed water extract.

Continued from LCMS/MS Profile

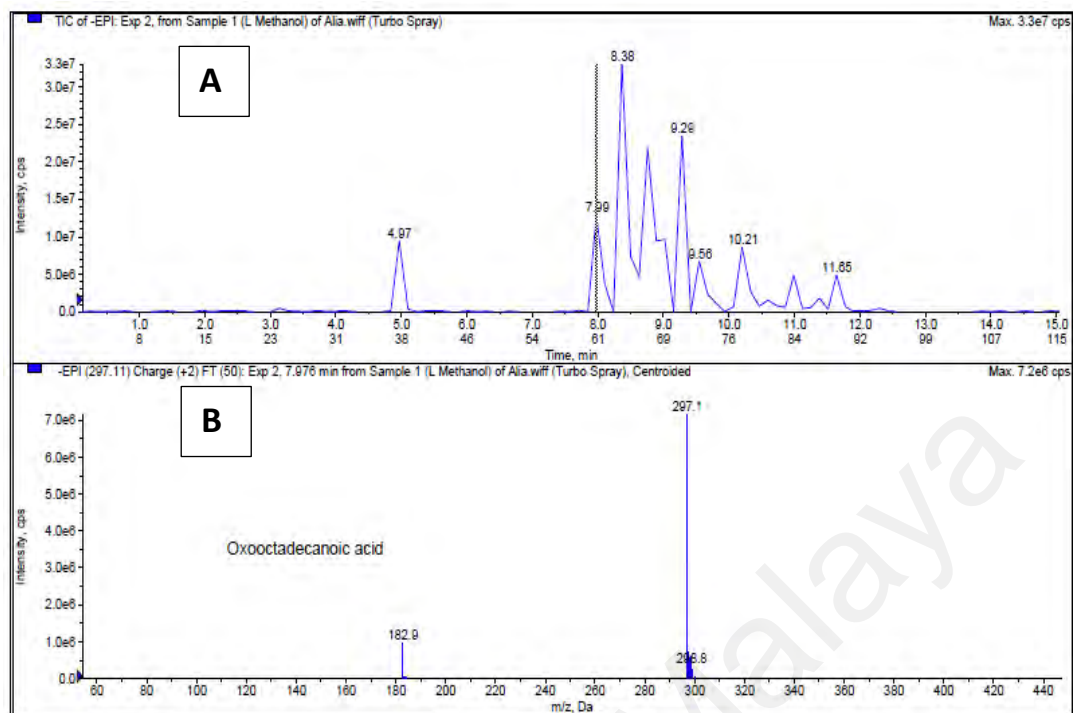


Figure 4.2 LCMS/MS of Oxooctadecanoic acid from *L. saribus* seed water extract.

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4.3 *In vitro* Antioxidant Analysis

4.3.1 Total Phenolic Content (TPC)

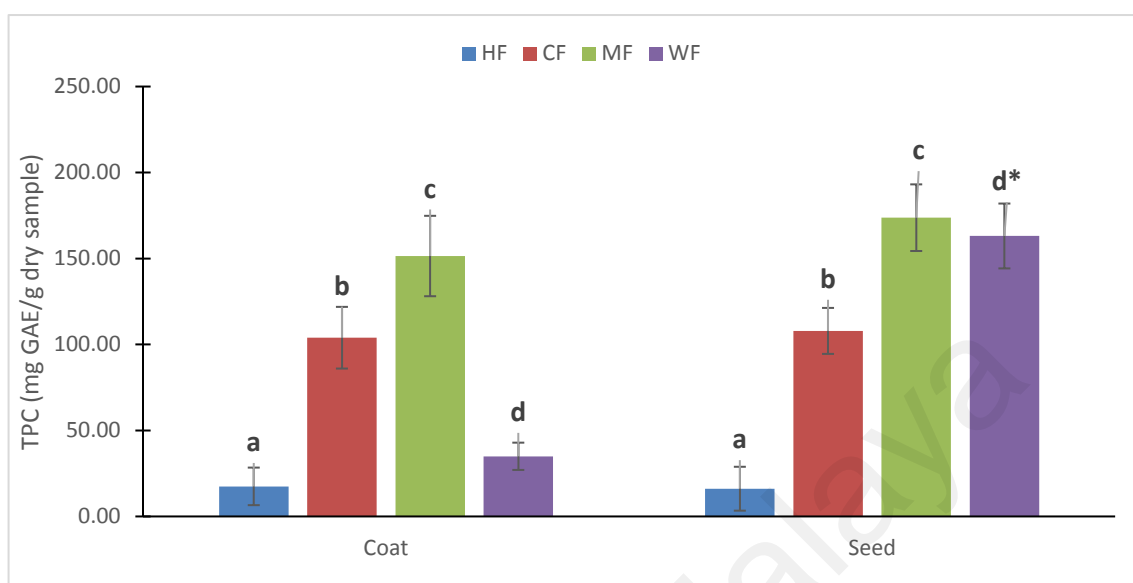


Figure 4.3: Total Phenolic Content (TPC). Similar letter representation of a, b, c and d between similar fraction with different fruit part (coat or seed) indicate non significance between the data (ANOVA $p > 0.05$). (*) representation indicates significant difference in TPC between similar fraction with different fruit part (ANOVA $p < 0.05$). The error bars represent Standard Deviation (SD) values ($n=3$). HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; and WF: water fraction.

Table 4.4: The average of Total Phenolic Content (TPC) in *L.saribus* extracts.

Fraction	Coat (TPC mg GAE/g sample)	Seed (TPC mg GAE/g sample)
HF	17.48 ± 10.92 ^a	16.13 ± 12.76 ^a
CF	103.94 ± 17.93 ^a	107.90 ± 13.37 ^a
MF	151.44 ± 23.38 ^a	173.73 ± 19.38 ^a
WF	34.98 ± 7.94 ^a	163.10 ± 18.86 ^b

The ± indicates Standard Deviation (SD) values ($n=3$). Means with different lower case letters (a and b) in the same row are significantly different (ANOVA $p < 0.05$). HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; and WF: water fraction.

4.3.2 Total Flavonoid Content (TFC)

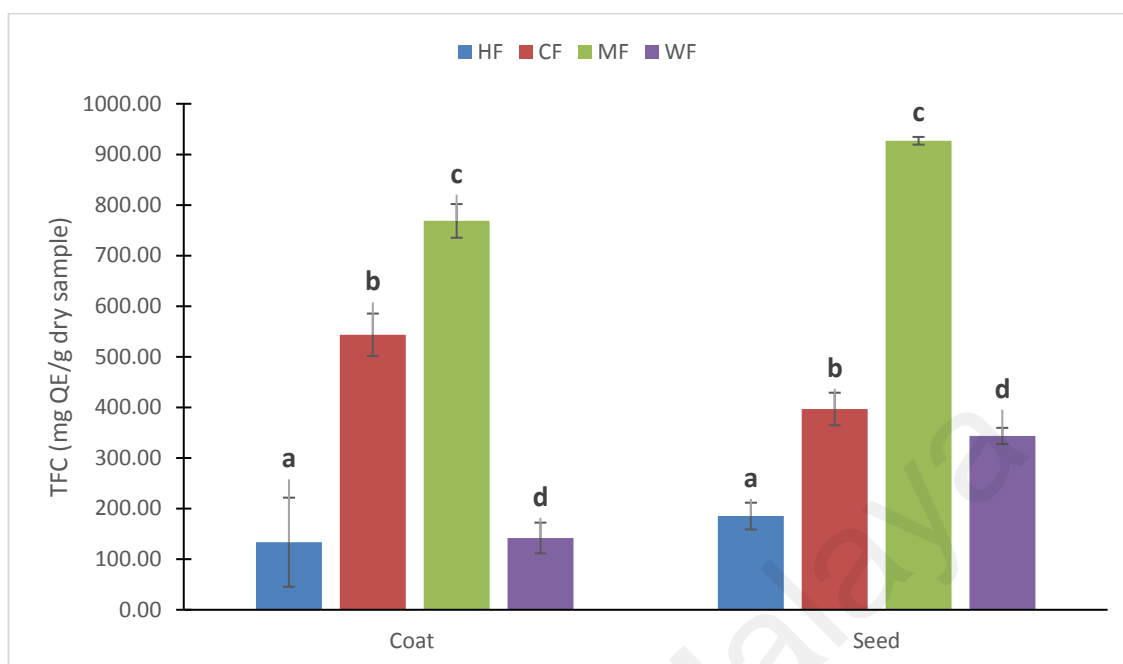


Figure 4.4: Total Flavonoid Content (TFC). Similar letter representation of a, b, c and d between similar fraction with different fruit part (coat or seed) indicate non significance between the data (ANOVA $p > 0.05$). (*) representation indicates significant difference in TFC between similar fraction with different fruit part (ANOVA $p < 0.05$). The error bars represent Standard Deviation (SD) values ($n=3$). HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; and WF: water fraction.

