EVALUATION OF *IN VITRO* ANTIOXIDANT AND CARDIOPROTECTIVE PROPERTIES OF SELECTED PLANT METHANOLIC EXTRACTS FROM SARAWAK, MALAYSIA

by

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ABSTRACT

Plants contain secondary metabolites especially antioxidant phenolic compounds that form part of their natural defence against harmful UV radiations. Studies on the antioxidant contents in local plants are growing steadily in Malaysia. The rich biodiversity of Sarawak's rainforest offers potential antioxidant sources, with the majority remains `untouched' and undiscovered. Thus, the aim of this study was to analyse the antioxidant properties of selected local edible plants in Sarawak using various *in vitro* methods.

The total phenolic content was determined using the Folin-Ciocalteau method. It was found that the methanolic extract of local edible leaves - `simpoh air' (*Dillenia suffruticosa* (Griff.) Martelli), `local bay leaf' (*Eugenia polyantha* Barb. Rodr.), and `singkil' *(Premna cordifolia* Roxb.) and singkil laut *(Premna serratifolia L.)* showed the highest total phenolic contents (4 – 15 mg gallic acid equivalent/100 mg dry sample). Similarly, these plants also contain flavonoids (1 – 2 mg quercetin equivalent/100 mg dry sample) as determined using the aluminium complexation method. Antioxidant activities, as assessed using the DPPH, ABTS and CUPRAC methods consistently suggested the presence of strong antioxidants in *D. suffruticosa* and *E. polyantha (*b) extract samples. Extracts of both samples showed more than 50 % more antioxidant activities than the rest of the plant samples, recording an average of 40 mg/L of EC_{50} value in the DPPH assay; 67 mg Trolox equivalent/100 mg dry sample in the ABTS assay, and 140 μmol Trolox equivalent/100 mg dry sample. Of the three antioxidant assays, only the ABTS antioxidant activity value showed significant correlation ($p<0.05$, $r = 0.9557$) with the total phenolic content.

Rat myoblast H9C2 (2-1) cell line was used to mimic the cardiomyocytes or cardiac muscle cells. The cytotoxicity and potential cardioprotective effect of the extracts with the strongest antioxidant activities (*D. suffruticosa* and *E. polyantha*) *(b)* were tested on these cells. *D. suffruticosa* (extract was found to be ten times more toxic to the cells than the *Eugenia polyantha (b)* extract (LD_{50} is 60 mg/L and 649 mg/L, respectively). Working with concentrations below the LD_{50} (*D. suffruticosa*: 15 & 30 mg/L; *E. polyantha* (b) *a*: 60 & 120 mg/L), both extracts were found to be able to provide some level of protection against cell deaths in the presence of a free radical inducer, H_2O_2 at 250 and 500 μM. The protection was more pronounced in the *E. polyantha* (b) extract, restoring more than 70 % of cell viability when exposed to H_2O_2. It was concluded that all selected plants had acceptable antioxidant properties while *E. polyantha (b)* show prospect in cardio-protection at low concentrations.

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DECLARATION

I hereby declare that the thesis presented, contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome. To the best of my knowledge, the document does not contain material previously published or written by another person except where due reference is made in the text of this thesis.

(ISURIY ADASURIYA)

DATE: 12/3/2018

In my capacity as the Principal Coordinating Supervisor of the candidate's thesis, I hereby certify that the above statements are true to the best of my knowledge.

(IRINE RUNNIE ANAK HENRY GINJOM)

DATE: 12/3/2018

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LIST OF ABBREVIATIONS

3T3-F442A	Non-cancer Swiss mouse embryo fibroblast cells
ABTS	2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid
ATCC	American Type Culture Collection
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAT	Catalase
CCL-81	Kidney epithelial cells
CUPRAC	Cupric ion reducing antioxidant capacity
DE	Dry extract
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubucin
DPPH	2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl
DW	Dry Weight
EC_{50}	Half maximal effective concentration
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FCR	Folin-Ciocalteau reagent
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FW	Fresh weight
GR	Glutathione reductase
GSH-Px	Glutathione peroxidase
GST	Glutathione-S transferase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HeLa	Cervical adenocarcinoma cell line
HPLC	High Performance Liquid Chromatography
HT29	Human colon cancer cell line
LD_{50}	Half maximal lethal dose
LDH	Lactate dehydrogenase
LDH	Lactate dehydrogenase
MCF-7	Breast cancer cell line
MDA	Malondialdehyde
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium
NADPH	Nicotinamide adenine dinucleotide phosphate
NCDs	Non-communicable diseases
NFκB	Nuclear factor κB
NHMS	National Health & Morbidity Survey
O_2	Oxygen
O^{2-}	Superoxide anion

OH^-	Hydroxyl radical
PG	Propylgallate
r	Pearson's coefficient
RCS	Reactive chlorine species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TFC	Total flavonoid content
TPC	Total phenolic content
UV	Ultraviolet
WHO	World Health Organisation

1 INTRODUCTION

1.1 Background

Chronic diseases are considered the major cause of mortality and morbidity in the world. According to the World Health Organisation (WHO), without proper treatment and prevention, fatalities due to chronic diseases are estimated to increase up to 52 million by 2030 (World Health Organization 2014). WHO recorded that in 2002, chronic diseases contributed towards 71 % of all deaths in Malaysia. Out of these, about half are due to four major non-communicable diseases (NCDs) i.e. cardiovascular diseases (21 %), cancers (12 %), chronic respiratory diseases (6 %), and diabetes (3 %). In 2014, WHO projected that the probability of Malaysians dying between the ages 30 and 70 years from the four main NCDs is about 20 %, with the highest falls within the cardiovascular diseases category (~10 %) (World Health Organization 2014). Recent National Health & Morbidity Survey (NHMS) 2015 findings highlighted that about 50 % of Malaysian adults have hypercholesterolemia, 30 % have hypertension, and about 18 % have diabetes (Institute for Public Health (IPH) Malaysia 2015). Malaysians lifestyle appears to be unhealthy, as about half of Malaysian population above 18 years old are either overweight or obese. While more than 60 % of the population are considered physically active, only 6 % are consuming the recommended 5-6 servings of fruits and vegetables per day (Institute for Public Health (IPH) Malaysia 2015).

There are increasing evidence linking many chronic diseases, such as type 2 diabetes, hypertension, cardiovascular diseases, cancers and neurodegenerative diseases e.g. Parkinson and Alzheimer with the damaging effects of oxidative damage/stress to biological structural components such as DNA (Aguilar, Navarro & Pérez 2016; Aruoma 1998; Höhn et al. 2017). Our body is continuously being exposed to oxidative stress, both from normal endogenous processes (e.g. metabolic process, response to inflammation, physical and mental stress, etc.), as well as from the exogenous or environmental sources (e.g. air pollutants, foods, drugs, radiations, etc.). Fortunately, human bodies have natural coping mechanisms to neutralize the harmful effects caused by oxidative stress; one of which includes the possession of endogenous antioxidants such as glutathione, coenzyme-Q, ferritin, uric acid, bilirubin, catalase, superoxide dismutase (SOD), and glutathione peroxidase (Aguilar, Navarro & Pérez 2016; Höhn et al. 2017). Other than that, antioxidants can also be obtained from exogenous source i.e. from our diet. One such example is antioxidant vitamins i.e. vitamin A, C and E in foods (Dasgupta & Klein 2014). More recently, the presence and activity of lesser-known nutrients such as phytochemicals are getting increased attention due to their antioxidant properties (Roleira et al. 2015).

Healthy lifestyle habits such as good dietary practice and plenty of appropriate physical activities tend to maintain the good balance of oxidative stress and antioxidant levels. Unhealthy lifestyle, increased exposure to pollutants and/or radiation, and diseased condition could skew the balance unfavourably towards oxidative stress. This should be avoided or remedied. Failure to do so would lead to increased cell damages and in turn, increased risk of diabetes, hypertension, and Parkinson (Aruoma 1998). One popular approach is to supplement the antioxidant through food sources or dietary supplements (Pham-Huy, He & Pham-Huy 2008). Due to public awareness and education, healthy lifestyles which incorporate consumption of more fruits and vegetables are now trending among many countries including Malaysia. Thus it has the potential to create a high market value for plant based food products. This also increases the demand for reliable and well-defined research on plant based food products and their health-boosting effects.

Many fruits and vegetables are utilized for the production of functional foods worldwide. However, the numerous health claims for products derived from unpopular food sources can be misleading to consumers as they are based upon traditional practices passed upon orally from one generation to another (Wong, Leong & Williamkoh 2006). It is not recommended to consume such products especially, if the identity of active compounds, dosages and mechanisms are not specified or backed up with scientific studies. In the race to increase their antioxidant intake, consumers may instead introduce `anti-oxidative stress' to their body, a condition where there is an overabundance of available antioxidants. Again, this subjects the body to a skewed oxidative stress-antioxidant balance. Other potential ill-effect of overconsumption of such supplement is the potential toxic effects on the liver or other organs. Therefore, it is vital to validate such health claims in a scientifically accurate manner which is accepted internationally (Clydesdale 1997; Lau et al. 2012).

1.2 Problem statement

Malaysians have prioritized their food choices based on health, natural content, weight control and convenience. With the increase in population size and presence of middle and upper income group families and individuals, the demand for a wide variety of processed food and drinks have increased among communities (Lau 2014; Prescott et al. 2002). Although a positive drive towards healthy living is seen, setbacks such as price inflation of imported in-demand processed products, weak value of currency (Ringgit) and spike in diseases such as cardiovascular disease, cancer and diabetes still exist. Therefore, it would be favourable if locally sourced foods are abundantly made available in both urban and rural markets. Despite being the home of millions of underutilized edible plant species, research on their potentials as source of nutraceutical that provide protection against diseases in general is scarce in Sarawak.

Apart from being consumed as typical vegetable for basic sustenance in small communities, some of the edible plants also include additional health claims based on traditional knowledge. Thus, this study aims to add the scientific value to traditional medicinal claims and bring about awareness on their cardioprotective potential, allowing them to be recognised as a valuable crop.

1.3 Rationale

Free radicals along with antioxidants exist in endogenous forms in both plant and animal systems. Plant defence systems are naturally equipped with a variety of powerful antioxidants which help them to fight against oxidative-stress related diseases and infection by scavenging or neutralizing free radicals and reactive species. Since human endogenous antioxidant activity declines with age, plants make an ideal extra source of external antioxidants for the build-up of defence systems in the body against oxidative stress related diseases. Hence, in this study the following is hypothesized:

1) All plants have high phenolic content.
2) There is a strong correlation between phenolic content and antioxidant activities.
3) Plants with high antioxidant activities can protect H9c2 (2-1) mammalian cells against free-radical-induced damages.

1.4 Research aims and objectives

This research aims to screen selected local edible leafy plants for antioxidant activities. To achieve this, the following research objectives were carried out:

(i) Extraction of phenolic antioxidants from 10 local edible plants.

(ii) Determination of the total phenolic and flavonoid contents of plant extracts using the Folin-Ciocalteau method and aluminium-complexation reaction assay respectively.

(iii) Determination of antioxidant activity of selected plant samples using *in vitro* antioxidant assays - i.e. DPPH, ABTS and CUPRAC.

(iv) Determination of the cytotoxicity and antioxidant protective effects of selected plants extracts against H_2O_2-induced oxidative damage on H9c2 (2-1) rat cardiomyocyte cell culture model.

The results of this study expect to find positive correlation between the polyphenolic content and the antioxidant activities obtained using the leaf extracts. The assays which were used to measure antioxidant activities help to take into account for both hydrophilic and lipophilic antioxidants. This offers a better physiological-relevant perspective on their contribution against free radicals. The data obtained during this study would also help to reveal the health promoting effects of underutilized plant leaf based food as most of the plant leaves mentioned have not been subjected

to broad quantitative *in vitro* methods such as cell culture based assays. Findings from this study could help to encourage Malaysians to fulfil their recommended fruits and vegetables servings by consuming local plant produce.

1.5 Thesis outline

This thesis comprised of the following chapters.

Chapter 1 provides a brief overview of the study. It presents the background of the work and the rationale behind the aim and objectives of the study.

Chapter 2 provides a summary of previous research related to the role of free radicals in the aetiology of certain types of diseases, especially cardiovascular diseases; therapeutic role of antioxidants, especially plant phenolic compounds; and relevant and common methods of assessing the antioxidant activities of the plants substances of interest.

Chapter 3 outlines the procedures involved in assessing the antioxidant activity of selected plant samples, beginning with the collection of the plant samples, treatment involved in the preparation of the methanolic extracts, determination of the phenolic contents, assessment of antioxidant activity using several spectrophotometric methods, and assessment of the plant extracts' ability to protect cardiac cell model from free radical (induced by H_2O_2) toxicity and the subsequent cell death.

Chapter 4 presents the results and discussion of this study. The phenolic contents and antioxidant activities of the plant samples will be presented and compared with each other and the values reported previously in the literature. A correlation analysis will be conducted to assess the potential association between the different phenolic content and antioxidant activities. Finally, the cardio-protective effect of the plant sample with the most antioxidant activity will be presented based on the H9c2 (2-1) cell culture assay.

Chapter 5 concludes the thesis with a summary of the results obtained and presented, contribution of this work to the body of knowledge in the field of antioxidant therapy, followed by the recommendation for future work.

Chapter 6 list the references cited in this thesis.

1.6 Significance of the study

Plants have been the source for healing purposes in indigenous groups since ancient times due to their abundancy and renewable nature. The traditional uses and safety of such medicinal plants have been passed down many generations mainly via trial and error, which nowadays would be classified as a low throughput screening. Even in the present day, the demand for plants and herbs as food sources or traditional medicine is as high as that for chemically developed drugs due to globalization. This demand raises the need for agronomically favourable, affordable and sustainable plants that could be grown locally (Cordell 2014). While cultural preservation of orally shared nutritive and medicinal information on traditionally used herbal plants, it is of utmost importance that such knowledge is optimized and tested via evidence based scientific approach. Such documentation allows understanding the nature of pharmacologically active compounds such as antioxidants from the plants, responsible for anti-inflammatory, anti-cancer, anti-diabetic and anti-microbial effects. While these studies could contribute to natural drug discovery, it could also be incorporated into primary health care in the form of a food source (Baghel & Thakar 2010).

Therefore, the outcomes of this study would hopefully signify the importance of locally resourced plants for their potential medicinal purpose against cardiovascular disease raised due to oxidative stress, thereby paving path to conservation and cultivation of those plants into economically valuable crops.

1.7 Limitations of the study

The current thesis initially explored the total phenolic contents of ten underutilized leafy plants in Sarawak as plants are well documented to be rich in phenolic compounds such as phenolic acids and flavonoids. Five of these plants with high TPC and TFC, were subjected to *in vitro* colourimetric assays to measure their radical scavenging ability (DPPH) and antioxidant activities in two methods (ABTS, CUPRAC). Two of the five plants, those with the best overall antioxidant activities, were further analysed on their potential toxicity on healthy mammalian cardiac cells, followed by their cardioprotective nature on oxidatively stressed cardiac cells. While these methods and aims had helped to recognize the potential cardioprotective capability of antioxidants from plant extracts, several limitations must be addressed.

First, any inferences in TPC or subsequent antioxidant activities due to individual environmental factors such as soil type, rainfall and available sunlight were not made as the samples were only collected twice from a single location (per plant) and processed immediately. However, such data,

along with the assessment of various parts of the plants could have given a better perspective on the plant structure as well as geological effect on antioxidant contents.

This study used aqueous alcohol (ethanol or methanol) as the main solvent to extract the phenolic compounds. Both solvents can dissolve both polar and non-polar substances, making them the best solvent for extraction in this study. Non-polar solvent such as hexane or chloroform were not used in this study as the leaf samples were expected to contain only small amount of non-polar or oil substance. If any non-polar was present, it would have been extracted by the alcohol, regardless. This work also did not involve any fractionation following the crude alcoholic extraction. As this work was still at its preliminary stage, it makes more sense to screen the crude extract that contains all the bioactive compounds at once. Furthermore, all of these plants are taken or consumed whole (not extract or supplements), thus, the crude extract represents the best form of test material.

More solid quantitative analysis on the phenolic composition using chromatography methods (e.g. High Performance Liquid Chromatography, HPLC) would have provided a better inference to be made on the specific compound or group of phenolic compounds responsible for the measured activities. This would have involved the use of multiple reference standards, which was rather costly at this early stage of the study.

The coupling of intracellular enzyme detection with the MTS cell viability assay was not carried out in this thesis, due to the slow growth rate of the cells upon revival from cryopreservation and risk of contamination. Solid inferences could have been made on cardioprotective effects with the aid of such assays.

Although, the above limitations may be identified as setbacks, they could be incorporated as part of the further work for the current thesis.

2 LITERATURE REVIEW

2.1 Introduction

Plants are rich in phenolic compounds, which protect them from pests and diseases, as well from the damaging effect of UV rays from the sun. Since the sun is directly over the equator, UV rays will only travel short distance through the atmosphere to reach these areas. As a result, the UV radiation in the equatorial regions, including Sarawak, Malaysia is typically high (UV index >8) (World Health Organization 2002). Thus, the living organisms living in this area should have a natural defence system that protects them from these harmful levels of UV radiation. For plants, it is suggested that their phenolic compounds are those that mainly responsible for the protective effects, typically through their antioxidant properties (Dehariya et al. 2011).

The following literature review will define some of the basic terms that are used in this study, especially those related to the role of free radicals in cardiovascular disease progression, the therapeutical properties of antioxidants, especially those of plant origin, and the common laboratory methods that can be used to assess the plant's antioxidant capacities. The review will look into these areas and discuss the current understandings so far are and what aspects are still lacking. Finally, how this project can contribute towards filling the gaps in knowledge in this field will also be discussed.

2.2 Free radicals and diseases

A free radical is a molecule where the molecule's outer shell consists of an unpaired electron. They are generated as by products from redox reactions, breakage of chemical bonds and cleavage of an existing radical (Pham-Huy, He & Pham-Huy 2008).

Free radicals are found in the human body due to both endogenous and exogenous activities. Endogenous sources include enzymatic and non-enzymatic reactions of metabolic processes such as electron transport chain and inactivity of intracellular enzymatic reactions. These processes involve the free radical production of Reactive Oxygen Species (ROS) (Table 1), Reactive Nitrogen Species (RNS) and Reactive Chlorine Species (RCS). From these categories, ROS are known to be the most commonly occurring type of radicals. At low levels, these free radicals are important for the regulation of intracellular activities and host defence mechanisms (Aruoma 1998; Mittler 2002; Valko et al. 2007). Both ROS and RNS are released by phagocytes to destroy foreign pathogenic microbes associated with disease and infection. They are also released by non-phagocytic NADPH oxidase isoforms, for the regulation of intracellular signaling cascades in cells such as endothelial cells, cardiac myocytes, fibroblasts and vascular smooth muscles to help

in their functions such as blood flow modulation, and neural activity (Pham-Huy, He & Pham-Huy 2008). Exogenous activities such as consumption of meals prepared by rancid oil, smoking, drinking alcohol, drugs and exposure to heavy metals, air pollution, industrial chemicals such as Fenton reagent and radiation can trigger the formation of free radicals (Young, Tsao & Mine 2011).

Table 1. Sources of intracellular reactive oxygen species (ROS)

Reactive Oxygen Species	Intracellular source of generation
Superoxide anion radical ($O_2^{\bullet -}$)	Reactions involving NADH/NADPH oxidase, xanthine oxidase, peroxidases in mitochondrial electron transport chain.
Hydroxyl radical (OH•)	Reaction of $O_2^{\bullet -}$ with H_2O_2 in the presence of Fe^{2+} or Cu^{+}
Hypochlorous acid (HOCl)	Oxidation of chloride ions in the presence of H_2O_2 by myeloperoxidase enzyme.
Hydrogen peroxide (H_2O_2)	Oxidation of hypoxanthine to xanthine and xanthine to uric acid by xanthine oxidase, monoamine oxidases in mitochondrial outer membrane.
Peroxyl radical (ROO•)	Catalysis of unsaturated fatty acids in the presence of lipoxygenase

Adapted from (Chamulitrat et al. 1991; Pham-Huy, He & Pham-Huy 2008).

