ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF *POLYGONUM MINUS*, *ALPINIA GALANGA* AND *ETLINGERA ELATIOR*

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ABSTRACT

The Malaysian traditional vegetables *Polygonum minus* (Kesum), *Alpinia galanga* (Lengkuas) and *Etlingera elatior* (Bunga Kantan) are commonly used in local cuisines and traditional medicine. In this study, antioxidant and anti-proliferative activities of extracts from these herbs were evaluated. This may provide insights on their nutritional and medicinal values. The herbs were dried and extracted sequentially using solvents of varying polarity (apolar to polar: hexane (HX) > ethyl acetate (EA) > methanol (MeOH) > water (W). The antioxidant potential was evaluated through these assays: Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Ferric Reducing Antioxidant Power (FRAP), 2,2'-azinobis (3-ethylbenzothiozoline-6-sulphonic acid) ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picryldydrazyl ($DPPH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$)-scavenging, nitric oxide ($NO^{\bullet}$)-scavenging and ferric ion-chelating (FIC) assays. The plant extracts were evaluated for their anti-proliferative activity through MTT assay using colon cancer cell line HCT116, with CCD841 as the non-cancerous cell line for comparison. The apoptosis-inducing property of selected extracts was evaluated using Apotox-Glo™ Triplex assay. Overall, the methanol extract of *P. minus* showed the most prominent antioxidant activity, demonstrating the highest values for TPC, TFC, FRAP, ABTS, DPPH and nitric oxide scavenging capacities. *P. minus*'s water extract also gave the highest value in $O_2^{\bullet-}$-scavenging assay. The methanol extract of *E. elatior* had the highest FIC activity. Polyphenols and flavonoid contents showed a strong positive correlation with antioxidant activities, suggesting their significant contribution to the observed antioxidant activity. LCMS-IT-TOF analysis for methanol extract of *P. minus* identified six flavonols (quercetin and kaempferol), a flavone (apigenin) and tannins. The following extracts showed selective cytotoxicity on HCT116 cells with the corresponding IC_{50} values: PM-HX (40.00±2.04 µg/mL), PM-EA (43.18±1.63 µg/mL)

and AG-EA (71.94±1.80 µg/mL). The apoptosis assay revealed that the AG-EA extract was able to induce a 30% increase in caspase 3/7 activity. This study demontrates that traditional herbs could be a potential source of natural antioxidant and anticancer compounds.

ABTSRAK

Ulam-ulaman tradisional Malaysia seperti *Polygonum minus* (Kesum), *Alpinia galanga* (Lengkuas) and *Etlingera elatior* (Bunga Kantan) yang sering digunakan dalam masakan tempatan dan perubatan tradisional. Ciri-ciri antioksidan dan anti-proliferatif daripada ekstrak herba-herba ini dinilai untuk mendapatkan gambaran nilai nutrisi dan perubatannya dalam kajian ini. Herba-herba ini dikeringkan dan diekstrak secara berjujukan menggunakan pelarut dengan pelbagai jenis kekutuban (daripada tidak berkutub kepada berkutub: heksana (HX) > etil asetat (EA) > methanol (EA) > air (W). Keupayaan antioksidan disiasat melalui ujian-ujian berikut: jumlah kandungan polifenol (TPC), jumlah kandungan flavonoid (TFC), kuasa antioksidaan penurunan ferik (FRAP), 2,2'-azinobis (3-ethylbenzothiozoline-6-sulphonic acid) ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picryldydrazyl (DPPH•), penghapusan anion superoksida ($O_2^{\bullet-}$), penghapusan nitric oksida (NO•) dan pengkelatan ion-ferus (FIC). Semua ekstrak dinilai aktiviti anti-proliferatif melalui uji kaji MTT menggunakan sel kanser kolon HCT116, beserta dengan sel kolon normal CCD841 sebagai perbandingan. Ciri-ciri pengaktifan apoptosis disiasat menggunakan ekstrak-ekstrak terplilih melalui ujian Apotox-Glo™ Triplex. Secara keseluruhannya, ekstrak metanol daripada *P. minus* menunjukkan aktiviti antioksidan yang paling kuat dengan nilai tertinggi untuk jumlah polifenol, flavonoid, FRAP dan keupayaan menghapuskan radikal $ABTS^{\bullet+}$, $DPPH^{\bullet}$, nitrik oksida yang tertinggi. Ekstrak larut-air daripada *P. minus* memberikan nilai tertinggi dalam keupayaan menghapuskan radikal anion superoksida. Ekstrak metanol daripada *E. elatior* pula menunjukkan aktiviti pengkelatan ion ferus yang tertinggi. Kandungan polifenol dan flavonoid menunjukkan positif korelasi yang kuat dengan ujian-ujian aktiviti antioksidan. Analisis LCMS-IT-TOF terhadap ekstrak methanol daripada *P. minus* telah mengenalpasti akan kewujudan enam flavonol (kuersetin dan kaempferol),

satu flavon (apigenin) dan tanin. Ekstrak- ekstrak yang berikut menunjukkan sifat kesitotoksikan berpilih terhadap sel HCT116 dengan nilai IC_{50} seperti berikut: PM-HX (40.00±2.04 μg/mL), PM-EA (43.18±1.63 μg/mL) dan AG-EA (71.94±1.80 μg/mL). Ujian apoptosis menunjukkan hanya ekstrak AG-EA menyebabkan peningkatan 30% aktiviti *caspases* 3/7. Kajian ini menunjukkan bahawa herba-herba tradisional berpotensi menjadi sumber komponen-komponen antioksidan dan antikanser.

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LIST OF SYMBOLS AND ABBREVIATIONS

ROS	-	reactive oxygen species
RNS	-	reactive nitrogen species
DNA	-	deoxyribonucleic acid
UV	-	ultraviolet
SOD	-	Superoxide dismutase
GSH	-	glutathione
NO	-	nitrogen oxide
NO_2	-	nitrogen dioxide
$OH^{\bullet}$	-	hydroxyl radical
$RO_2^{\bullet}$	-	peroxyl radical
$HO_2^{\bullet}$	-	hydroperoxyl radical
$RO^{\bullet}$	-	alkoxyl radical
$NO^{\bullet}$	-	nitric oxide radical
$NO_2^{\bullet}$	-	nitrogen dioxide radical
$LOO^{\bullet}$	-	lipid peroxyl radical
$O_2^{\bullet-}$	-	superoxide radical
H_2O_2	-	hydrogen peroxide
HOCl	-	hypochlorous acid
O_3	-	ozone
$L^{\bullet}$	-	lipid radical
$LO_2^{\bullet}$	-	lipid peroxyl radical
$LO^{\bullet}$	-	lipid alkoxyl radical
$RS^{\bullet}$	-	thiyl radical
$P^{\bullet}$	-	protein radical
LDL	-	low-density lipoproteins
H_2O_2	-	hydrogen peroxide
H_2O	-	water
O_2	-	oxygen
GSSG	-	glutathione disulfide
LOOH	-	lipid hydroperoxides
LOH	-	lipid alcohol
PLOOH	-	phospholipid hydroperoxides

PL-OH	-	phospholipid alcohol
AH	-	antioxidant
H^+	-	hydrogen proton
$^1O_2$	-	singlet oxygen
Prx	-	peroxiredoxin
GPx	-	glutathione peroxidise
Mn	-	manganese
Cu	-	copper
Se	-	selenium
HAT	-	hydrogen atom transfer
SET	-	single electron transfer
ET-PT	-	electron transfer followed by proton transfer
SPLET	-	sequential proton loss electron transfer
CAT	-	catalase
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
PBS	-	phosphate buffered saline
H_3PO_4	-	phosphoric acid
DMSO	-	dimethyl sulphoxide
SNP	-	sodium nitroprusside dihydrate
PMS	-	phenazine methosulphate
$K_2S_2O_8$	-	potassium persulphate
MTT	-	3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
$AlCl_3$	-	aluminium trichloride
EDTA	-	ethylenediaminetetraacetic acid
TPTZ	-	2,4,6-tri(2-pyridyl)-1,3,5-triazine
NADH	-	dihydronicotinamide adenine dinucleotide
NEDD	-	N-(1-naphthyl)ethylene-diamine dihydrochloride
ABTS	-	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
NBT	-	*p*-nitroblue tetrazolium chloride
$FeSO_4$	-	iron (II) sulphate
HCl	-	hydrochloric acid
FBS	-	Fetal Bovine Serum
ATCC	-	American Type Culture Collection
ANOVA	-	analysis of variance

g	-	gram
mL	-	millilitre
°C	-	degree Celcius
min	-	minute
G	-	gravity
nm	-	namometre
GAE	-	gallic acid equivalent
QE	-	quercetin equivalent
µL	-	microlitre
mg	-	milligram
µg	-	microgram
SD	-	standard deviation
mmol	-	millimole
µmol	-	micromole
%	-	percentage
TEAC	-	trolox equivalent antioxidant capacity
ER	-	endoplasmic reticulum

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1 Introduction

Plants sourced foods provides essential component of nutrients in human nutrition. The assessment of antioxidant and anticancer activity of plant sourced foods provides insights to their medicinal benefit. A plethora of phytochemical compounds have been isolated from plants with bioactivities that could potentially provide protection against chronic ailments such as cancer, Alzheimer's, diabetes and cardiovascular diseases (Basu, Temple, & Garg, 1999). These chronic diseases could have arisen from cellular damage owing to prolonged cellular redox imbalances. Plant sourced foods are rich in antioxidants and such dietary component may be helpful in preventing cellular damage by inhibiting oxidative stress or avert imbalances in the amount of free radicals. Antioxidant is defined as 'any substance that can prevent, reduce, or repair the reactive oxygen and nitrogen species (ROS/RNS)-induced damage to a target biomolecule' (Halliwell, 2007). Metabolic reactions in the living cells can produce ROS/RNS that can damage biological molecules such as lipid, DNA and proteins via oxidation (López-Alarcón & Denicola, 2013). This happens especially during oxidative stress, where the pro-oxidants:antioxidants ratio is increased, culminating from the disruption of cellular redox signalling and control (Jones, 2008). The traditional vegetables of the Malay community in Malaysia comprise of more than 120 species (Mansor, 1988). Studies have shown that many of these vegetables are rich in carbohydrates, proteins, minerals and vitamins (Ismail, 2000). *Polygonum minus* Huds, *Alpinia galanga* and *Etlingera elatior* are among the most widely used herbs in

Malaysian cuisines. In this study, antioxidant and anticancer activities of extracts from these herbs was evaluated.

P. minus Huds. is a tropical herbaceous plant from the family Polygonaceae. In Malaysia, it is locally known as *kesum,* and it is popularly used as *ulam* (traditional vegetable salad) and as flavouring enhancer in many dishes. Traditionally, *kesum* leaves have been used to address skin fungal infection, indigestion, dandruff, postnatal tonic, sprains and alleviating body aches (Burkill, 1966; Ong & Nordiana, 1999; Wiart, 2006). *P. minus* extract reportedly showed antiviral activities against *herpes simplex type*-1 (HSV-1) and *vesicular stomatitis (VSV)* viruses (Ali et al., 1996), anti-acetylcholinesterase (Noor Hashim, Abas, Shaari, & Lajis, 2012) and cytotoxicity towards HeLa cells (Mackeen et al., 1997).

Alpinia galanga is locally known as *lengkuas* and also commonly recognised as galangal. It is a rhizomatous herb with tuberous aromatic rootstocks from the Zingiberaceae family. It is a popular food flavouring ingredient in South East Asian cuisines due to its pleasant aroma with a hot and spicy taste. *A. galanga* have been reported to show anti-amnesiac, anti-inflammatory, anti-tumour, antibacterial and anti-ulcer activities (Chudiwal, Jain, & Somani, 2010; Hanish Singh, Alagarsamy, Sathesh Kumar, & Narsimha Reddy, 2011; Zaidi et al., 2012). In traditional Malay medicine, pieces of the rhizome is cut and dipped into kerosene and rubbed onto skin for anti-fungal treatment (Ong & Nordiana, 1999).

Etlingera elatior is locally known as *Kantan*, and it is the one of the most commonly found species of *Etlingera*. This plant is also known as 'torch ginger' or 'red ginger lily' due to the striking similarity of the inflorescence to a flaming torch (Yunus, Aziz, Kadir, & Rashid, 2012). It is native to South East Asia and belongs to the Zingiberaceae family. In Malaysia there are more than 15 species of *Etlingera* plants

recorded (Lim, 2001). *E. elatior* flower has a characteristic ginger like aroma and it is popularly used in local cuisines such as *asam laksa* and *kerabu* and also as flavouring enhancer. It has been traditionally used for the treatment of earache and assisting wound healing (Burkill, 1966). The flower extract have been reported to show antioxidant, hepatoprotective and cytotoxic activities (Chan, Lim, & Omar, 2007; Haleagrahara et al., 2010; Jackie, Haleagrahara, & Chakravarthi, 2011; Vairappan, Nagappan, & Palaniveloo, 2012).

In this study, we employed sequential extraction using non-polar to polar solvents system (hexane> ethyl acetate> methanol> water) to obtain extracts of the plant materials containing phytochemicals that have been separated according to their relative polarity. This extraction method could potentially recover a wider spectrum of components from the plant materials. To the best of our knowledge, no other studies have adopted this extraction method to characterise the activity of the plant materials used in this study. To our knowledge, the anti-proliferative activity of *A. galanga* and *E. elatior* extracts on normal colon CCD841 cell line, and colon cancer cells HCT116 have never been reported.

1.2 Objectives of the study

1.2.1 Main Objective

To study the bioactivities of extracts from *P. minus (Pokok Kesum)*, *A. galanga* (*Lengkuas*) and *E. elatior* (Kantan).

1.2.2 Specific Objectives:

1. To evaluate antioxidant activity of the selected plant extracts.
2. To investigate the effect of the selected plant extracts on the proliferation normal and cancerous colon cell lines
3. To identify the anti-cancer/antioxidant component of the selected plant extracts using MTT and *in vitro* antioxidant assays and followed by LCMS-IT-TOF.

CHAPTER TWO

LITERATURE REVIEW

2.1 Free radicals

The theory of free radicals and free radical reaction were stated as early as in the eighteenth century. In 1896, the chemist Oswald stated that the existence of free radicals is impossible due to its highly reactivity and very short half-life. Thereafter, a relatively stable free radical, triphenyl-methyl, was discovered for the first time by Gombeth in 1900 (Olinescu & Smith, 2002). The next milestone in understanding the concept of the free radical was in 1937, when Hey and Waters published a review explaining the products and kinetic chain reactions mechanism of the free radical (Nonhebel & Walton, 1974). Today the discussions on free radicals are still ongoing, due to the complexity of biological systems, the high reactivity of free radicals, and to the variety of experimental conditions to understand free radicals and its influence on living organism.

Free radicals are defined as molecules that possess one or more unpaired electrons in the outer orbit, often denoted by the symbol "•" (Asmus & Bonifačić, 2000; Fang, Yang, & Wu, 2002; Olinescu & Smith, 2002). The radicals include transition metal ions (e.g.), the hydrogen atom, organic molecules and a few common inorganic compounds, for instance nitrogen oxide (NO) and nitrogen dioxide (NO_2) (Southorn & Powis, 1996). Free radicals are formed in various situations and are able to participate in intermediary chemical reactions. Their life spans are different depending on the

complexity of the free radical structure. The simpler the structure, the shorter the life span. Complex radicals are more stable and have longer life span.