Table 4.5: Data are mean of Total Flavonoid Content (TFC) in *L.saribus* extracts.

Fraction	Coat (TFC mg QE/g sample)	Seed (TFC mg QE/g sample)
HF	133.50 ± 88.08 ^a	185.17 ± 26.46 ^a
CF	343.50 ± 41.93 ^a	396.83 ± 32.15 ^b
MF	768.50 ± 33.29 ^a	926.83 ± 7.64 ^b
WF	141.83 ± 30.41 ^a	343.50 ± 16.07 ^b

The ± indicates Standard Deviation (SD) values ($n=3$). Means with different lower case letters (a and b) in the same row are significantly different (ANOVA $p < 0.05$). HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; and WF: water fraction.

4.3.3 DPPH Radical Scavenging

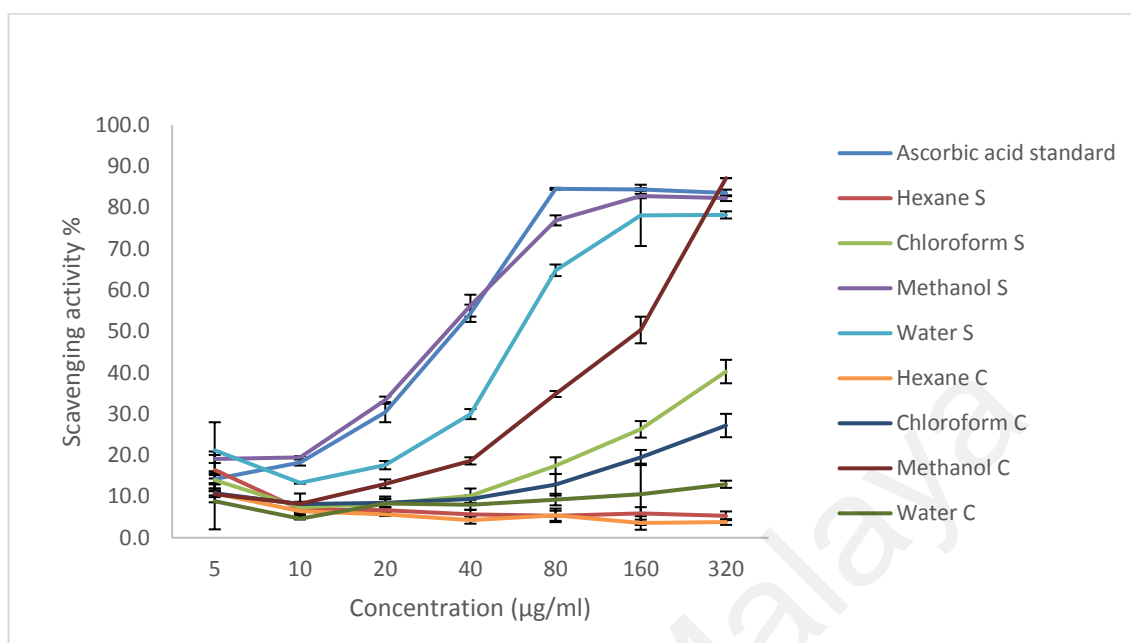


Figure 4.5: Percent (%) DPPH Scavenging Activity. Ascorbic acid was used as standard. The error bars represent Standard Deviation (SD) values (n=3). C: coat and S: seed.

Table 4.6: Data are 50% Inhibitory concentration (IC_{50}) of DPPH of *L. saribus* extracts \pm SD (n=3).

Fraction	Coat (DPPH IC_{50} μ g/ml)	Seed (DPPH IC_{50} μ g/ml)
HF	ND	ND
CF	ND	ND
MF	123.30 \pm 17.51 ^a	33.18 \pm 4.17 ^b
WF	ND	61.99 \pm 12.15

Ascorbic acid standard IC_{50} 33.24 \pm 4.57

The \pm indicates Standard Deviation (SD) values (n=3). IC_{50} values were calculated using GraphPad Prism 7.00. Means with different lower case letters (a and b) in the same row are significantly different (Non parametric paired t-test $p < 0.05$). DPPH: 2,2 diphenyl-1-picrylhydrazyl; HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; WF: water fraction and ND: not detected.

4.3.4 Ferric Reducing Antioxidant Power (FRAP)

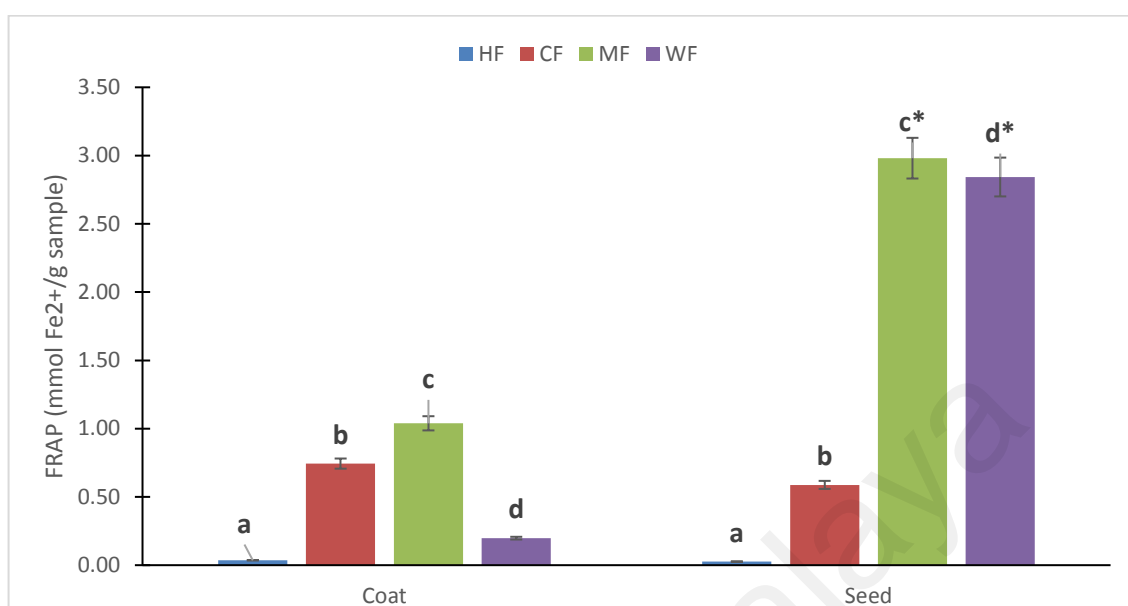


Figure 4.6: Ferric Reducing Antioxidant Power (FRAP). Similar letter representation of a, b, c and d between similar fraction with different fruit part (coat or seed) indicate non significance between the data (ANOVA $p > 0.05$). (*) representation indicates significant difference in ferric reducing between similar fraction with different fruit part (ANOVA $p < 0.05$). The error bars represent Standard Deviation (SD) values ($n=3$). HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; and WF: water fraction.

Table 4.7: Data are mean of Ferric Reducing Antioxidant Power (FRAP) in *L.saribus* extracts.