The presence of free radicals in our body is highly regulated. For example, ROS and RNS generated from resting and contracting skeletal muscles help to maintain the norm and contractility of the muscles. However, when the ROS is generated in excess, this will result in muscle weakness and fatigue tied to contractile dysfunction (Rahal et al. 2014). The state when the level of free radicals exceeds the threshold that the body can no longer neutralize or remove is generally known as `oxidative stress'. The role of oxidative stress in the etiology of several lifestyle-related diseases such as pulmonary, cardiovascular, autoimmune and inflammatory diseases such as rheumatoid arthritis, cancer, diabetes and neurodegenerative diseases are well documented (Aruoma 1998; Dillard & German 2000; Duthie et al. 1996; Fearon & Faux 2009; Pham-Huy, He & Pham-Huy 2008; Valko et al. 2007).

The key reason is that cells exposed to prolonged oxidative stress can lead to extensive damage to their structural components (DNA, protein and lipid) of organs in the body. In the state of oxidative stress, the radicals add double bonds to DNA bases and abstract a hydrogen atom, causing the DNA to undergo deletion, and cleavage. The same applies to mitochondrial DNA; all

of which leads to change in functionaity of both enzymatic and structural proteins and transcription factors. These changes modifies the DNA binding activities, and thereby altering gene expression, all of which, is implicated in mutagenesis, carcinogenesis, and aging (Cooke et al. 2003; Rahal et al. 2014).

Lipid peroxidation of polyunsaturated fatty acids is one of the most common form of damage initiated by excessive free radicals. End products such as malondialdehyde (MDA) and F2-isoprostanes are generated as a result of chain reaction propogation of the lipid peroxidation process (Rahal et al. 2014). Neurodegenerative diseases are some of the diseases where lipid peroxidation plays a major role. F2-isoprostanes, the lipid peroxidation product, is in fact, used as one of the major biomarkers for the detection of multiple sclerosis, Alzheimer's disease, Huntington's disease, and Creutzfeldt-Jakob diseases (Milatovic, Zaja-Milatovic & Gupta 2014; Mittler 2002).

Oxidative stress can be generated due to genetic, diet and health routine issues (Csányi & Miller 2014). However, they can also be generated as a side effect of anti-cancer anthracycline drugs such as danorubucin, doxorubicin and epirubucin. This is obtained first by the generation of quinone-semiquinone cycle production as a result of DOX transformation to semiquinone. Followed by the activation of NADP(H) oxidases, interference of non-enzymatic reactions in the presence of iron and metabolism of DOX (Granados-Principal et al. 2010).

2.3 Free radicals and cardiovascular diseases

Cardiovascular disease is a leading cause of natural death in the world. As described earlier in Section 2.1, oxidative stress is considered to play a crucial role in bringing about toxicity leading to cardiovascular diseases such as cardiovascular ischemia, heart failure, atherosclerosis and hypertension (Bhattacharya, Ahmed & Chakraborty 2011) .

Cardiovascular organ system is made up of vascular systems such as endothelial, smooth muscle (vascular) and adventitial cells. ROS can be produced in these cells from the NADPH oxidase activity (Table 1). Elevated levels of these ROS are associated with hypertension. As a result, high levels of by-products from lipid peroxidation are observed. It also weakens endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Furthermore, it is also known to alter membrane fluidity of the mitochondrial lipid bilayer via lipid peroxidation and cause functional disabilities in surrounding membrane proteins and receptors (Mizutani et al. 2005; Xu et al. 2001). This leads to a loss of energy production and oxidation of DNA in mitochondria leading to its dysfunction (Oliveira & Wallace 2006).

The role of free radical in the pathology of cardiomyocytes cell damage is also very significant during the reperfusion process. Reperfusion is the restoration of blood flow to the cells or tissue following an event of ischaemia or restriction of blood flow to the tissue. The process of reperfusion reintroduces oxygenated blood flow, which in turn promote ROS generation. The ROS can induce cellular damage via localized inflammation, and compromise plaque stability via activation of matrix and vascular remodelling enzymes such as metalloproteinases (Chen et al. 2000).

It is further responsible for the formation of atherosclerotic lesions in cardiomyocytes. The formation of nuclear transcription factors (NFκB) is also a sign of oxidative stress due to cardiovascular ischemia. Due to the cardiotoxicity brought upon by oxidative stress, research has been carried out on the cardio-protective effects from food sources (Dludla et al. 2014; Hosseinzadeh et al. 2011; Kumral et al. 2015; Tan et al. 2016; Valko et al. 2007) , all of which show promising results.

2.4 Free radicals in plants

Green leafy plants contain more oxygen compared to animals. This coupled with abiotic stress creates a highly hypoxic environment for the plant (Das et al. 2015). Hence, similar to animals, plants generate free radical species such as NADPH oxidases, amine oxidases and cell wall bound peroxidases during metabolic processes such as photosynthesis and aerobic respiration. Such free radical species strictly regulated and have been identified as essential for pathogen defence, along with programmed cell death as they help to activate signal transduction pathways against abiotic stress conditions such as UV-radiation, heat, salinity, nutrient deprivation and infection (Mittler 2002). While these free radicals are important for maintenance of plant health, over accumulation can cause damage to plant cells. Therefore, plants contain naturally occurring antioxidants to control the adverse effects of free radicals.

2.5 Antioxidants

Antioxidants play an important role in regulating the balance in reactive oxygen and other free radical species. The antioxidants can stabilize the radical, disintegrate the radical into an non-harmful product, or reduce the rate of chain initiation (Pham-Huy, He & Pham-Huy 2008). All of this is achieved by the antioxidants as they are able to: 1) scavenge ROS/RNS that initiate peroxidation, 2) prevent generation of reactive species and decomposition of lipid peroxides by chelating metal ions, 3) quench superoxide radical to prevent formation of peroxide, 4) break the auto-oxidative chain reaction and 5) reduce localized oxygen concentration (Brewer 2011).

Endogenous antioxidants are the first line of defence against oxidative stress. These include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR), alpha-lipoic acid, coenzyme-Q, and uric acid (Aguilar, Navarro & Pérez 2016; Höhn et al. 2017; Pham-Huy, He & Pham-Huy 2008; Valko et al. 2007) . On top of that, our body also obtain its antioxidant supply from exogenous sources, especially from our diet.

2.5.1 Mechanism of plant antioxidants

Plants are naturally equipped with molecular oxidative protection including molecules such as flavonoids and carotenoid. These molecules are generated as secondary metabolites within the plant system as a response to situations such as abiotic stress. As an example, flavonoids play a vital role in protecting plants from UV-B radiation damage. They act as a UV filter by absorbing light in the UV region (280-320 nm), preventing photosynthetic damage to tissues by stabilizing and protecting the lipid phase of the thylakoid membrane (Ravindran et al. 2010). Flavonoids also act as quenchers for chlorophyll (in its excited triple state). Plants have adapted two major pathways for the scavenging of ROS. First, is the use of scavenging enzymes such as catalase, superoxide dismutase, dehydroascorbate, glutathione peroxidase and phenylalanine ammonia lyase. Second, is the use of molecules such as ascorbic acid, α-tocopherols, glutathione and proline (Mittler 2002). Furthermore, plants also contain metal binding proteins which help to sequester free copper and iron in the plant cells. Both of the metals are well-known for their ability to catalyse oxidative reaction. Metal binding proteins include albumin, ferritin and lactoferrin (Kumar 2014). Shown below in Table 2 are naturally occurring antioxidant compounds in plants and their mode of actions.

Table 2. List of antioxidant compounds in well-known herbs, fruits and vegetables and their modes of action.

Herb	Scientific name	Antioxidant compounds	Mode of action
Rosemary	*Rosmarinus officinalis* L.	Carnosol, carnosic acid, rosmanol, rosmadial, diterpenes (epirosmanol, isorosmanol, rosmaridiphenol, rosmariquinone, rosmarinic acid)	Superoxide radical scavenger, lipid antioxidant and metal chelator
Sage	*Salvia officinalis L.*	Carnosol, carnosic acid, rosmanol, rosmadial, methyl and ethyl esters of carnosol, rosmarinic acid	Free radical scavenger
Oregano	*Origanum vulgare* L.	Rosmarinic acid, caffeic acid, protocatechuic acid, 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy] phenylpropionic acid, flavonoids (apigen, eriodictyol, dihydroquercetin, dihydrokaempherol), cavacrol, tymol	Free radical scavenger
Thyme	*Thymus vulgaris* L.	Thymol, cavacrol, ρ-cumene-2,3-diol, gallic acid, caffeic acid, rosmarinic acid, phenolic diterpenes, flavonoids	Free radical scavenger
Marjoram	*Majorana hortensis* Moench	Beta-carotene, beta-sitosterol, caffeic-acid, carvacrol, eugenol,hydroquinone, linalool-acetate plant 3–17, myrcene, rosmarinic-acid, terpinen-4-ol	Free radical scavenger
Grapes	*Vitis vinifera* L.	Caffeic acid, tyrosol acid, vanillic acid, myricetin,ρ-coumaric acid, ferulic acid, gallic acid, ellagic acid, quercetin,catechin, (-)-epicatechin	Free radical scavenger, hydroxylation of monophenols
Blackberry leaves	*Rubus fruticosus* L. ex Dierb.	Chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, gallic acid, rutin, ellagic acid, quercetin-3-d-glucoside,(+)-catechin, (-)-epicatechin, epicatechingallate and procyanidin B1	Free radical scavenger, lipid antioxidant
Kale	*Brassica oleracea* var. *Ssabellica* L.	Quercetin-3-O-sophoroside-7-O-D-glucoside, kaempferol-3-O-sophoroside-7-O-D-glucoside, isorhamnetin, 3-caffeoylquinic acid	ROS scavenger
Green Tea	*Camellia sinensis* (L.) Kuntze	Gallic acid, caffeic acid, (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG)	Free radical scavenger, inducing antioxidant enzymes

Adapted from (Aybastıer et al. 2013; Burin et al. 2014; Çelik & Gökmen 2014; Embuscado 2015; Fiol et al. 2012; Gramza-Michałowska et al. 2016; Hagen et al. 2009; Huang et al. 2012; Riebel et al. 2017).

2.5.2 Dietary antioxidants

Ageing of a body causes a reduction in the cellular antioxidant potential and plasma resulting in poor activity of intracellular enzymes (Young, Tsao & Mine 2011). Hence it is important to obtain antioxidants of exogenous means. Natural food and beverages are excellent sources of antioxidants in comparison to processed foods. Fruits, vegetables, grains and herbs contain various antioxidants which are also referred to as phytochemicals (Dimitrios 2006). The most common natural antioxidants are the phenolic compounds (Brewer 2011; Kwon et al. 2000; Lady 2015; Young, Tsao & Mine 2011).

Natural antioxidants are usually preferred over synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which are associated with carcinogenic effects (Agrawal, Kulkarni & Sharma 2011). Natural antioxidants are synthesized by plants during shikimate, 2-C-methyl-D-erythritol 4-phosphate (MEP) and acetate-malonate pathways as secondary metabolites, to withstand abiotic stress (Della Penna & Pogson 2006; Rice-Evans, Miller & Paganga 1997).

2.5.2.1 Phenolic compounds

Phenolic compounds are compounds that have one or more hydroxyl groups attached directly to an aromatic ring. The entire group is in fact, based on phenol structure (Figure 1). When there are more than one phenolic hydroxyl group is attached to one or more benzene rings, then the compound is known as polyphenols. Phenolic compounds are considered among the best antioxidants in nature due to their ability to readily donate H atoms to free radicals from their hydroxyl group.

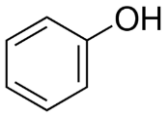
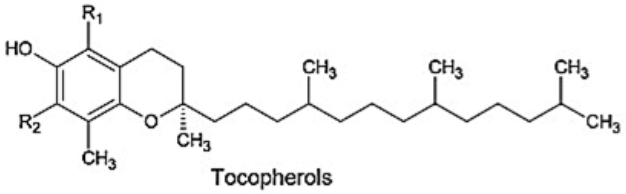
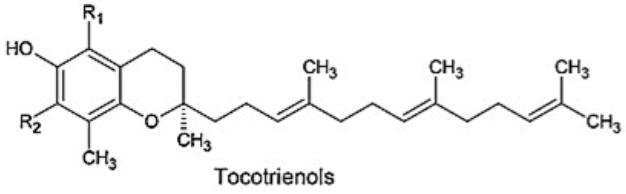


Figure 1. Structure of phenol (Sigma-Aldrich 2017)

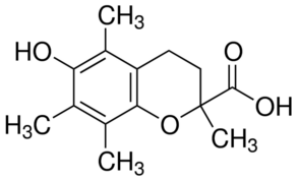
Phenolic compounds cover a very large and diverse group of chemical compounds. Common biologically active phenols include tocopherol and tocotrienols (Figure 2), which are also known as vitamin E. These lipid soluble phenols are ubiquitously found in vegetable oils, nuts, seeds, and green leafy vegetables. In food industry, vitamin E is commonly added to polyunsaturated-rich oils such as the vegetable oils (sunflower oils, corn oil, grapeseed oil, etc.) to prevent lipid oxidation, and hence, prolong their shelf life. In our body, vitamin E is particularly important in protecting the long-chain polyunsaturated fatty acids commonly found in the membrane phospholipids of the nervous system against oxidation (Traber & Atkinson 2007). Trolox (Figure

2) is a water-soluble analog of vitamin E and is commonly used as a reference standard in antioxidant assays.



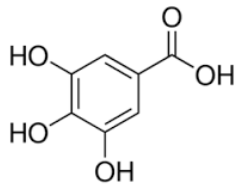


Tocopherol/ Tocotrienol	R^1	R^2
α–	CH_3	CH_3
β–	CH_3	H
λ–	H	CH_3
γ–	H	H

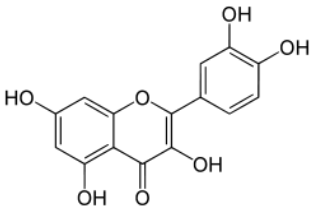


Trolox

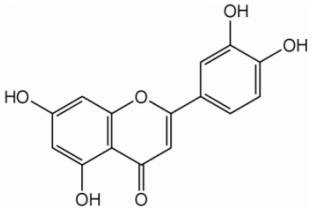
Figure 2. Tocopherol, tocotrienol and Trolox structures (Bartosińska, Buszewska-Forajta & Siluk 2016)



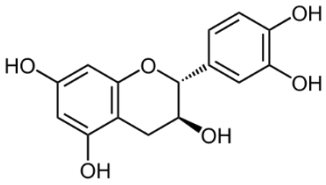
Phenolic acid - Gallic acid



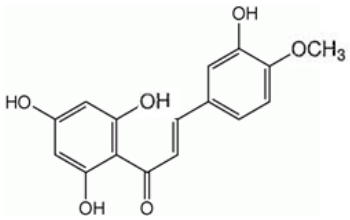
Flavonol - Quercetin



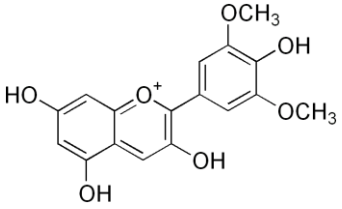
Flavone - Luteolin



Flavonol - Catechin



Flavanone - Hesperitin



Anthocyanidin - Malvidin

Figure 3. Structures of phenolic acid and flavonoids (Goertz & Ahmad 2015; Pauwels & Kostkiewicz 2009)

Other commonly found biologically active phenol groups are phenolic acids (e.g. gallic acid, caffeic acid) and flavonoids (Figure 3). Flavonoids are the most abundant type of antioxidants found in plants and researched on extensively (Brewer 2011; Young, Tsao & Mine 2011). Structurally, most flavonoids share the same backbone structure that includes two benzene rings linked by a chromane ring. Flavonoids can be further classified into flavonols (quercetin, kaempferol), flavones (e.g. luteolin, apigenin), flavonols (e.g. catechins, epicatechin, epicatechin gallate), flavanones (e.g. hesperitin, naringenin), anthocyanidins (malvidin, cyanidin, delphinidin) and isoflavonoids (e.g. genistein, daidzein) (Figure 3). Glycosylation patterns and other structural features between different flavonoids determine their respective antioxidant activity strength. These phenolics are commonly found in leafy green vegetables such as kale, lettuce, cabbage, spinach and watercress (Young, Tsao & Mine 2011). It has been widely reported that the flavonoids act as anti-inflammatory agents against cardiovascular disease and cancer (Russo et al. 2012).

2.6 Dietary and lifestyle trends in Malaysia

2.6.1 Food, lifestyle and disease

The National Morbidity Health Survey conducted in Malaysia, revealed that cardiovascular disease is the main contributor to 73 % of deaths among Malaysian population (Institute for Public Health (IPH) Malaysia 2015). The survey further revealed that high cholesterol, high body-mass index (BMI), smoking and hypertension were contributory factors to the above number.

The advancement of food production technology allows a variety of foods to be mass produced and also kept afresh for a longer period of time. Foods that contain high levels of unsaturated fats (e.g. vegetable oil) tend to be subjected to lipid peroxidation, referred to as rancid fats. Other than that, other food components, such as proteins, vitamins and pigments can also be oxidised, resulting in loss of food quality. Food additives, in the form of antioxidant are commonly added to prevent these oxidative damages and to extend the shelf-life of the foods. Currently, synthetic phenol antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propylgallate (PG) and tert-butylhydroquinone (TBHQ) are widely used due to their availability, low production cost, high purity and constant activity (Berdahl, Nahas & Barren 2010; Makahleh, Saad & Bari 2015). These are typically added in the stabilization of high fat ingredients and/or products such as vegetable oils, potato chips, animal fats. Despite their advantages, these synthetic antioxidants usage is also cause of concern due to some inconclusive studies. For example, BHA and BHT can cause liver damage and cancer. However, these studies were

conducted on laboratory animals and the dosage used was unrealistically high (Makahleh, Saad & Bari 2015).

Following these reports, some countries have limited the usage of BHT in some products (Makahleh, Saad & Bari 2015). While possibly not scientifically sound, this has caused consumers and lobbyist to doubt the safety of these synthetic antioxidants and instead, demand for more natural food products.

In the past decade, the food and medicine trend of consumers have gradually shifted to organic eating habits, vegetarian/vegan food and nutraceuticals. The Google food trends 2016 report shows that consumers in the United States are more focused on healthy foods instead of cakes, cupcakes, wheat free bread, and bacon cinnamon rolls (Pina et al. 2016). The demand for functional foods is on the rise, with the spotlight belonging to foods such as turmeric, apple cider vinegar, jackfruit, manuka honey, coconut milk, bitter melon and cumin seeds. It is interesting that the demand to experience global cuisine has also increased (Pina et al. 2016). This creates a potential to introduce local food from Sarawak to the rest of the world.

2.7 Edible plants in Sarawak as antioxidant source

Natural rain forests are rich with diverse eco-systems that flourish many fauna and flora. The Malaysian state of Sarawak is considered as one such treasure trove due to its vast coverage of natural forests and many of its plant species are endemic to the island of Borneo.

Many of the edible indigenous fruits and vegetables are not commercially farmed and the supply is generated from wild trees. Traditionally, Sarawakian Dayak communities (Iban, Bidayuh and Orang Ulu ethnic groups) consume edible parts of certain fruits, vegetables and herbs fresh, cooked as curries or fermented into sweets or drinks (Ting, Tan & Nastassia 2017). Some of the ingredients in local food have also been used as traditional medicine to treat certain health ailments (Table 3).

Table 3. Collective data on several local plants used in traditional medicine in Malaysia.

Scientific name	Local name	Part used	Claimed health benefit	Reference
Blumea balsamifera (L.) DC.	Sembung	Leaves, roots	• Relieves stomach ache	(Grosvenor, Agus & David 1995)
Cosmos caudatus Kunth	Ulam raja	Leaves	• Improves bone health • Cleanses the circulatory system	(Abas et al. 2006)
Dillenia suffruticosa (Griff.) Martelli	Simpoh air / Buan	Leaves, stem bark	• Heals topical wounds • Relieves rheumatoid pain • Reduces cancerous growth	(Sabandar, Jalil & Ahmat 2015)
Durio zibhetinus L.	Durian	Stem bark	• Reduce fever	(Diba et al. 2013)
Moringa oleifera L.	Drumstick tree	Leaves	• Improves vision and prevents eye diseases	(Liu, Perera & Suresh 2007)
Premna cordifolia Roxb.	Singkil	Leaves	• Reduce fever and asthma • Stimulate milk production in lactating mothers	(Mohd Nazri et al. 2011)
Sauropus androgynous (L.) Merr.	Cekur manis	Leaves	• Improves vision and prevents eye diseases	(Liu, Perera & Suresh 2007)
Stenochlaena palustris (Burm. F.) Bedd.	Midin	Leaves	• Relieves ulcers and skin diseases	(Chai et al. 2012)
Eugenia polyantha Wight or *Syzygium polyanthum* (Wight) Walp.	Bungkang	Stem bark	• Relieves stomach ache	(Diba et al. 2013)

These edible plants are typically consumed in the form of raw salads; stir fried, boiled or as flavouring agents to meat or rice. Some of the plants (*P. cordifolia, C. caudatus, S. palustris, D. suffruticosa*) had been subjected to antimicrobial activity, total phenolic, DPPH-radical scavenging and nutritional studies (Chai et al. 2012; Mohd Nazri et al. 2011; Sulaiman et al. 2011; Voon & Kueh 1999).