Free radicals could actually arise from natural processes that occur in living organism, including humans. For example, the existence of superoxide dismutase (SOD) enzyme is closely correlated to the presence of superoxide radical, $O_2^{\bullet -}$ common oxygenated radical species but its concentration is tightly regulated to prevent undesirable cellular oxidation. However, when there is a deficiency of radical species, it may also bring undesirable outcome. For example, cervical cancer cells have been reported to be almost devoid of radical species, whereas quite a measurable radical concentrations are observed in healthy cells (Asmus & Bonifačić, 2000).

Generally, free radicals are unstable and very reactive. They have variety of charge such as neutral, electropositive or electronegative. Due to such unstable condition, the radicals have great tendency and reactivity to react with other molecules to achieve stability, and this is where it could potentially cause oxidation of biomolecules such as lipid bilayer, DNA or other organic components of the cells and this could cause cellular injury and when prolonged, could become a factor that contributes to chronic disease development. This is where antioxidant molecules may be important in preventing cellular oxidation and providing health benefits.

2.1.1 Source of free radicals

Free radicals could come from two main sources: exogenous and endogenous sources. The exogenous sources arise from chemical pollutants exposure, UV/ionizing radiation, heavy metals, drugs consumption, herbicides, pesticides, and unhealthy lifestyles such as smoking and indulgence of fast-food products. These could provide sources of potentially health damaging compounds, since when they are metabolized, levels of free radical intermediates could increase, and thus oxidative damage to living tissues is promoted.

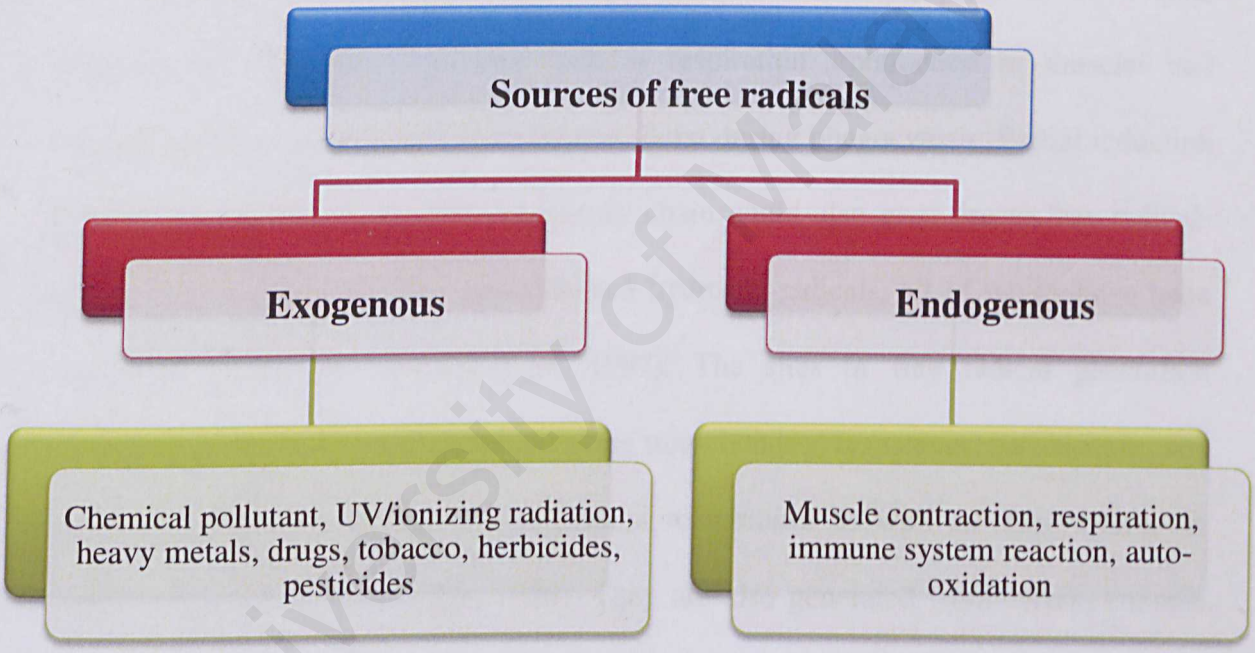

Figure 2.1: Sources of free radicals.

Exogenous sources of free radicals generation could come from radioactive energy. The decay of radioactive elements could release alpha and beta particles, as well as gamma radiation. The ionizing radiation released could act on cellular components such as water molecules or solutes (protein, nucleic acid, carbohydrate, and inorganic components), leading to 'radiolysis'. Since water molecules are abundantly found in living cells, it could be ionized and dissociated into hydroxyl radicals and hydrogen

radicals (Halliwell, 2005). Hydroxyl radicals are known to be highly reactive. Oxygen molecules in the cell can also fuse with hydrogen radicals and forming hydrogen peroxide. Excessive number of H_2O_2 in the cells is also harmful and intoxicate the organism. Light sensitive compounds in the body could also undergo photolysis process due to UV light exposure, which then generate free radicals (Diplock, Symons, & Rice-Evans, 1991). Exposure to asbestos and silica initiate an inflammatory process that leads to increased production of free radicals and other reactive oxygen species (Heffner & Repine, 1989; Vallyathan, Shi, Dalal, Irr, & Castranova, 1988).

Endogenous free radicals are generated from the by-products of normal processes in the living organism, such as respiration, contraction of muscles and immune defense activity such as respiratory burst during phagocytosis. Partial reduction of oxygen in the mitochondrial respiratory chain could also give rise to free radicals such as superoxide, hydrogen peroxide and hydroxyl radicals, all of which have been implicated in oxygen toxicity (Sies, 1997). The sites of free radical generation encompass all cellular constituents including mitochondria, lysosomes, peroxisomes and nuclear, endoplasmic reticulum and plasma membranes as well as sites within the cytosol (Halliwell & Gutteridge, 1990). They are also generated from certain enzyme activities such as peroxidases, lipoxygenases, dehydrogenases, oxidases and cyclo-oxygenases. Electron transfer from metals such as iron to oxygen-containing molecules can also produce free radicals.

2.1.2 Type of free radical and oxidative-damage related disease

There are numerous types of free radicals that can be formed within the body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are among the free radicals which are known to have both deleterious and beneficial effects in the body (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). Superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) are the examples of ROS and RNS radicals, while hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3) are examples of non-radical forms of ROS. These non-radicals are acting as oxidants by Valko et al. (2006) and could easily to lead free radical reactions in living organisms.

Table 2.1: Reactive radical and non-radical species

Radicals		Non-radicals	
$O_2^{\bullet -}$	Superoxide	H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical	$^1O_2$	singlet oxygen
$HO_2^{\bullet}$	hydroperoxyl radical	LOOH	lipid hydroperoxide
$L^{\bullet}$	lipid radical	Fe=O	iron-oxygen complexes
$LO_2^{\bullet}$	lipid peroxyl radical	HOCl	hypochlorite
$LO^{\bullet}$	lipid alkoxyl radical		
$NO_2^{\bullet}$	nitrogen dioxide		
$NO^{\bullet}$	Nitric oxide		
$RS^{\bullet}$	thiyl radical		
$P^{\bullet}$	protein radical		

Oxidative stress leads to oxidative damage and this arises from imbalances in ROS/RNS ratio. The imbalance could arise from the failure of antioxidant defenses, and thus leading to lipids, proteins, and nucleic acids oxidation. A number of factors such as trauma, heat injury, toxin, infection, hyperoxia and excessive exercise may cause a short-term oxidative stress in the tissues. Radicals-producing enzymes (e.g., cyclooxygenase, lipogenase and xanthine oxidase), could also induce cellular injury

from excessive ROS/RNS propagation reactions. Oxidative damage may also arise from chronic disease such as cancer, ionizing radiation exposure and chemotherapy. Prolonged oxidative stress contributes toward the development of chronic diseases with excessive inflammation (e.g. lupus erythematous, arthritis, glomerulonephritis, vasculitis, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, emphysema, gastric ulcers, hypertension, neurological disorders (Alzheimer's disease, Parkinson's disease) and many others (Lobo, Patil, Phatak, & Chandra, 2010).

2.2 Antioxidant

Halliwell & Gutteridge (1995) proposed antioxidant as 'substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. This definition was used to categorize when an item is considered to be having antioxidant capacity. The levels of potentially damaged target (oxidizable substrate), the molar amount of the species that could quench the free radicals, could affect whether an item qualifies to be called as an antioxidant agent. For example, plasma albumin has antioxidant capacities to chelate copper ions and prevent oxidative damage of extracellular biomolecules such as low-density lipoproteins (LDL), but since albumin is in great molar excess compared to LDL levels in the blood, it is not considered to be having antioxidant activity, based on the earlier definition. The same definition also excludes chaperones activity, oxidative-damaged DNA repair mechanism, methionine sulfoxide protein-repair mechanism and reactive species generation inhibitors. Therefore, to be more comprehensive, Halliwell (2007) proposed a new definition for "antioxidant" agent, as 'any substance that can prevent, reduce, or repair the reactive oxygen and nitrogen species (ROS/RNS)-induced damage to a target biomolecule'.

With the newer definition, antioxidants could come from two main sources: endogenous and exogenous sources, as illustrated in Figure 2.2. The endogenous antioxidants are those synthesized in our body, including enzymatic reactions that are antioxidative, such as catalases, thioredoxin, peroxiredoxin (Prx), glutathione peroxidise (GPx) and superoxide dismutase (SOD); and non-enzymatic ones such as ferritin, transferrin, albumin, bilirubin, urate and glutathione (Gough & Cotter, 2011; Halliwell & Gutteridge, 2007). On the other hand, exogenous antioxidants are diet-derived antioxidants where the body cannot synthesize them *de novo*. They include natural and

synthetic antioxidants. Examples include phenolic compounds, nitrogen compounds (chlorophyll derivatives, alkaloids, amino acids and amines), carotenoids and the vitamins A, C and E (Velioglu, Mazza, Gao, & Oomah, 1998). Fresh fruits and vegetables form the major dietary sources of antioxidants (Maxwell & Lip, 1997).

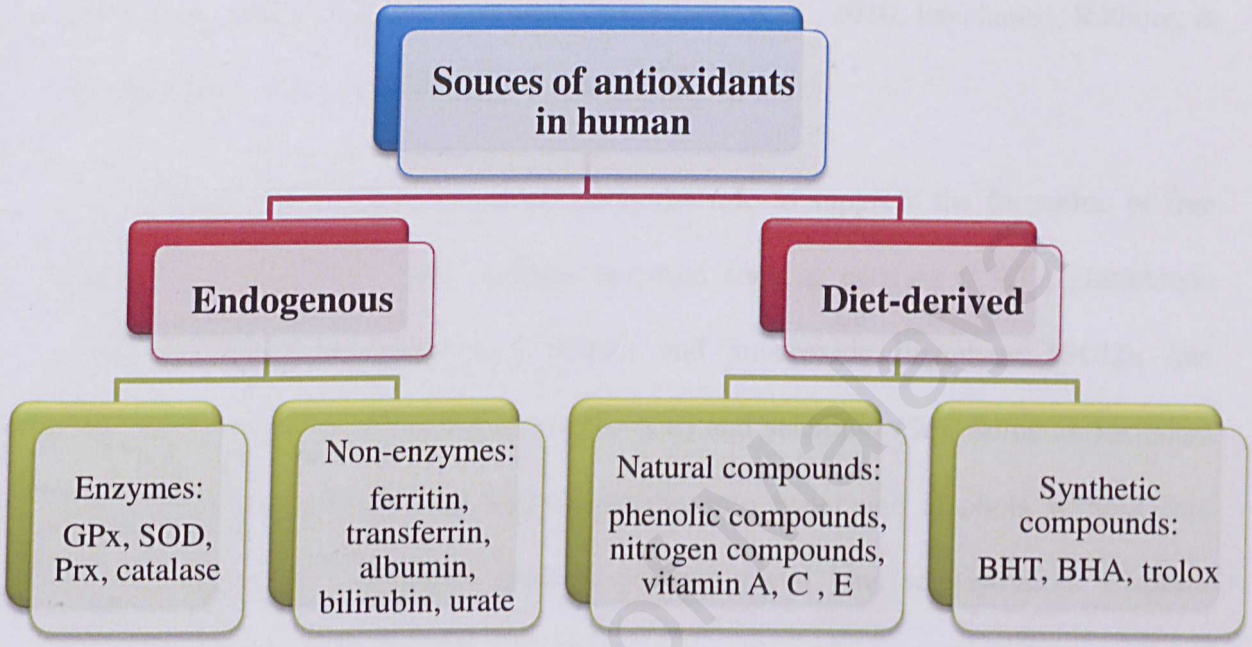

Figure 2.2: Sources of antioxidants in human. GPx, glutathione peroxidise; SOD, superoxide dismutase; Prx, peroxiredoxins.

Antioxidants could be sub-classified into two types, depending on their solubility in water (hydrophilic) and in lipids (lipophilic). Water-soluble antioxidants react with oxidant in the cytosol and blood plasma, while lipid-soluble antioxidants protect cell membranes and lipoproteins from lipid peroxidation. Vitamin C (ascorbate) is the best known water-soluble antioxidant which has powerful electron donor capability. Its oxidized form (dehydroascorbate) could be changed into the reduced form (ascorbate) intracellularly (Maxwell & Lip, 1997). Vitamin E (α-tocopherol) is an example of lipid-soluble antioxidant that commonly found in lipoprotein and acting as a powerful radical chain-breaker (Maxwell & Lip, 1997).

2.2.1 Mechanism of action of antioxidants

The mechanism of action of antioxidants could be classified into 4 main categories: preventive antioxidant, radical scavenging, 'repair and *de novo* enzymes' and enzyme inhibitor antioxidants; which showed in Table 2.2 (Charles, 2013; Jebakumar, Nondo, George, & Manoj, 2012; Lobo et al., 2010; Panchawat, Rathore, & Sisodia, 2010; Prior, Wu, & Schaich, 2005).

Firstly, preventive antioxidant plays the role to suppress the formation of free radicals and ROS/RNS. This includes enzymes such as catalase (CAT), glutathione reductase, glutathione peroxidase (GTX) and superoxide dismutase (SOD); and minerals such as manganese (Mn), copper (Cu) and selenium (Se). Some antioxidants reduce hydrogen peroxide and hydroperoxides into water and alcohols without free radicals production; and some proteins perform metal ions sequestration. Catalase catalyses the hydrogen peroxide (H_2O_2) decomposition into oxygen and water. SOD decomposes superoxide anion, and GTX catalyses the H_2O_2 and lipid peroxide reduction during lipid peroxidation.

Secondly, radical scavenging antioxidant inhibits chain initiation and/or break the chain propagation of the harmful radicals. Such type of antioxidant includes flavonoids, carotenoids, glutathione (GSH), albumin, vitamin C, vitamin E, bilirubin and uric acid. Vitamin C, GSH and β-carotene. These are good scavenging agents acting on various radicals such as superoxide anion, hydroxyl radicals and lipid hydroperoxides. These antioxidants scavenge free radicals through several mechanisms such as hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET or ET-PT), and the sequential proton loss electron transfer (SPLET). HAT mechanism involves a rapid hydrogen transfer from the antioxidant to the radicals, independent from the reaction pH and solvent. SET mechanism involves the transfer of

one electron to the radicals, carbonyls and metals in the pH dependent condition. SPLET mechanism is the same as HAT mechanism, in terms of antioxidant net result, but it is a long reaction involving ionization of the active site of antioxidant and electron transfer.

Thirdly, repair and *de novo* antioxidant is made up from a complex group of enzymes that can terminate the chain propagation of peroxyl lipid radical; and repair damaged DNA, protein, peroxides and oxidized lipids. These enzymes could repair damaged biomolecules and regenerate the cell membrane. The fourth type of antioxidant mechanism involves enzyme inhibitors. These are able induce the formation of free radical signal to produce and transport appropriate antioxidants to specific target site.