Fraction	Coat (mmol Fe ²⁺ /g sample)	Seed (mmol Fe ²⁺ /g sample)
HF	0.036 ± 0.0032 ^a	0.026 ± 0.0079 ^a
CF	0.74 ± 0.096 ^a	0.59 ± 0.086 ^a
MF	1.04 ± 0.036 ^a	2.98 ± 0.12 ^b
WF	0.20 ± 0.011 ^a	2.84 ± 0.15 ^b

The ± indicates Standard Deviation (SD) values ($n=3$). Means with different lower case letters (a and b) in the same row are significantly different (ANOVA $p < 0.05$). HF: *n*-hexane fraction; CF: chloroform fraction; MF: methanol fraction; and WF: water fraction.

4.3.5 Ferrous Ion Chelation

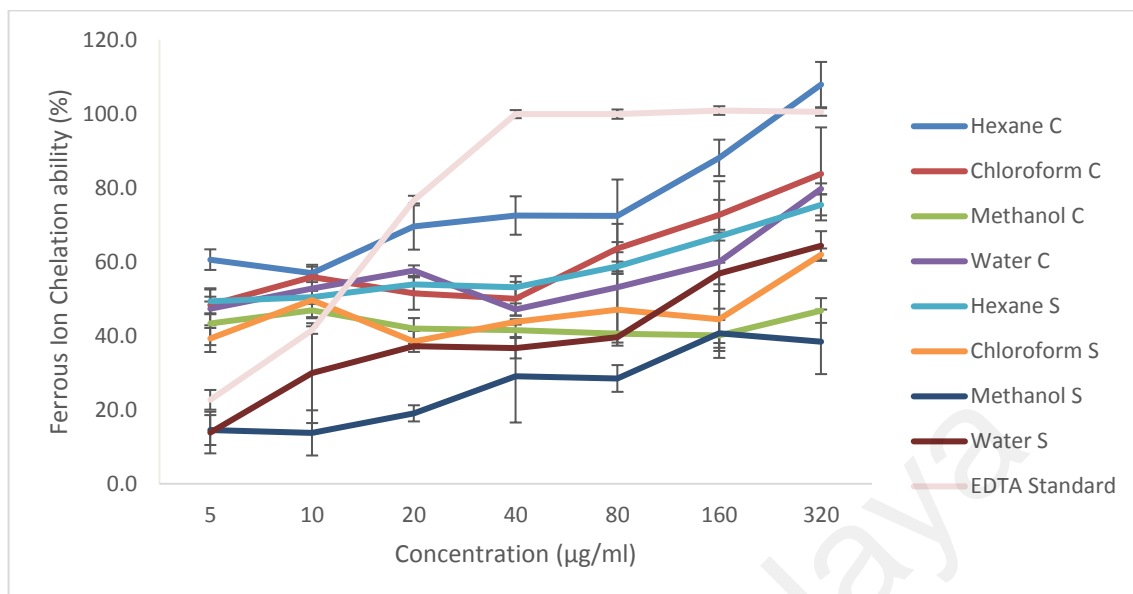


Figure 4.7: Percent (%) ferrous chelation ability. EDTA was used as standard. The error bars represent Standard Deviation (SD) values (n=3). C: coat and S: seed.

Table 4.8: Data are 50% Inhibitory concentration (IC₅₀) of ferrous ion chelation of *L. saribus* extracts.

Fraction	Coat (Ferrous Chelation IC ₅₀ µg/ml)	Seed (Ferrous Chelation IC ₅₀ µg/ml)
HF	3.92 ± 2.61 ^a	10.05 ± 3.62 ^b
CF	9.92 ± 4.91 ^a	117.20 ± 107.50 ^b
MF	ND	ND
WF	8.24 ± 4.33 ^a	106.80 ± 24.31 ^b
EDTA Standard IC₅₀ 10.87 ± 0.70		

The ± indicates Standard Deviation (SD), values (n=3). IC₅₀ values were calculated using GraphPad Prism 7.00. Means with different lower case letters (a and b) in the same row are significantly different (Non parametric paired t-test p < 0.05). HF: n-hexane fraction; CF: chloroform fraction; MF: methanol fraction; WF: water fraction and ND: not detected.

4.4 *In vivo* Antioxidant Analysis

4.4.1 Oral Acute Toxicity

The oral acute toxicity test was conducted according to the guidelines of the 423 OECD for Acute Oral Toxicity. The experimental rats showed no signs of toxicity throughout the testing period. 300 mg/kg body weight was subjected to experimental rat analysis.

University of Malaya

4.4.2 Serum Superoxide Dismutase (SOD) Activity

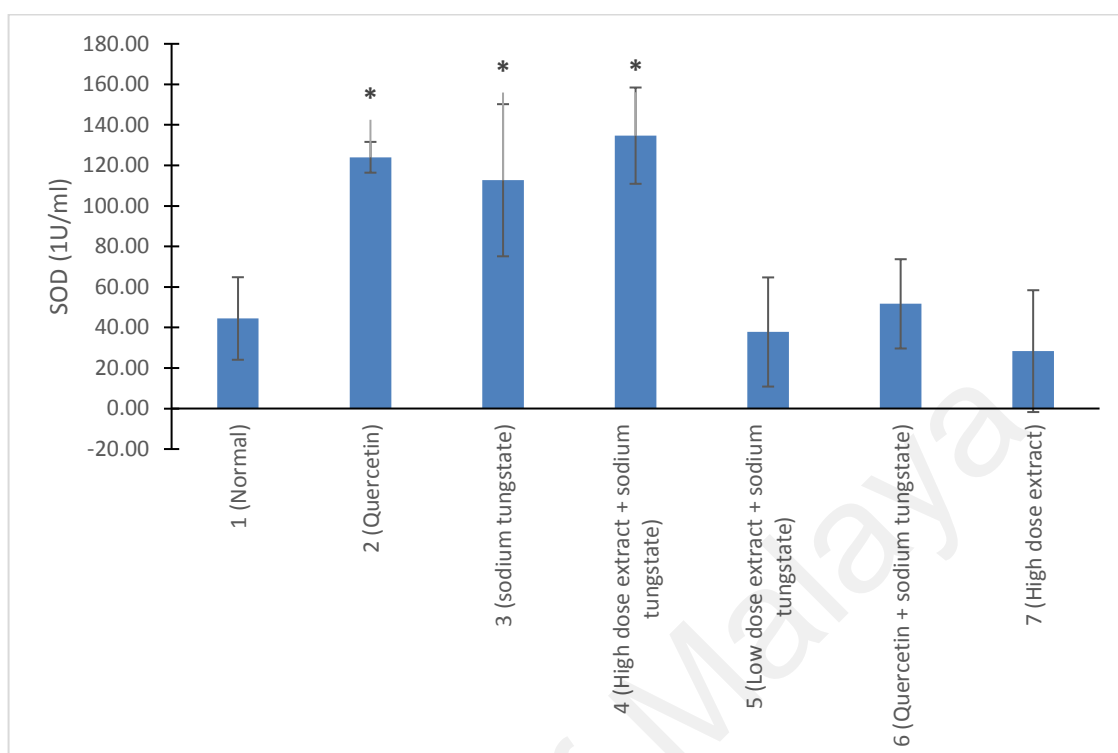


Figure 4.8: Serum Superoxide Dismutase (SOD) activity. * indicates significant difference when compared to Group 1 (Normal) (ANOVA $p < 0.05$). The error bars represent Standard Deviation (SD) values ($n=6$). SOD: Superoxide dismutase

Table 4.9: Serum Superoxide dismutase (SOD) activity in rats.