Voon and Kuek (1999) reported that *E. polyantha* or *S. polyanthum* ('bungkang') has high energy content (115 kcal per 100 g of its edible portion) compared to 25 other leafy vegetables. Used ethno-medically as a remedy for diarrhoea and diabetes, *S. polyanthum* leaves, stems and bark have indicated mild traits of *Staphylococcus aureus* inhibition, high phenolic and high DPPH free radical scavenging activity (Grosvenor, Agus & David 1995; Lelono, Tachibana & Itoh 2009; Wong, Leong & Williamkoh 2006). High energy foods helps to minimize the constant urge to eat that arise from the internal need to replenish energy levels in the body (Ledikwe et al. 2006). Therefore, these findings make this plant not only a source of antioxidants but also an ideal "health food" for individuals concerned about dieting, without the need for starvation.

Similarly, other plants of interest such as *P. cordifolia, D. suffruticosa* and *S. palustris* show promising properties in terms of phenolic contents, radical scavenging activities and antibacterial (Table 4) activities against microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.*

However, information on further analysis such as those involving the cell culture assays, particularly to mammalian cardiomyocyte cell lines are still very scarce. Therefore, given their potential health values, it is beneficial to conduct such study as to add more knowledge to these basic antioxidant potentials and in turn, add more value to the plants.

Table 4. Antioxidant activities and other properties of edible leafy plants in Sarawak.

Scientific name	Local name	Activity						Reference
		Antimicrobial	TPC[a]	DPPH (IC_{50})	TFC[b]	TAC[c]	Cytotoxicity (LD_{50})	
Blumea balsamifera (L.) DC.	Sembung	Weak inhibition of *S. aureus, E.coli*	29.00 ± 0.50 g GAE/g dw	72 g/mL	-	-	HepG2 cancer cell line specific toxicity	(Grosvenor, Agus & David 1995; Norikura et al. 2008; Pang et al. 2014)
Cosmos caudatus Kunth	Ulam raja	Moderate inhibition of *S. aureus* and *E.coli*	1.7 ± 0.8 mg GAE/g dw	21.31 ± 0.13 µg/mL	27.7 ± 1.0 mg QE/g dw	4.71 ± 0.19 µmol TE/g fw	-	(Ghimire et al. 2011; Rasdi et al. 2010; Sulaiman et al. 2011)
Dillenia suffruticosa (Griff.) Martelli	Simpoh air / Buan	-	432.02 ± 13.79 mg GAE/g extract	31.33 ± 71.15 mg/mL	6.05 ± 0.20 mg RE/g extract	156.57 ± 9.36 mg TE/g extract	128.00 ± 6.00 µg/mL (HeLa cancer cell line)	(Armania et al. 2013)
Eugenia polyantha Wight	Bungkang	Moderate inhibition of *S. aureus*	856 ± 28 mg GAE/g extract	0.181 ± 0.04 mg/mL	26.5 ±7.1 mg RE/g extract	449 ± 23.5 mg AA/g extract	53.5 µg/mL (Vero epithelial cell line)	(Grosvenor, Agus & David 1995; Lelono, Tachibana & Itoh 2009; Perumal et al. 2012)
Premna cordifolia Roxb.	Singkil	Weak-moderate inhibition of *S. aureus* and *P. aeruginosa*	21.9 ± 0.4 mg GAE/g dw	200 ppm	6.1 ± 0.3 mg QE/g dw	-	-	(Mohd Nazri et al. 2011; Sulaiman et al. 2011)
Premna serratifolia L.	Singkil Tree	-	143.67 ± 2.37 mg GAE/g extract	-	22.07 ± 2.04 mg QE/g extract	-	Ehrlich Ascites Carcinoma cell line specific toxicity	(Ghimire et al. 2011; Sridharan et al. 2011)
Stenochlaena palustris (Burm. F.) Bedd.	Midin	-	51.69 ± 1.28 mg GAE/g dw	99.21 ± 0.17 µg dw/mL	58.05 ± 0.30 mg CE/g dry matter	4.71 ± 0.19 (µmol TE/g FW)	-	(Andarwulan et al. 2010; Chai et al. 2012)

[a]TPC refers to the Total Phenolic Content, [b]TFC refers to the Total Flavonoid Content, [c]TAC refers to the Total Antioxidant Capacity.

2.8 Laboratory methods for screening and antioxidant activity assessments

The current study focuses on screening methanolic plant leaf extracts for total polyphenolic and flavonoid content for more specific *in vitro* colourimetric techniques that will help to make inferences on antioxidant activities such as DPPH radical scavenging, ABTS radical cation decolourization, Cupric Ion Reducing Antioxidant Capacity and their effect on mammalian heart cells under oxidative stress. These tests will help to evaluate the safety and efficacy of the plant based antioxidants and their potential food products.

2.8.1 Drying of plant leaves

Post-harvest processing methods that are used for preservation of herbal substances such as leafy plants. Removal of moisture is an essential component for preservation, as relative humidity (<70%) promotes growth of microorganisms such as yeast, bacteria and fungi. It also aids in decomposition (Müller & Heindl 2006). Therefore, drying methods such as air-drying, drum-drying and freeze drying have been used to protect sensitive active ingredients such as antioxidants. Of these, freeze drying has shown to be the most effective drying method of preservation opposed to other types of drying as they decline total phenolic contents, ascorbic acid equivalent antioxidant capacity and ferric reducing capacity (Chan et al. 2009). While the presence of heat and humidity in the air-drying method aids to little cell rupture, freeze drying leads to higher extraction efficiency for phenolic compounds such as tannins as the process develops ice crystals within the plant matrix, allowing better access for solvents and extraction (Abascal, Ganora & Yarnell 2005; Asami et al. 2003).

2.8.2 Extraction of bioactive compounds

Most antioxidant-plant based studies require a good solvent for phytochemical extraction that involves characteristics as optimal solubility with active compounds, ease of evaporation, degree of preservation and low toxicity leading to the degradation of the cell wall (Tiwari et al. 2011). The polarity of solvents such as methanol, ethanol, acetone and water vary from medium polar to polar. This allows the extraction of hydrophilic antioxidants such as phenolics and flavonoids. Hydrophobic antioxidants are better extracted with organic solvents such as mixtures of hexane with methanol or ethyl acetate with ethanol (Xu et al. 2017). Typically, aqueous methanol (80 %) is used as the extraction solvent of choice as it is deemed the most effective in comparison to other percentages of methanol or solvents such as ethanol. Often, its high percentage yield and optimal antioxidant activity were achieved following 30-min incubation during sonication (Ahmad et al. 2009; Annegowda et al. 2012).

The solvent, coupled with maceration, sonication and centrifugation leads to a successful extraction as it assists in the destruction of the cell wall and enhances mass transport of bioactive compounds (Tiwari et al. 2011; Xu et al. 2017).

2.8.3 Total phenolic content (TPC) assay

Polyphenolic contents in plant material are normally quantified using Folin-Ciocalteau assay as first described by Singleton & Rossi (1965). The Folin-Ciocalteau reagent (FCR) consists of sodium tungstate and sodium molybdate, hydrogen chloride, phosphoric acid and lithium sulphate tetrahydrate. When the acidified F-C solution is introduced to lithium sulphate tetrahydrate, it gives the solution its signature yellow colour (Sanchez-Rangel et al. 2013). The total polyphenolic content assay measures the ability of phenolic contents from the extracted sample to transfer electron to reduce free radical (Yoo et al. 2012). When this electron transfer takes place the pH of the solution is ~3. By the addition of sodium carbonate, the basic conditions are elevated to a pH of ~10 allowing the phenolic proton to dissociate and form phenolate ions. These ions are able to reduce to Folin-Ciocalteau agent resulting in blue colour following incubation for 2 hours (Cicco & Lattanzio 2011). Generally, gallic acid is used as the equivalent standard to represent the total polyphenols present in a sample (Atoui et al. 2005; Brighente et al. 2007; Ginjom et al. 2010).

The TPC assay also detects any reducing agents such as reducing sugar present in the sample(Cicco & Lattanzio 2011; Yoo et al. 2012). Although this is considered a disadvantage, the TPC method is still commonly used by researchers as a primary screening test to indicate the presence of phenolic antioxidants, before proceeding to any other antioxidant related assays.

2.8.4 Total flavonoid content (TFC) assay

The total flavonoid content assay is another primary screening test that accounts for the detection of the flavonoid content in food sources such as plants and other phytomedicines (da Silva, Pezzini & Soares 2015). The colourimetric assay quantifies them by the intensity of the yellow coloured complex molecule that forms due to the interaction of 2-10 % (w/v) of aluminium chloride in the presence of water or methanol. The reaction between the aluminium ion and the hydroxyl and keto groups belonging to the flavonols and flavones gives a measurable intensity between 404-430 nm following an incubation between 2 – 60 minutes (Pontis et al. 2014). The type of reference standard used depends on the type of main groups of flavonoids expected in the sample. For example, in samples expected to contain high flavonol content, quercetin or rutin or galangin are commonly used.

For samples expected to contain high flavan-3-ol contents such as those in tea, catechin is normally used as the reference standard (Pękal & Pyrzynska 2014).

2.8.5 *In vitro* antioxidant activity assays

Antioxidants can be divided into lipophilic and hydrophilic, based on their solubility. The antioxidant activities related to these can be assessed by their electron transfer (ET) or Hydrogen atom transfer (HAT) with antioxidants. Table 5, shows the different methods of measuring antioxidant activities, some of which will be discussed in detail within the current literature review.

Table 5. Types of measurement of *in vitro* antioxidant activities

Characteristic	Assay of antioxidant activity	
	Hydrogen atom transfer (HAT)	**Electron transfer (ET)**
Mechanism	The donation of protons from an antioxidant to quench free radicals	Reduction of ROS or RNS by the transfer of a single electron by antioxidants
Type of radical scavenged	The antioxidant and substrate competes for thermally generated peroxyl radicals through the decomposition of azo-compounds	Measures the capacity of an antioxidant in the reduction of an oxidant, which is then correlated with the antioxidant concentrations
Examples	• Oxygen radical absorbance capacity (ORAC) • Total radical trapping antioxidant parameter (TRAP) • Crocin bleaching assays	• TPC, TFC • ABTS assays • Ferric ion reducing antioxidant power (FRAP) • DPPH radical scavenging assay • CUPRAC assay

Adapted from (Apak et al. 2007; Mawalagedera 2014).

2.8.5.1 DPPH radical scavenging assay

One of the main characteristics for an *in vitro* antioxidant activity test is using free radical traps. The DPPH (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl) radical scavenging assay is one such method that is simple, inexpensive and rapid. It only involves two basic chemicals i.e. the DPPH powder and the solvent used to dissolve the DPPH (e.g. ethanol or methanol). The DPPH reagent avoids dimerization due to its delocalized spare electron. This allows the molecule to be stable in solution form and gives its distinctive deep purple colour. The donation of an hydrogen atom by antioxidants allow the purple DPPH molecule to reach its reduced form that is yellow in colour in a 30 minute reaction frame (Alam, Bristi & Rafiquzzaman 2013). The purpose of this assay is to discover the concentration of the sample which is able to effectively reduce 50 % of the DPPH radical concentration.

Due to the stable nature of the nitrogen radical in the DPPH molecule (Figure 4), the rate of kinetics for many of the reacting antioxidants may differ, possibly with no reaction at all. Typically, an incubation time of 30 minutes is used (Ginjom et al. 2010). The resulting EC_{50} values may vary from one lab to another, as there are a few variations (e.g. solvents, DPPH radical final concentrations, incubating temperatures, and incubation periods) of this method are being used by different researchers. Low readings of antioxidant capacities may be recorded due to the reversible conversion of $DPPH^{2\bullet+}$ to $DPPH^{\bullet}$ form and the subsequent low reaction between the DPPH molecule and the antioxidant (Mishra, Ojha & Chaudhury 2012). However, this method is still widely used due to its simplicity and ability to rank the different samples within the same laboratory conditions.

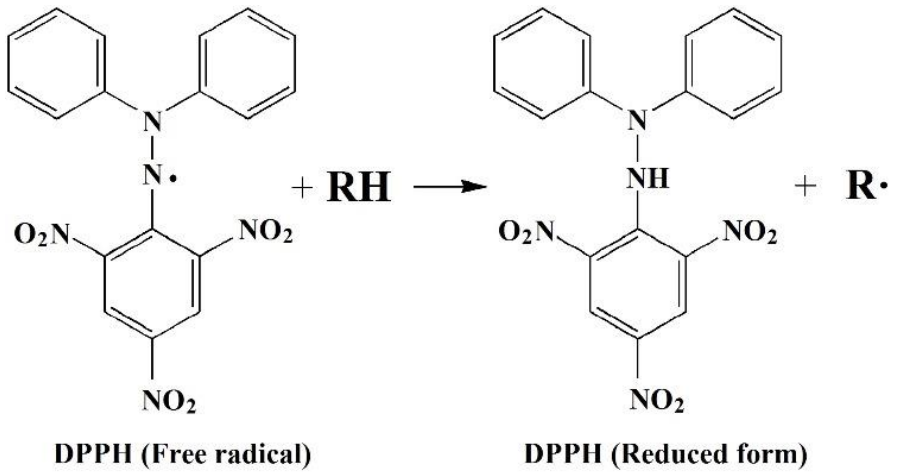


Figure 4. Structure of DPPH in its radical and free radical form (Alam, Bristi & Rafiquzzaman 2013).

2.8.5.2 ABTS radical cation decolourizing assay

There are two versions of the ABTS assay. One of which (Miller & Rice-Evans 1997) involves the oxidation of the ABTS (2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) by hydroxyl radicals generated by metmyoglobin-H_2O_2 followed by the ABTS radical's reaction with antioxidants. However, this method is ambiguous due to its overestimation o antioxidant activity(Miller & Rice-Evans 1997).

The other ABTS decolourization assay, involves the generation of the stable, blue-green chromophore, ABTS radical cation by oxidising ABTS with potassium persulphate, followed by its reduction by antioxidants (Figure 5). This process determines the antioxidant activity by measuring the extent of decolourization at a fixed point of time (Zheng et al. 2016).

In this assay it is vital for the ABTS acid to react with potassium persulphate at a stoichiometric ration of 2:1. Although oxidation begins immediately upon addition, it requires a lengthy incubation of 12 hours for the concentrated solution mixture reaches its maximum plateau (Thaipong et al. 2006). The radical solution is diluted to obtain an absorbance of 0.700 AU at 734 nm as it would give less interference from plant pigments (Apak et al. 2007).

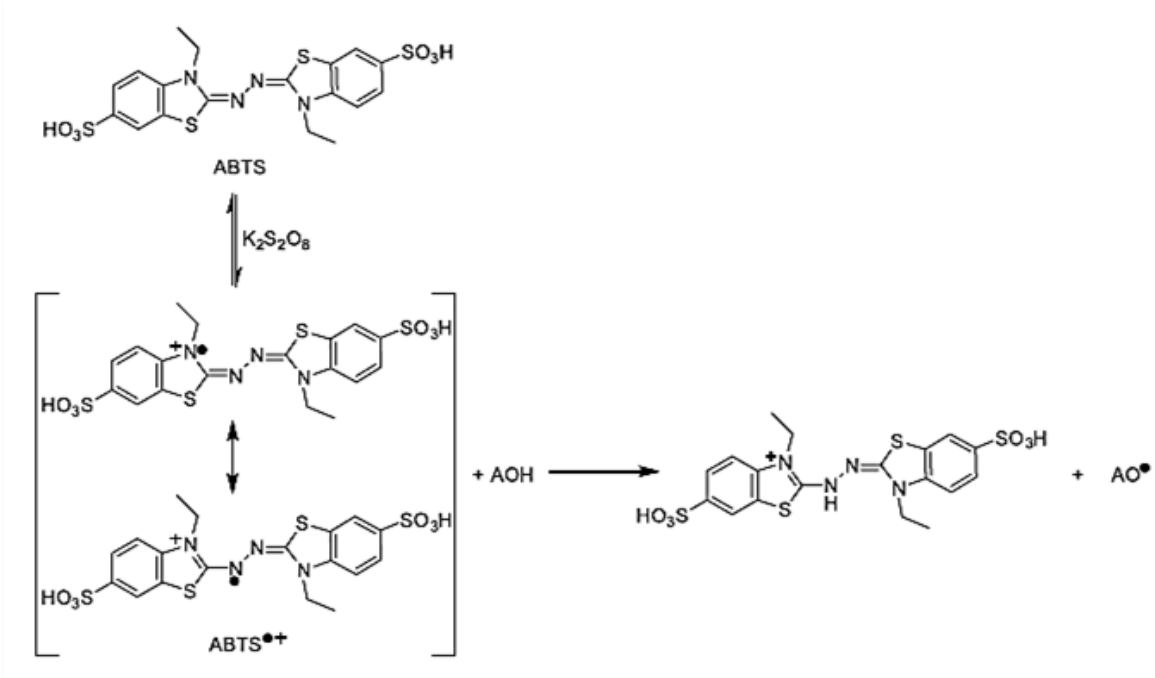


Figure 5. Structure of ABTS in its radical and free radical form (Oliveira et al. 2014)

2.8.5.3 Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Assay

Due to the majority of aqueous (alcohol-water) extraction techniques, most antioxidant activity assays such as DPPH and FRAP (Ferric Reducing Antioxidant Power) account for hydrophilic antioxidants. The cupric ion reducing antioxidant capacity or CUPRAC method use cyclodextrins to detect both hydrophilic and hydrophobic antioxidants. Cyclodextrins are oligosaccharides which have a hydrophilic exterior and hydrophobic cavity to bind both lipophilic and hydrophilic antioxidants (Özyürek et al. 2008) to enhance their solubility. Several advantages of this method over other conventional antioxidant activity assays are that its closeness of reaction pH to the physiological value (pH 7), stability of reagents and simplicity (Apak et al. 2014). As shown in Figure 6, the chromogenic redox reagent bis(neocuproine)copper(II) [Cu(II)-Nc] chelate is reduced by the polyphenolic antioxidants into an orange solution, where the colour intensity is directly proportional to the amount of chelate used for the reaction. The excess protons generated at the end of the reaction are neutralized by ammonium acetate buffer.

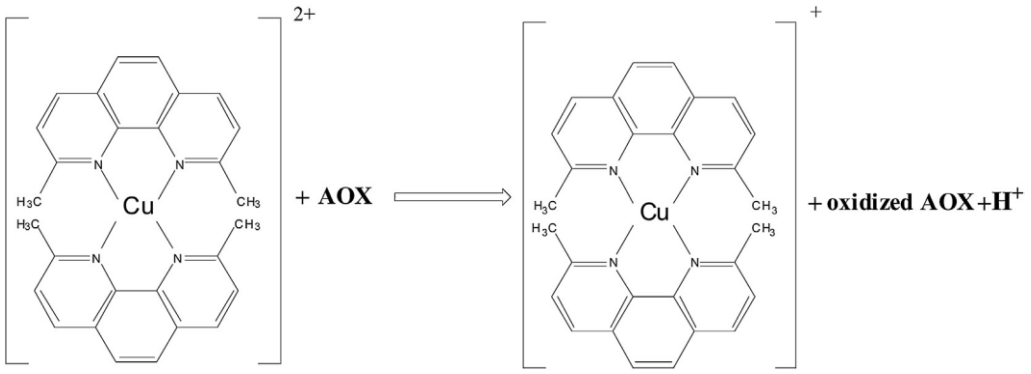


Figure 6. Reaction between the blue bis(neocuproine)copper(II) chelate and antioxidants (Apak et al. 2014).