Table 2.2: Antioxidant mechanism of action against oxidative damage

Antioxidant mechanism against oxidative damage	
1. Preventive antioxidants: suppress the formation of free radicals	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide	
Catalase	Decomposition of hydrogen peroxide $2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ $LOOH + 2GSH \rightarrow L\text{-}OH + H_2O + GSSG$
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipid hydroperoxides $PLOOH + 2GSH \rightarrow PL\text{-}OH + H_2O + GSSH$
Phospholipid hydroperoxide glutathione peroxidase (PHGPx)	Decomposition of phospholipid hydroperoxides $2GSH + L\text{-}OOH \rightarrow GSSG + L + 2H_2O$
Peroxidase	Decomposition of hydrogen peroxide and phospholipid hydroperoxides $LOOH + AH_2 \rightarrow LOH + H_2O + A$ $H_2O_2 + AH_2 \rightarrow 2H_2O + A$
Glutathione S-transferase	Decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
Carotenoids, vitamin E	Quenching singlet oxygen β-carotene + $^1O_2 \rightarrow$ β-carotene endoperoxide

2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation

(a) Hydrogen atom transfer (HAT) mechanism:

$AH + ROO^{\bullet} \rightarrow A^{\bullet} + ROOH$

(b) single electron transfer followed by proton transfer (SET or ET-PT), including metals, carbonyls and radicals:

(i) $ROO^{\bullet} + AH \rightarrow ROO^{-} + AH^{\bullet+}$

$AH^{\bullet+} \overset{H_2O}{\rightleftharpoons} A^{\bullet} + H_3O^{+}$

$ROO^{-} + H_3O^{+} \rightarrow ROOH + H_2O$

(ii) $Metal(III) + AH \rightarrow AH^{+} + Metal(II)$

(c) sequential proton loss electron transfer (SPLET):

$AH \rightleftharpoons A^{-} + H+$

$A^{-} + ROO^{\bullet} \rightarrow A^{\bullet} + ROO^{-}$

$ROO^{-} + H^{+} \rightarrow ROOH$

3. Repair and *de novo* enzymes: repair the damage and reconstitute membranes

Methionine sulphoxide reductase, protease, DNA repair enzymes, lipase, transferase

4. Enzyme inhibitor antioxidants: These induce production and reaction of free radicals and the transport of appropriate antioxidants to appropriate active site

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H_2O_2, hydrogen peroxide; H_2O, water; O_2, oxygen; GSH, glutathione; GSSG, glutathione disulfide; LOOH, fatty acid hydroperoxides; L-OH, lipid alcohol; PLOOH, phospholipid hydroperoxides; PL-OH, phospholipid alcohol ; AH, antioxidant; L; A, antioxidant $O_2^{\bullet-}$, superoxide anion; H^{+}, hydrogen proton; $^1O_2$, singlet oxygen; $ROO^{\bullet}$, peroxy radical; ROOH, organic hydroperoxide.

2.2.2 Antioxidants from the plant kingdom

Higher plants synthesized a vast number of phytochemicals - more than 200,000 that have been isolated individually. These constituents are further categorized into primary and secondary metabolites. The primary metabolites play an essential role/ function for the growth, development and reproduction of plants, which include sugars, amino acids, fatty acids and nucleotides (Fiehn, 2002; Wu & Chappell, 2008). Secondary metabolites are distinguished constituents that are present in specialized cells and are not crucially involved in some of the basic biochemical pathways/cellular housekeeping chores. These secondary metabolites may have specialised functions, for example as a defense mechanism against microbes, predators or competing plants. They can also act as a signalling component for pollination and protect plants against UV rays and harmful oxidants (Lattanzio, 2013). Many studies have been documented to characterize these compounds of their antioxidant potential. These natural constituents are good radical scavengers, enzyme inhibitors, peroxide decomposers, and synergists (Larson, 1988). Numerous studies have demonstrated the strong association of the benefits of vegetable/fruit consumption in the prevention of various degenerative diseases (Gropper & Smith, 2008).

Ginkgo biloba, for example, is a well-known medicinal herb with potent antioxidant activities due to its flavonoids/polyphenolic components (Marcocci, Packer, Droy-Lefaix, Sekaki, & Gardès-Albert, 1994; Vanhaelen & Vanhaelen-fastre, 1988). *G. biloba* is able to prevent cardiovascular and degenerative diseases and demonstrated radical scavenging activities (Maitra, Marcocci, Droy-Lefaix, & Packer, 1995; Punkt, Welt, & Schaffranietz, 1995; Ramassamy, Christen, Clostre, & Costentin, 1992). Flavonoids in *G. biloba* showed potent ability to scavenge various free radicals such as superoxide anion, hydroxyl radicals and nitric oxide (Haramaki, Packer, Droy-Lefaix, &

Christen, 1996; Marcocci et al., 1994; Sichel, Corsaro, Scalia, Di Bilio, & Bonomo, 1991). The low incidence of coronary artery disease in Japan is believed to be explained by the high consumption of flavonoids present in green tea (Hertog, 1995). Furthermore, a study found that asthma was less common in adults of British population who consumed more apples, more red wine and had a high selenium intake. Apples and red wine are rich in ascorbate and polyphenol such as quercetin, tannin and catechin (Shaheen et al., 2001).

Endogenous antioxidants maybe insufficient to provide protection against oxidative damage especially when the antioxidant:oxidant ratio is low, hence, studies are on-going to search for sources of dietary antioxidants to complement the endogenous antioxidant defense system.

2.2.2.1 Polyphenolic antioxidant

Phenolic constituents or polyphenols are the vital bioactive compounds that are widely distributed in the plants and comprised more than 8,000 distinguished chemical structures. Polyphenols are commonly found in fruits, vegetables, nuts, seeds, grains, flowers, and bark. Their categories including simple phenols, phenolic acids (rosmarinic, gallic, protocatechuic, carnosic and caffeic acid), anthocyanins (delphinidin), hydroxybenzoic acids (vanillic acid), hydroxycinnamic acid (chlorogenic and ferulic acid), tannins (tannic acid and procyanidin), lignans (sesaminol), stilbenes (resveratrol), coumarins, essential oils (limonene, thymol, menthol and eugenol), flavonoids (apigenin, quercetin, catechin, and rutin), lignans and quinones (El Gharras, 2009; Li & Beta, 2013).

Many studies reported that polyphenols possesses powerful anticarcinogenic, antiviral, antibacterial, anti-inflammatory, antiatherosclerotic, and antioxidant activities (Báidez, Gómez, Del Río, & Ortuño, 2007; Han, Shen, & Lou, 2007; Veeriah et al., 2006). Most of the plants' phenolic compounds exhibit powerful antioxidant capacities which have been verified by various assay procedures and such properties are beneficial to health by protecting the human body from free radicals. For example, most of the phenolic compounds which isolated from hot pepper fruit showed strong antioxidant activities in the β-carotene-linoleic acid and DPPH-radical scavenging system (Materska & Perucka, 2005). Flavonoids are able to inhibit the enzymes which are involved in ROS/RNS generation, such as protein kinase C, lipoxygenase, mitochondrial succinoxidase, cyclooxygenase and xanthine oxidase (Pietta, 2000). Flavonoids are also able to efficiently chelate metals such as copper and iron, thus it is able to prevent the synthesis of aggressive hydroxyl radicals that arise from transition metal ions pro-oxidant activities (Pietta, 2000). The presence of multiple hydroxyl groups in plant polyphenolic have contributed to their redox capacities, which allow them to act as singlet oxygen quenchers, hydrogen donor, reducing agent, ferryl haemoglobin reductant and metal chelating agent (Gebicka & Banasiak, 2009; Rice-evans, Miller, Bolwell, Bramley, & Pridham, 1999; Rice-Evans, Miller, & Paganga, 1997). Several studies reported that a high intake of polyphenol-rich food plants improve endothelial dysfunction and decrease vascular oxidative stress, which reduced risk of cardiovascular diseases (Heitzer, Just, & Munzel, 1996; Motterlini, Foresti, Bassi, & Green, 2000).

2.3 Cancer

Cancer is often the focal point of media attention world-wide due to the serious mortality rate from such a disease, affecting both men and women, rich and poor, all over the globe. The modern 'fast-food' diet and 'couch-potato' lifestyles are among the reasons that could have contributed to the increase in the number of cases and deaths from cancer world-wide. Carcinogenesis is also currently considered as 'a rapid genetic evolution' that could appear as people age, and also as a consequence from the loss of control in cellular proliferation (Ebadi, 2007). World Health Organization (WHO) has made various suggestions through seminars and numerous representatives organizations to improve the outcome of cancer treatment (Miller, Hoogstraten, Staquet, & Winkler, 1981). Among the suggestions include comprehensive data documentation (information of the individuals, the tumour, laboratory and radiologic data), with the outcomes records properly archived (after each treatments and therapy, staging of toxicity, responses, recurrence and disease-free interval). These recommendations may provide crucial information that are pivotal in preventing cancer incidence and could contribute towards the reduction of cancer related mortality world-wide.

2.3.1 Colon cancer

National Cancer Society Malaysia reported that colon or colorectal cancer is the second most common cancer affecting Malaysians each year, across all age groups (Hassan and Lim, 2010). Although it can affect anyone, older men (over the age of 50 years) are said to be at a higher risk to have the disease. Colon cancer could arise from either in the large intestine (colon) or the rectum (end of the colon). The disease could start when a benign adenomatous polyp (the small outgrowths in the lining of the colon) develops into an advanced adenoma with high-grade dysplasia (HGD). The abnormal cells eventually divide uncontrollably, especially when mutations occurred in the

gene(s) that control cellular division. This could eventually lead to an invasive cancer and making the disease malignant. This occur when metastasis takes place, where the cells from the primary tumour site migrate and colonize other parts of the body, such as the liver or lung.

In early stages of colon cancer, a mutation that deactivates the *APC* gene (adenomatous polyposis coli) could occur. *APC* is a tumor-suppressor gene, and its inactivation allowed uncontrolled cellular division and leading to the formation of a polyp. Later, the mutation in *Ras* oncogene could occur. *Ras* mutation commonly takes place in the larger-sized polyp, and this situation stimulates the tumour to grow even larger. Other tumor-suppressor genes such as p53 could also be mutated during the disease progression. Additional genetic mutations / lesions in the tumour allowed the cancer cells to invade and spread to other parts of the body. The mutation of genes involve in DNA repair could make the condition worse. Numerous rare inactivating mutations of genes which maintained chromosomal stability could also occur in colon cancer cells. Colorectal cancer is different from some other cancers, as it does not commonly involve amplification of gene copy number or gene rearrangement (Markowitz & Bertagnolli, 2009).

2.4 Phytochemicals and cancer therapeutics

Previous studies on the plant phytochemicals has revealed that phytochemicals with antioxidant activity may reduce the risk of cancer and improve health (Kushi et al., 2006; Liu, 2003).

A wide variety of bioactive phytochemicals are found in plants which include alkaloids, flavonoids, tannins and triterpenes. These are some of the best known phytochemicals that could be used in cancer therapeutics. Although traditional folks had no idea about the existence of such compounds, various folklores have benefitted from the medicinal plants healing or poisonous properties through the ages. Plants and vegetables have been shown to have antioxidant activity that is able to prevent DNA damage and prevent transformation of normal cells into cancerous ones. For example, *Syzygium jambolanum* leaf extract was shown to have anti-proliferative activity on cancer cells (Thliamplam, Kuppusamy, Kanthimathi, & Wiart, 2006). The proliferation of HT29 and MCF-7 (colon and breast cancer cell lines, respectively) cell lines was inhibited when strawberry extract was added to the cell cultures (Olsson, Andersson, Berglund, Gustavsson, & Oredsson, 2005).

Phenolic compounds are the major constituent from plants with antioxidant and anticancer properties. A number of phenolic compounds such as caffeic and gallic acid are known to show antioxidant, anti-proliferation and cytotoxic properties. A study on phenolic compounds suggested that the existence of three free phenolic hydroxyl groups besides a carboxyl group would be able to induce apoptosis (Inoue et al., 1994). Lignans are estrogenic-like polyphenols that have shown inhibitory effects on several types of cancer including breast cancer (Hirano et al., 1990). Flavonoids are polyphenolic agents which are widely present in plant products. Flavonoids have anti-cancer properties through different mechanisms such as carcinogen inactivation, cell cycle arrest,

antiproliferation, induction of apoptosis and differentiation, antioxidation and inhibition of angiogenesis (Ren, Qiao, Wang, Zhu, & Zhang, 2003). Alkaloids are secondary metabolite of many organisms including plants which contain nitrogen atom in their structure. Solamargine, an alkaloid extracted from the Chinese herb *Solanum inacum*, was reported to induce apoptosis (Hsu, Tsai, Lin, Yen, & Kuo, 1996) and was able to down regulate HER2/neu expression in breast cancer cell lines (Shiu, Liang, Huang, Sheu, & Kuo, 2008). Many other chemical components such as polysaccharides, glycoproteins, lectins, terpenoids, isoprenoids and quinines found in plants that have been reported to show apoptotic effects on cancerous cells to some extent, as well as cancer prevention properties (Deepak & Salimath, 2006; Taraphdar, Roy, & Bhattacharya, 2001).

Novel plant materials could be used for targeted therapy, which is an alternative way of cancer treatment, and this is usually utilized as an adjuvant therapy alongside chemotherapy. Targeted therapy could be achieved using specific components from plants which preferentially induce apoptosis of cancerous cells. Examples of this sort of treatment include: direct targeting of expression of specific proteins such as HER2/neu, epithelial growth factor receptor (EGFR), or vascular endothelial growth factor (VEGF) in order to cut down the tumour growth rate and inducing cell apoptosis. Apoptosis induction is one of the key event that must be fulfilled by a novel drug or adjuvant molecule to qualify itself to be a potential agent of cancer treatment. Apoptosis may be initiated by signals from intra or extracellular part of the cells, involving a cascade of biochemical reactions which eventually leading to cell death. Apoptosis causes activation of certain enzymes and biological pathways, which include activation of caspases (a family of proteases), endogenous endonucleases and caspase-activated DNase. Such activation leads to chromatin cleavage and cell shrinking. It has been

shown that several anti-tumor agents were able to selectively induce apoptosis mainly in neoplastic cells (Taraphdar et al., 2001).

Due to uncontrolled divisions in cancerous cells, gene expression and protein expression patterns are significantly different compared to normal cells. Plant extracts and its components are capable of influencing gene expression in mammalian cells (Coldren et al., 2003). New methods are being developed to manipulate gene expression/protein expression patterns to inhibit tumour growth (Wang et al., 2005). *Selaginella tamariscina*, is a traditional medicinal plant which has been used to treat advanced stage cancer and it was demonstrated to have a modifier effects on gene expression (Kuo et al., 1998). The p53 gene expression, which plays a substantial role in tumour suppression by regulating the apoptosis process, and other genes such as bcl-2, which is considered as an anti-apoptosis gene can be regulated by plants' phytochemicals (Hsieh et al., 1998; Lee, Nishikawa, Furukawa, Kasahara, & Kim, 1999).