Group	SOD (IU/ml)
1 (Normal)	44.44 ± 20.37
2 (Quercetin)	124.00 ± 7.60 *
3 (Sodium tungstate)	112.67 ± 37.54*
4 (High dose extract + sodium tungstate)	134.67 ± 23.76 *
5 (Low dose extract + sodium tungstate)	37.78 ± 26.94
6 (Quercetin + sodium tungstate)	51.67 ± 22.03
7 (High dose extract)	28.33 ± 30.06

4.4.3 Serum Catalase (CAT) Activity

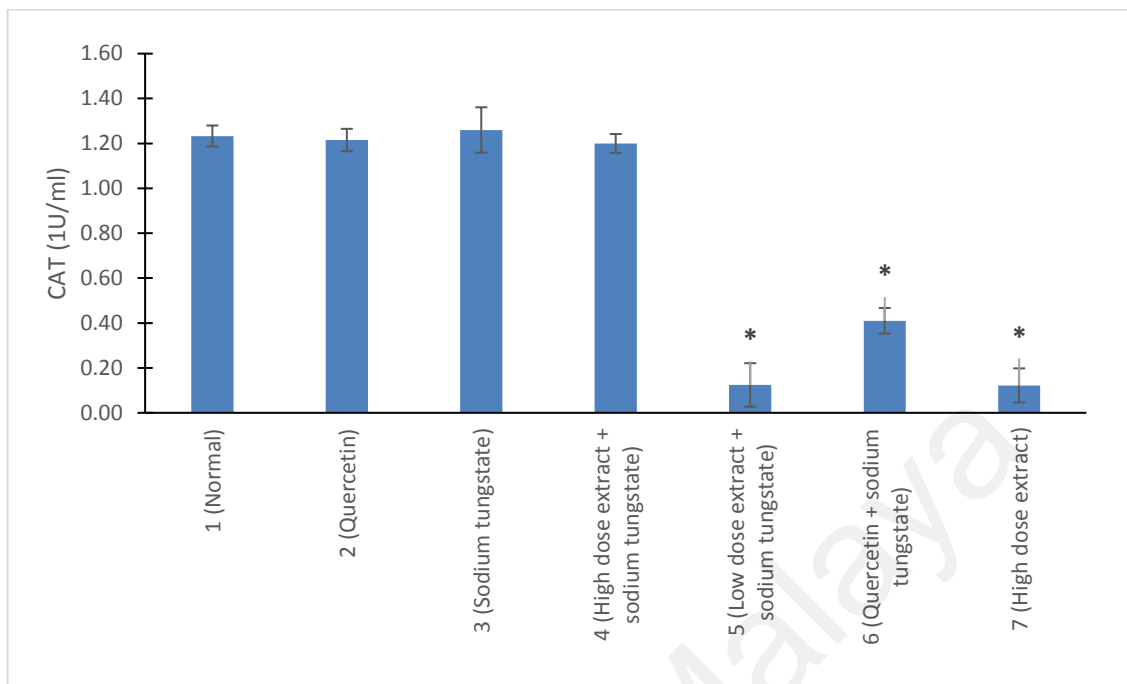


Figure 4.9: Serum Catalase (CAT) activity. * indicates significant difference when compared to Group 1 (Normal) (ANOVA $p < 0.05$). The error bars represent Standard Deviation (SD) values ($n=6$). CAT: Catalase.

Table 4.10: Serum catalase (CAT) activity in rats.

Group	CAT Activity (IU/ml)
1 (Normal)	1.23 ± 0.047
2 (Quercetin)	1.22 ± 0.050
3 (Sodium tungstate)	1.26 ± 0.10
4 (High dose extract + sodium tungstate)	1.20 ± 0.042
5 (Low dose extract + sodium tungstate)	0.12 ± 0.097 *
6 (Quercetin + sodium tungstate)	0.41 ± 0.057 *
7 (High dose extract)	0.12 ± 0.076 *

4.4.4 Serum Glutathione Peroxidase (GPx) Activity

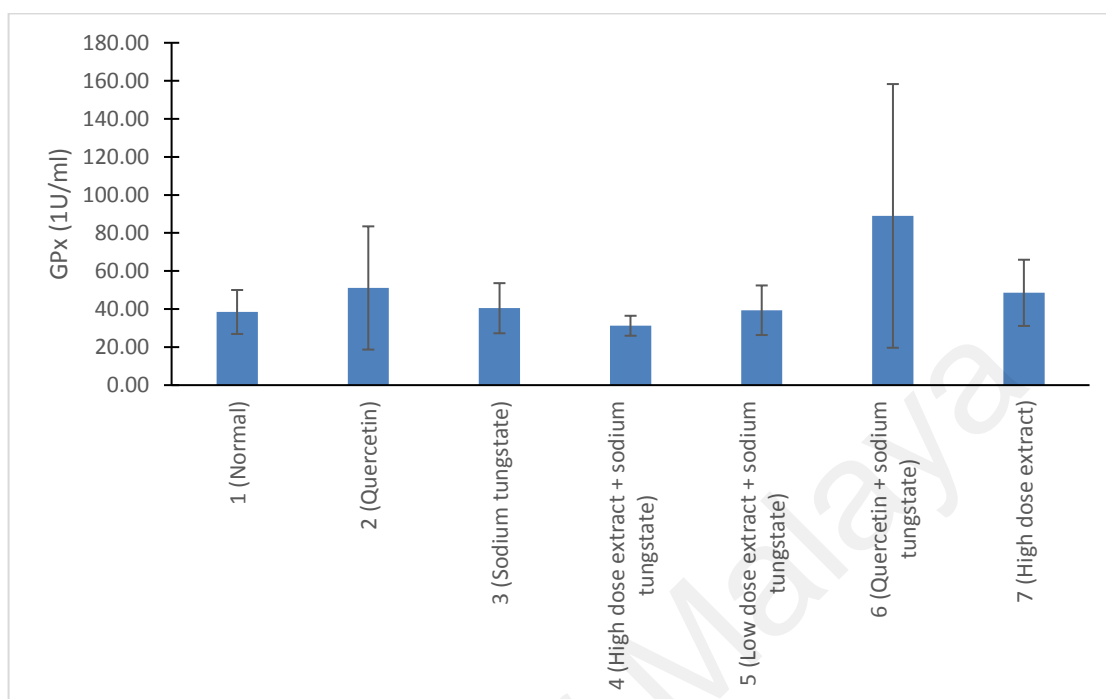


Figure 4.10: Serum Glutathione Peroxidase (GPx) activity. No significant difference was observed between the groups (ANOVA $p > 0.05$). The error bars represent Standard Deviation (SD) values ($n=6$). GPx: Glutathione Peroxidase.

Table 4.11: Serum Glutathione peroxidase (GPx) activity in rats.

Group	GPx Activity (1U/ml)
1 (Normal)	38.46 ± 11.6
2 (Quercetin)	51.09 ± 32.40
3 (Sodium tungstate)	40.45 ± 13.18
4 (High dose extract + sodium tungstate)	31.23 ± 5.25
5 (Low dose extract + sodium tungstate)	39.38 ± 13.04
6 (Quercetin + sodium tungstate)	88.97 ± 69.32
7 (High dose extract)	48.52 ± 17.41