2.8.6 Cell culture model

The above *in vitro* chemical methods are most generally used due to their simple, rapid and low operating costs. In contrast, cell culture based assays are costly and time consuming. However, it helps to significantly reflect the nature of biological responses (e.g. absorptions and metabolisms) of any bioactive compounds, under true physiological conditions (Wan et al. 2015). Malignant cell lines such as HeLa cells have been the preferred choice for chemotherapeutic drug based cell culture studies due to its robust nature and fast cell growth and division. However, it is not a suitable choice of cell line for studies that require the understanding of healthy metabolic pathways (i.e. redox signalling and mechanisms). Therefore, primary cell culture, where cells are harvested and grown from tissue is recommended for such studies (Halliwell 2014). Cell lines such as BDIX, MHEC5, RCVC originating from cardiac tissues; C2C12, RCMH, CMG originating from skeletal tissues; and RCSN, NT2, CNh originating from neuronal tissues, are commonly used for *in vitro* disease pathology, cytotoxicity and drug development studies (Allen et al. 2005). It is vital that the user, maintains a clean and sterile environment when handling any type of cell line to minimize risks such as contamination from mycoplasma, genetic and phenotypic instability due to poor passaging (Geraghty et al. 2014).

2.8.6.1 H9c2 (2-1) cell line

The H9c2 (2-1) cardiomyocyte cell line used in this assay is a sub-clonal derivative of the BDIX embryonic rat heart cell line (American Type Cell Culture 2016). The large, flat, spindle shaped and mononucleated cells are used widely as a cell culture model in cardiotoxicity and in studying adaptive mechanisms as they are easy to obtain, have a longer life span, are phenotypically homogenous making it easier for repetitive usability and have little or no involvement of ethical issues. Due to their vagueness of molecular phenotypes, complicated biological responses may arise (Lenčo et al. 2015). Adult H9c2 heart cells are more susceptible to oxidative stress related damage than atrial HL-1 cells due to their utilization of free fatty acids and aerobic nature. Whereas fetal mammalian hearts are at less risk due to their high dependency on glycolysis. H9c2 cells possess a higher phosphorylation state of AMP-activated protein kinase and show similarity to primary cardiac cell lines in terms of energy metabolism (Kuznetsov et al. 2015). These characteristics make the H9c2 cardiomyocytes an ideal cell model for oxidative stress studies.

H9c2 (2-1) cardiomyocytes grow in the presence of Dulbecco's Modified Eagle's Medium (DMEM) which consists of essential nutrients such as amino acids, vitamins, ferric nitrate, sodium pyruvate and glucose. DMEM also consists of buffering agents such as sodium bicarbonate for CO_2 buffering, phenol red as a pH indicator (low pH range exhibits red colour), and antibiotics such as penicillin and streptomycin to minimize bacterial and mycoplasma

contamination (Collection 2012). Furthermore, the cells require fetal bovine serum (FBS) which is a complex mixture of biomolecules including protein components, fatty acids, hormones, growth promoting factors and trace elements including transition metals, responsible for different, physiologically balanced growth-promoting and growth-inhibiting activities (Brunner et al. 2010). While culture media is vital for growth, transitional metals such as iron (Fe^{3+}) could react with antioxidants such as ascorbic acid and vitamin E to induce pro-oxidant conditions (Rahal et al. 2014).

Passaging or sub-cultivating of cells at its exponential growth phase (70-80% confluency) is required to maintain its viability, phenotypic and genetic stability. It is important to passage at this stage, as over-crowding due to 100% of monolayer confluency leads to lack of space, depletion of nutrients, followed by cell aggregation. This is usually overcome by disrupting intracellular cell to surface protein attachment bonds with the aid of trypsin/EDTA (a proteolytic enzyme) solution (Collection 2012). It is also vital to be observant of the passage number, as too many passages could lead to altered gene expression responsible for morphological and functional changes in cells (Hughes et al. 2007).

2.8.6.2 Trypan blue assay

This dye exclusion assay is an easy and the most common method that is practised for cell counting (Menyhárt et al. 2016). Using a haemocytometer, it enables to distinguish between live and dead cells in a cell suspension. Non-viable cells are able to uptake the blue diazo dye as their cell membranes are disrupted leaving them blue and swollen, under a light microscope. However, the negatively cell membranes of viable cells remain intact and due to their selective membrane the cells are unable to take up the dye, therefore appear small, round and colourless (Tran et al. 2011). The counting can be done with the aid of a click counter or using commercially available automated counting systems such as Coulter Vi-CELL™ XR Cell Viability Analyzer, Nexcelom Bioscience Cellometer Vision® and the Invitrogen Countess™. Although this method accounts for non-viable cells, it is unable to take account for cell death due to apoptosis or necrosis (Louis & Siegel 2011).

2.8.6.3 Cryopreservation of cells

Preservation is an important component in mammalian cell culture as it helps to minimize cell passaging frequency and conservation of usage. Cryopreservation is a technique that integrates ultra-low temperatures to help preserve the structure and function of suspended cells, small proteins and tissues for many years and prevent the formation of ice crystals in the presence of a cryoprotective agent (CPA) such as dimethylsulfoxide (DMSO), glycerol or hydroxyethyl starch (HES) (Giugliarelli et al. 2016; Jang et al. 2017). Of these, DMSO is a low molecular weight

(<400 Da) CPA, passively diffusible through the cell membrane, which allows to protect cells from cryopreservation induced stress during post-thawing procedure. However, CPAs such as DMSO is associated with possible toxicity in a time and temperature dependent manner (Chatterjee et al. 2017). This setback could be minimized with effective cooling temperature mechanisms such as initial overnight cooling at -70ºC to -90ºC due to its slow cooling rate of -1ºC / minute, followed by cryogenic cooling in liquid nitrogen at -196ºC (American Type Cell Culture 2010).

2.8.6.4 MTS assay

In order to measure cell proliferation and test for cytotoxicity of compounds in cell-based assays tetrazolium salt based assays are carried out. Tetrazolium salts such as MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide] have been commonly used for cell cytotoxicity studies in cell culture as it accumulates in its reduced form within cells due the reaction with mitochondrial dehydrogenase. More recently, the MTT is replaced with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent as it is faster and reduces the error for loss of cells and more stable than the MTT reagent (Huang, Chen & Walker 2004). MTS has a net negative charge that contributes to its cell impermeability and does not require organic solvents to produce formazan intermediates unlike MTT reagent and allows real-time measurement of metabolic activity

Metabolism in viable cells produces reducing equivalents such as NADPH or mitochondrial dehydrogenase succinate. The electrons of these molecules are passed to an intermediate electron transfer reagent such as phenazine methyl sulphate (PMS) or phenazine ethyl sulphate (PES) that reduces the MTS into a soluble purple formazan product (Figure 7). Upon cell death, cells rapidly lose the ability to reduce tetrazolium products. Cell death could be triggered by oxidative stress, mycotoxins and silver nanoparticles The number of viable cells in a culture is considered to be directly proportional to the intensity of formazan produced (Promega Corporation 2017).

It has been reported that the activity of succinate dehydrogenase maybe spike when a reaction takes place between a MTT and phytochemicals such as epigallocatechin-3-gallate (EGCG), kaempferol and iodoacetic acid present in plant extracts (Hsu et al. 2005; Huang, Chen & Walker 2004). Due to the surge of succinate dehydrogenase produced by the reaction between extract EGCG and MTT (or MTS), it results in the increased rate of reduction of MTT from a single cell to produce more formazan, resulting in an overestimation of cell viability (Stepanenko & Dmitrenko 2015). Studies have shown that washing cells with PBS buffer, helps to reduce interferences caused by reactants.

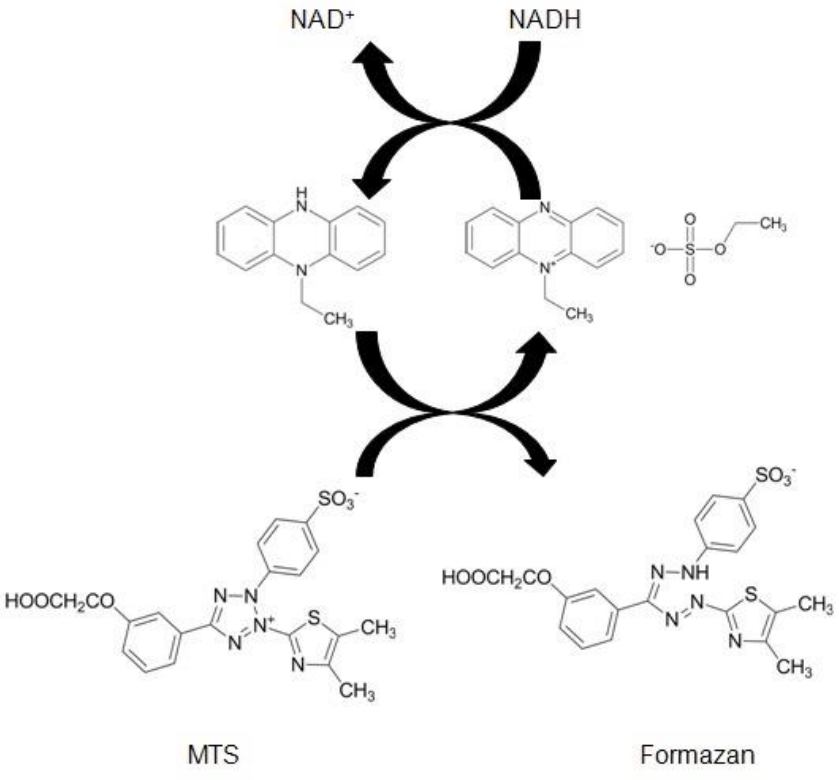


Figure 7. Reduction of MTS for the formation of formazan due to the electron transfer between NADH and phenazine ethyl sulphate (PES) (Promega Corporation 2017).

2.8.6.5 Hydrogen peroxide assay

Hydrogen peroxide (H_2O_2) is a common ROS generated in excess as a result of oxidative stress in the body. In cell culture media it is seen as a stable molecule. In the presence of cells, the H_2O_2 is able to penetrate the cellular membrane. Although the H_2O_2 can be detoxified by the intracellular catalase, at high concentrations, the catalase activity diminishes, leading to an increase in the hydroxyl radical (Gille & Joenje 1992). Similarly, in the presence of copper or iron in the media or extract, the H_2O_2 would react to produce more hydroxyl radical. This is also known as the metal ion-catalyzed Fenton and Haber-Weiss reaction (Özyürek et al. 2010). In both instances, the radical causes damage to cell components including the cellular membrane, resulting in cell death. In cell culture, this scenario coupled with the MTS assay, allows the evaluation of the lethal dose (LD_{50}) of the hydrogen peroxide able to reduce 50 % of the cell line population. Once the LD_{50} is determined, a suitable H_2O_2 concentration is selected and a concentration range of selected extracts are added to cells to determine their ability to uphold the cell viability. The LD_{50} value varies greatly with the type of cell lines it is being tested on. For example, the LD_{50} of H_2O_2 on P388 mouse leukemic cells is much lower (LD_{50} = 30 μM, more toxic) than those in the RC-5 lung fibroblast cells (LD_{50} = 500 μM, less toxic) (Table 6).

Table 6. Hydrogen peroxide induced cell cytotoxicity and extract induced cell viability in the presence of H_2O_2 in different cell lines.

Cell Line	LD_{50} for H_2O_2	Extract/ chemical	Cell viability post-extract treatment (%)	Reference
PC12 neuronal cell	200 µM	Digested *Camellia sinensis* 0.625 µg/ml (24 h)	80 % in the presence of 200 µM H_2O_2	(Okello et al. 2011)
RC-5 lung fibroblast cells	500 µM	*Ribes nigrum* L. 10 mg/ml (24 h)	86.0 % in the presence of 500 µM H_2O_2	(Jia et al. 2014)
P388 mouse leukemic cells	~30 µM	Naringin 1 mM (15 min)	93.6 % in the presence of 30 µM H_2O_2	(Kanno et al. 2003)

2.9 Conclusion

Oxidative stress brought upon by intracellular and unhealthy dietary and lifestyle habits lead to many diseases including cardiovascular disease. The build-up of excessive free radical species attacks cell membrane via lipid peroxidation and weakens the intracellular defence system of endogenous antioxidants which are responsible of neutralizing free radicals. Hence, the need for an external source of antioxidants is required. Food and beverages of plant origin are abundant in naturally occurring antioxidants belonging to the groups of phenolic compounds, especially the flavonoids. These serve as excellent sources of exogenous antioxidants. Various *in vitro* assessments have been widely practised to extract antioxidants form plant sources, detect the presence of antioxidant compounds (TPC and TFC) and their antioxidant activities (ABTS, DPPH, CUPRAC) due to their efficiency, colourimetric reactions, availability, novelty and rapid detection. Moreover, it is important to understand and identify the nature of free radicals and antioxidants sources on mammalian cells as they are the closest physiological representation as an *in vitro* biological model. H9c2 (2-1) cardiomyocytes make an ideal model of study for oxidative stress as they are more susceptible to such conditions and are easy to maintain and grow.

The literature review suggests that although traditional medicinal claims and ample research has been carried out on microbial and antioxidant activities of some Malaysian plants. However, underutilized edible plants in Sarawak have had less exposure to antioxidant activity detection methods such as CUPRAC and especially cell culture studies related to cardiomyocytes. With the understanding of mechanisms for the above tools of detection, this thesis aims to deliver a scientific insight into the presence of antioxidants, antioxidant activities, cytotoxicity and cardio-protection against free radicals in selected edible leafy plants in Sarawak.

3 MATERIALS AND METHODS

The current study was conducted in three stages of screening- Stage I: Phenolic content; Stage II: Antioxidant activity; and Stage III: Cell-based cardio-protection. The three stages screened different types of plant samples, with lesser number of samples for each subsequent stage. The subsequent stages only analysed plant samples with high phenolic and antioxidant contents. The following flowchart (Figure 8) summarises the methodology involved in this study.

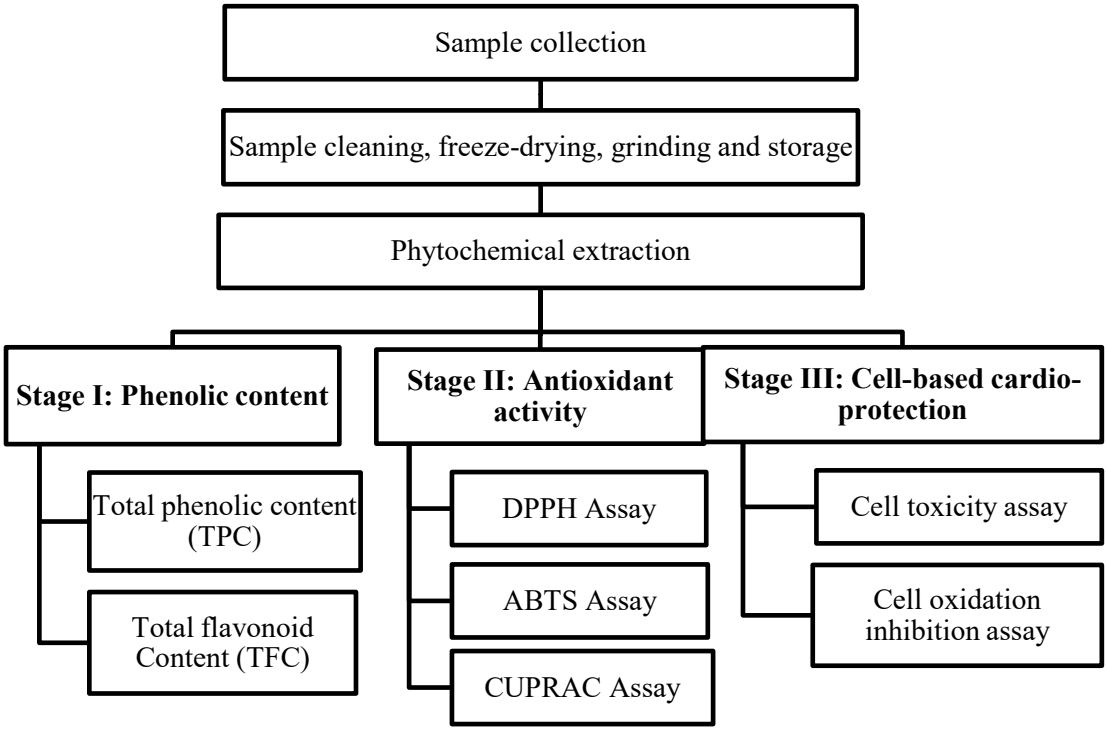


Figure 8. Methodology flow chart.

3.1 Plant materials

Various plant materials (Table 7, Figure 9) were collected from Agriculture Research Centre (ARC) in Semenggok, Sarawak; from personal donations; as well as from local farmer's market in Kuching, Sarawak.

The identities of the plants were confirmed by Research Officer Dr. Maclin Dayod from the ARC. The edible parts i.e. shoot or young leaf and stem of the collected plant samples were cleaned, rinsed with distilled water and briefly dried at room temperature. Once dried, they were chopped into smaller pieces, weighed and further freeze-dried for 36 - 48 h using a Labconco 7753030 FreeZone 6L Console Freeze Dry System (Labconco Corp., Kansas City, MO).

The dried plant samples were weighed once more to obtain their dry weight and then ground into powder with a kitchen grinder. All of the dried powder was stored in airtight containers at -20°C in the dark until analysis.

Table 7. List of edible leafy plants used in the study.

	Scientific name	**Code name**	**Local name (dialect)**
1.	*Dillenia suffruticosa* (Griff.) Martelli**	*D. suffruticosa*	Simpoh air (Malay), buan (Bidayuh)
2.	*Eugenia polyantha* Wight/ *Syzygium polyanthum* (Wight) Walp. *	*E. polyantha* (2 types): (a) Small leaf (wild) (b) Soft leaf (common)	Indonesian bay leaf (English), salam/ serai kayu (Malay), bungkang (Iban)
3.	*Premna serratifolia* L.*	*P. serratifolia*	Singkil laut (Malay)
4.	*Premna cordifolia* Roxb.*	*P. cordifolia*	Singkil/bebuas (Malay), sikel (Bidayuh)
6.	*Brassica juncea (*L.*)* Czern. ***	*B. juncea*	Local mustard green (English), ensabi (Iban), sabih (Bidayuh)
7.	*Passiflora foetida* L.***	*P. foetida*	Goat-scented passion flower (English), daun letup (Malay)
8.	*Morus alba* L.**	*M. alba*	Mulberry leaves (English)
9.	*Stenochlaena palustris* (Burm. f.) Bedd. ***	*S. palustris*	Paku midin/lemidin (Iban/Malay)
10.	*Blumea balsamifera* (L.) DC. **	*B. balsamifera*	Sembung/Sambong (Malay)

Plant source: *Semenggok Agriculture Research Centre (ARC) herbarium; ** Dr. Irine Ginjom; ***Batu Kawa Farmer's market.

D. suffruticosa

E. polyantha (a)

E. polyantha (b)

P. serratifolia

P. cordifolia

B. juncea

P. foetida[1]

M. alba

B. balsamifera

S. palustris

Figure 9. Images of leafy plants subjected to extraction.

All names are given in their code name as given in Table 7. Image sources: [1](Kaakkara 2012); others from own collection.

3.2 Chemicals and reagents

All chemicals and reagents were of analytical grade. Methyl-β-cyclodextrin (M-β-CD), 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), neocuproine and cupric chloride dehydrate (Cu(II)Cl), trypan blue, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; methanol from PC Lab; aluminium chloride, $AlCl_3$ from Acros, ethanol from Scharlau; methanol and acetone from Merck; gallic acid from MPBio; anhydrous sodium carbonate, hydrogen peroxide (30 % v/v) and Na_2CO_3 from Bendosen; Folin-Ciocalteau phenol reagent from Nacalai; potassium persulfate, $K_2S_2O_8$ from HmbG; ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) from Amresco; and ammonium acetate, NH_4Ac from Unichem; Fetal Bovine Serum (FBS), Penicillin-Streptomycin (10,000 units), 0.25 % Trypsin-EDTA, Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffer saline (PBS) from Gibco®; and CellTiter 96® Aqueous Non-Radioactive Cell Proliferation assay kit from Promega.