2.5 Profile of selected plants from tropical biomass

2.5.1 *Polygonum minus* Huds

Polygonum minus Huds (*P. minus*) is a tropical herbaceous plant that belongs to the family *Polygonaceae*, and locally known as 'pigmy knot weed' in English and *Kesum, Cenohom, Jarak Belanda, Kelima Paya* and *Kunyit Jawa* in Malay. This creeping-type plant is found in Southeast Asian countries namely Malaysia, Indonesia, Thailand and Vietnam, particularly nearby rivers and lakes, as well as in the wet and damp areas such as the side ditches. This plant can grow up to a height of 1.0 m in lowland and up to 1.5 m in highland areas. The leaves (Figure 2.3) are about 5-7 cm long and 0.5-2.0 cm wide with narrow and lanceolate shape, dark green in color and very aromatic (Bunawan, Talip, & Noor, 2011). The stem is cylindrical, dark green with a little reddish color and having short internodes and nodal segments of simple roots. Influorescence is apical, flowers are small white purple-colored of 1.5 to 2.0 mm long and it could have lenticular black or dark brown coloured fruits (Bunawan et al., 2011). This plant has a sweet and pleasent aroma (like lemonade unique aroma), hence it is popularly used as flavouring ingredient in Malay cuisines. It could also be consumed directly as vegetable salad (*ulam*). *P. minus* has profitable market value in food additive and perfume industries because it is relatively rich in aliphatic aldehydes (Baharum, Bunawan, Ghani, Mustapha, & Noor, 2010).

Kesum is thought as having a significant impact as a traditional therapeutic agent. It has long been used by traditional practitioners by many ancient cultures. Traditionally, *kesum* leaves is used to help alleviate indigestion, prevention from dandruff and also as tonic drink for mothers after giving childbirth (Baharum et al., 2010; Burkill, 1966; Lachumy, Sasidharan, Sumathy, & Zuraini, 2010; Wiart, 2006). It has been reported that Sarawak folklore have used *Kesum* to treat sprains and body

aches (Wiart, 2006). Moreover, the leaves are used to as antifungal agent, by applying the leaves to the infected skin with a small amount of kerosene (Ong & Nordiana, 1999).

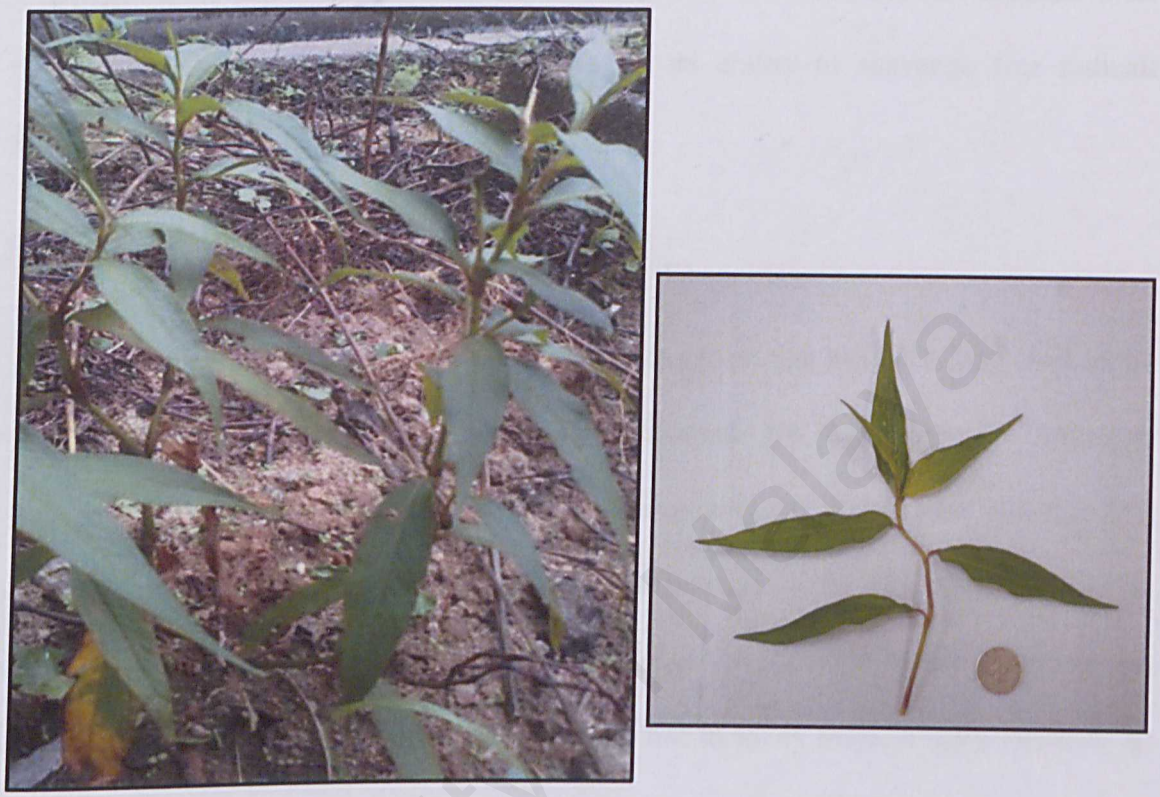

Figure 2.3: *P. minus* plants

P. minus has been reported to exhibit various biological activities such as antiviral activity towards v*esicular stomatitis (VSV)* and *herpes simplex type*-1 (HSV-1) viruses; antibacterial activities against *Bacillus subtilis*, *Helicobacter pylori* and *Pseudomonas aeruginosa*; and antifungal properties towards *Colletotrichum gloeosporioides* (Ali et al., 1996; Jamal, Karim, Abdullah, Raus, & Zuhanis, 2011; Johnny, Yusuf, & Nulit, 2011; Mackeen et al., 1997). Other studies have reported that *Kesum* showed antiacetylcholinesterase activity (Noor Hashim et al., 2012) and was cytotoxic on human cervical carcinoma (HeLa) cells (Mackeen et al., 1997). In addition, *P. minus* has been described to contain essential oils such as aldehydes (68.624 %), hydrocarbons (13.489 %), alcohols (9.857 %), esters (0.071 %) and furans (0.004

%) (Baharum et al., 2010). Gallic acid, rutin, coumaric acid, quercetin, vitamins, calcium, and flavonoids are some of the compounds reportedly found in *P. minus* based on *in vitro* cell culture study (Baharum et al., 2010; Huda-Faujan, Noriham, Norrakiah, & Babji, 2007). *P. minus* extract was also reported to inhibit ethanol induced ulcer formation and strong antioxidant activities by its ability to scavenge free radicals (Narasimhulu & Mohamed, 2014).

2.5.2 *Alpinia galanga*

Alpinia galanga (L.) Willd. is a rhizomatous plant that has been long used in the local cuisines and traditional medicine. The synonyms for *A. galanga* are *Amomum galanga* (L.) Lour, *A. viridiflora* Griff, *Maranta galanga* (L.), *Languas galanga* (L.) Stuntz and *Languas vulgare* J. Koeni. This plant belongs to the Zingiberaceae family, and also known as greater galangal in English and as '*lengkuas*' in Malay. Although the cultivation of this perennial herb is now carried out in many tropical areas of Asia, *A. galanga* was mainly harvested from India. *A. galanga* could grow to the maximum height of 129.4 cm with more than 48 tillers per clump and 13 leaves per tiller. Arambewela and Wijesinghe (2013) described *A. galanga* leaves as oblong-lanceolate, acute, glabrous, green colour on the adaxial surface and paler green colour on the abaxial surface, with slightly callus white margins, while its sheaths are long and glabrous, ligule are short and rounded. This plant has flowers of greenish white in colour, with 30 cm panicles and ovate-lanceolate bracts. The calyx is tubular and irregularly 3-toothed. In addition, it has orange red fruits. The roots are adventitious, in groups, fibrous, persistent in dried rhizomes and are about 0.5 to 2.0 cm long and 0.1 to 0.2 cm in diameter and yellowish brown in colour. The rhizomes are cylindrical, branched, with about 2 to 8 cm in diameter, longitudinally ridged with prominent rounded warts (remnants of roots) marked with fine annulations (Chudiwal et al., 2010).

A. galanga rhizome has light yellow or white flesh and found to be slightly aromatic. Harvested rhizomes can be carefully washed and cut into small segments for consumption or dried for storage (Figure 2.4).

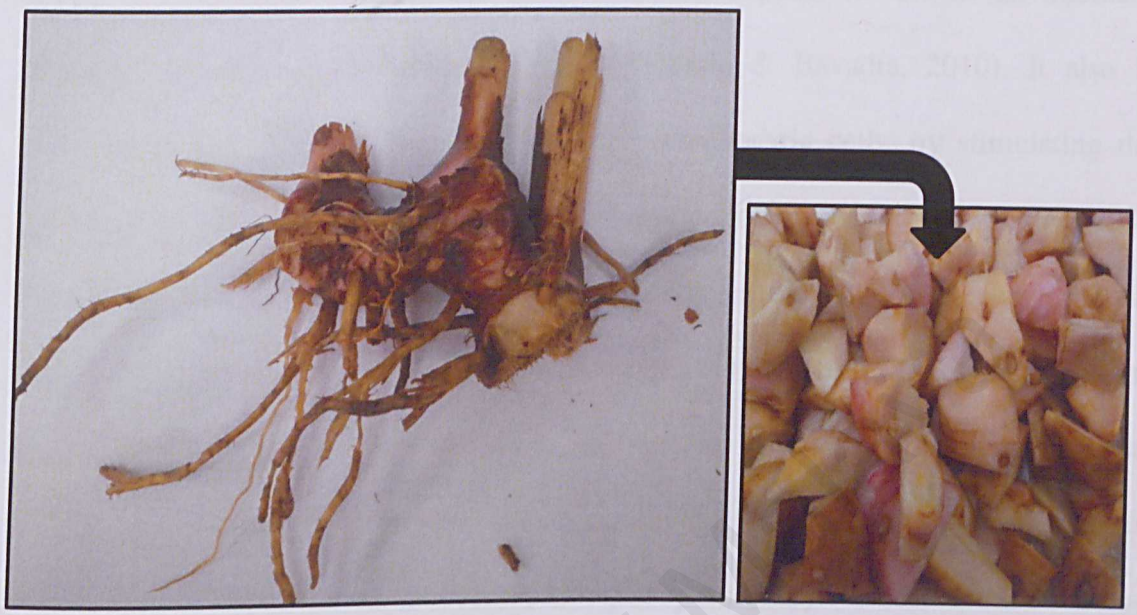

Figure 2.4: *A. galanga* rhizomes that have been washed and cut into small pieces.

A. galanga has been used in culinary, cosmetics and medication for centuries. Rhizomes of this plant has a pungent, hot and spicy taste with aromatic ginger odour (Chudiwal et al., 2010), hence it is widely used as a condiment and food flavouring in Southeast Asian delicacies including soups and curries. *A. galanga* is also used in Ayurvedic practise, and other traditional medicine practise from various regions including Unani, Chinese and Thai. *A. galanga* rhizome is widely used in traditional medicine, among which are: diabetes, heart disease, rheumatic pains, sore throat, stomachic, diarrhoea and vomiting (Chudiwal et al., 2010; Oonmetta-aree, Suzuki, Gasaluck, & Eumkeb, 2006). *A. galanga* is also used in Malay traditional medicine for skin fungal infection (Ong & Nordiana, 1999).

A number of studies have highlighted *A. galanga* therapeutic values such as antiviral, antibacterial, antifungal, antiameobic, antiplasmid, antileishmanial, anti-platelet, antioxidant, antitumour/anticancer, antimelanogenic, anti-inflammatory,

antiulcer, antiallergic, gastroprotective, immunostimulating effect, hypolipidemic properties (Chan, Ng, Tan, & Low, 2011; Chudiwal et al., 2010; Kaushik, Yadav, Kaushik, Sacher, & Rani, 2011; Muangnoi et al., 2007). *A. galanga* extract exhibited antidiabetic property *in vitro* and *in vivo*, which support its use as an alternative medicine for diabetes (Srividya, Dhanabal, Satish, & Bavadia, 2010). It also has immunoenhancing effects on phagocytic and lymphocytic cells, by stimulating their proliferation in the peritoneal cavity (Bendjeddou, Lalaoui, & Satta, 2003). Furthermore, a recent study suggested that the extracts of *A. galanga* showed antiamnesiac activity through its antioxidant property (Hanish Singh et al., 2011). Some of the major compounds isolated from *A. galanga* are: 1'S'-1'-hydroxychavicol acetate, 1'S'-1-acetoxyeugenol acetate, 1'S'-1'-acetoxychavicol acetate, trans-*p*-coumaryl alcohol, trans-*p*-hydroxycinnamaldehyde, trans-*p*-coumaryl diacetate and trans-*p*-hydroxy cinnamyl acetate (Chudiwal et al., 2010; Kaushik et al., 2011; Mayachiew & Devahastin, 2008).

2.5.3 *Etlingera elatior*

Etlingera elatior (Jack) R. M. Smith (*E. elatior*) is another widely used plant in traditional medicine and local cuisines. It belongs to the family Zingiberaceae and locally known '*kantan*' in Malay. Its synonyms are *Alpinia acrostachya* Steud., *Alpinia elatior* Jack, *Amomum tridentatum* (Kuntze) K. Schum., *Nicolaia elatior* (Jack) and *N. imperialis* Horan. This plant is also known as 'torch ginger' or 'red ginger lily' due to the striking similarity of the inflorescence to a flaming torch (Yunus et al., 2012). *E. elatior* can grow up to 5-6 m tall and usually cultivated in clusters or gaps. In contrast to other rhizomatous plants, *E. elatior* produced aromatic stout rhizomes that are subterranean or above ground (Chan et al., 2007). Each rhizome can produce leafy shoots or on erect shoots near the base of the plant. This relatively large ginger plant has

antiulcer, antiallergic, gastroprotective, immunostimulating effect, hypolipidemic properties (Chan, Ng, Tan, & Low, 2011; Chudiwal et al., 2010; Kaushik, Yadav, Kaushik, Sacher, & Rani, 2011; Muangnoi et al., 2007). *A. galanga* extract exhibited antidiabetic property *in vitro* and *in vivo*, which support its use as an alternative medicine for diabetes (Srividya, Dhanabal, Satish, & Bavadia, 2010). It also has immunoenhancing effects on phagocytic and lymphocytic cells, by stimulating their proliferation in the peritoneal cavity (Bendjeddou, Lalaoui, & Satta, 2003). Furthermore, a recent study suggested that the extracts of *A. galanga* showed antiamnesiac activity through its antioxidant property (Hanish Singh et al., 2011). Some of the major compounds isolated from *A. galanga* are: 1'S'-1'-hydroxychavicol acetate, 1'S'-1-acetoxyeugenol acetate, 1'S'-1'-acetoxychavicol acetate, trans-*p*-coumaryl alcohol, trans-*p*-hydroxycinnamaldehyde, trans-*p*-coumaryl diacetate and trans-*p*-hydroxy cinnamyl acetate (Chudiwal et al., 2010; Kaushik et al., 2011; Mayachiew & Devahastin, 2008).

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beautiful leaves with conspicuous bracts (size could be up to 80 × 18 cm) and entirely green in colour, although the young leaves are sometimes flushed pink in their colour (Figure 2.5). *E. elatior*'s inflorescences and leaves released a distinctive pleasantly fragrant aroma. This plant is broadly cultivated throughout Southeast Asia especially Malaysia and Indonesia, especially the inflorescence is widely used in local cuisines (Chan et al., 2011c). In Malaysia, there are more than 15 different species of *Etlingera* plants that have been recorded (Chan et al., 2007; Lim, 2001).