4.4.5 Lipid Peroxidation (TBARS-MDA) Analysis

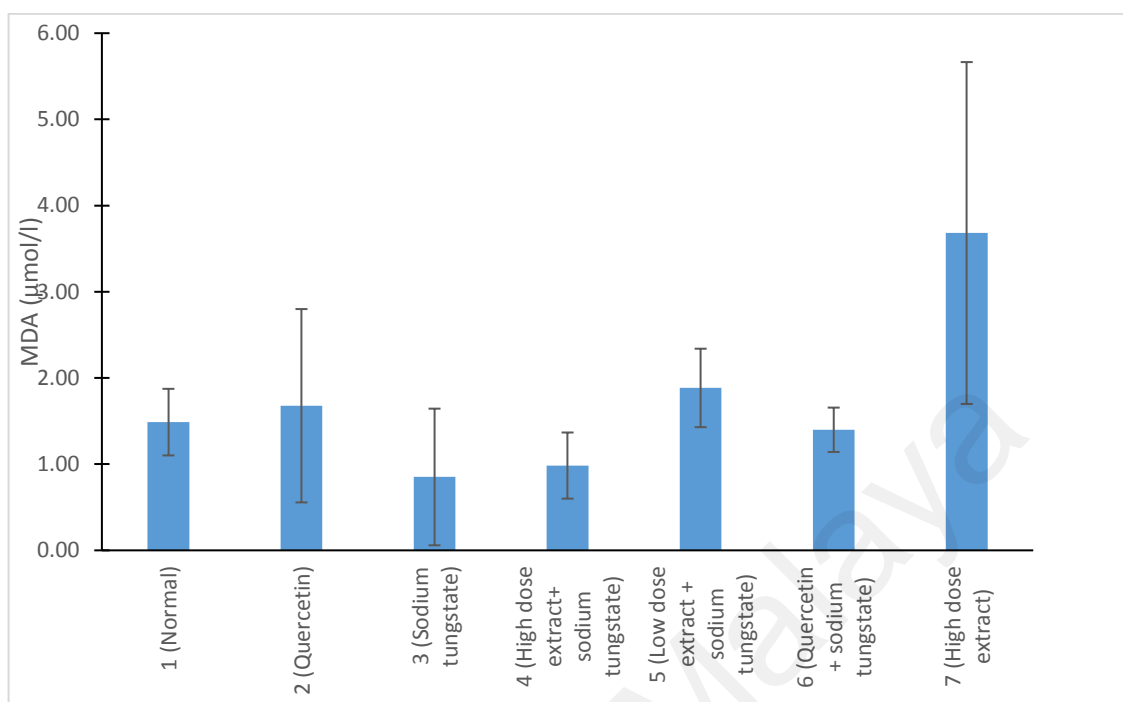


Figure 4.11: Thiobarbituric acid reactive substances (TBARS) Analysis. The error bars represent Standard Deviation (SD) values (n=6). The test was done in duplicate. Samples were tested in duplicate.

Table 4.12: Lipid peroxidation analysis based in MDA levels in rats.

Group	Average MDA Concentration (µmol/min/l)
1 (Normal)	1.49 ± 0.39
2 (Quercetin)	1.68 ± 1.12
3 (Sodium tungstate)	0.85 ± 0.79
4 (High dose extract + sodium tungstate)	0.98 ± 0.38
5 (Low dose extract + sodium tungstate)	1.88 ± 0.46
6 (Quercetin + sodium tungstate)	1.40 ± 0.26
7 (High dose extract)	3.68 ± 1.98

CHAPTER 5

DISCUSSION

5.1 Phytochemical Analysis using TLC

Table 4.1.1 illustrates the TLC analysis of *L. saribus* chloroform coat fraction separation using 10% methanol in chloroform. There were eighteen (18) bands identified. Based on the screening, a number of possible compounds were identified such as phenolic, terpenes and alkaloids under visible light and ultraviolet (UV) light. Overall, four (4) bands of terpenoid were identified at R_f 0.317, 0.350, 0.533 and 0.667. While ten (10) phenolic bands were identified when TLC plate was sprayed with anisaldehyde reagent. Terpenoid appeared to be colorless in visible light but changed to green, purple and brown when sprayed with sulfuric vanillin reagent. Terpenoid is the primary constituent of essential oil that is responsible for giving scent and are generally lipid soluble and are located in the cytoplasm of plant cell. For instance, D- limonene, one of the major components of citrus peel oil, belongs in the monoterpenoid group has demonstrated anti-carcinogenic effects in inhibiting mammary carcinomas in rats (Putignani et al., 2013). The rationale of using vanillin reaction is to detect the presence of terpenoids compounds, anisaldehyde reagent to detect phenolic compounds while the dragendorff reagent to detect alkaloids.

Table 4.1.2 shows the TLC analysis of *L. saribus* chloroform seed fraction separation using 10% methanol in chloroform. Six (6) bands were identified in which four of them were classified as phenolic compounds. One (1) alkaloid band was identified at R_f 0.833. Alkaloids contain one or more nitrogen atoms as part of a cyclic system. Well known alkaloids include morphine, quinine, ephedrine and nicotine. The visible green bands indicate chlorophyll, that is usually hydrophobic. Other than that, there were two (2) terpenoid bands at R_f 0.667 and 0.900 and four (4) phenolic bands at

R_f 0.583, 0.750, 0.767 and 0.833. In general, TLC screening of chloroform coat and seed revealed that chloroform coat had more phytochemicals than chloroform seed.

Table 4.1.3 demonstrates *L.saribus* *n*-hexane seed fraction using 10% methanol in chloroform. Six (6) bands were observed in which four of those exhibited as phenolic compounds. Two (2) terpenoid bands were observed at R_f 0.700 and 0.900; 1 (one) alkaloid band at R_f 0.833 and 4 (four) phenolic bands at R_f 0.550, 0.683, 0.733 and 0.833. TLC analysis was also done on other fractions with different combinations of mobile phases; however, no band / non discrete bands were observed.

5.2 LC-MS/MS Analysis

Two compounds were specifically detected from *L. saribus* seed water extract, 2(3,4 – Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid and oxooctadecanoic acid both at 8.38' and 7.99' retention time and molecular ion 311.1 m/z and 297.1 m/z molecular ion respectively as portrayed in Figure 4.2.1 and 4.2.2. This analysis reveals that *L. saribus* water seed extracts contained mainly phenolic acid class compounds. Meanwhile, the other five peaks, 4.97', 9.29', 9.56', 10.21' and 11.65' indicated presence of other separated but unidentified compounds.

2(3,4 – Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid is classified as carboxylic acid from the class of phenylpropanoids. At room temperature it is a white, crystalline solid with a sweet, floral odour. Due to this characteristic, 2(3,4 – dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid is widely used in many industrial application such as food and pharmaceuticals (Korneev, 2013). Meanwhile oxooctadecanoic acid is a type of stearic acid. *L. saribus* is a type of palm fruit, therefore stearic acid is detected in this fruit as many stearic acid also found in many palm fruits such as palm and coconut.

Both of the identified compounds are phenolic acids that are widely spread throughout the plant kingdom. They are responsible in signaling and structural roles, seed dispersal and as defense mechanism from stress such as wounding, infection and UV light (Costa et al., 2015). These compounds contribute to plants' color, stability, flavor and bitterness. Furthermore, polyphenolic compounds are well recognized for its antioxidant, anti-cancer, anti-inflammatory and anti-viral properties (Carocho & Ferreira, 2013).

5.3 *In vitro* Analysis

5.3.1 Total Phenolic Content (TPC)

Gallic acid (IUPAC : 3,4,5 Trihydroxybenzoic acid), a phenolic acid was used as the standard calibration to measure the phenolic content. The calibration of absorbance versus concentration had linear responses from three trials in which the correlation was $R^2 = 0.9499$ when measured at 725 nm wavelength. Based on figure 4.3.1, seed methanol fraction had the highest TPC of 173.73 ± 19.38 mg GAE/g dry sample, followed by seed water fraction with 163.10 ± 18.86 mg GAE/g dry sample as second highest TPC. While, seed and coat hexane fractions displayed the lowest TPC of 16.13 ± 12.76 mg GAE/g dry sample and 17.48 ± 10.92 mg GAE/g dry sample respectively. From the observation, water fraction of coat and seed had most apparent significant difference in phenolic content (ANOVA $p < 0.05$, $p = 0.0004$). While other fractions did not demonstrate significant difference in phenolic extraction in both coat and seed parts.

Essien et al. (2017), they reported that the total phenolic content of the *L. chinensis* palm nut extracted with macerated 95% methanol yielded 245.00 ± 0.02 mg GAE/g. This is due to the antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived

radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (termination of the Fenton reaction).

Based on a study conducted on antioxidant activity and profiles of common fruits in Singapore, the hexane seed fraction of *L. saribus* being the lowest TPC (16 mg GAE/dry sample) in this study content supercedes the TPC content of commonly consumed fruits including banana (2.17 mg GAE/g fresh weight (FW)), guava (1.75 mg GAE/g FW), red grape (2.94 mg GAE/FW), mangosteen (2.69 mg GAE/FW) and orange (1.57 mg GAE/FW).