3.3 Preparation of sample extraction

The powdered plant material (0.1 g, dry weight) was immersed in 20 mL 80 % v/v ethanol and the extraction was carried out in a Bransonic 5510 ultrasonic bath (Branson Ultrasonics Corporation, USA) for 20 min at room temperature. The extract solution was centrifuged using Eppendorf 5702 centrifuge 2,500 rpm (Eppendorf AG, Hamburg, Germany) at 2,500 rpm for 10 mins. The resulting supernatant was filtered using a Whatman No.1 filter paper into a 25 mL volumetric flask. The volume was made up to 25 mL with the extraction solvent (80 % ethanol). Each sample was extracted in triplicates. The final concentration of the extracted stock solution was 4,000 mg/L (dry sample weight/solvent volume). Except for the CUPRAC assay, the prepared extracts were analysed immediately or stored in tight containers in the dark at -20°C until further analysis (within 3 days). For CUPRAC sample, 5 mL of the extract was subjected to solvent removal using Labconco 7810037 Acid-Resistant CentriVap Centrifugal Vacuum Concentrator (Labconco Corp., Kansas City, MO) for 200 min at 35°C. The remaining solution was then removed using the freeze-dryer. The weight of the dried extract was recorded and the sample was stored at -20°C until CUPRAC analysis.

A total of 10 plant extract samples were subjected to total phenolic content assay. Plants that showed high phenolic contents (top 5) were further subjected to the total flavonoid assays and the antioxidant assays. Then out of these five samples, only the top 2 samples with the highest antioxidant activities were further tested for their cardio-protective capacities.

3.3.1 Percentage yield

Twenty-five millilitres of selected methanolic plant extracts (4,000 mg/L) were subjected to Rotary Evaporation (Yamato Scientific, Japan), at 35°C, 150 rpm in the dark until the total volume was halved. Afterwards, the extracts were placed in the Labconco 7810037 Acid-Resistant CentriVap Centrifugal Vacuum Concentrator (Labconco Corp., Kansas City, MO) for 200 min at 35°C until the extract was concentrated into 2 mL. Rotary evaporation and Vacuum Concentration was required to remove the presence of highly volatile methanol solvent present in the extract.

The concentrated sample was then subjected to freeze drying for approximately 1 hour in order to remove any remaining moisture within the sample and was continued to do so until constant weight was achieved upon weighing. The percentage yield for the lyophilized dry extract sample was calculated using the following equation:

$$\text{Percentage yield} = \frac{\text{Weight of final dry extract}}{\text{Weight of initial dry powder}} \times 100$$

3.4 Stage I: Screening for phenolic contents

3.4.1 Total phenolic content (TPC)

The total phenolic content of all plant extracts were determined based on a method described previously (Ginjom et al. 2010) with slight modification.

Prior to analysis, the extract stock solution was thawed. The solution was then diluted accordingly to 1 % or 5 % (40 or 200 mg/L, respectively) with distilled water. The reference phenolic standard, i.e. gallic stock solution (1,000 mg/L) was prepared in 80 % ethanol. Prior to analysis, the gallic acid stock was diluted into 0 - 40 mg/L solution with distilled water.

In brief, the total phenolics assay was initiated by adding 100 µL of Folin-Ciocalteau Reagent into 600 µL of sample (diluted extract, diluted gallic acid, or distilled water as blank). The mixture was briefly vortexed and left to stand for 2 min. Then, 300 µL of saturated Na_2CO_3 (20 %) was added and the reaction mixture was mixed again and incubated for 2 hours at room temperature (26°C) in the dark. The appearance of blue-violet colour signified the presence of phenolic compounds. The absorbance at 760 nm was measured on a Genesys 20 Visible Spectrophotometer (Thermo Fisher Scientific, Germany). Quantification was performed using a standard curve generated from the gallic acid solutions (0-40 mg/L) and the total phenolic content was expressed as mg gallic acid equivalent per 100 mg of dry sample weight (mg GAE/100 mg).

3.4.2 Total flavonoid assay (TFC)

The TFC of the plant extracts were determined using aluminium-complexation reaction assay (Amado et al. 2014) with slight modifications. Briefly, the extract stock solution was diluted with distilled water to 10 % (*P. cordifolia* and *D. suffruticosa*) and 20 % (*P. serratifolia* and *E. polyantha*) of its original stock concentration. The reference flavonoid standard, i.e. quercetin stock solution (1,000 mg/L) was prepared in 80 % ethanol. Prior to analysis, the quercetin stock was diluted into 0-60 mg/L solution with distilled water.

The assay involved the mixing of 1 mL of sample (diluted extract, quercetin or distilled water as a blank) to 1 mL of 2 % $AlCl_3$. The development of yellow colour represented the formation of aluminium chloride – flavonoid complex. After 10 min incubation at room temperature (26°C) in the dark, the absorbance readings of the mixture were measured at 415 nm. The total flavonoid content was quantified using the quercetin solution standard curve (0-60 mg/L) and total flavonoid was expressed as mg of quercetin equivalent per 100 mg of dry sample weight (mg QE / 100 mg).

3.5 Stage II: Screening for antioxidant activities

3.5.1 DPPH assay

The selected plant extracts were subjected to DPPH (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl) assay based on the method previously described (Ginjom et al. 2010) with slight modifications.

The DPPH stock solution (80 mg/L) was prepared by dissolving 2 mg of DPPH powder in 25 mL of absolute methanol, immediately before analysis. The plant extracts were diluted into the following range of concentrations: 20 - 120 mg/L for *E. polyantha a* and *D. suffruticosa;* 40 - 240 mg/L for *E. polyantha b*; 50-300 mg/L for *P. cordifolia*; and 200 - 1,200 mg/L for *P. serratifolia*. The stock solutions (1,000 mg/L) of the reference standards prepared with absolute methanol were diluted into the following ranges of concentrations: 1-6 mg/L gallic acid, 4 - 20 mg/L quercetin and 10 - 60 mg/L Trolox.

The DPPH assay was performed on at least 5 different dilutions of each sample (plant extracts and reference standards). A high throughput absorbance reading method was used, with Synergy HT Multi-Detection Microplate Reader (BioTek, USA). All of the solution addition and mixing was conducted in a 96-well flat bottomed clear polystyrene microplate (Corning, USA).

In brief, 100 µL of methanolic solution of DPPH was added to 100 µL of diluted extract solutions or standards. For blank sample, the extract sample was replaced with distilled water. The microplate containing the solution was programmed in the microplate reader to be incubated for

30 min incubation at 25°C in the dark, shaken before and after the incubation period, and the absorbance measured at 517 nm against sample blank. A colour change from purple to yellow signified the presence of antioxidants. The radical scavenging activity (RSA) of the plant extract was calculated using the following formula:

$$\% \text{ RSA} = (1 - A_{sample}/A_{control}) \times 100$$

Where, $A_{control}$ is the absorbance of the blank sample and A_{sample} is the absorbance of the sample or reference standard. The % RSA was used to generate a dose-response curve for all samples. This was then used to determine an `effective concentration' or EC_{50} value. The curve fitting for the EC_{50} estimation was based on non-linear regression fit as generated using GraphPad Prism software. A lower EC_{50} value represents a higher antioxidant activity and the results were expressed as a function of the sample concentration (mg/L).

3.5.2 ABTS assay

The total antioxidant capacity of the plant extracts using the ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) radical cation ($ABTS^{\bullet+}$) was determined according to the method described previously (Re et al. 1999) with few modifications.

In the modified method, the ABTS free radical was prepared by dissolving 19.2036 mg of ABTS reagent in 5 mL of distilled water (final concentration was 7 mM). A 140 mM potassium persulfate, $K_2S_2O_8$, was prepared by dissolving 378.5 mg of $K_2S_2O_8$ in 10 mL of distilled water. Then, 5 mL of 7 mM ABTS solution was mixed with 88 mL of the 140 mM $K_2S_2O_8$ solution and allowed to react for 16 h to form the stable ABTS radical cation. The resulting solution was dark green in colour. On the day of analysis, the $ABTS^{\bullet+}$ solution radical was further diluted with distilled water (1:88) to obtain an absorbance of 0.70 ± 0.02 at 734 nm.

The selected plant extract stock solutions were diluted with distilled water to 0.1 % to 0.3 % of its original concentration. The reference standards, i.e. Trolox and gallic acid 1,000 mg/L stock solutions were prepared in 80 % ethanol. Prior to analysis, the stock solutions were diluted with distilled water (Trolox: 0 - 10 mg/L; gallic acid: 0 - 1.8 mg/L).

For the ABTS assay, 1 mL of $ABTS^{\bullet+}$ solution was added to 0.5 mL of sample (diluted plant extract, standard or distilled water as control), mixed and incubated at room temperature (26°C). Distilled water was used as a reference blank. A colour change from green to colourless signifies the presence of antioxidants (quenching of ABTS radical). The absorbance of the mixture after

30 min of incubation was determined at 734 nm. The antioxidant activity or radical scavenging activity (RSA) of each sample was calculated according to the equation:

$$\% \text{ RSA} = (1 - A_{sample}/A_{control}) \times 100$$

Where $A_{control}$ is the absorbance value of ABTS solution containing distilled water, and A_{sample} is the absorbance due to the residual ABTS solution containing plant extract or reference sample (Trolox or gallic acid) sample. The % RSA of Trolox solutions (0-10 mg/L) and gallic acid (0-1.8 mg/L) solutions were used to generate 2 different standard curves.

The antioxidant activity of the plant samples was estimated based on the standard curve of the standards and were expressed as mg Trolox equivalent antioxidant capacity per 100 mg of dry sample weight (mg TEAC/100 mg) and mg gallic acid equivalent antioxidant capacity per 100 mg dry sample weight (mg GAAC/100 mg).

3.5.3 CUPRAC assay

The CUPRAC (cupric ion reducing antioxidant capacity) method (Apak et al. 2006; Özyürek et al. 2008) determines the ability of a sample to reduce neocuproine-cupric complex (CuII-Nc).

The CUPRAC reagents include 10 mM copper chloride solution, 1 M (pH7.0) ammonium acetate buffer solution, and 7.5 mM neocuprine (Nc) solution (prepared fresh in ethanol). The extract solvent solution was a 2 % methyl-beta-cyclodextrin (M-β-CD), prepared using water-acetone solution (1:9, v/v).

On the day of analysis, a 5 mL aliquot from the stock sample extract solutions (4,000 mg/L) were concentrated to a volume of 2 mL using the Rotary Evaporator (Yamato Scientific, Japan) and freeze dried for 1-2 hours until constant weight. The lyophilized extract sample was dissolved with 5 mL of 2 % M-β-CD which was further diluted to 5.5 mg/L for *E. polyantha b D. suffruticosa, P. cordifolia, P. serratifolia* and *11 mg/L for E. polyantha a.* Similarly, 1 mM stock solutions of the standard reference i.e. Trolox, gallic acid and quercetin, were prepared with 2 % M-β-CD. The standard solutions were diluted to prepare a working solution of 410 µM. The working solutions of each standard were further diluted to the following ranges in order to generate a standard calibration curve during the assay: Trolox (12 - 72 µM); gallic acid (5 - 30 µM) and quercetin (3 - 18 µM).

For the 2,050 µL reagent solution: 500 µL of 10 mM of $CuCl_2$, 500 µL of 7.5 mM Nc solution (freshly prepared in absolute ethanol), and 500 µL of ammonium acetate solution (1 M, pH 7.0) were added. Finally, 550 µL M-β-CD-containing standard or sample solutions in the ranges given

above were added. Following a 30 min of incubation at room temperature, the absorbance of the final solution at 450 nm was read using a UV-Vis Spectrophotometer (Agilent Technologies, USA) against a 2 % M-β-CD reagent blank. A colour change from light blue to yellow suggests the presence of antioxidant.

Trolox results were used to generate a standard curve to generate its molar extinction coefficient and the following equation was used to estimate the total Trolox equivalent antioxidant capacities (TEAC) of the plant extracts:

$$\text{TEAC (mmol TE/g)} = \left(\left(\frac{A_{final}}{\varepsilon_{TR}}\right) \times \left(\frac{V_{final}}{V_{sample}}\right) \times r\left(\frac{V_{CUPRAC}}{m}\right)\right) \times 100/$$

Where the alphabetical letters denote the following:

A_{final} = absorbance of final sample/standard solution
ε_{TR} = molar extinction coefficient of Trolox (1.67 x 10^4 1/mol/cm)
V_f = final (total) volume for reaction (mL)
V_s = sample volume for reaction (mL)
V_{CUPRAC} = initial volume of sample solution (mL)
M = mass of sample (g)
r = dilution ratio

Since the first two of the antioxidant assays were based on the mg weight of reference standard in 100 mg of sample, the final result of the CUPRAC assay was also converted to mg TE/100 g by multiplying the results with Trolox molecular weight (250.20 g/mol) and dividing the value with 10. The results were also compared to gallic acid and quercetin standard curve. The molecular weights of gallic acid and quercetin are 170.12 g/mol and 302.236 g/mol, respectively.

3.6 Stage III: Cell-based cardio-protective activities of selected plant extracts

3.6.1 Cell line, culture and cryopreservation

The H9c2 (2-1) rat cardiomyocyte cells (ATCC® CRL-1446™) were cultured, sub-cultured and cryopreserved as per ATCC® instruction (Collection 2012). The culture media was DMEM containing 10 % FBS and 1 % Penicillin / Streptomycin. Subsequent sub-culturing from the purchased H9c2 (2-1) cells (passage #24) was necessary to obtain cells that are suitable for the bioassay i.e. passage number between 26-30. This was performed in a 75-mL cell culture flask (Eppendorf, USA) where the cells were immersed in the culture media and was incubated at 37 ℃,

in humidified 5 % CO_2 atmosphere until 80 % confluency was reached (~3 days). On the third day, the culture medium was gently removed, and the cell layer was rinsed with 0.25 % (w/v) Trypsin-EDTA 1X solution to remove all traces of serum which contains trypsin inhibitor. The cell layer was dispersed by adding 2 – 3 mL of the Trypsin-EDTA solution to the flask. The detachment was observed under a Nikon Eclipse T*i*-S inverted microscope (Nikon Corporation, Japan), to ensure complete dispersal of cell layer (usually within 5 to 15 minutes). Incubation at 37°C could be used for cells that are difficult to detach. Once dispersed, 6 – 8 mL of culture media (DMEM with 10 % FBS) was added and the cells were gently aspirated. Cell number was estimated using the trypan blue method (Section 3.6.2) and appropriate aliquots of the normal cell suspension (8 x 10^4 cells/mL) were added to new culture vessels, which could be another 75-mL flask (i.e. 25 mL of normal cell suspension) for sub-culturing; or 96-well plate (i.e. 100 µL of normal cell suspension) for bioassay. Some of the cells were also cryopreserved for further use (section 3.6.3).

3.6.2 Cell counting using trypan blue method

Cell counting was required in order to proceed with cell plating and cryopreservation as both methods required a specific amount of cells. Once near confluent cell culture has been trypsinizsed and diluted with an appropriate volume of media (Generally, 5 mL or 10 mL for T-25 and T-75 respectively), 30 µL of the solution was mixed with an equal volume of trypan blue and homogenized using a micropipette. Afterwards, 10 µL of the trypan blue-culture solution was added under each side of the coverslip on the sterile haemocytometer. The haemocytometer was observed under the Nikon Eclipse T*i*-S inverted microscope (Nikon Corporation, Japan). The number of viable cells in each of the quadrants belonging to both chambers was counted using a click count.

The concentration of the original cell suspension, along with the suitable dilutions for cell plating and cryopreservation (8 x 10^4 cells / mL for cell plating and 8.5 x 10^5 cells / mL for cryopreservation) were calculated using the following equations:

$$Concentration\ of\ original\ cell\ suspension = \frac{No.\ of\ viable\ cells\ counted}{No.of\ quadrants\ counted} \times 10^4 \times dilution\ factor$$

$$Volume\ required\ for\ dilution = \frac{Final\ cell\ concentration\ \times Final\ volume}{no.of\ viable\ cells\ in\ original\ cell\ suspension}$$

3.6.3 Cryopreservation of cells

Near confluent cells (70 - 80 % confluency) were harvested by trypsinization. After cell counting, the cells were suspended in DMEM to a final cell density of 8.5 x 10^5 cells / mL. The cells were further pelleted via Eppendorf 5702 centrifuge (Eppendorf AG, Hamburg, Germany) at 15000 rpm for 5 mins. The process was repeated twice. After the removal of the DMEM supernatant, the cells (sediment) were suspended in 2 mL of 10 % of chilled DMSO and aliquoted 1 mL per cryovial. Following the storage of vials at -80^{o}C for 24 hours, they were then stored in a liquid nitrogen tank until revival of cells is required for future use.

3.6.4 Cell viability using MTS assay

Cell viability was determined using the MTS assay kit (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay Kit G5421, Promega). Briefly, after the cell in the well plate was incubated with the test sample, the media was discarded and cells were washed using 50 µL serum free DMEM. Afterwards each well received a 60 µL aliquot mixture containing 10 µL of MTS and 50 µL of serum free DMEM. After incubation at 37^{o}C, 5 % CO_2 for 4 hours, the absorbance reading was measured at 490 nm using Synergy HT Multi-Detection Microplate Reader (BioTek, USA). The percentage of viable cells was calculated using the following formula:

$$\% \text{ Viable cells} = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100$$

Where, A_{sample} is the absorbance of the sample, $A_{control}$ is the absorbance of the respective concentration of ethanol sample, and A_{blank} is the absorbance value of the averaged blanks containing just the MTS reagent and media.

3.6.5 Cell cytotoxicity study of selected extracts, Trolox and H_2O_2

Once the cells reached 80 % confluency upon subcultivation, 100 µL of the cells (8 x 10^4 cells/mL) cell density) were seeded on to a 96 well plate in triplicates and incubated for 24 hours at 37^{o}C, 5 % CO_2.

On the following day, test solutions for the plant extract (*D. suffruticosa* and *E. polyantha b*), reference standard (1000 mg/L, Trolox in absolute ethanol), negative control (absolute ethanol) and an oxidative stress inducer (1000 µM H_2O_2 in DMEM) were all prepared using the DMEM solution. The range of dilutions for each samples were: 15 – 960 mg/L for *D. suffruticosa* and *E. polyantha b*; 2 – 250 mg DE/L for Trolox: 0.02 – 1.0 % for ethanol, and 7.8-1,000 µM for H_2O_2. All of the solutions were diluted with serum free DMEM. The solutions were mixed thoroughly

prior to their addition to the cells in the well plate. After the 24 h incubation, the media from the 96 well plate was removed and the cells were gently washed with 100 µL PBS. Following the aspiration of the PBS, an aliquot of 120 µL from each test solution prepared earlier was introduced to the cells in triplicates. For sample blanks, serum free DMEM media was added to the cells instead of any of the test solutions. Cell blank well contained only the serum free DMEM and no cells. All assays were performed or repeated at least two times using the same cell passage number. Each plate would have contained a set (triplicates) of ethanol (negative control); test solution sets (triplicates) and the two types of blanks. An example of the sample arrangement in a well plate is shown in Table 8.

Table 8. An example of a 96-wellplate layout for the MTS assay with concentration ranges of *D. suffruticosa* and *E. polyantha b* added to H9C2 (2-1) cells.

Well no.	Ethanol (%)			Extract sample (mg DE/L)*						Blank samples		
				D. suffruticosa			*E. polyantha b*					
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.00			960			960			B1: Cells + media		
B	0.50			480			480			B2: Media only		
C	0.30			240			240			Empty cells		
D	0.13			120			120					
E	0.06			60			60					
F	0.03			30			30					
G	0.02			15			15					
H	0.01			7.5			7.5					

*Highest concentration is 960 mg/L for both extract samples. Except for wells B10-12 and the empty wells, all of the other wells were seeded with cells.

The well plate containing the cells and the test solutions was then further incubated for 24 h at 37°C, 5 % CO_2. Then, the MTS assay was performed as described earlier (Section 3.6.4). The percentage of viable cells and the test sample concentration were used to generate a dose-response curve for all samples. This was then used to determine the half maximal inhibitory concentration (LD_{50}) value of the test sample.

3.6.6 Induction of oxidative stress and inhibition by plant extracts and Trolox

In this assay, the cells were pre-incubated with the plant extracts (*D. suffruticosa, E. polyantha b*) or Trolox, and then subjected to oxidative stress, as induced by H_2O_2. The ability of the extracts

or Trolox to protect the cells from the damaging effect (cell death) of H_2O_2 would infer their potential as antioxidant and also cardio-protective agents.

The assay was initiated by seeding the H9c2 (2-1) cells in the 96-well plate and incubated at 37ºC and 5 % CO_2 for 24 hours. After washing, the cells were further incubated for 24 h with *D. suffruticosa* (7.5, 15 and 30 mg DE/L), *E. polyantha b* (60, 120 and 240 mg DE/L), and Trolox (2 and 3.9 mg/L) in the same method as described in Section 3.6.3. After the 24 h incubation, the cells were washed using 100 µL serum free DMEM and it was replaced with varying concentrations of freshly prepared H_2O_2 solution in DMEM (250 – 1000 µM) to induce oxidative stress. The treated cells were incubated for another 24 h at 37ºC and 5 % CO_2 and MTS assay was performed thereafter to assess the % cell viability.