Figure 2.5: *E. elatior* plants and its inflorescences

This plant is used traditionally as flavouring agent and also in traditional medicine (Mohd Jaafar, Osman, Ismail, & Awang, 2007). The mature fruits of *E. elatior* are edible but sour, and are reputed to have antihypertensive activity. The hearts of young shoots and inflorescence is consumed as vegetable salad (*ulam*) that is also often called as 'Kerabu'. It is also a main ingredient for a locally popular cuisine called 'Laksa'. Furthermore, the young inflorescence is an essential ingredient of curry, sour and spicy dishes. In traditional medicine, the decoction of the fruits is used to treat earache, and decoction of the leaves are used to clean wounds. The consumption of

young shoots by thought to help to remove the unpleaseant body odour (Mohamad et al., 2005). In Indonesia, this plant is used to eliminate the odour of fish; and to inhibit pathogenic bacteria and fungus growth in foods (Andarwulan, Batari, Sandrasari, Bolling, & Wijaya, 2010). The inflorescence aroma with its brilliant pink color has also made it appealing enough to be sold as commerical flowers in Hawaii and Australia (Chan, Lim, & Wong, 2011).

Several studies using various parts of this plant showed that *E. elatior* possess beneficial bioactivities such as antimicrobial, antitumour, antioxidant, tyrosinase inhibition, hepatoprotective and cytotoxic activities (Chan et al., 2011b; Habsah et al., 2005; Haleagrahara et al., 2010; Jackie, Haleagrahara, & Chakravarthi, 2011; Lachumy, Sasidharan, Sumathy, & Zuraini, 2010). Diarylheptanoids, labdane diterpenoids and steroids have been isolated from the rhizomes of *E. elatior*. Phytochemical screening of the inflorescence showed presence of terpenoids, tannins, phenolic acid, anthocyanin, flavonoids, saponins and carbohydrates (Chan et al., 2011b). The presence of flavonoids and caffeoylquinic acids were also reported in the leaves (Chan et al., 2009). A recent study showed that *E. elatior* extract has protective effect against lead-induced damage of bone marrow cells, and thus demonstrating its potential therapeutic ability in treating lead poisoning, most possibly from its lead chelating ability (Haleagrahara et al., 2010).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Research Materials

3.1.1 Chemicals

Sigma-Aldrich:

i. 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

ii. Gallic acid

iii. L-ascorbic acid

iv. Iron (III) chloride tetrahydrate

v. Quercetin

vi. Phosphate Buffered Saline (PBS)

vii. Phosphoric acid (H_3PO_4)

viii. Dimethyl sulphoxide (DMSO)

ix. Trypan blue (cell culture tested)

x. Sodium acetate trihydrate ($CH_3NOONa.3H_2O$)

xi. Sodium nitroprusside dihydrate (SNP)

xii. 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt (ferrozine)

xiii. Sulphanilic acid

xiv. Phenazine methosulphate (PMS)

xv. Potassium persulphate ($K_2S_2O_8$)

xvi. Penicillin and streptomycin

xvii. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent

xviii. Potassium acetate (CH_3CO_2K)

xix. Aluminum trichloride ($AlCl_3$)

xx. Ethylenediaminetetraacetic acid (EDTA)

Merck Millipore :

i. Sodium Chloride

ii. Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)

iii. 2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ)

iv. Dihydronicotinamide adenine dinucleotide disodium salt (NADH-Na_2)

v. Folin-Ciocalteu's phenol reagent

vi. N-(1-Naphthyl)ethylene-diamine dihydrochloride (NEDD)

vii. Trolox

viii. Hydrogen peroxide

ix. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), NH_4 (ABTS™) chromophore, diammonium salt

x. *p*-Nitroblue tetrazolium chloride (NBT)

xi. *n*-Hexane

xii. Methanol

xiii. Ethyl acetate

xiv. Glacial acetate acid

xv. Iron (II) sulphate ($FeSO_4.7H_2O$)

xvi. Hydrochloric acid 6M

JR Scientific :

i. Trypsin 0.25 % (1X) solution

ii. Fetal Bovine Serum (FBS)

Nacalai Tesque :

i. Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/Ham's F-12) with HEPES, sodium pyruvate and L-glutamine

Thermo Fisher Scientific :

i. Dimethyl sulphoxide (DMSO)

3.1.2 Kits

i. ApoTox-Glo™ Triplex Assay Kit (PROMEGA)

3.1.3 Cell Culture

i. Human colon carcinoma cell line, HCT 116 (ATCC® CCL-247™)

ii. Human normal colon cell line, CCD 841 CoN (ATCC® CRL-1790™)

3.1.4 Sample

i. *Polygonum minus* Huds. (*P. minus*), (leaves) (pokok kesum)

ii. *Alpinia galanga* (L.) Willd. (*A. galanga*), (rhizome) (lengkuas)

iii. *Etlingera elatior* (Jack) R.M.Sm. (*E. elatior*), (flower) (bunga kantan)

3.2 Research Methodology

3.2.1 Samples preparation

Fresh and healthy plant materials (*E. elatior* inflorescence, *A. galanga* rhizome, *P. minus* leaves) were purchased from the local wet market and processed on the same day. The identity of the samples were confirmed by Dr. Sugumaran Manickam (Rimba Ilmu, ISB, Faculty of Science). Each sample was cleaned with water, sliced into smaller pieces and dried in a 40 °C oven until no weight reduction was observed. The samples were powdered using a table blender and stored at -20 °C until further analysis.

3.2.2 Extraction of plant materials

Samples were extracted sequentially using the following solvents in the order indicated: hexane>ethyl acetate>methanol>water. 30 g of dried plant material was extracted using 300 mL of solvent in an incubator shaker at 37 °C for 8 hours and centrifuged at 200G for 5 min. The supernatant was filtered through Whatman filter paper (No. 4). The extraction was repeated twice, and the plant material residues were dried. The extraction was continued using the same method for each of the remaining solvents following the sequence as indicated above. Each of the 900 mL solvents collected was evaporated at 40 °C using a rotary evaporator. The filtrate of the water extract was dried using a freeze-dryer. The plant extracts were dissolved in 10% dimethyl sulfoxide (DMSO) at 2 mg/mL and kept at -20 °C until needed for the assays.

3.2.3 Analyses for antioxidant potential

3.2.3.1 Total phenolic content (TPC)

Total polyphenol content (TPC) was determined using Folin-Ciocalteu reagent (FCR) (Singleton & Rossi, 1965). 500 µL of aqueous FCR (10% v/v) was mixed with 100 µL of the plant extract and incubated for 5 min. 350 µL of 1 M Na_2CO_3 was added to the mixture and incubated at room temperature for 2 h, followed by absorbance measurement at 765 nm. A standard calibration curve was constructed using gallic acid using concentrations up to 0.2 mg/mL. All experiments were done in triplicate and results were expressed as mg gallic acid equivalents (GAE) per gram of raw material.

3.2.3.2 Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined according to Chang, Yang, Wen, and Chern (2002) with slight modification. 5 µL aluminium trichloride (10% w/v) and 5 µL of potassium acetate (1 M) were mixed with 25 µL of plant extract. 75 µL of ethanol (95% v/v) and 140 µL of distilled water were added to the mixture successively and mixed. The mixture was incubated for 30 minutes at room temperature followed by absorbance reading at 415 nm. Quercetin was used as a standard and flavonoid values were expressed as µg quercetin per gram of raw material. All determinations were done in triplicate.

3.2.4 Analyses for antioxidant activity

3.2.4.1 Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) was determined according to Benzie and Strain (1996) with minor modifications. FRAP reagent was freshly made by mixing the following solutions in a 10:1:1 volume ratio (v/v/v) – acetate buffer (300 mM, pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and $FeCl_3.6H_2O$ (20 mM). 10 µL of the plant extract was mixed with 300 µL of FRAP reagent and absorbance reading at 593 nm was taken 30 min thereafter. A calibration curve was plotted using $FeSO_4$ solution (0–1 mM). The results were expressed as µmol Fe^{2+} per gram of raw material. Quercetin, gallic acid, ascorbic acid and trolox were used as positive controls. All determinations were done in triplicate.

3.2.4.2 $ABTS^{\bullet+}$ radical-scavenging activity

The $ABTS^{\bullet+}$ (2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity was determined according to Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) with minor modifications. $ABTS^{\bullet+}$ radical cations was made by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate and left in the dark for 12-16 h at room temperature. The resulting solution absorbance at 734 nm was adjusted to 0.7 using ethanol. 20 µL of plant extract was mixed with 180 µL of $ABTS^{\bullet+}$ reagent and the absorbance at 734 nm was taken after 30 minutes. Quercetin, gallic acid, ascorbic acid and trolox were used as positive controls. The TEAC values were calculated for the extracts using trolox calibration curve (0–1.6 mM). Results were expressed as µmol trolox equivalents (TE)/g raw material. All experiments were done in triplicate.

3.2.4.3 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical-scavenging activity

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity was determined according to Brand-Williams, Cuvelier, and Berset (1995) with minor modifications. 100 µM DPPH solution was prepared in methanol and left in the dark for at least 30 min. 20 µL of plant extracts at different concentrations (up to 1000 µg/mL) was mixed with 150 µL of 100 µM DPPH solution. After 30 min, the absorbance of the reaction mixture was taken at 515 nm. Quercetin, gallic acid, ascorbic acid and trolox at concentrations up to 1000 µg/mL were used as positive controls. A calibration curve was constructed using Trolox at concentrations up to 400 mM. DPPH radical scavenging activity of the extracts was expressed as µmol trolox equivalents (TE) per gram of raw material. All experiments were done in triplicate. The DPPH radical-scavenging rate of test compounds was calculated using the formula $[(A_0 - A_x) / A_0] \times 100\%$, where A_x and A_0 are the absorbance in the presence and absence of test compounds, respectively. When significant scavenging activity was detected, the EC_{50} value was also estimated.

3.2.4.4 Superoxide anion radical ($O_2^{\bullet -}$)-scavenging activity

Superoxide anion radical-scavenging activity was evaluated according to Robak and Gryglewski (1988) with slight modifications. Reagents consisted of 150 µM nitroblue tetrazolium (NBT), 468 µM nicotinamide adenine dinucleotide (NADH) and 60 µM phenazine methosulphate (PMS) in 0.1M phosphate buffered saline. 50 µL of extract, at different concentrations (0–1000 µg/mL), was mixed with 50 µL of NBT, 50 µL of NADH and 50 µL of PMS. After incubation in the dark for 10 min at room temperature, the absorbance was read at 570 nm. Gallic acid was used as positive control. All determinations were done in triplicate. Results were expressed as percentage inhibition

of $O_2^{\bullet -}$ radicals. The EC_{50} was estimated when significant $O_2^{\bullet -}$ scavenging activity was observed.

3.2.4.5 Nitric Oxide ($NO^{\bullet}$) radical-scavenging activity

Nitric oxide scavenging activity was measured according to Awah and Verla (2010) with minor modification. Twenty-five microlitres of the extract, at different concentrations (0–1000 µg/mL), were mixed with 25 µL of freshly prepared 5 mM sodium nitroprusside solution in phosphate buffered saline (pH 7.3). The mixture was then incubated for 60 min under a visible polychromatic light (150 W). Griess reagent (50 µl), containing equal volumes of 1% sulfanilamide in 5% phosphoric acid (H_3PO_4) and 0.1% of naphthylethylenediamine dihydrochloride was added to the mixture and incubated for 5 min before absorbance was read at 550 nm. Gallic acid was used as positive control. All determinations were done in triplicate. Results were expressed as percentage of NO radical-scavenging activity. The EC_{50} was estimated when significant NO scavenging activity was observed.

3.2.4.6 Ferrous (Fe^{2+}) ion-chelating (FIC) ability

The FIC ability was determined according to the method of Decker and Welch (1990) with minor modifications. For each determination, the following solutions were added & mixed sequentially in a 96-well microplate: 50 µL of each of the extracts (0-2000 µg/mL), 20 µL of 0.5 mM $FeCl_2$, 160 µL of distilled water and 20 µL of 2.5 mM ferrozine. The well mixed mixture was allowed to stand for 10 min at room temperature. The ferrous iron–ferrozine complex formation was then monitored by taking the absorbance at 562 nm against a blank reaction mixture, which uses 50 µL of distilled water instead of the tested extracts. EDTA was used as a positive control. All

measurements were performed in triplicate. EC_{50} value is the concentration (mg/ml) at which the chelating activity of the tested compound was at 50%.

3.2.5 Statistical analyses

Unless otherwise specified, each determination was performed in triplicate. Results were presented as means ± standard deviation and analysed by SPSS statistical software (version 20.0, SPSS Inc.). One-way analysis of variance (ANOVA) and Tukey test were used to compare means among groups (three and more groups). Independent *t*-test was calculated to compare mean between two groups. Pearson correlation were used to investigate the correlation between antioxidant components and the antioxidant activities. An observation is considered statistically significant if the *p* value was less than 0.05 ($p<0.05$).

3.2.6 MTT cell proliferation assay

The anti-proliferative activity of the extracts was evaluated through MTT (3,(4,5-dimethythiazol-2-yl)-2,3-diphenyl tetrazolium bromide) assay according to Twentyman and Luscombe (1987) with slight modification. HCT 116 (CCL-247, colorectal carcinoma) and CCD841 (CRL-1790, normal colon) cell lines were cultured and maintained according to the protocol specified by ATCC. The cells (~5.0×10^3) were incubated overnight in a 100 µL culture media/96-well plate and thereafter treated with 20 µL of plant extracts at different concentrations. After 48 hr of incubation, 20 µL of MTT solution was added into each well and incubated for another 4 hr. The culture solution was then discarded and 150 µL DMSO was added to solubilize the formazan crystals. The absorbance at 595 nm was then taken using a microplate reader. Each

determination was done in six replicates. When significant anti-proliferative activity was observed, the IC_{50} value was estimated.

3.2.7 Apoptosis: caspase – 3/7 activation assay

Detection of apoptosis was measured by measuring the caspase 3 and 7 activities using Apotox-Glo™ triplex kit (Promega). The HCT116 cells were seeded at a density of 10,000 cells/well in an opaque 96-well plates, and incubated for 24 hours to allow cells adherence. Selected plant extracts with different concentrations (untreated, ½ × IC_{50}, 1 × IC_{50} and 2 × IC_{50}) were added to appropriate wells for final volume of 100 µL per well. The cells were exposed with each of the extracts for 3 different time durations (8, 16 and 24 hours). Then, 100 µL of Caspase-Glo reagent was added to each well and mixed with orbital shaker (500 rpm for 30s). The mixture was then incubated at room temperature for 30 min, after which luminescence reading was taken using GloMax®-Multi Microplate Multimode Reader (Promega). Each determination was done in triplicate

3.2.8 Analysis of selected extracts using Liquid Chromatography Ion-Trap Time-of-Flight mass spectrometry (LCMS-IT-TOF)

2 mg of the dried extract was dissolved in 1 mL of methanol (HPLC grade) and filtered using 0.25 µm nylon filter membrane. The extract was analyzed on Shimadzu LCMS-IT-TOF system, equipped with a PDA UV detector (220, 254, and 350 nm) and Ion Trap TOF mass spectrometer. The analysis was performed in positive and negative ion modes, using Waters XBridge BEH C_{18} column (PN 186003085, 2.1 x 50 mm, 2.5 µm) with these parameters: solvent A - 0.1% formic acid; solvent B – acetonitrile with 0.1% formic acid; flow rate - 0.25 mL/min; column temperature: 40°C; injection volume - 25 µL; gradient: 0-100 % over 14 minutes. The compounds were identified by comparison of the MS data to those reported in the literature.