Meanwhile, a Malaysian based study on antioxidant capacity and total phenolic content on *Malaysian* underutilized fruits revealed that *belimbing buloh*, *rambai sarawak*, *buah melaka*, *asssam kelubi*, *buah bidara*, *sentol tempatan* and *sentol bangkok* have more than 10 mg GAE/g edible portion of total phenolic content. Other listed fruits on the study such as *isau*, *durian tutong*, *assam gelugor*, and *bacang* demonstrated low content of phenolic content that is less than 10 mg GAE/g (Ikram et al., 2009). These fruits do not match *L.saribus*'s lowest phenolic fraction at 16 mg GAE/ dry sample. However, *Labisia pumila* (Kacip Fatimah) demonstrated high content of phenolic content at 274 mg GAE/g sample when extracted with 100% water (Chua et al., 2011). This amount supercedes the highest extracted phenolic content of *L. saribus* seed using methanol that is at 173 mg GAE/g sample. Kacip fatimah has been widely used in South East Asia to treat many kinds of illnesses and used as health supplements.

The Folin-Ciocalteu method possess limitations for estimating TPC since the total phenolic measurement may have contributed by non-phenolic antioxidants, aromatic amino amine (tyrosine and tryptophan) and reducing substances such as glucose, fructose, ascorbic acid and sulfites that are inherently present in fruits and vegetables (Medina, 2011). Also, studies have demonstrated that phenolic content varies

according to climatic changes, fertilizers, soil, water stress, harvest time, stage of growth of the fruit during harvest and harvest practices (Passo Tsamo et al., 2015; Routray & Orsat, 2014).

5.3.2 Total Flavonoid Content (TFC)

Quercetin (IUPAC: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) was used as the standard calibration to measure the flavonoid content. The calibration of absorbance versus concentration was linear from triplicates in which the correlation was $R^2 = 0.9846$ when measured at 510 nm wavelength. The TFC results for different fractions are shown in Figure 4.3.2. All fractions of *L. saribus* coat and seed demonstrated significant difference in TFC between fractions (ANOVA $p < 0.05$), except for the hexane fraction (ANOVA $p > 0.05$; $p = 0.091$). Seed methanol fraction had the highest TFC of 926.83 ± 7.64 mg QE/g dry sample, followed by coat methanol fraction at 768.50 ± 33.29 mg QE/g dry sample. While, coat hexane fraction and coat water fraction exhibited the lowest TFC at 133.50 ± 88.08 mg QE/g dry sample and 141.83 ± 30.41 mg QE/g dry sample respectively.

In comparison, *L. saribus*'s total flavonoid content (TFC) of both coat and seed superceded the flavonoid content of tropical pineapple, *Ananas comosus* L., Merr. methanolic extract. The numeration as such that *L. saribus* TFC coat is 519 mg QE/g and *L. saribus* TFC seed is 359 mg QE/g as to pineapple TFC at 55mg QE/g (Hossain & Rahman, 2011). Also, Lipoi, *Vitaceae tetrastigma* that is used by the local ethnic Murut in Sabah for treatment of menstrual disorders and asthma has low flavonoid content as compared to *L. saribus* extracts (Hossain et al., 2011). The methanolic extract of Lipoi has 72 mg QE/g as compared to high *L. saribus* flavonoid of both coat and seed methanol fraction as mentioned above. Not only that, bunga kantan, *Etlingera elatior* that is used in cooking and folklore medicine demonstrated low amount of flavonoid

content, especially in methanolic fraction, which is only at 8mg QE/g (Wijekoon et al., 2011). These findings suggest that *L. saribus* has concentrated flavonoid content compared to other medicinal plants, which could potentially be used as a potent dietary antioxidant supplement.

High consumption of fruits and vegetables is linked to protection against cardiac and neurodegenerative diseases, in which flavonoid compounds are responsible for such actions (Fraga & Oteiza, 2011; Williams & Spencer, 2012). A study on the effects of flavonoid antioxidant supplementation on gestation rats *in vivo* suggested that flavonoid compounds assist as preemptive trigger to antioxidant defense system that decreases oxidative stress induced DNA damage in rats liver (Vanhees et al., 2013). This proposes that *in utero* supplementation of flavonoid is beneficial in having a long-lasting effect of protection against oxidative stress induced diseases. It is proven time and again that dietary intake of antioxidant especially from fruits and vegetables tremendously help in maintaining overall health by means of combatting oxidative stress.

From the TPC results, it can be deduced that the distribution of phenolic and flavonoid compounds vary between parts (coat or seed) and types of fractions. Such is as expected based on observations that different parts exhibit different colors and structures; hence, they are differ in nutritional content.

5.3.3 DPPH Radical Scavenging

DPPH radical scavenging analysis was utilised to examine the antioxidant capability of *L. saribus* extracts to neutralize DPPH radicals. IC₅₀ values are used to express inhibitory action of the extracts in achieving halfway antioxidant response between baseline and maximum after a period of time. Low IC₅₀ values indicate high antioxidant capacity. Figure 4.3.3 summarizes the trends of antioxidant capacity in

scavenging DPPH· over increasing concentration of extracts and ascorbic acid standard (5 to 320 µg/mL).

Zeng et al. (2013) reported that the phenolics isolated from the roots of *L. chinensis* (2*R*,3*R*)-3,5,6,7,3',4'-hexahydroxyflavane, phenanthrene-2,4,9-triol, (-)-catechin, and naphthalen-2-ol yielded showed obvious free radical scavenging activity on DPPH with IC₅₀ values of 2.11 ± 0.18, 2.89 ± 0.28, 3.58 ± 0.18, 10.70 ± 1.18 µM, respectively.

From the result showed that at low concentration of 5 µg/ml, all the extracts had percentage of inhibition of 10% to 20%. As the concentration of extracts increase, the percentage of inhibition also increase. This was caused by the antioxidants scavenging the free radicals of DPPH. The more antioxidants content the more scavage reactions. At concentration of 80 µg/ml, the ascorbic acid graph has become plateau as all the DPPH radicals was consumed by the ascorbic acid. Methanol from seed extracts had almost the same IC₅₀ as ascorbic. This showed that methanol seed extract has had the highest content of antioxidants compared to other type of extracts from different solvent used. Methanol and water extract had the highest content of antioxidants because antioxidants are more readily dissolved in those solvents. Hexane extracts had the lowest IC₅₀ compared to other type of extracts because antioxidants hardly dissolved in petroleum-based solvent.

L-Ascorbic acid (IUPAC: (5*R*)-[(1*S*)-1,2-Dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one) was used as the reference for the evaluation inhibitory . It is important to note that the antioxidant capacity of seed methanol fraction matches the ascorbic acid standard based on their IC₅₀ values of 33.18 ± 4.17 µg/mL and 33.24 ± 4.57 µg/mL respectively. Meanwhile, IC₅₀ of seed water fraction was at 61.99 ± 12.15 µg/mL and coat methanol fraction at 123.30 ± 17.51 µg/mL. Other fractions did not achieve at least

50% antioxidant activity, hence no IC₅₀ values were assigned. Overall, the data suggests that the antioxidant capacity is as follows: seed methanol fraction > ascorbic acid > seed water fraction > coat methanol fraction.

Ascorbic acid is one of the most commonly used standards for DPPH assay among many others like butyrate hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA) and trolox. The antioxidant activities in plant extracts are associated with the presence of phytochemicals.

5.3.4 Ferric Reducing Antioxidant Power (FRAP)

Figure 4.3.4 summarizes the antioxidant capacity of *L. saribus* crude extracts to reduce Fe(III)(TPTZ)₂ into Fe(II)(TPTZ)₂ over increasing concentration of ferrous sulphate (FeSO₄) standard solution 0 - 1000 µg/mL, $r^2 = 0.9974$. Ferrous sulfate (IUPAC: Iron (II) sulfate). The ferric reducing capacity is significantly different in coat and seed of methanol fraction, ANOVA $p < 0.05$; $p = 1.08 \times 10^{-5}$ and water fraction $p = 7.09 \times 10^{-6}$. Seed methanol and seed water fractions had the highest reducing capacity at above 2.0 mol Fe²⁺/g, recorded at 2.98 ± 0.12 mol Fe²⁺/g and 2.84 ± 0.15 Fe²⁺/g respectively. While coat and seed *n*-hexane fractions demonstrated the lowest reducing capacity of 0.04 ± 0.003 mol Fe²⁺/g and 0.03 ± 0.008 Fe²⁺/g respectively. Since seed methanol and seed water extracts exhibited the highest phenolic content, it suggests the phenolic compounds might be responsible for high ferric reducing capacity and strong DPPH scavenging activity. However, methanol coat fraction ferric reducing capacity was not as high as compared to seed methanol and seed water fractions, which is at 1.04 ± 0.036 mol Fe²⁺/g, although the methanol coat fraction had the highest flavonoid content and DPPH scavenging activity. That suggests each fraction exhibit different mechanism of antioxidant capacity.