3.7 Statistical analysis

All results were expressed as mean ± standard deviation of at least three different experimental repetitions per sample. The Student's t-tests, simple linear regression and correlation analysis statistical analysis was performed using the Microsoft® Excel Analysis ToolPak (Microsoft® Professional Plus 2010) while the curve fitting for the EC_{50} and LD_{50} estimation based on non-linear regression fit was performed using GraphPad Prism® version 6.01 for Windows (GraphPad Software, la Jolla California USA). In all cases, statistical significance was set at $p<0.05$.

4 RESULTS AND DISCUSSION

4.1 Sample drying and extraction of phenolic compounds

4.1.1 Moisture content

The drying process was achieved using the freeze-drying method. The plant samples contained between 63 % (mulberry leaves, *M. alba*) to 94 % (`daun ensabi', *B. juncea*) moisture content (Table 9). This shows the high range of moisture content in various leaves sample, which is common in plants. In the following antioxidant analysis, the values were expressed based on both wet- or fresh weight (FW) and dry weight (DW) basis. The dry weight basis normalised the values so that comparisons could be made without the dilution effect of water or moisture in the sample. The fresh weight basis provided the value that is present in fresh samples, in the form where these plants or leaves are commonly consumed.

Table 9. Moisture content of plant leaves used after the freeze-drying process.

Local name	Code name	Moisture content (%)
Buan/Simpoh Air	*D. suffruticosa*	74.8 ± 2.2
Bungkang (Small leaf)	*E. polyantha (a)*	67.2*
Bungkang (Soft leaf)	*E. polyantha (b)*	72.0 ± 0.3
Daun ensabi	*B. juncea*	93.7 ± 2.7
Daun letup	*P. foetida*	77.5 ± 2.3
Mulberry leaves	*M. alba*	62.7 ± 12.9
Pucuk midin	*S. palustris*	81.6 ± 7.7
Sembung	*B. balsamifera*	79.1*
Singkil laut	*P. serratifolia*	76.8 ± 0.3
Singkil / Buas	*P. cordifolia*	73.9 ± 1.7

Values are means ± standard deviation (n=3). *Values from a single determination.

4.2 Stage I: Screening for phenolic contents

4.2.1 Total phenolic and total flavonoids contents

Total phenolic content (TPC) in plants typically includes phenolic acids, flavonoids and diterpenes. While different plant species will have different phenolic content and composition, the phenolic contents obtained from same plant species can vary due growth conditions such as soil nutrient and environmental conditions such as exposure to light, carbon dioxide levels, temperature, biotic and abiotic factors (Jaafar et al. 2008; Briskin & Gawienowski 2001; Alam, Bristi & Rafiquzzaman 2013.

In this assay, the total phenolic content was expressed as gallic acid equivalent (mg GAE/100 mg) while the total flavonoid content was expressed as quercetin equivalent (mg QEA/100 mg). Based on the weight data obtained from the drying and extraction process, all of the TPC and TFC values were also expressed based on the fresh weight (FW), dry weight (DW) and dry extract weight (DE). This range of values allow for easier comparison with other literature values that expressed their values in either one of these weights. The calibration curve used to estimate the TPC and TFC of the plant samples shown in Appendix A (p.78).

Preliminary TPC screening results (**Table 10**) showed that the TPC values ranged from 1.3 ± 0.1 mg GAE/100mg DW to 15.6 ± 0.6 mg GAE/100mg DW, with the highest recorded in the soft leaf-type `bungkang' (*E. polyantha* b). The top five plants with the highest TPC contents (*E. polyantha (b), D. suffruticosa*, *P. cordifolia*, *E. polyantha (a)* and *P. serratifolia*) were chosen to be further analysed for the determination of extraction yield, total flavonoids other antioxidant activity assays.

The TPC values of the top 5 samples in the current study constantly showed higher phenolic contents than those reported in other studies. The TPC value of both types of *E. polyantha* recorded in this study is approximately 7 to 14 times higher than those reported in another study (Wong, Leong & Williamkoh 2006). Another study reported the high TPC contents in the roots and fruits of `simpoh air' (*D. suffruticosa*) compared to its leaves (23.6 ± 2.37 mg GAE/100 mg leaf extract) (Armania et al. 2013). However, this is only half of the concentration of TPC found in the present study for *D. suffruticosa* and three times less than *E. polyantha (b*). The TPC per estimated extract of `singkil' or *P. cordifolia* is about 4 times higher than those reported for the same sample (2.19 ± 0.4 mg GAE/100 mg DW) (Sulaiman et al. 2011). Ghimire et al. (2011) reported a TPC content of 14.3 ± 2.37 mg GAE/ 100 mg DE in the bark of `singkil laut' or *P. serratifolia.* This value is comparable to the TPC value of 13.2 ± 0.4 mg GAE/ 100 mg DE found for *P. serratifolia* leaves in this study.

Table 10. Summary of total phenolic content (TPC) and total flavonoid content (TFC) of selected plant extracts.

Extract	Percentage yield (%, w/w)	Average of total phenolic content			Average of total flavonoid content		
		(mg GAE/ 100mg DW)	(mg GAE/ 100mg FW)	(mg GAE/ 100mg DE)	(mg QE/ 100mg DW)	(mg QE/ 100mg FW)	(mg QE/ 100mg DE)
*E. polyantha (b)**	32.8	15.6 ± 1.0^{a}	4.4 ± 0.3^{ac}	48.7 ± 1.6^{ad}	1.0 ± 0.2^{d}	0.2 ± 0.0^{d}	3.4 ± 0.4^{d}
*D. suffruticosa**	23.4	15.0 ± 0.6^{ab}	3.8 ± 0.1^{b}	64.2 ± 2.5^{b}	1.9 ± 0.1^{a}	0.5 ± 0.1^{a}	8.5 ± 0.1^{e}
*P. cordifolia**	33.4	8.1 ± 0.7^{bce}	2.1 ± 0.2^{ced}	24.2 ± 2.3^{c}	1.7 ± 0.0^{a}	0.5 ± 0.0^{a}	5.1 ± 0.0^{a}
*E. polyantha (a)**	29.9	7.6 ± 0.9^{ce}	2.5 ± 0.3^{d}	27.6 ± 2.3^{d}	1.0 ± 0.1^{cd}	0.3 ± 0.1^{bcd}	3.2 ± 0.4^{cd}
*P. serratifolia**	32.2	4.3 ± 0.1^{dfg}	1.0 ± 0.0^{eh}	$13.\ 2 \pm 0.4^{e}$	1.1 ± 0.3^{bcd}	0.3 ± 0.1^{bd}	3.5 ± 0.8^{bcd}
S. palustris	-	4.3 ± 0.3^{fg}	0.8 ± 0.1^{ghi}	-	-	-	-
B. balsamifera	-	4.1 ± 1.7^{gh}	0.9 ± 0.4^{hij}	-	-	-	-
P. foetida	-	2.7 ± 0.7^{h}	0.6 ± 0.2^{ij}	-	-	-	-
M. alba	-	1.2 ± 0.1^{ij}	0.5 ± 0.1^{j}	-	-	-	-
B. juncea	-	1.3 ± 0.1^{j}	0.1 ± 0.0^{k}	-	-	-	-

All values are expressed as mean ± standard deviation (n=3). *Samples chosen for antioxidant activity assays. DW refers to the dry sample weight; FW refers to the fresh sample weight; and DE refers to the dry extract weight. Means in a column followed by different letters differ significantly ($P < 0.05$) as analysed using Student's T-test.

Profound research carried out on green tea and black tea antioxidants have shown that they are rich sources of antioxidants. Atoui et al. (2005) reported that Chinese green tea and Ceylon black tea possessed high phenolic contents, with TPC values of 67.5 ± 1.7 mg GAE/ 100mg DW and 47 ± 0.49 mg GAE/100mg DW, respectively. These are 3 to 4 times higher than the TPC value of *E. polyantha (b)*, the top TPC sample in the present study. However, the TPC values of black and green tea can vary. For example, a Turkish black tea was found to have the TPC value of 5.6 ± 0.17 mg GAE/100 mg DW (Turkmen, Sari & Velioglu 2006) while an unspecified type of green tea contained 13.5 mg GAE/100 mg DW. In this case, the TPC value of *E. polyantha (b)* in the current study is 2.8 times and 1.2 times higher than the reported TPC in the Turkish black tea and green tea, respectively. Apak et al. (2013) reported the TPC of some common vegetables such as red onion, red pepper and garlic. Red onion showed the highest TPC with value of 1.56 mg GAE/100 mg FW. This is only slightly higher that *P. serratifolia* (TPC = 1 mg GAER/ 100 mg FW), which is ranked the 5th highest TPC sample in the current study.

In this assay, out of the five plants selected, the two highest total flavonoid content (TFC) were found in *D. suffruticosa* and *P. cordifolia* leaves at 1.9 ± 0.1 mg QE/100 mg DW and 1.7 ± 0.0 mg QE/100 mg DW, respectively. This is an interesting point as their respective TPC values (Table 2) were lower in rank than the *E. polyantha (b)* leaf extract. A relative comparison of the total flavonoid fraction of the five samples analysed shows that most of the plant sample phenolic extract contained more than 10 % flavonoids, except for *E. polyantha* (a), where only 6 % of its phenolic compounds are flavonoids. This is not surprising as the composition of actual groups of phenolic compounds in different plant species varies.

When compared to the other studies, most of these values are comparable or higher than those reported in similar samples. For *P. cordifolia* shoot, the current study recorded more than 7 times more TFC content than those reported by Sulaiman et al. (2011). Ghimirie et al. (2011) analysed the bark of *P. serratifolia* and reported a lower TFC value (2.21 ± 2.04 mg QE/100 mg DE) compared to the leaves of *P. serratifolia* in the current study (3.5±0.8 mg QE/100 mg DE).

Total flavonoids contents were also performed on other types of plants, such as *Ziziphus jujuba* L (Chinese/Korean date or jujube), which is traditionally consumed to cure various diseases. The TPC and TFC analysis on *Z. jujuba* (Al-Saeedi, Al- Ghafri & Hossain 2016) reported values that are 10 to 1000 times lower (TPC = 6.8 mg GAE/100 mg DE; TFC = 0.009 mg QAE/100 mg DE) than those reported for *D. suffruticosa* in this study.

The phenolic and flavonoid contents in the selected plant samples were found to be comparable, and in many cases, are far superior to other previously reported plants. Thus, they can be considered as good source of phenolic compounds.

4.3 Stage II: Screening for antioxidant activities

4.3.1 Determination of radical scavenging activity based on DPPH assay

The effective concentration or EC_{50} values were estimated using the quadratic equation from the curve generated for each phenolic reference standard solution and plant extract sample solution (Figure 10 and Figure 11). The EC_{50} value is based on the concentration of the test solution depicting 50 % radical scavenging activity (% RSA). Lower EC_{50} value means higher antioxidant activity.

Based on the EC_{50} values, the three reference compounds all showed very low values (1.4 – 13.2 mg DW/L), indicating a very high radical scavenging or antioxidant activity. None of the crude plant extract is within the EC_{50} range of the three reference compound, which is expected due to the crude nature of the extract. Of the five extracts analysed, *E. polyantha (b)* extract has highest antioxidant activity, as denoted by the lower EC_{50} value of 37.8 ± 9.9 mg DW/L. This is about 3 times weaker than Trolox or 27 weaker than gallic acid. Another DPPH analysis on *E. polyantha* reported an antioxidant activity that was 1.8 times stronger than those reported for the same sample in the current study (Perumal et al. 2012).

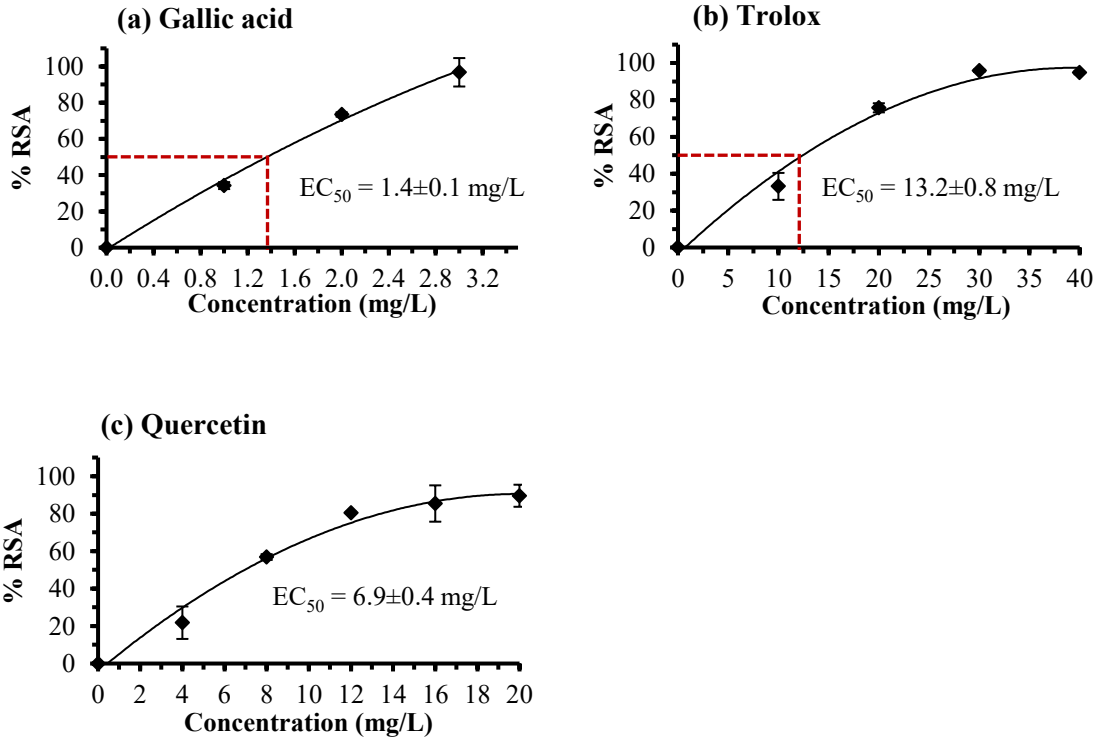


Figure 10. Dose response curve of phenolic reference standards: (a) gallic acid, (b) Trolox, and (c) quercetin, as determined using the DPPH assay. Data shown are mean ± standard deviation (n=3).

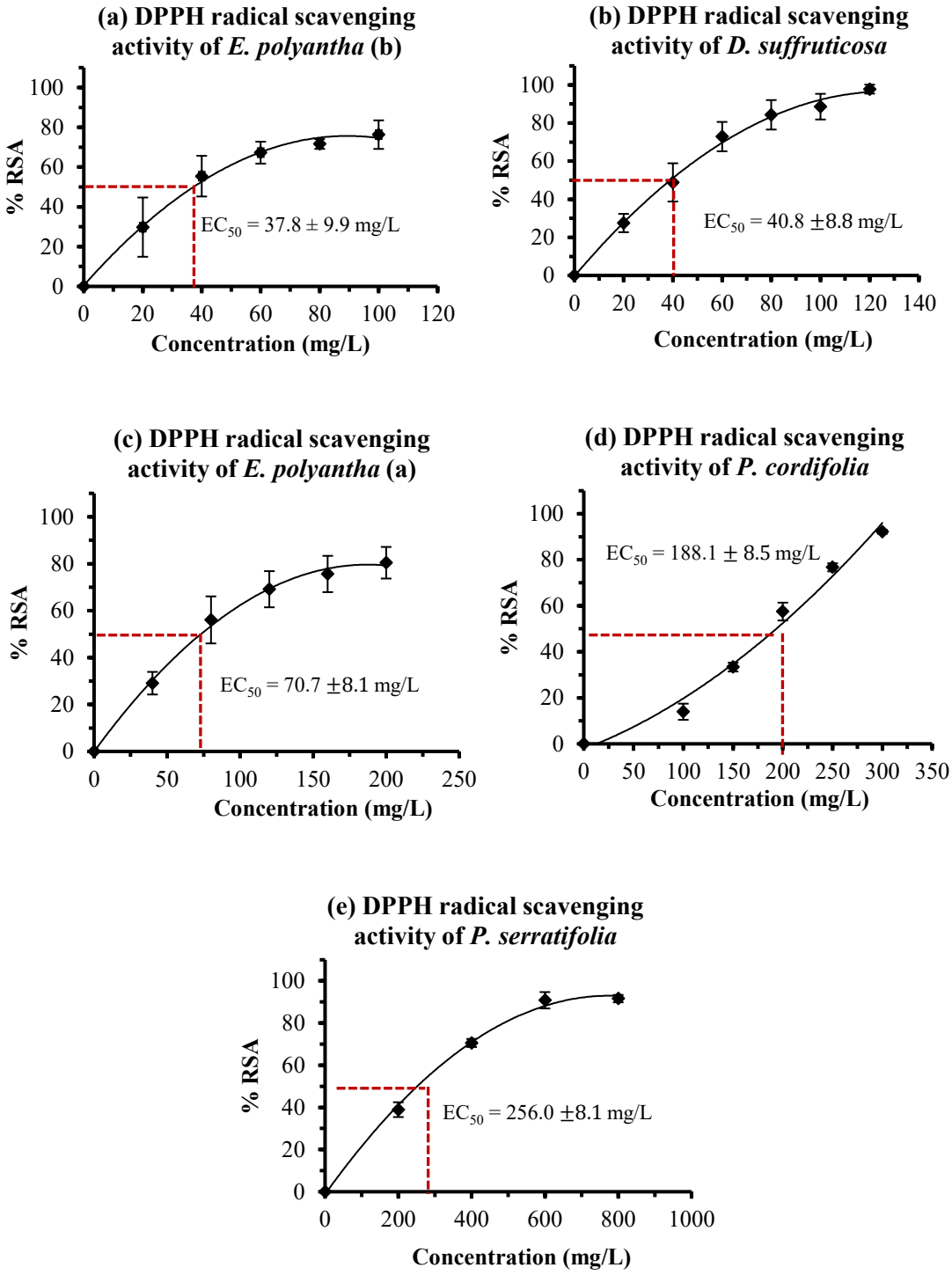


Figure 11. Dose response curve of plant extracts: (a) *E. polyantha* b, (b) *D. suffruticosa*, (c) *E. polyantha* a, (d) *P. cordifolia*, and (e) *P. serratifolia*, as determined using the DPPH assay. Data shown are mean ± standard deviation (n=3).

Green tea remains a powerful source of antioxidant. The Japanese green tea was reported to have an EC_{50} value of 5.95 mg/L when assessed using the similar DPPH assay (Mongkolsilp et al. 2004) (Mongkolsilp et al. 2004). This value suggested an antioxidant activity which is comparable to those of pure quercetin, and 6 times stronger than those of *E. polyantha* (b), the best antioxidant in the current study. However, when compared to lemongrass, which is a common herb used in local cooking, all of the plants in the current study appeared to possess superior amount of antioxidants. The antioxidant activity of lemongrass, based on its EC_{50} value of 1,140 mg/L (Mongkolsilp et al. 2004) is at least 4 times weaker than any of the plant samples analysed in the current study.

While it may not be as `powerful' as the Japanese green tea, all of the five plants samples selected in the current study are decent source of antioxidants.

4.3.2 Determination of antioxidant activity based on ABTS assay

This assay is another assay that estimated the antioxidant activity of a phenolic compound based on its ability to reduce the coloured ABTS radical. The presence of an antioxidant is denoted by the loss of green colour of the ABTS radical, and measurable at 734 nm.

In addition to the common standard, Trolox for the reference standard, gallic acid was also added as another reference compound as it was also used as the reference in the previous total phenolic content assay. The calibration curve of both Trolox and gallic acid are shown in Figure 12 while the respective antioxidant activity of each plant extract is summarised in Table 11.

Based on the results obtained, *D. suffruticosa* leaves show the highest antioxidant activity of 292.6 mg TE/100 mg DE and 55.6 GAE mg/100 mg DE in terms of Trolox and gallic acid equivalency, respectively. This value is about 4 times higher than those reported earlier on the root extract of *D. suffruticosa* which was 75.1 mg TE/100 mg DE (Armania et al. 2013). It is also apparent that the TAC values in terms of Trolox equivalency (TE) in all plants seems to be higher than that with gallic acid (GAE). This is possibly because per gram gallic acid possess a much higher antioxidant activity that per gram of Trolox. This was further established by the data from the DPPH assay earlier.