CHAPTER FOUR

RESULTS

4.1 Antioxidant components analyses

4.1.1 Extraction yield and total phenolic and flavonoid contents

The edible parts of *P. minus* (PM), *A. galanga* (AG) and *E. elatior* (EE) plants were dried and extracted using four solvents in ascending order of polarities, namely hexane, ethyl acetate, methanol, and water. The antioxidant components were ascertained by determining the extracts TPC and TFC values. The extraction yield and antioxidant components in *P. minus*, *A. galanga* and *E. elatior* are as shown in Table 4.1. Results were expressed as means of triplicates ± standard deviation (SD). The yield was presented as percentage of starting material (dry weight). The phenolic content (TPC) was expressed as mg gallic acid equivalents (GAE) in 1 g of raw material ± SD. The flavonoid (TFC) content was expressed as mg quercetin equivalents (QE) in 1 g of raw material ± SD.

Extraction of *P. minus* with methanol showed the highest yield at 15.41% and this was followed by water (7.09%), ethyl acetate (1.85%) and hexane (1.56%). Among the extracts of *A. galanga*, the methanol extract had the highest yield of 24.01%, followed by hexane (4.43%), water (4.25%) and ethyl acetate (0.97%) extracts. For *E. elatior*, the hexane extract had the highest yield of 12.18%, and this is followed by water (9.11%), methanol (5.64%) and ethyl acetate (1.56%) extracts. Most of the extracts produced more than 1% yield. The sum of yields (total extractable materials)

using the four solvents from each of *P. minus*, *A. galanga* and *E. elatior* was 25.91%, 33.66% and 28.31% respectively.

The methanol extract of *P. minus* exhibited the highest total phenolic content at 174.00 ± 0.31mg GAE/g, while the water extract showed the second highest TPC value at 73.90 ± 0.23 mg GAE/g. The *P. minus* ethyl acetate had a TPC value of 37.18 ± 0.23 mg GAE/g while the hexane extract had the lowest TPC value of 2.45 ± 0.08 mg GAE/g. The polyphenol contents of the *A. galanga* extracts ranged from 9.36-20.87 mg GAE/g. As for *E. elatior* extracts, the methanol extract had the highest polyphenols content at 59.89 ± 0.48 mg GAE/g, followed by (in descending order) ethyl acetate (12.95 ± 0.28 mg GAE/g) > water (11.15 ± 0.10 mg GAE/g) and hexane extract (2.29 ± 0.16 mg GAE/g). The result showed that the phenolic compounds were present in all of the extracts although the TPC values were not the same among them. The sum of polyphenols from all of the extracts for *P. minus*, *A. galanga* and *E. elatior* were 288.49 ± 0.86, 57.27 ± 1.30 and 86.28 ± 1.02 mg GAE/g, respectively.

In this study, flavonoids were detected in all of the plant extracts. *P. minus* exhibited prominent flavonoid contents in ethyl acetate, methanol and water extracts, which were 47.22 ± 1.23, 53.19 ± 1.23 and 43.65 ± 0.62 mg QE/g extract respectively. As for the *A. galanga* extracts, ethyl acetate extract had the highest flavonoid content at 17.73 ± 0.21 mg QE/g. Only two of the extracts from *E. elatior* showed prominent TFC values, and the values were 16.43 ± 0.16 and 18.98 ± 0.28 mg QE/g for ethyl acetate and methanol extracts, respectively. The sum of flavonoid contents from all of the extracts (from apolar to polar fractions) for each of *P. minus*, *A. galanga* and *E. elatior* were 154.26 ± 3.44, 28.94 ± 0.59 and 48.61 ± 0.86 mg QE/g respectively

Table 4.1: The yield and antioxidant components of the extracts of *P. minus, E. elatior* and *A. galanga*.

	Extraction solvent				
	Hexane	Ethyl acetate	Methanol	Water	Total
Yield (%)					
PM	1.56	1.85	15.41	7.09	25.91
AG	4.43	0.97	24.01	4.25	33.66
EE	12.18	1.38	5.64	9.11	28.31
Polyphenol (mg GAE/g extract)[a]					
PM	2.45 ± 0.08^{a}	37.18 ± 0.23^{b}	174.00 ± 0.31^{c}	73.90 ± 0.23^{d}	288.49 ± 0.86
AG	9.36 ± 0.33^{e}	20.87 ± 0.20^{f}	15.99 ± 0.49^{g}	11.04 ± 0.27^{h}	57.27 ± 1.30
EE	2.29 ± 0.16^{a}	12.95 ± 0.28^{i}	59.89 ± 0.48^{j}	11.15 ± 0.10^{h}	86.28 ± 1.02
Flavonoid (mg QE/g extract)[b]					
PM	10.18 ± 0.34^{a}	47.22 ± 1.23^{b}	53.19 ± 1.23^{c}	43.65 ± 0.62^{d}	154.26 ± 3.44
AG	2.50 ± 0.13^{e}	$17.73 \pm 0.21^{f,g}$	2.12 ± 0.16^{e}	$6.57 \pm 0.08^{h,i}$	28.94 ± 0.59
EE	7.68 ± 0.25^{h}	16.43 ± 0.16^{f}	18.98 ± 0.28^{g}	5.50 ± 0.16^{i}	48.61 ± 0.86

Results are expressed as means ± standard deviation (n=3). TPC is expressed as mg gallic acid equivalents (GAE) in 1 g of dried extract (DE) ± SD. TFC is expressed as mg quercetin equivalent (QE) in 1 g of dried extract ± SD.

4.2 Antioxidant activities analyses

4.2.1 Ferric Reducing Antioxidant Power (FRAP) activities

Table 4.2 shows the FRAP values of the plant extracts. Gallic acid, the positive control used in the experiment showed the highest FRAP value, followed by (in descending order) ascorbic acid, quercetin and trolox. Among the plant extracts, generally, ethyl acetate, methanol and water extracts showed prominent FRAP values, which were more than 90 μmol Fe^{2+}/g extract. However, all of the hexane extracts had low FRAP values, which was lower than 35 μmol Fe^{2+}/g extract. Among the *P. minus* extracts, methanol extracts had the highest FRAP value at 1728.33 ± 1.66 μmol Fe^{2+}/g extract, followed by (in descending order) water (898.33 ± 4.16 μmol Fe^{2+}/g)> ethyl acetate (351.14 ± 5.61 μmol Fe^{2+}/g) and hexane (26.11 ± 0.48 μmol Fe^{2+}/g) extracts. The *P. minus* methanol extract is the only one with a FRAP value higher than 1000 μmol Fe^{2+}/g extract. Although *P. minus* methanol extract had the highest FRAP activity, it was approximately 3.7 times lower compared to that of trolox. Methanol extracts of *A. galanga* and *E. elatior* gave the highest FRAP values compared to the other extracts of the same plant, and the values were 158.33 ± 3.33 and 556.66 ± 8.66 μmol Fe^{2+}/g extract respectively.

Table 4.2: Ferric reducing antioxidant power (FRAP) of *P. minus*, *A. galanga* and *E. elatior* extracted sequentially with hexane, ethyl acetate, methanol and water.

	Extraction solvent			
	Hexane	Ethyl acetate	Methanol	Water
FRAP (μmol Fe^{2+}/g extract)				
P. minus	26.11 ± 0.48^{a}	351.14 ± 5.61^{b}	1728.33 ± 1.66^{c}	898.33 ± 4.16^{d}
A. galanga	31.66 ± 0.83^{a}	130.83 ± 1.66^{e}	158.33 ± 3.33^{f}	91.38 ± 0.48^{g}
E. elatior	14.21 ± 2.65^{h}	107.77 ± 0.48^{i}	556.66 ± 8.66^{j}	138.05 ± 3.46^{e}
FRAP activities of positive controls (μmol Fe^{2+}/g extract)				
	Quercetin	Gallic acid	Ascorbic acid	Trolox
	12265.60±651.81	23186.24±951.53	19399.65±473.16	4590.23±55.44

Results are expressed as means ± standard deviation ($n = 3$). Values with different superscripts (lower case letters,$^{a,b,c,...}$) are significantly different ($p < 0.05$). Ferric reducing antioxidant power is expressed as μmol Fe^{2+} per gram of dried extract.

4.2.2 ABTS$^{\bullet+}$ radical scavenging activities

The ABTS$^{\bullet+}$ radical-scavenging activity of the extracts was expressed as TEAC and as IC_{50} values (Table 4.3). Results were the average of triplicate ± SD. Trolox, quercetin, gallic acid and ascorbic acid were used as positive controls. The IC_{50} value was determined by referring to the concentration of extract at which 50% of the free radicals were inhibited. The scavenging activity was also expressed by TEAC value by comparing the activity of radical-scavenging of the extracts to that of trolox. The higher the TEAC value, the better the antioxidant potential of the corresponding extract.

Based on the results, only 4 extracts had IC_{50} values <2000 µg/mL. The methanol extract of *P. minus* showed the lowest IC_{50} value of 159.62 ± 25.14 µg/mL, and this is followed by the water extract of *P. minus* (IC_{50}, 198.36 ± 26.44 µg/mL), methanol extract of *E. elatior* (IC_{50}, 1308.27 ± 186.27 µg/mL) and ethyl acetate extract of *E. elatior* (IC_{50}, 1621.69 ± 5.60 µg/mL). For the positive controls, gallic acid had the lowest IC_{50} value, followed by quercetin, trolox and ascorbic acid, and their corresponding IC_{50} values were 4.73 ± 0.19, 10.12 ± 0.43, 38.02 ± 0.99 and 61.10 ± 2.53 µg/mL, respectively.

The TEAC values for different extracts from different plants ranged from 25.43 ± 1.77 to 226.25 ± 7.36 µmol trolox equivalents (TE) per gram of extract. The PM-methanol extract had the highest ABTS$^{\bullet+}$ radical-scavenging activity of 226.25 ± 7.36 µmol TE/g extract. The PM-water extract showed the second highest ABTS$^{\bullet+}$ radical-scavenging activity at 190.6 ± 4.29 µmol TE/g extract. Among the *A. galanga* extracts, the water extract had the highest ABTS$^{\bullet+}$-radical scavenging value, followed by (in descending order) ethyl acetate>methanol and hexane extracts. For *E. elatior*, the ABTS$^{\bullet+}$ radicals scavenging activity of the extracts (in descending order) were: methanol > water > ethyl acetate > hexane.

Table 4.3: $ABTS^{\bullet+}$ radical scavenging activities of three plants extracted with hexane, ethyl acetate, methanol and water.

Extracts	$ABTS^{\bullet+}$ radical scavenging activity	
	TEAC value (µmol TE/g extract)	IC_{50} (µg/mL)
P. minus (HX)	25.43 ± 1.77	NA
P. minus (EA)	133.23 ± 1.59	NA
P. minus (MeOH)	226.25 ± 7.36	159.62 ± 25.14
P. minus (W)	190.60 ± 4.29	198.36 ± 26.44
A. galanga (HX)	94.95 ± 0.44	NA
A. galanga (EA)	123.34 ± 3.74	NA
A. galanga (MeOH)	103.79 ± 6.8	NA
A. galanga (W)	129.21 ± 6.34	NA
E. elatior (HX)	26.10 ± 1.86	NA
E. elatior (EA)	90.94 ± 2.63	1621.69 ± 5.60
E. elatior (MeOH)	150.52 ± 6.17	1308.27 ± 186.27
E. elatior (W)	112.78 ± 5.59	NA
Trolox	standard curve	38.02 ± 0.99
Quercetin	12977.3 ± 120.13	10.12 ± 0.43
Gallic Acid	42107.87 ± 78.30	4.73 ± 0.19
Ascorbic acid	1567.27 ± 3.24	61.10 ± 2.53

Result are expressed as means ± standard deviation (n=3). NA: not available. HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water. TEAC, trolox equivalent antioxidant capacity, expressed as µmol trolox equivalents (TE) per gram of dried extract. IC_{50}, the concentration of the extracts that inhibited 50% of free radicals.

Figure 4.1(a-c) shows the $ABTS^{\bullet+}$-scavenging capacities of extracts and the antioxidant standard or positive controls. Most of the extracts showed a concentration-dependent radical-scavenging activity relationship. The activity curves of the positive controls demonstrated prominent scavenging activities, and they reached 100% inhibition at concentrations <200 µg/mL (except ascorbic acid). Among the extracts, the water and methanol extracts of *P. minus* exhibited the most prominent $ABTS^{\bullet+}$ radicals inhibition activity and they both reached 100% $ABTS^{\bullet+}$ radicals inhibition at 2000 µg/mL. The hexane extract of PM and EE are less reactive, showing inhibition of <20% at the highest concentration of the extracts. However, at the highest concentration of the extracts used in the assay (2000 µg/mL), none of them had $ABTS^{\bullet+}$ scavenging activity that was lower than 10%.