The Figure 4.3.4 showed higher FRAP activity in seed compared to coat. This indicated that more antioxidants and bioactive compound contained in seeds. The seed *L. saribus* is where the location of nutrients storage. Palm fruits usually have shell. *L. saribus* shell is called the seed coat or husk. Within this coat, there is a thin layer located beneath the shell which enveloped very tightly against the seed coat. The husk constitutes mostly from hard insoluble fiber. This coat contains less FRAP activity because it has less antioxidants or bioactive compounds.

5.3.5 Ferrous Ion Chelation

EDTA (IUPAC:2-(2 [Bis (carboxymethyl) amino] ethyl (carboxymethyl) amino) acetic acid) was used as the standard reference to measure ferrous chelating ability because of its strong metal chelating strength. Figure 4.3.5 illustrates the chelation abilities of *L. saribus* fractions. All fractions of coat and seed demonstrated significant difference between fractions within group. Data indicated that coat and seed of hexane fraction, coat chloroform fraction, and coat water fraction had lower effective concentration in comparison to standard EDTA's IC_{50} at $10.9 \pm 0.7 \mu\text{g/ml}$. Therefore, that suggests the phytochemicals in the those fractions exhibited powerful chelating ability despite low antioxidant activity in DPPH and FRAP.

Both coat and seed methanol fractions did not achieve 50% chelating activity, hence IC_{50} values were not assigned. The principle of this assay is ferrozine chelates with Fe^{2+} to form a red colored complex. The reaction is limited by in the presence of other chelating compounds that results in the decrease of Fe^{2+} -ferrozine red color. Therefore, the paler the color of resulting reaction, the higher is the chelating ability. Coat and seed in experimented fractions exhibited significant difference in ferrous ion chelation ability, ANOVA $p < 0.05$.

From the figure 4.3.5, at lower concentration of 5µg/ml, hexane coat had the highest chelation ability with 60%, this was followed by hexane seed, chlorofoam coat and water coat with chelating ability of almost 50%. Methanol seed and chlorofoam seed had chelation ability at the range of 40%. The lowest chelation ability were from chlorofoam coat and methanol seed with 17% chelation ability. At concentration of 40 µg/ml, the EDTA chelation ability was 100%, at this point all of the EDTA has been used to chelate the ferrous ions. As the concentration increases, chlorofoam coat, water coat, water seed and chlorofoam seed showed dose-dependent which indicated by increase of chelation ability with the increase of dose.

5.4 *In vivo* Antioxidant Analysis

5.4.1 Oral Acute Toxicity

300mg extract per body weight (300mg/kg) was set as experimental acute oral toxicity. It was determined according to the guidelines of the 423 OECD for Acute Oral Toxicity. The experimental rats showed no signs of toxicity such as writhing, gasping, response to touch, and decreased respiratory rate or for any lethality throughout the testing period.

5.4.2 Serum SOD Activity

SOD activity in blood serum was assessed in inhibiting the oxidation of epinephrine at alkaline pH. In this analysis, SOD reacts with the O_2^- during the oxidation of epinephrine to cause a slowdown in the adrenochrome formation and the amount that is formed. Throughout time, the adrenochrome formation disappears and brown, insoluble products in the reaction mixture.

Rats in group 4, exhibited the highest unit SOD activity at 134.67 ± 23.76 U SOD/ml when treated high dose extract (300 mg/kg) with sodium tungstate (50 mg/kg). While rats in group 7 demonstrated the lowest SOD activity at 28.33 ± 30.06 U

SOD/ml when treated with only high dose extract (300 mg/kg). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Group 2, 3 and 4 displayed significant difference in SOD unit activity than the normal group, group 2: 124.00 ± 7.60 SOD U/ml, group 3: 112.67 ± 37.54 SOD U/ml and group 4: 134.67 ± 23.76 SOD U/ml respectively as compared to normal group, 44.44 ± 20.37 SOD U/ml. (ANOVA $p > 0.05$, group 2 $p = 0.000178$; group 3 $p = 0.0125$; group 4 $p = 0.00048$).

Group 1, the normal group SOD activity was indicated at 40 (U/ml) range. This SOD value was a value of normal SOD activity without given any treatment of drugs of plant extracts. Group 4 expressed highest SOD activity compared to other groups. Increased SOD suggests that significant enhancement of endogenous enzymatic antioxidants by *L. saribus*. It was believed that synergistic expression of high dose of *L. saribus* extract and sodium tungstate would result in more SOD generated. Group 3 in which the rats were treated with only sodium tungstate (50 mg/kg) had one of the highest of SOD activities. Sodium tungstate was an effective medium to produce endogenous free radicals in liver rats. Sodium tungsten exert toxic effects to rats as reported by Sachdeva et al. (2013). The third highest SOD activity was represented by group 2. In this group, all the rats were given quercetin. Quercetin is a type of flavonoids. The result showed that there was an increased of SOD activity in group 2 compared with the normal group.

Group 5, 6 and 7 exhibited low SOD activities, which nearly match the normal group. These groups were treated with different dose of *L. saribus* extracts and chemicals. However, the SOD activities showed near similar responses to the normal group, Group 1 at (37.78 ± 26.94 for group 5), (51.67 ± 22.03 for group 6), and ($28.33 \pm$

30.06 for group 7) respectively. The low SOD activities in those groups represent low inhibition of epinephrine. The possible explanation to these results may be due to the inability of SOD to fully react to O_2^- formed during the reaction. In order for SOD to fully react with O_2^- , a condition of ideal pH is needed so that a maximum substrate can be generated *in situ*.

5.4.3 Serum Catalase Analysis

Catalases are the enzymes that prevent cell oxidative damage by degrading hydrogen peroxide into water and oxygen. In this assay, catalase activity was measured in serum samples against H_2O_2 standard curve 10 to 160 $\mu\text{mol/l}$. The higher the catalase activity, the lower is the H_2O_2 concentration, hence the lower is the fluorescent product.

Figure 4.4.3, shows that group 3 exhibited the highest catalase activity at 1.26 ± 0.10 IU/ml when treated with only sodium tungstate (50 mg/kg). While group 1, 2, and 4 demonstrated near similarity in catalase activity, that is at 1.2mmol/min/ml. On the other hand, group 5, 6 and 7 had significant difference in catalase activity as compared to the normal group. (ANOVA $p < 0.05$; Group 5: $p = 5.75 \times 10^{-8}$, Group 6: $p = 0.375$, Group 7: $p = 2.11 \times 10^{-8}$).

The presence of other H_2O_2 degrading enzyme such as glutathione peroxidase may also counter react with ROS which may have interfered this catalase analysis (Glorieux et al., 2015). In theory, a low H_2O_2 concentration in a system corresponds to a high catalase activity. The SOD activity analysis revealed that rats in group 3 exhibited amongst the highest epinephrine oxidation. This may be due to the decrease of glutathione peroxidase activity (Figure 4.4.4) that would reduce the erythrocyte ability to react with hydrogen peroxide and thus lead to an increase in CAT activity (Blum and Fridovich, 1985).

Group 4 or treated group with high dose of *L. saribus* and sodium tungstate resulted in increased catalase activity. *L. saribus* may detoxify hydrogen peroxide and convert lipid hydroperoxides to non-toxic substances. *L. saribus* exhibit significant antioxidant activity as detected in antioxidant assays. Antioxidants such as phenolic, alkaloids, flavonoids and terpenes found in *L. saribus* were able to express high level in group 4 by preventing oxidative injury resulting from high levels of ROS, which are able to oxidize all macromolecules within cell and impair metabolism.