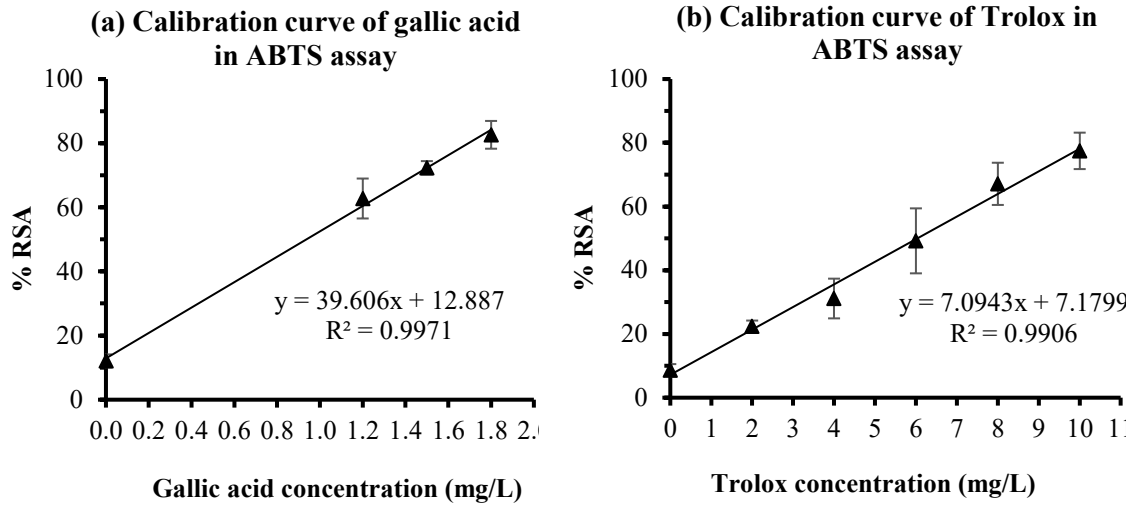


Figure 12. Dose response curve of (a) gallic acid and (b) Trolox for radical scavenging activity (RSA) as determined by the ABTS assay. Data shown are mean ± standard deviation (n = 3).

Table 11. The summaries of total antioxidant capacities of selected plant extracts as determined using the ABTS assay.

Plant sample extract	Antioxidant activity (mg TE/100 mg)		
	DW	**FW**	**DE**
D. suffruticosa	68.4 ± 6.5^{d}	17.2 ± 1.3^{d}	292.6 ± 22.2^{d}
E. polyantha (b)	64.7 ± 3.9^{cd}	18.1 ± 0.9^{cd}	197.4 ± 9.6^{c}
E. polyantha (a)	30.7 ± 6.2^{b}	10.1 ± 2.7^{b}	102.7 ± 27.9^{b}
P. cordifolia	15.5 ± 1.3^{a}	4.0 ± 0.7^{a}	46.3 ± 8.2^{a}
P. serratifolia	13.9 ± 0.2^{a}	3.3 ± 1.1^{a}	43.1 ± 14.8^{a}
	Antioxidant activity (mg GAE/ 100 mg)		
	DW	**FW**	**DE**
D. suffruticosa	13.0 ± 1.0^{d}	3.3 ± 0.5^{d}	55.6 ± 8.0^{d}
E. polyantha (b)	12.3 ± 0.6^{cd}	3.4 ± 0.5^{cd}	37.4 ± 5.3^{c}
E. polyantha (a)	5.8 ±1.1^{b}	1.9 ± 0.3^{b}	19.4 ± 3.1^{b}
P. cordifolia	2.9 ± 0.3^{a}	0.8 ± 0.2^{a}	8.7 ± 2.7^{a}
P. serratifolia	2.6 ± 0.0^{a}	0.6 ± 0.3^{a}	8.1 ± 4.2^{a}

All values are expressed as mean ± standard error (n=3). DW= Dry weight of powdered sample, FW = fresh weight of powdered sample, DE = dry extract weight of powdered sample. Means in a column followed by different letters differ significantly ($P < 0.05$) as analysed using Student's T-test.

4.3.3 Determination of antioxidant capacity based on CUPRAC assay

The cupric ion reducing antioxidant capacities were analysed in this assay in terms of Trolox gallic acid and quercetin equivalencies (TEAC, GEAC and QEAC respectively). The calibration curves of these standards are shown below in Appendix B.

The antioxidant activity as determined using the CUPRAC assay is typically expressed as Trolox equivalence. In the current study, the antioxidant activity was also expressed as gallic acid and quercetin equivalence. The antioxidant activities of the selected plant extracts are summarised in Table 12.

The highest antioxidant activity in terms of Trolox equivalency was reported in the *E. polyantha* (b) extract (46.6 ± 6.0 mg TE/100 mg DW); while the lowest was recorded in the *P. cordifolia* extract (3.0 ± 1.6 mg TE/100 mg DW). All three *D. suffruticosa*, *E. polyantha* (b) and *E. polyantha* (a) samples consistently recorded the top three highest antioxidant activities in the three assays – DPPH, ABTS and CUPRAC. In both DPPH and ABTS assays, the antioxidant activity of *D. suffruticosa* > *E. polyantha* (b) > *E. polyantha* (a). In the CUPRAC assay, the order was slightly different. It was recorded that the antioxidant activity of *E. polyantha* (b) > *E. polyantha* (a) > *D. suffruticosa*.

All three assays measure the antioxidant activities of chemicals via electron transfer (ET) mechanisms (Apak et al. 2007). This shows that even though the assays measure the same antioxidant mechanisms, variation still exist due to the different types and composition of the chemicals present in the sample. These result in different chemical structure, their solubility in the test solution and hence the resulting measurable antioxidant activities (Apak et al. 2007; Shahidi & Zhong 2011). The addition of methyl-beta-cyclodextrin in the CUPRAC assay allows the assay to measure both hydrophilic and hydrophobic antioxidants. Thus, it is possible that *E. polyantha* sp. especially *E. polyantha* (a) contains a higher fraction of hydrophobic antioxidants which were not effectively measured in the previous DPPH and ABTS assays. In summary, the present result suggests that the CUPRAC assay was able to differentiate the antioxidant activity of samples having different phenolic compositions slightly different antioxidant components compared to the DPPH and ABTS assay.

Table 12. The summaries of total antioxidant capacities of selected plant extracts as determined using the CUPRAC assay.

Plant sample extract	Antioxidant activity (mg TE/100 mg)		
	DW	**FW**	**DE**
E. polyantha (b)	46.6 ± 6.0[a]	19.9 ± 1.0[a]	142.2 ± 18.3[a]
E. polyantha (a)	39.4 ± 7.7[a]	19.8 ± 11.0[ac]	131.7 ± 25.9[a]
D. suffruticosa	30.1 ± 2.2[a]	16.5 ± 2.1[a]	128.4 ± 9.4[a]
P. serratifolia	11.5 ± 1.7[b]	0.5 ± 0.0[bc]	35.7 ± 5.3[b]
P. cordifolia	3.0 ± 1.6[c]	10.2 ± 0.0[bc]	9.1 ± 4.7[c]
	Antioxidant activity (mg GAE/ 100 mg)		
	DW	**FW**	**DE**
E. polyantha (b)	11.6 ± 1.5[a]	4.9 ± 0.3[a]	35.3 ± 4.5[a]
E. polyantha (a)	9.8 ± 1.9[a]	4.9 ± 0.3[ac]	32.7 ± 6.4[a]
D. suffruticosa	7.7 ± 0.5[a]	4.1 ± 0.5[a]	31.9 ± 2.3[a]
P. serratifolia	2.9 ± 0.4[b]	0.5 ± 0.0[bc]	8.9 ± 1.3[b]
P. cordifolia	0.8 ± 0.4[c]	2.5 ± 0.0[bc]	2.3 ± 1.2[c]
	Antioxidant activity (mg QE/ 100 mg)		
	DW	**FW**	**DE**
E. polyantha (b)	13.4 ± 1.7[a]	5.7 ± 0.3[a]	41.0 ± 5.3[a]
E. polyantha (a)	15.0 ± 6.5[a]	5.7 ± 3.2[ac]	37.9 ± 7.5[a]
D. suffruticosa	8.7 ± 0.6[a]	4.8 ± 0.6[a]	37.0 ± 2.7[a]
P. serratifolia	3.3 ± 0.5[b]	0.6 ± 0.0[bc]	10.3 ± 1.5[b]
P. cordifolia	0.9 ± 0.5[c]	2.9 ± 0.0[bc]	2.6 ± 1.4[c]

All values are expressed as mean ± standard error (n=2). DW= Dry weight of powdered sample, FW = Fresh Weight of powdered sample, DE = Dry Extract Weight of powdered sample. Labels within the same column with different letters represent significantly different ($P<0.05$) values as analysed using Student's T-test (Results not shown).

It is also apparent that the CUPRAC antioxidant values of all the plant samples in this study were exceptionally higher (3.0 – 46.6 mg TE/100 mg DW) than those recorded for common western herbs such as rosemary, oregano, thyme, sage and peppermint leaves (0.1 - 0.2 mg TE/ 100 mg DW) (Luminița 2015). However, when compared to Ceylon black tea (110.4 mg TE/100 mg DW), the antioxidant activity of selected plant extracts are still weaker (Apak et al. 2006), by 2 to 36 times.

4.3.4 Correlation analysis between total phenolic and antioxidant activities of selected plant extracts

Potential correlations between various assays used in this study were evaluated using the Pearson's regression analysis. Of the ten possible pairs (Table 13), only one showed statistically significant correlations ($p<0.05$), which was the total phenolic content (TPC) and the ABTS pair ($r = 0.9557$, *p*-value = 0.0111). DPPH also showed some positive correlation with the TPC value ($r = 0.8437$) but the relationship was not statistically significant (*p*-value = 0.0724). Similarly, CUPRAC data also showed some positive correlation ($r = 0.6793$) with the TPC values, but the correlation was not statistically significant. Total flavonoid content (TFC) showed very weak and insignificant correlation ($r = 0.2827$) with the TPC. This suggested that the ABTS-based antioxidant activity is strongly affected by the total phenolic content present in the test sample.

In general, the total phenolic contents are better predictor of the antioxidant activity of the samples when compared to the total flavonoid contents. This is possibly due to the presence of non-flavonoid phenolic components that also contribute towards the plant samples' antioxidant activity. Among the three antioxidant assays, ABTS and CUPRAC values were more correlated ($r = 0.4582$) to each other compared to other pairings. However, the data was not statistically significant (*p*-value = 0.4377). Lack of strong correlations among the three antioxidant assays further indicated that multiple antioxidant analysis is needed to represent the `true' antioxidant activity of a compound or sample. This is especially true when dealing and comparing samples of different genera or even different species.

The CUPRAC antioxidant values shows very weak correlations with all the other parameters, suggesting that it possibly measure antioxidants that are of phenolic- as well as non-phenolic based. It is possible that the presence of other plant phenolic substances such as tannins or terpenes such as tocopherols and tocotrienols along with non-sulphur compounds found in leaves such as saponins were responsible for such activity (Dimitrios 2006; Zheleva-Dimitrova, Nedialkov & Kitanov 2010).

In this study, the five plant samples selected for the antioxidant assays came from 3 distinct genera – *Dillenia* spp. (*D. suffruticosa*), *Eugenia* spp. (*E. polyantha* a and b), and *Premna* spp. (*P. serratifolia* L. and *P. cordifolia*). Of the three genera, *Dillenia* spp. and *Eugenia* spp. consistently showed high antioxidant activities in all antioxidant assays, while the *Premna* spp. always falls behind. Hence, it is further confirmed that different plant genera and species lead to different phenolic content, composition and antioxidant activities.

The methanol used for solvent extraction may have played an important role for the strong correlation between TPC and DPPH as it is more efficient in extracting lower molecular weight polyphenols found in plant leaves such as ellagic acid, ellagitannins, hydroxycinnamic acid and chlorogenic acid (Dai & Mumper 2010; Oszmiański et al. 2011).

Table 13. Correlation of all phenolic contents and antioxidant activities of *E. polyantha* (b), *D. suffruticosa*, *P. cordifolia*, *E. polyantha* (a), and *P. serratifolia*.

	TPC (mg GAE/100 mg DW)	**TFC (mg QE/100 mg DW)**	**DPPH (EC_{50}, mg DW/L)**	**ABTS (mg TE/100 mg DW)**
TFC (mg QE/100 mg DW)	0.2827 (0.6449)			
DPPH (EC_{50}, mg DW/L)	0.8437 (0.0724)	0.0462 (0.9412)		
ABTS (mg TE/100 mg DW)	0.9557 (**0.0111**)	0.1800 (0.7721)	0.2682 (0.6627)	
CUPRAC (mg TE/100 mg DW)	0.6793 (0.2072)	0.3618 (0.5496)	0.0919 (0.8831)	0.4582 (0.4377)

Values expressed as Pearson correlation coefficient (r) with *p*-value in brackets. Values in bold are significantly different at $P<0.05$.

In summary, the TPC helps to make the initial inferences about antioxidant activities, especially the antioxidant activity based on the ABTS assay. All of the three assays used in this study should be considered to provide a more comprehensive data on the antioxidant activity of a sample. This is especially important when the samples to be compared are of different genera and species. However, the results of the ABTS and the DPPH assays are almost similar, at least in terms of antioxidant ranking. Thus, when analysing sample of the same species, it is possible to omit one of the assays. DPPH is much simpler to conduct compared to the ABTS assay, thus ABTS assay could be omitted. The CUPRAC should be included as it provides a slightly different outcome, which allow the set of antioxidant assay to be as inclusive as possible. This is because; the antioxidant in each plant sample varies, in terms of composition and their mechanism or antioxidant actions.

4.4 Stage III: Cell-based cardio-protective activities of selected plant extracts

4.4.1 Cell cytotoxicity assessment of test solutions

To find the suitable working concentration of H_2O_2 and the plant extracts, a series of dose-response assays was performed on the H9c2 cells, and their viability after a 24-hour incubation in the test solution was assessed using the MTS assay. Treatment with increasing H_2O_2 concentration reduced the cell viability (Figure 13). The concentration of H_2O_2 that resulted in 50 % lethality (LD_{50}) was estimated to be 124 μM. A 250 and 500 μM of H_2O_2 were used in the subsequent experiments. These correspond to 40 %, 20 % and 8 % cell viability, respectively. H9c2 (2-1) cells have previously exhibited necrosis between 250-600 μM, while apoptosis was observed at 250 μM or below (Chen et al. 2000). While these data correspond with the lethal dose obtained in this study, interestingly, evidence provided by Chen et al. (2000) further suggests that cells exposed to hydrogen peroxide for more than 5 hours, exhibited hypertrophy which has been a cause for heart failure.

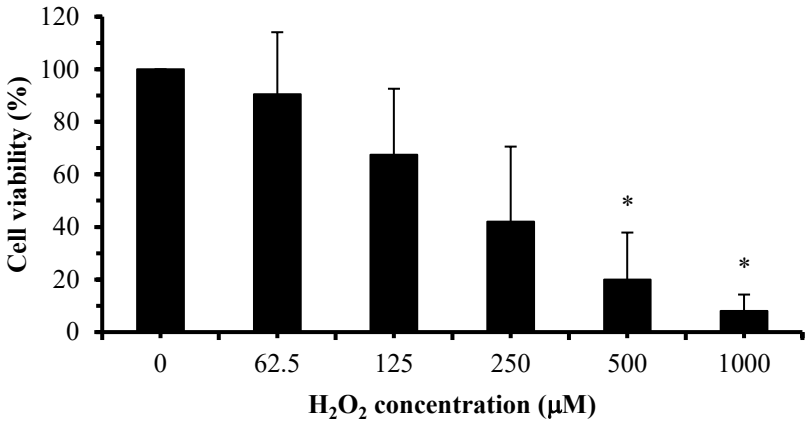


Figure 13. Concentration effects of H_2O_2 on the survival of H9c2 (2-1) rat cardiomyocyte cells. Data shown are mean ± standard deviation (n=3). Mean with asterisk (*) are significantly different ($p<0.05$) from control at 0 μM.

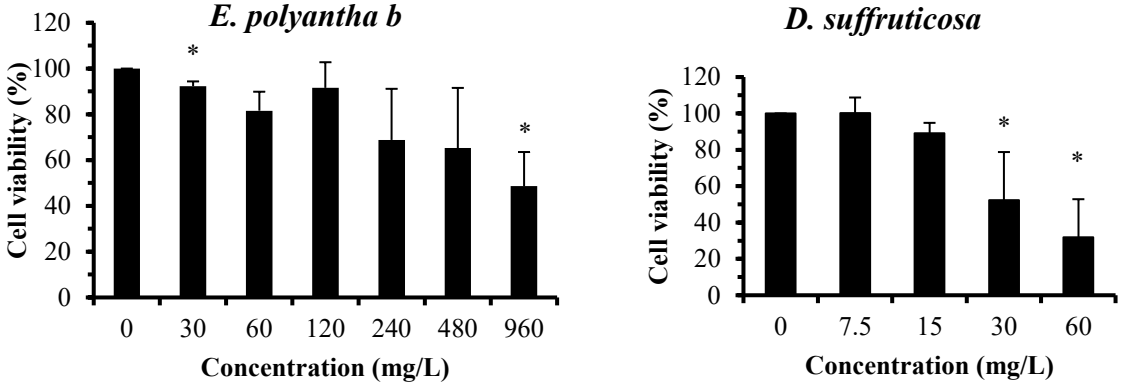


Figure 14. Concentration effects of (a) *E. polyantha b* and (b) *D. suffruticosa* on the survival of H9c2 (2-1) rat cardiomyocyte cells. Data shown are mean ± standard deviation (n = 3). Mean with asterisk (*) are significantly different ($p<0.05$) from control at 0 mg/L.

Of the five plant extracts assessed for their antioxidant activities, only the *E. polyantha* (b) and *D. suffruticosa* extracts were selected for Stage III cell culture assays due to their constantly high antioxidant activities in all assays. Of the two samples, *E. polyantha* extract was less toxic to the cells compared to *D. suffruticosa* extract (Figure 14). The LD_{50} value for the *E. polyantha* was 649 mg/L while for *D. suffruticosa*, it was ten times more toxic with LD_{50} value of 60.3 mg/L. For the subsequent experiments, concentrations below the LD_{50} values were used. For the *E. polyantha* extract, concentrations of 60, 120 and 240 mg/L were used. For *D. suffruticosa*, concentrations of 15 and 30 mg/L were used. Trolox was found to be non-toxic at concentration range of 0 to 250 mg/L while ethanol solution was found to be non-toxic at concentrations below 1 % (results not shown). The *D. suffruticosa* extract had similar cytotoxicity in comparison to the LD_{50} of BJLN rice extracts (64.6 mg/L) on H9c2 cells (Tan et al. 2016).

Cytotoxicity of the two plant extracts have not been reported before on H9c2 cell lines. However, the leaves of *D. suffruticosa* obtained from Terengganu state if Malaysia have also been tested against cancer cell lines such as human breast cancer cells (MCF-7), human cervical adenocarcinoma (HeLa) and human colon cancer (HT29) cells and non-cancer Swiss mouse embryo fibroblast cells (3T3 F442A) (Armania et al. 2013). The study showed that *D. suffruticosa* was not only cytotoxic towards all the cancer cell lines, but was also highly toxic towards non-cancer adipocyte cells, 3T3-F442A (LD_{50} = 30 mg/L). Similarly, extract of *Nerium oleander* and *Thevetia nerifolia* from South Africa have exhibited both cardioprotective and cardiotonic effects on Guinea pig heart cells (Akinmoladun, Olaleye & Farombi 2014).This pointed that *D.*

suffruticosa can be used as an anticancer agent, but it is also potentially toxic to cardiac cells as well as adipose tissues.

Therefore, thorough studies should be conducted on the bioactive compounds in *D. suffruticosa* to evaluate the full potential of this plant as a therapeutic agent and to avoid any toxicity effect.

The Vero cell line CCL-81 (kidney epithelial) has been previously tested with *E. polyantha* leaf extracts. The LD_{50} has been recorded at 53.5 mg/L (Perumal et al. 2012), suggesting that the extract was also quite toxic to normal kidney cells. This shows that the cytotoxicity effect of the plant samples varies with different type of cell lines.