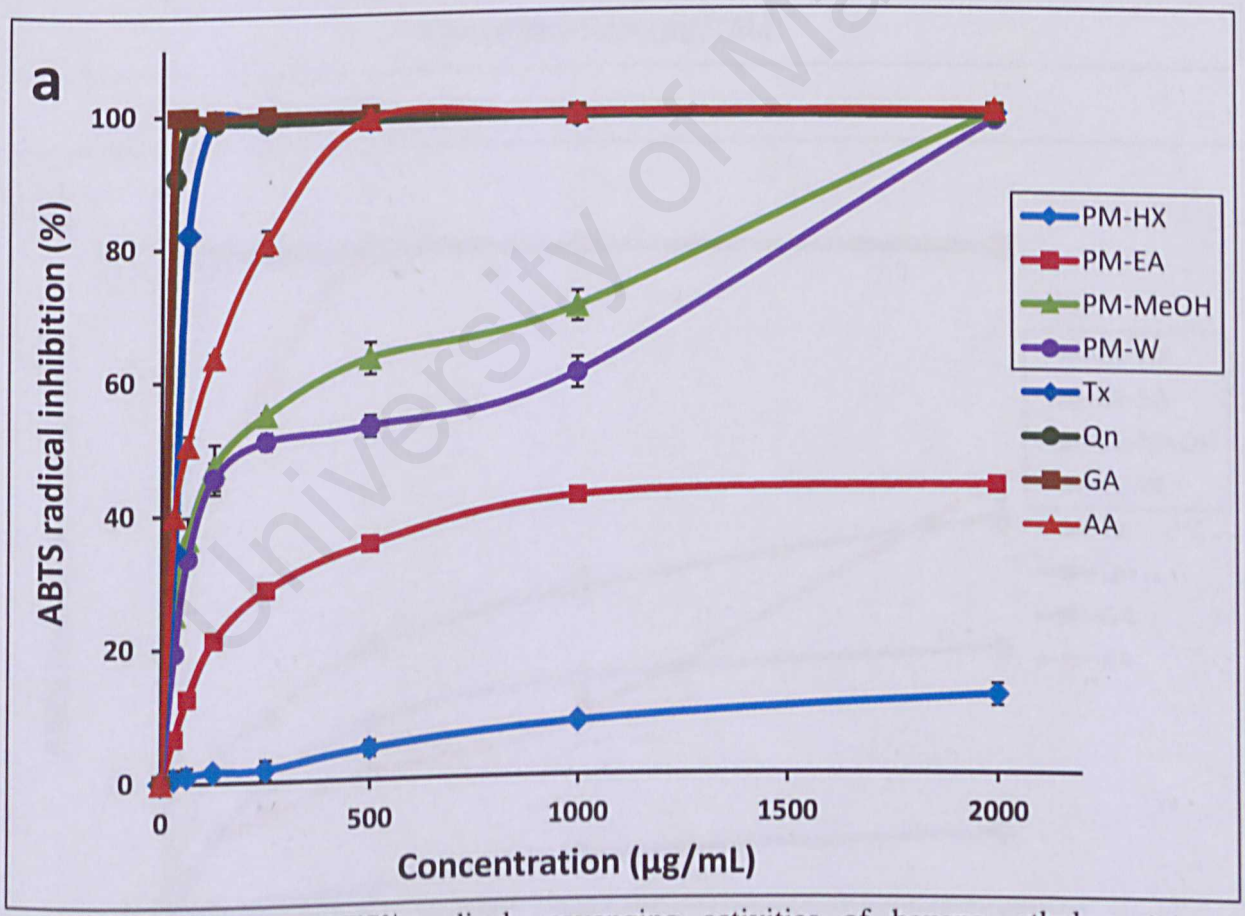
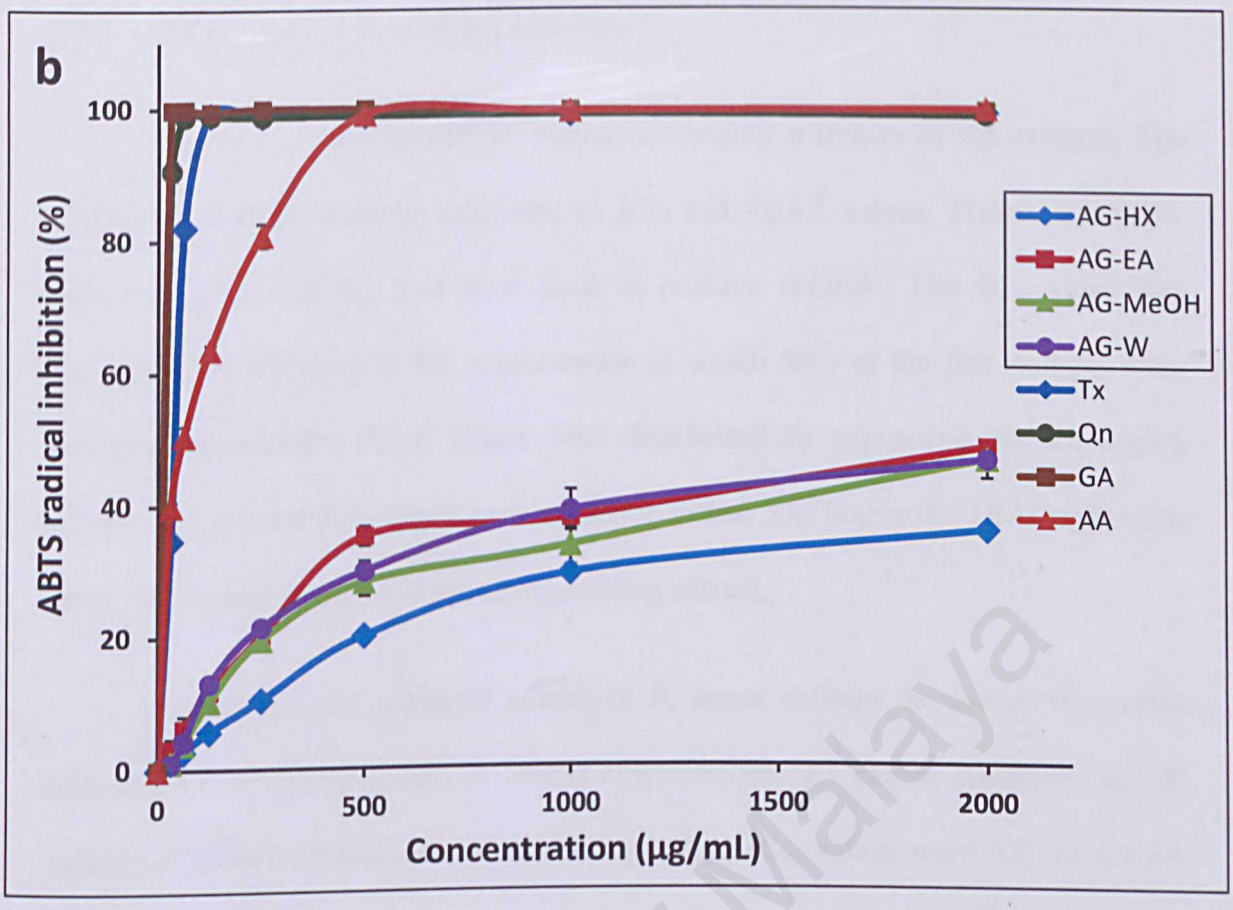


Figure 4.1(a-c): $ABTS^{\bullet+}$ radical scavenging activities of hexane, ethyl acetate, methanol and water extracts of *P. minus*, *A. galanga* and *E. elatior* (0-2000 µg/mL). The results were expressed as means percentage inhibition of $ABTS^{\bullet+}$ ± SD (n=3). PM, *P. minus*; AG, *A. galanga*; EE, *E. elatior*; HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water; Tx, trolox; Qn, quercetin; GA, gallic acid; AA, ascorbic acid.



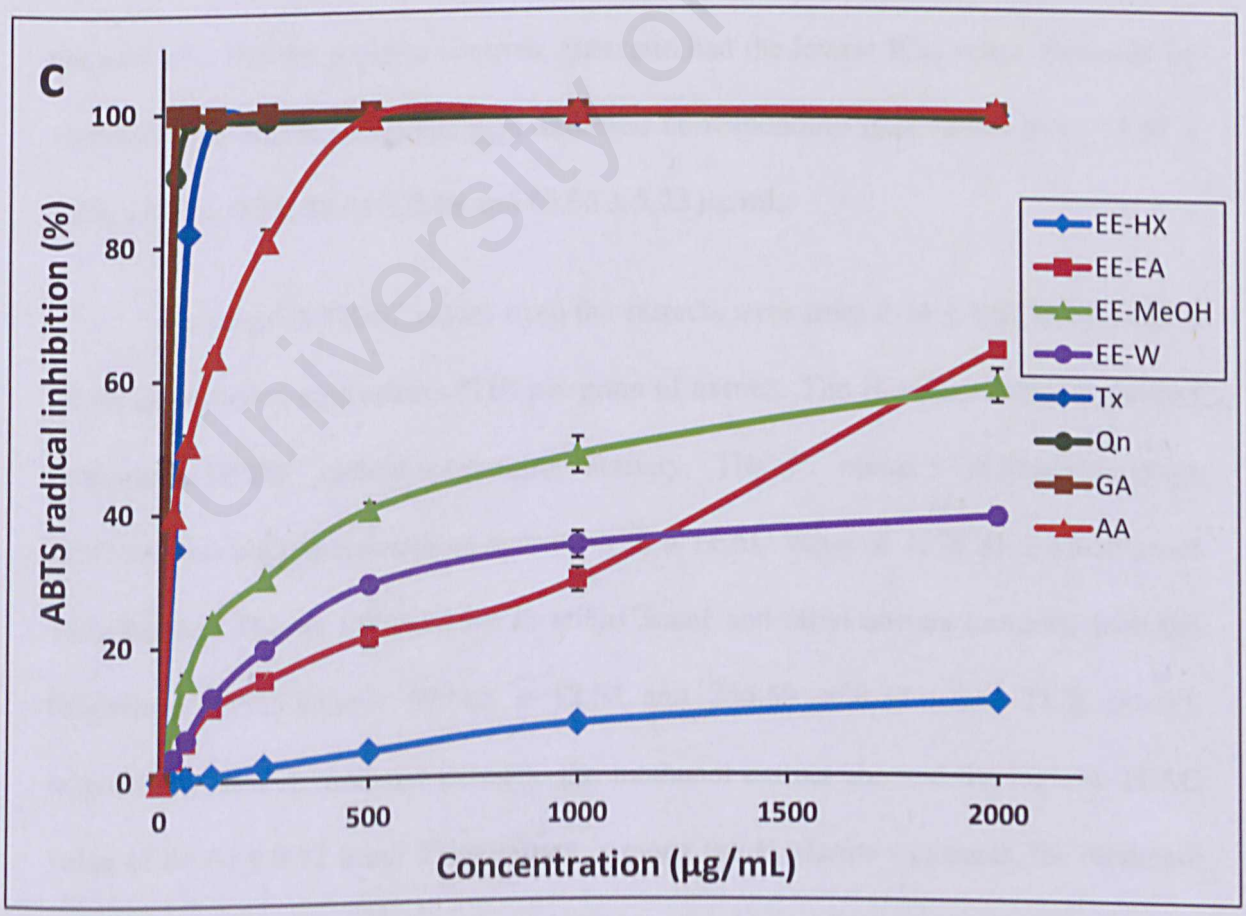


Figure 4.1(a-c), continued.

4.2.3 DPPH• radical scavenging activities

Table 4.4 shows the DPPH• radical-scavenging activities of the extracts. The scavenging-activity could be expressed as IC_{50} and TEAC values. Trolox, quercetin, gallic acid and ascorbic acid were used as positive controls. The IC_{50} value was determined by referring to the concentration at which 50% of the free radicals were inhibited. Meanwhile, TEAC values were determined by expressing the scavenging activity of 1 gram of the extract as compared to trolox. The higher the TEAC value, the better antioxidant potential of the corresponding extract.

In table 4.4, the methanol extract of *P. minus* showed the lowest IC_{50} value followed by *P. minus*-water, *P. minus*-ethyl acetate, *E. elatior*-methanol and *A. galanga*-methanol extracts; and their corresponding IC_{50} values were 125.51 ± 8.14, 210.16 ± 2.34, 602.82 ± 19.62, 766.77 ± 77.33 and 1825.41 ± 14.65 µg/mL, respectively. For the positive controls, quercetin had the lowest IC_{50} value, followed by ascorbic acid, trolox and gallic acid and their corresponding IC_{50} values were 17.99 ± 0.89, 23.23 ± 0.33, 38.41 ± 2.69 and 65.05 ± 5.23 µg/mL.

The range of TEAC values from the extracts were from 4.74 ± 1.92 to 1276.81 ± 12.26 µmol trolox equivalents (TE) per gram of extract. The *P. minus* extracts showed prominent DPPH• radical-scavenging activity. The *P. minus*'s methanol extract exhibited the highest scavenging activity with a TEAC value of 1276.81 ± 12.26 µmol TE/g extract. This is followed by *P. minus* water and ethyl acetate extracts, with the following TEAC values: 699.83 ± 12.54 and 255.59 ± 8.33 µmol TE/g extract, respectively. For *A. galanga* extracts, the methanol extract showed the highest TEAC value of 83.40 ± 0.72 µmol TE/g extract. Among the *E. elatior*'s extracts, the methanol extract give the highest TEAC value of 232.74 ± 11.34 µmol TE/g extract.

Table 4.4: DPPH• radical-scavenging activities of extracts from *P. minus*, *A. galanga* and *E. elatior*.

Extracts	DPPH• radical scavenging activity	
	TEAC value (µmol TE/g extract)	IC_{50} (µg/mL)
P. minus (HX)	7.57 ± 1.16	NA
P. minus (EA)	255.59 ± 8.33	602.82 ± 19.62
P. minus (MeOH)	1276.81 ± 12.26	125.51 ± 8.14
P. minus (W)	699.83 ± 12.54	210.16 ± 2.34
A. galanga (HX)	6.74 ± 2.38	NA
A. galanga (EA)	63.24 ± 0.74	NA
A. galanga (MeOH)	83.40 ± 0.72	1825.41 ± 14.65
A. galanga (W)	47.00 ± 2.96	NA
E. elatior (HX)	4.74 ± 1.92	NA
E. elatior (EA)	62.47 ± 2.55	NA
E. elatior (MeOH)	232.74 ± 11.34	766.77 ± 77.33
E. elatior (W)	41.26 ± 1.33	NA
Trolox	standard curve	38.41 ± 2.69
Quercetin	9201.22 ± 580.56	17.99 ± 0.89
Gallic Acid	21653.16 ± 512.11	65.05 ± 5.23
Ascorbic acid	6464.54 ± 50.17	23.23 ± 0.33

Results are expressed as means ± standard deviation (n=3). NA: not available. HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water. TEAC, trolox equivalent antioxidant capacity, expressed as µmol trolox equivalents (TE) per gram of dried extract. IC_{50}, the concentration of the extracts that inhibited 50% of DPPH• radicals.

Figure 4.3(a-c) shows the DPPH radical scavenging activities of the plant extracts, as well as the antioxidant standards, which were quercetin, gallic acid, ascorbic acid and trolox. Most of the extracts showed a concentration-dependent radical-

scavenging activity relationship. In Figure 4.3(a), the PM-methanol and PM-water extracts showed were among the highest in DPPH radical-scavenging activity, comparable to the antioxidant standard/ positive controls activities, and they reached 100% scavenging activity at ~500 µg/mL. In Figure 4.3(b), all extracts showed linear scavenging activities up to 1000 µg/mL, but most of the extracts did not reach 50 percent radical inhibition at the maximum concentration of the extracts. In figure 4.3(c), only methanol extract of EE showed prominent scavenging activity ($IC_{50,}$ 766.77 ± 77.33 µg/mL). The hexane extracts from the three plants were the least reactive in scavenging the DPPH• radicals.