Meanwhile group 1 or normal group has had equally high SOD activity even without any treatment. It was possible that increased CAT activity seen even in normal group could be due to higher production of hydrogen peroxides or interruption by many external and internal factors such as stress, inability of rats to adapt or illness or infection during period of this experiment conducted. This internal and external factors would produce Catalase as a counter reaction to those factors. The catalase would then protect SOD inactivation by hydrogen peroxide that led to increase in SOD activity as explained by Blum and Fridovich, 1985.

5.4.4 Serum Gluthathione Peroxidase (GPx) Activity

Gluthathione is an important endogenous antioxidant which is active in removing hydrogen peroxide (Yagi, 1987). The activity of endogenous glutathione peroxidase is regarded as the first line of defence against ROS and the decrease in their activity contribute to oxidative stress (Sekar et al., 2005). The current experiment was showed that the augmentation of glutathione peroxidase in vitro. In this study, group 6, rats treated with quercetin and sodium tungstate exhibited the highest glutathione peroxidase activity at 88.97 ± 69.32 IU/ml.

While group 4 (high dose extract and sodium tungstate), group 5 (low dose extract) and group 7 (high dose extract) demonstrated low GPx activity. As such the

glutathione peroxidase activities at 31.23 ± 5.25 IU/ml, 39.38 ± 13.04 IU/ml and 48.52 ± 17.41 IU/ml respectively. No significant difference between was observed amongst the groups when compared to Group 1, the normal group (ANOVA $p < 0.05$). Similar finding was also reported by a study, Sachdeva et al (2013) demonstrated that no significant alteration on GPx and catalase activities were observed in any of sodium tungstate exposed groups compared to the normal group.

Group 4 (rats supplemented with sodium tungstate) had the lowest glutathione peroxidase value at 31.23 ± 5.25 IU/ml. Its low activity may be due to alteration of modulation of glutathione peroxidase status. This alteration can be attributed to changing in enzymatic or non enzymatic process as reported by Schafer and Buettner, (2001) where oral administration of tungstate will inhibit glutathione peroxidase and oxidised glutathione (GSSG) ratio.

The results showed that antioxidant activity of *L. saribus* in rats were influenced by enzymatic and non-enzymatic factors that can be a key player in detoxification of hydrogen peroxides. Whilst the level of glutathione peroxidase is an indicator of stress and oxidative stress sensor, it is intracellular accumulation of individual ROS and reactive nitrogen species (e.g. nitric oxide, NO; peroxynitrite, ONOO⁻), ROS-producing enzymes, antioxidants and their oxidation/reduction states all contribute to the general redox homeostasis. This investigation tested the hypothesis that aqueous extract of *L. saribus* contains useful phytochemicals such as alkaloids, terpenoids and phenolics detected thin layer chromatography, LCMS and several antioxidants assays that could maintain intracellular glutathione levels by augmenting glutathione peroxidase.

5.4.5 Lipid Peroxidation – TBARS Analysis

The thiobarbituric acid reactive substances (TBARS) analysis measures malondialdehyde (MDA) concentration in the sample as well as MDA produced from

the unsaturated fatty acid peroxidation process in the hydrolytic conditions of the reaction. MDA forms an adduct with thiobarbituric acid and produces TBA-MDA complex that is measured by spectrophotometer or microplate reader. A decrease in lipid peroxidation index signifies a reduction in oxygen free radicals in physiological system. However MDA assay must not be confused with antioxidant assay because MDA assay gives an indication the degree of oxidative stress related to lipid peroxidation (i.e. quantity/degree of ROS being generated) and not the antioxidant activity.

The supplementation of high dose of *L.saribus* fruit extract to rats (group 7) had shown the highest MDA level of $3.68 \pm 1.98 \mu\text{mol/l}$ compared to the other groups. This group was supposed to have a low MDA level. The current study involved a measure of the capacity of *L.saribus* extract to inhibit lipid peroxidation induced *in vivo*. One of the cause might be a high proteins or amino acids in rats serum which interferes with the readings when detected at 532nm. This interference was explained by Hodges et al. (1999) where a relative high contents of sugars, phenols and proteins could interfere the mesure of oxidative stress at lipidic phase, giving higher MDA indexes. Another interruption might be the presence of aldehydes from contamination of samples. A minute presence of aldehydes can give false high MDA index as MDA is an aldehyde itself, thus effect the result of MDA-TBARS assay.

Second highest MDA level was group 5 (quercetin and sodium tungstate) at 1.88 ± 0.46 . While the third highest MDA index was group 1, normal group (fed with quercetin) at $1.49 \pm 0.39 \mu\text{mol/l}$. No significant difference was observed amongst the groups when compared to Group 1, the normal group (ANOVA $p < 0.05$). The lowest MDA index recorded was group 3 (sodium tungstate) at $0.85 \pm 0.79 \mu\text{mol/min/ml}$. The supplementation of sodium tungstate did not produce high MDA index. The lower

MDA index in group 3 may be due to higher antioxidant capacity of rats to counteract with ROS.

The variation of MDA index seen in group 1, 2, 3, 4, 5 and 6 were due to different enzymatic activity of SOD, catalase and glutathione peroxidase. These different enzymatic activities gave different expression of MDA index. Some previous studies have made several hypotheses that glycooxidation, nonenzymatic glycation of protein, shifts in redox balances toward polyol pathway, activation of protein kinase C, decreased tissue concentrations of low molecular weight antioxidants, and impaired activities of antioxidant enzymes were contributors to variations of free radicals in physiological system (Baynes & Thorpe, 1999; Brownlee, 2001).

CHAPTER 6

CONCLUSION

Livistona saribus fruit of different fractions exhibited distinct antioxidant capacity and mechanism. From this investigation, methanol seed fraction demonstrated the highest phenolic content and the most potent ferric reducing capacity and DPPH radical scavenging; however, it performed poorly in chelating ferrous ion. On the other hand, methanol coat fraction had the most abundant flavonoid content and demonstrated potent DPPH radical scavenging; but, it performed poorly in ferric reducing and chelating ferrous ion. Based on this observation, it can be deduced that the chemical constituents in coat and seed have different mechanistic pathways in neutralizing oxidants.

Water seed fraction was selected for *in vivo* study due to it had relatively high phenolic content, strong DPPH radical scavenging and ferric reducing ability. Moreover, phytochemical - water extracting approach is far *natural* to everyday life, that it can be done at home by boiling. Assessment of bioactive compound with thin layer chromatography showed presence of terpenoids, alkaloid, and phenolic contents. The results also in tandem with the assessment of LC-MS/MS. LC-MS/MS scan revealed that *L.saribus* water seed extract contained mainly phenolic acid class compounds which explains the high phenolic content from the TPC analysis.

The supplementation of 300 mg/kg water seed extract with induced oxidative stress rats (Group 4) was found to have heightened SOD activity and demonstrated lowest lipid peroxidation. This finding suggests that the dosage of 300 mg/kg every other day was able to confer protection against oxidation in lipid. However, the dosage caused increased formation of hydrogen peroxide, which indicated that the rats did experience physiologic oxidative stress. The data suggested that the inhibitory effect of

L.saribus water extract with regards to lipid peroxidation was related to phenolic compound in the extract.

An important concern pertaining to the study is that the duration of *in vivo* study (2 weeks) may have not permitted ample time for nutrient absorption in rats. Hence, the results presented here may not wholly portray the exact effect of treatment. Other than that, minor error in rat handling may have caused incomplete dose feeding when the rats were agitated. Future work may include the assessment of serum glutathione peroxidase to further validate the effect of hydrogen peroxide reduction into water in comparison to catalase, as they exhibit similar function. Also, effective dosage for consumption should be discerned through future studies to determine correct dosage and timeline for oxidative protection to take effect. A comparison between *L.saribus* and *L.chinensis* antioxidant capacity should also be assessed to gauge its effectiveness.

This study can be supplemented with other *in vitro* work by incorporating other oxidative stress assays like nitric oxide and beta-carotene. Furthermore, gene expression studies can be conducted to map out antioxidative pathways and functions. In conclusion *L. saribus* extracts have the capacity to combat oxidative stress may be beneficial to the development of new strategies for prevention of oxidative related illness

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