4.4.2 Protective effect of Trolox against H_2O_2-induced cell death

Two concentrations of Trolox were chosen to be tested against two concentrations of H_2O_2 pre-treated H9c2 cells. In the previous section, it was noted that higher concentrations of H_2O_2 (500 µM and above) are cytotoxic towards the cells. Figure 15 shows the cardioprotective effect of Trolox at increasing concentrations. Trolox at 2 mg/L in 250 µM H_2O_2 treated cells had increased the cell proliferation 1.7 times (20 % to 86.5 %) in comparison to Trolox untreated cells, whereas the increase in cell proliferation for 500 µM H_2O_2 treated cells was 4.3 times higher (42 % to 74.8 %). A similar pattern was seen between the untreated cells and the cells treated with 4 mg/L, with the Trolox restoring more than 80% of the cell viability at both H_2O_2 concentrations.

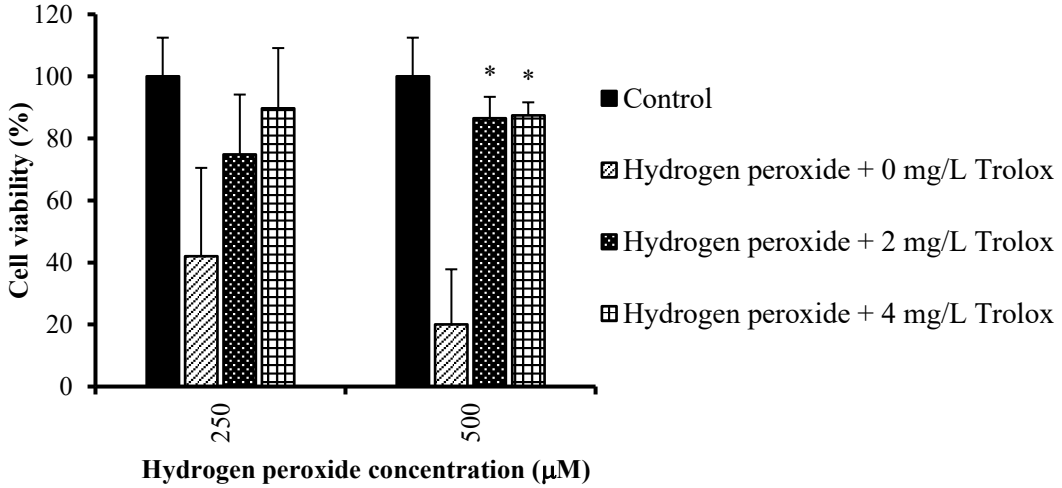


Figure 15. Protective effect of Trolox against H_2O_2-induced cell death at two different concentrations of H_2O_2. Data shown are mean ± standard deviation (n = 3). Mean with asterisk (*) are significantly different ($p<0.05$) from mean when Trolox added was 0 mg/L.

Studies have reported the protective effects of Trolox on H9c2 and rat astrocyte cells (Mojzisova et al. 2009; Yu et al. 2015). Higher concentrations of Trolox (10 mM) have shown protection against acrylonitrile induced cytotoxicity in rat astrocyte cells by significantly increasing the release of lactate dehydrogenase (LDH) in comparison to that of 5 mM Trolox (Mojzisova et al. 2009). While Trolox shows positive cell proliferation in oxidative damage, substances such as quercetin, naringenin and caffeic acid phenethyl esters in their individual forms or mixtures are deemed as more powerful protectors of different cell lines from toxicity due to radioactivity and anthracycline drugs (Bai et al. 2014; Mojzisova et al. 2009).

4.4.3 Protective effect of plant extract against H_2O_2-induced cell death

For *D. suffruticosa*, concentrations below the LD_{50} (60 mg/L) was used i.e. 15 mg/L and 30 mg/L. The results as shown in Figure 16 suggested a dose-dependent response of the protective effect of *D. suffruticosa* trended similar to Trolox. However, the effect was not statistically significant. This is mainly due to the high variations (high standard deviation) observed in the control (0 mg/L) data.

HPLC studies show that majority of *D. suffruticosa* leaves contain oleamide (an amide derivative of fatty acids), Vitamin E and palmitic acid (Jalil et al. 2015; Thooptianrat et al. 2017). It is possible that such compounds may have been responsible for the cell proliferation trend. For example, Vitamin E is an antioxidant, thus, this protect the cells against the oxidative damage caused by the H_2O_2.

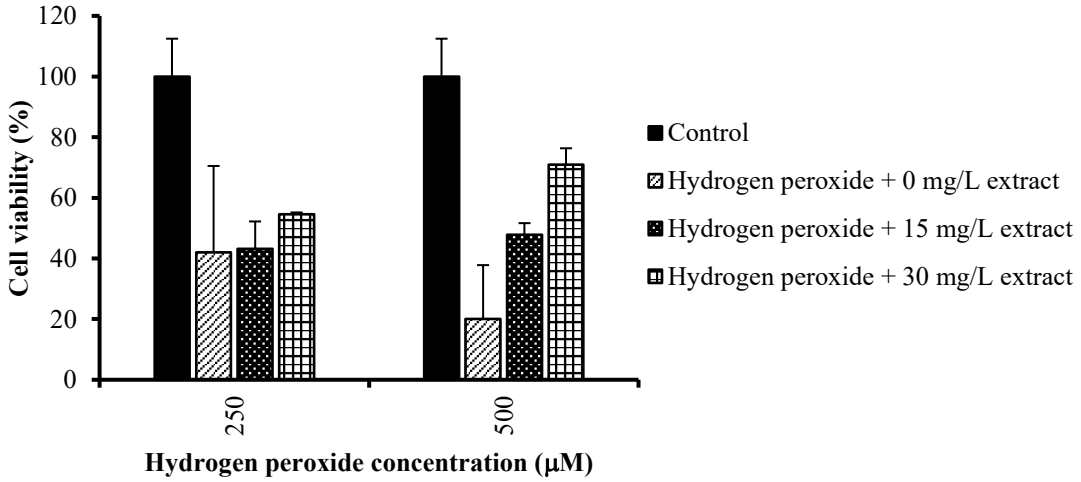


Figure 16. Protective effect of *D. suffruticosa* against H_2O_2-induced cell death at two different concentrations of H_2O_2. Data shown are mean ± standard deviation (n = 3). Mean with asterisk (*) are significantly different ($p<0.05$) from mean when *D. suffruticosa* extract added was 0 mg/L.

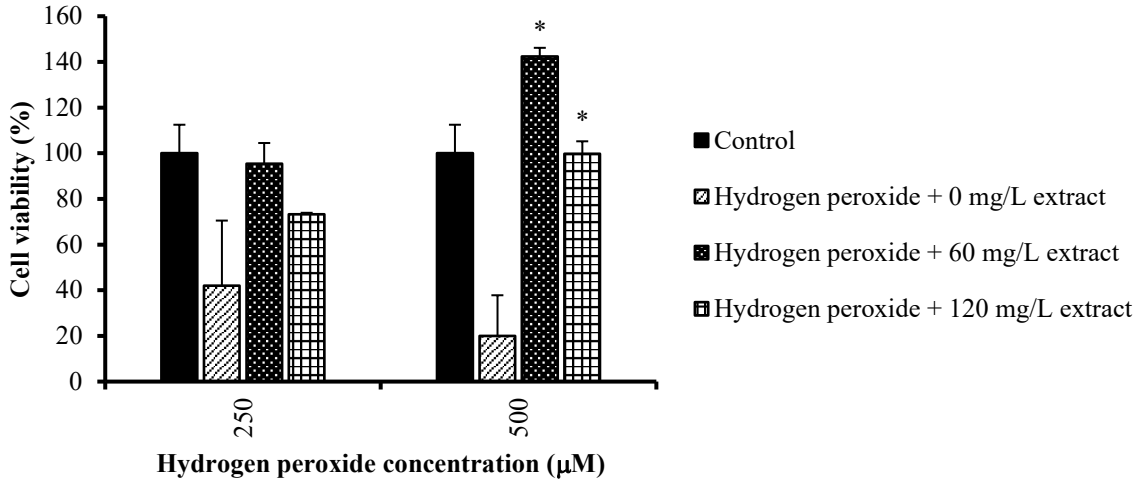


Figure 17. Protective effect of *E. polyantha* (b) against H_2O_2-induced cell death at two different concentrations of H_2O_2. Data shown are mean ± standard deviation (n = 3). Mean with asterisk (*) are significantly different ($p<0.05$) from mean when *E. polyantha* (b) *suffruticosa* extract added was 0 mg/L.

Both concentrations of *E. polyantha* (b) at 60 and 120 mg/L were able to protect the cells against the damaging effects of H_2O_2 at both 250 μM and 500 μM concentrations (Figure 17). However, only those that were tested against 500 μM H_2O_2 showed statistically significant effect. The lack of significant effect at 250 μM H_2O_2 could be attributed to the high standard deviation value of the control (0 mg/L) results. The result for the 500 μM H_2O_2 treatment seems to produce interesting results. Based on the MTS assay, the treatment, in the presence of 60 mg/L *E. polyantha* suggested that the cells were not only protected but seem to be growing more than their original density. Somehow, the protective effect was reduced at higher concentration (120 mg/L), possibly due to the cytotoxicity effect, as observed earlier (Figure 14). Another possibility is there could exist a pro-oxidative effect of the phenolic compounds in the extract. This may result in a cumulative damaging effect to the cell viability, especially in the presence of H_2O_2, another source of free radicals (Rahal et al. 2014; Tan et al. 2016).

The results showed that on its own, *D. suffruticosa* is quite toxic towards the H9c2 (2-1) cells. However, when the cells were incubated with the extract, and then subjected to oxidative stress, the extract seems to protect the cells to some extent from the oxidative damages, and cell death. For *E. polyantha* (b) extract, the protective effect was more pronounced, to the extent that the cell viability was almost fully restored (>90% viability) when 60 mg/L of the extract was added to the cells prior to the H_2O_2 treatment. The effect seems to be reduced when more dosage (120 mg/L) was added. As shown by *E. polyantha* extract, excess amount of dosage appeared to have an opposite effect.

These results demand for more studies such as testing on different cell lines, both cancer and non-cancer cells, to be conducted to get a better perspective of the potential of the two plant extracts as a therapeutic agent. Most importantly, the specific bioactive compound needs to be identified, so that potential proper cell specific cytotoxicity, dose-response effect, similar to those conducted on drugs, can be performed. As an example, suppression of cancer cells by functional group modification of bioactive compounds with boronic acid or esters have been effective as it inhibits hydrolytic membrane disruption enzymes such as matrix metalloproteinases (Peng & Gandhi 2012). These pro-drugs allow masking of toxicity from the effective agent on normal tissue while the active drugs are released on specific cancer cells upon reaction with a ROS such as hydrogen peroxide.

Since the plant extracts studied above are consumed by locals, it is highly suggested to further investigate the anti-inflammatory effect of digested plant extracts on intestinal epithelial cells such as CMT93 and enterocyte-like Caco-2 cell monolayers (Bribi et al. 2016; Olejnik et al. 2015).

4.5 Summary

Ten leafy plants from Sarawak were screened for total phenolic contents. Five plants (*E. polyantha (b), D. suffruticosa, P. serratifolia, E. polyantha (a)* and *P. cordifolia*) out of the ten were selected for the investigation of their antioxidant activities as they exhibited high total phenolic contents (4 – 15 mg GAE/100 mg DW). While all plants exhibited low TFC, two of them (*D. suffruticosa* and *E. polyantha (b)*) consistently showed high antioxidant activities for ABTS (68.4 and 64.7 mg TE/ 100 mg DW) and CUPRAC assays (31 and 46.6 mg TE/ 100 mg DW) and contained strong effective concentrations against scavenging DPPH radicals (40.8 and 37.8 mg DW/ L). Strong correlations were seen between the assays. Both plants were selected for the cell-culture based H9c2 (2-1) cell line cardioprotective assay. The cell cytotoxicity of *E. polyanthum (b)* and *D. suffruticosa* extracts were at 649 mg/L and 60.3 mg/L respectively. Although quite cytotoxic, *D. suffruticosa* extract provided slight protection against oxidative stress damage in the cardiomyocyte cells. The effect was however, not statistically significant ($p >$ 0.05). The cardioprotective effect was more prominent and was statistically significant in *E. polyantha (b)* extract when the cells were treated at higher H_2O_2 concentration (500 μM). This study suggested that *E. polyantha* (b) is a good candidate for further cardioprotective agent as it was less toxic to the cardiac cells compared to the *D. suffruticosa*, and afforded protection against H_2O_2-induced oxidative damage.

5 CONCLUSION

Five out of ten plants were selected to be screened for the investigation of antioxidant activity due to their high total phenolic contents (4 – 15 mg GAE/100 mg DW) and two of these (*D. suffruticosa* and *E. polyantha (b)*) consistently showed high antioxidant activities and were selected for the cell-culture based cardioprotective assay. Some correlations were seen between these assays, with the positive correlation between TPC and ABTS antioxidant value being statistically significant ($p < 0.05$). The total flavonoid content was relatively low in all selected samples. The cell cytotoxicity of *E. polyantha (b)* and *D. suffruticosa* extracts were at 649 mg/L and 60.3 mg/L respectively. Cardioprotective properties were observed from both 2 mg/L *E. polyantha (b)* and more prominently in 4 mg/L *D. suffruticosa* extracts as they effectively increased cell proliferation of cells subjected to oxidative stress induced by 250 µM and 500 µM H_2O_2.

Since all selected plants show acceptable antioxidant properties, it is important to understand its therapeutic effect on cardiac cells and potential therapeutic properties. This would help to build a profound image on the benefits of antioxidants against free radical mediated diseases in humans. It would also help to uplift the value of the local plants in Sarawak

5.1 Further work

Widening the experimental scope, helps to build a more profound research. It helps to give more insight into the understanding of oxidative stress induced cell damage. Although it requires more time and funding, the following suggestions are made as further work for the current research.

Antioxidant activities and cardio-protective nature of different parts of the plants could be investigated as they contain amounts of antioxidants and show activities that differ from one another (Ali et al. 2014; Armania et al. 2013; Sulaiman et al. 2011). More detailed information of the different fractions of the crude extract could be explored. This could be done by using different solvents such as ethyl acetate, hexane, acetone and their combinations with ethanol or methanol to extract different groups of plant secondary metabolites. The extraction method could be varied to optimise the extraction yield (Ahmad et al. 2009; Tiwari et al. 2011; Xu et al. 2017). For some assays, such as the TFC, more comprehensive method that is able to evaluate the specific identity and content of the phenolic compound could be employed. For example, the use of high performance liquid chromatography (HPLC).

As the results in this study shows promising cardioprotective properties, it would be worthwhile to assess the effects on DNA damage, protein oxidation, lipid peroxidation and diabetes by the plant extracts (Kwon et al. 2000; Sabu & Kuttan 2002; Verma et al. 2010; Verma, Shrivastava & Kumar 2015). While the use of assays to estimate antioxidant activities and cell viability are important, better inferences could be made to explain its results by also measuring intracellular enzymes such as lactate dehydrogenase (LDH), superoxide dismutase (SOD) and catalase (CAT) following the study into apoptotic pathways by measuring caspase-3 activity (Chen et al. 2000; Liang et al. 2010; Oyama, Takahashi & Sakurai 2011). The caspase cascade aggregates into macromolecular complexes that bring out controlled destruction of cellular components. Apoptotic pathways could be measured by immunoprecipitation, immunoblotting and flow cytometry coupled with antibodies and fluorescent probes (Menyhárt et al. 2016).

Both plant extracts show some level of cardio-protective properties. However, for *D. suffruticosa,* the extract seems to be quite cytotoxic. Therefore, this extract could be assessed on its ability to reduce the proliferation activity of different cancer cell lines. On top of that, different types of non-cancer cell lines should also be used to evaluate the cytotoxicity effect of both extracts on different types of tissues. This is to ensure that before any claim is to be made on the therapeutic effects of these plant, extensive and comprehensive study need to be done on their safety in general, and dosage amount, in particular.

In addition to their safety, all plant samples that were studied in this thesis should also be subjected to studies that assess their bioavailability, bioactivity and stability in their edible forms (raw, boiled, and infused with curries or herbs). For a start, an *in vitro* gastrointestinal models that mimic human digestive system could be used (Mawalagedera 2014; Pavan, Sancho & Pastore 2014). Upon fruitful results, the study could be extended towards the bioavailability of plant antioxidants in ethically approved human or animal *in vivo* studies. This would help to build an overall perspective of how plant antioxidants behave once inside a mammalian system, in comparison to *in vitro* studies.

In summary, the current study has provided a glimpse of what these plants can offer towards human wellness, especially in the antioxidant aspects. As this was only a preliminary study, it is obvious that there are many opportunities and need for further research in this area. Such collective, well-designed research and well-documented information are needed before any bold claim could be made on their potential therapeutic uses.

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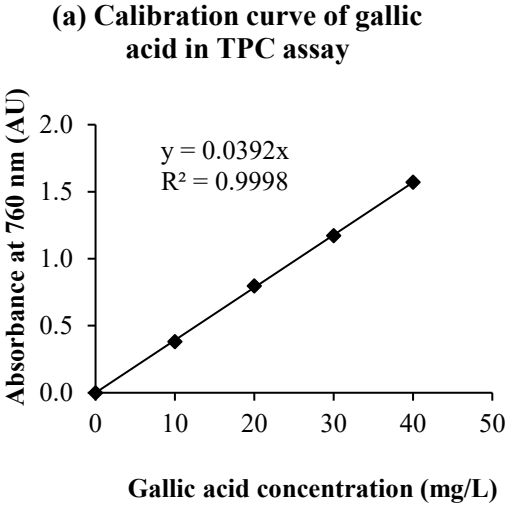
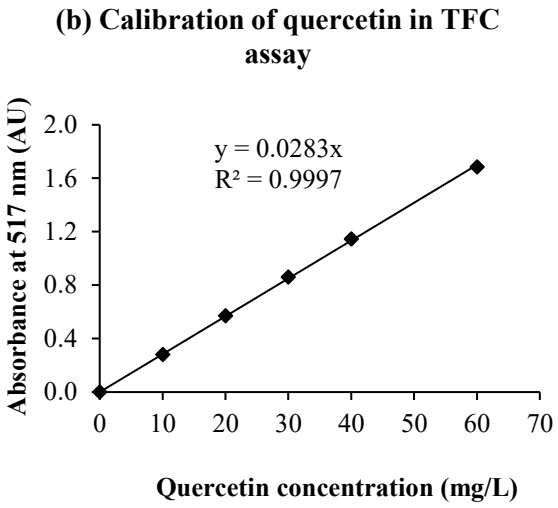
7 Appendices

Appendix A

Calibration curve of gallic acid and quercetin for total phenolic content (TPC) and total flavonoid content (TFC) assays. Data shown are mean ± standard deviation (n = 3).

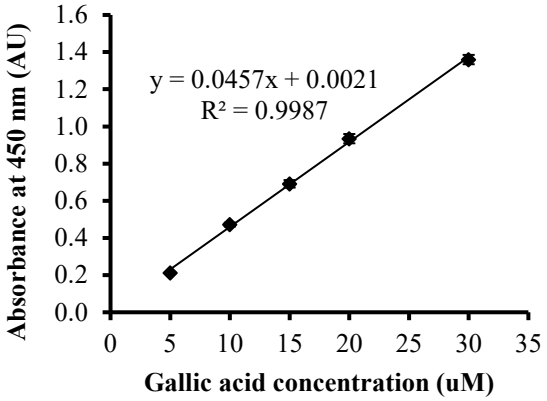




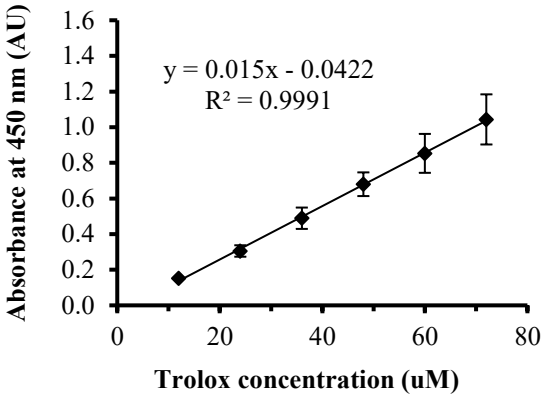
Appendix B

Calibration curve of (a) gallic acid, (b) Trolox and (c) quercetin for antioxidant activity as determined by the CUPRAC assay. Data shown are mean ± standard deviation (n = 3).

(a) Gallic acid



(b) Trolox



(c) Quercetin