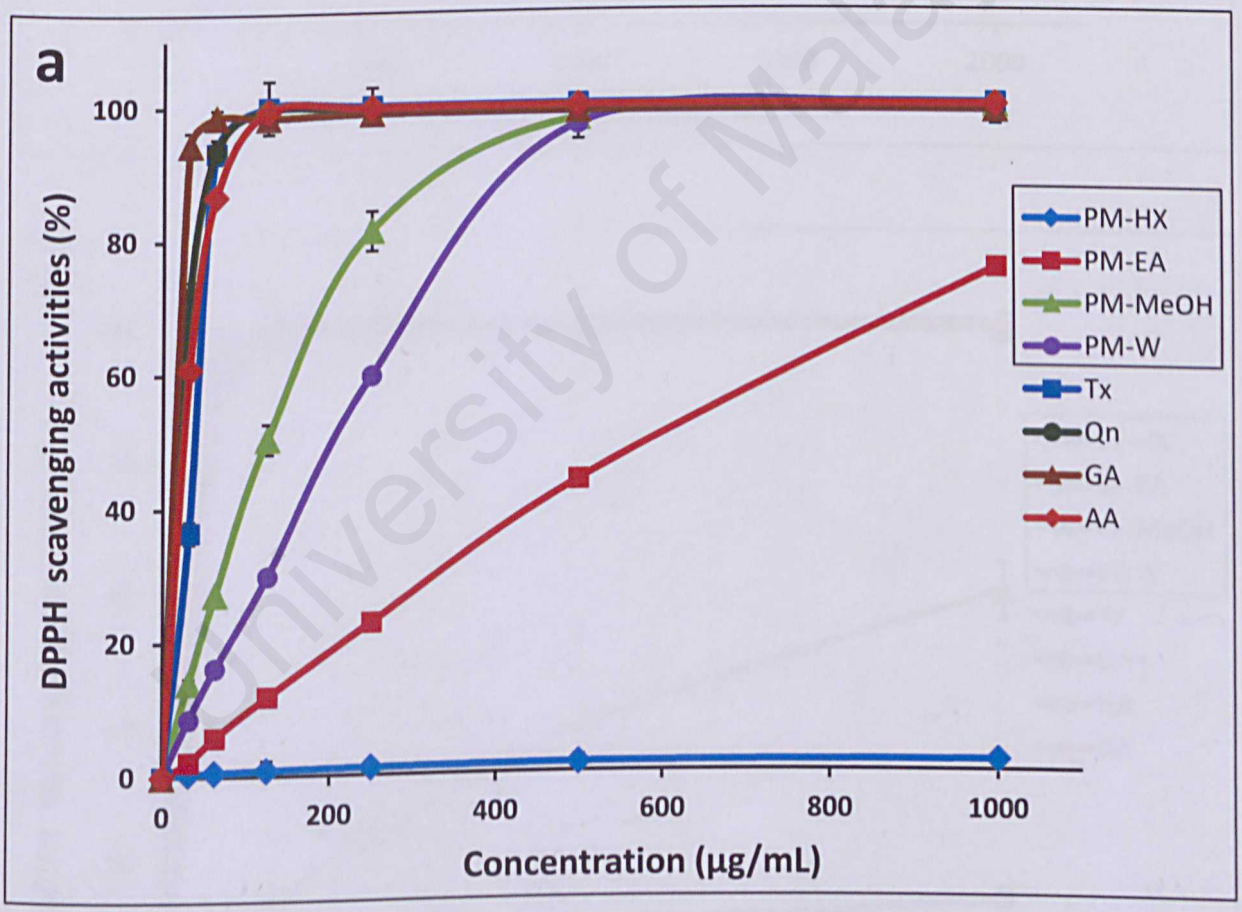
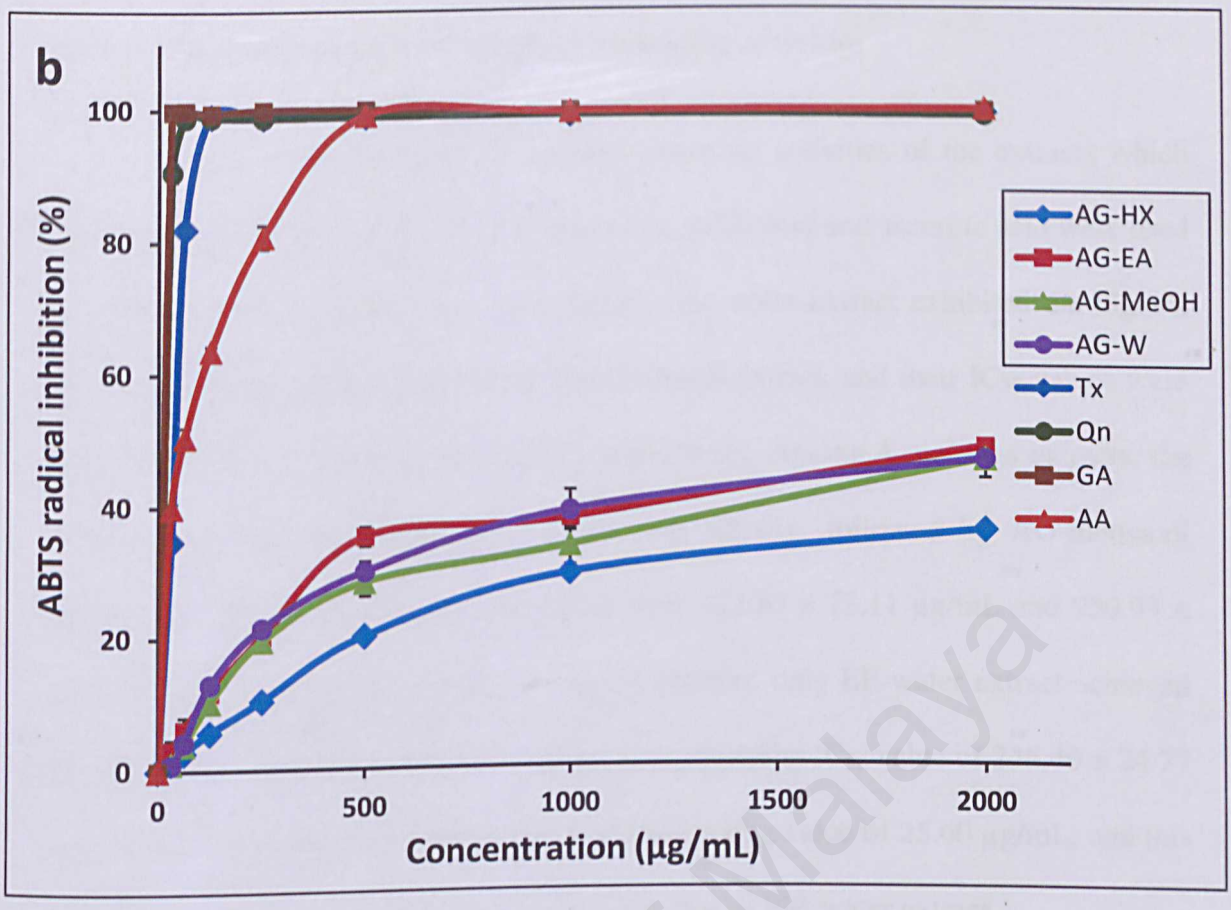


Figure 4.2(a-c): DPPH• radical scavenging capacity of hexane, ethyl acetate, methanol and water extracts of *P. minus*, *A. galanga* and *E. elatior*. The result were expressed as means percentage (%) of DPPH• scavenging ± SD (n=3). PM, *P. minus*; AG, *A. galanga*; EE, *E. elatior*; HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water; Tx, trolox; Qn, quercetin; GA, gallic acid; AA, ascorbic acid.



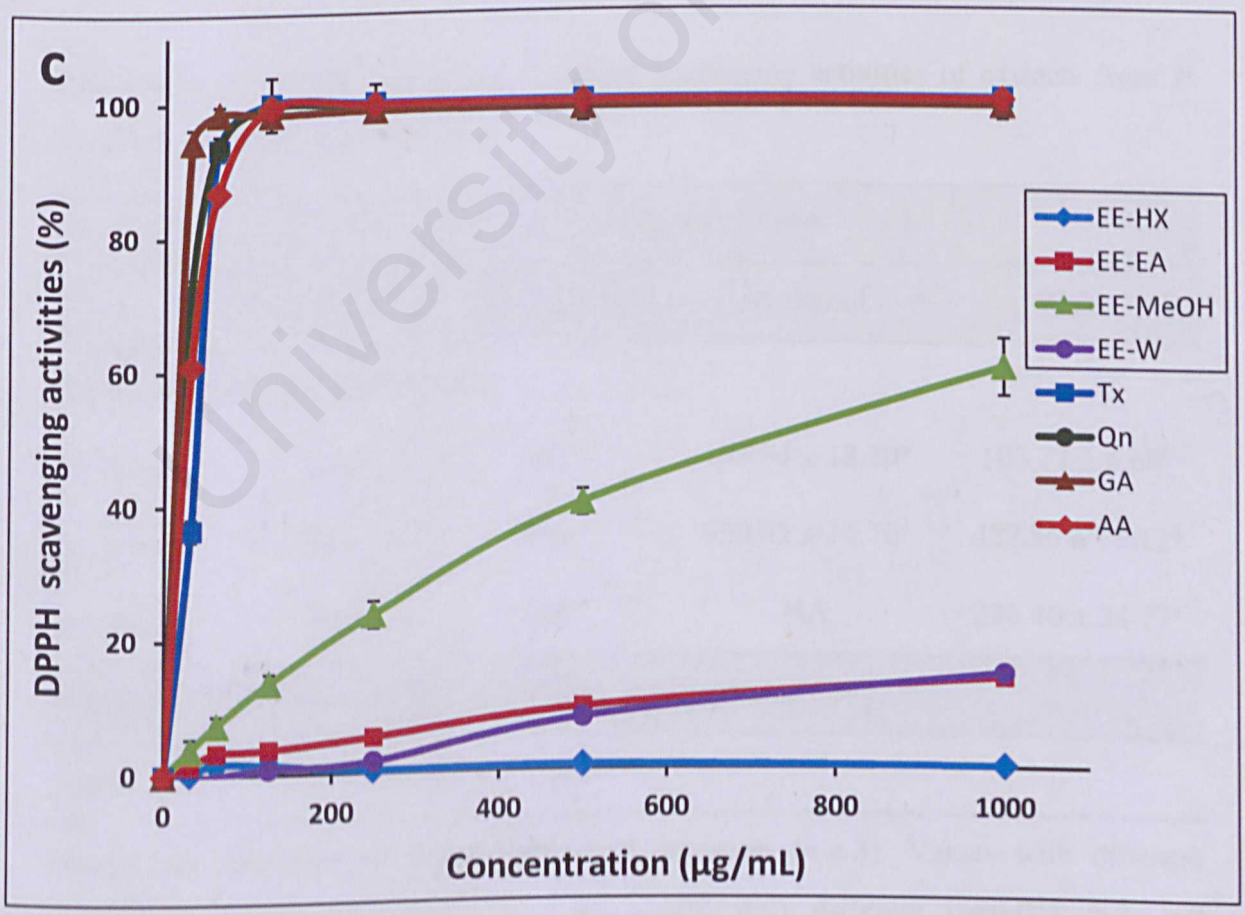


Figure 4.2(a-c), continued

4.2.4 Superoxide anion ($O_2^{\bullet -}$) radical scavenging activities

Table 4.5 showed the $O_2^{\bullet -}$ radical-scavenging activities of the extracts which were expressed as IC_{50} values. Trolox, quercetin, gallic acid and ascorbic acid were used as positive controls. Among *P. minus* extracts, the water extract exhibited the highest $O_2^{\bullet -}$ scavenging activity, followed by PM-methanol extract, and their IC_{50} values were 103.77 ± 6.68 and 103.77 ± 6.68 µg/mL, respectively. Among *A. galanga* extracts, the water extract had the highest $O_2^{\bullet -}$ scavenging activity, followed by AG-methanol extract, and their correspondng IC_{50} values were 422.85 ± 73.11 µg/mL and 950.93 ± 14.70 µg/mL, respectively. Among *E. elatior* extracts, only EE-water extract achieved 50 percent $O_2^{\bullet -}$ radicals inhibition, with the corresponding IC_{50} value of 236.40 ± 24.77 µg/mL. For the positive controls, gallic acid had an IC_{50} value of 25.60 µg/mL, and this means its activity was about 4 times higher than that of PM-water extract.

Table 4.5: Superoxide anion ($O_2^{\bullet -}$) radical scavenging activities of extracts from *P. minus*, *A. galanga* and *E. elatior*.

	Extraction solvent			
	Hexane	Ethyl acetate	Methanol	Water
Superoxide, IC_{50} (µg/mL extract)				
P. minus	NA	NA	195.64 ± 18.30[a]	103.77 ± 6.68[b]
A. galanga	NA	NA	950.93 ± 14.70[c]	422.85 ± 73.11[d]
E. elatior	NA	NA	NA	236.40 ± 24.77[a]
Superoxide inhibition of positive controls, IC_{50} (µg/mL extract)				
Gallic acid	25.60±0.00			

Results are expressed as means ± standard deviation ($n = 3$). Values with different superscripts (lower case letters,[a,b,c,...]) are significantly different ($p<0.05$). NA: not available. IC_{50}, the concentration of the extracts that inhibited 50% of free radicals.

Figure 4.3(a-c) showed the $O_2^{\bullet -}$ scavenging activities of the plant extracts. Most extracts showed dose-dependent inhibition. Gallic acid, PM-W and PM-MeOH extracts showed prominent activity, where their activity curves were relatively steep and reaching >50% percentage of $O_2^{\bullet -}$ inhibition at a lower range of concentrations (<300 µg/mL). The AG-MeOH, AG-water and EE-water extracts also showed dose-dependent inhibition activity up to their maximum concentration tested (1000 µg/mL). These extracts could be said to have a relatively moderate $O_2^{\bullet -}$ radicals scavenging activity. In this study, it is observed that the methanol and water extracts from the plants showed high $O_2^{\bullet -}$ radical-scavenging activities. The remaining were considered to have low as the $O_2^{\bullet -}$ scavenging activities, as they did not reach 50% inhibition at the maximum concentration tested. However, AG-HX, AG-EA, EE-HX and EE-EA extracts showed negative $O_2^{\bullet -}$ inhibition as their concentrations increased, indicating pro-oxidant behaviour at such elevated concentrations.

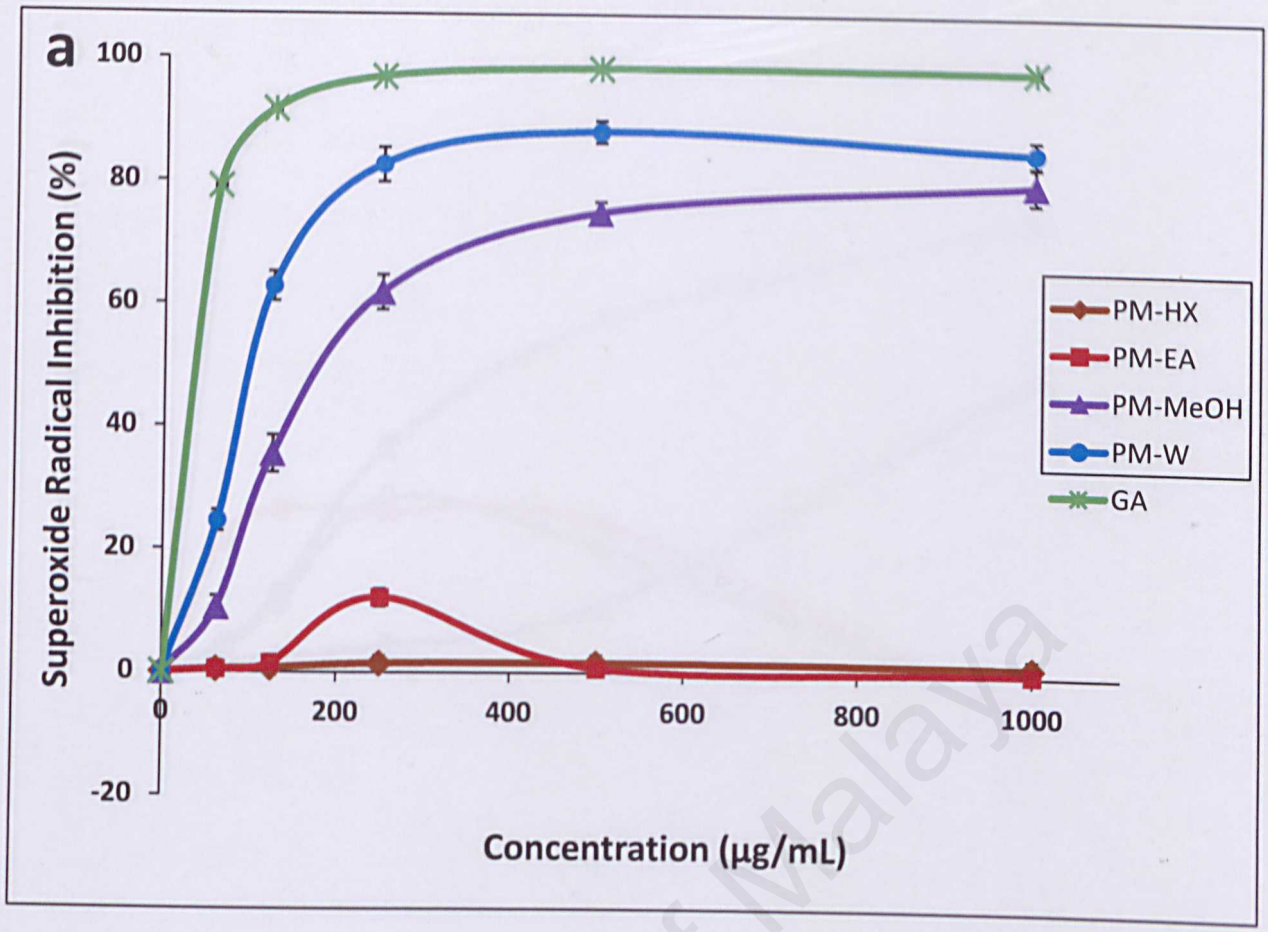
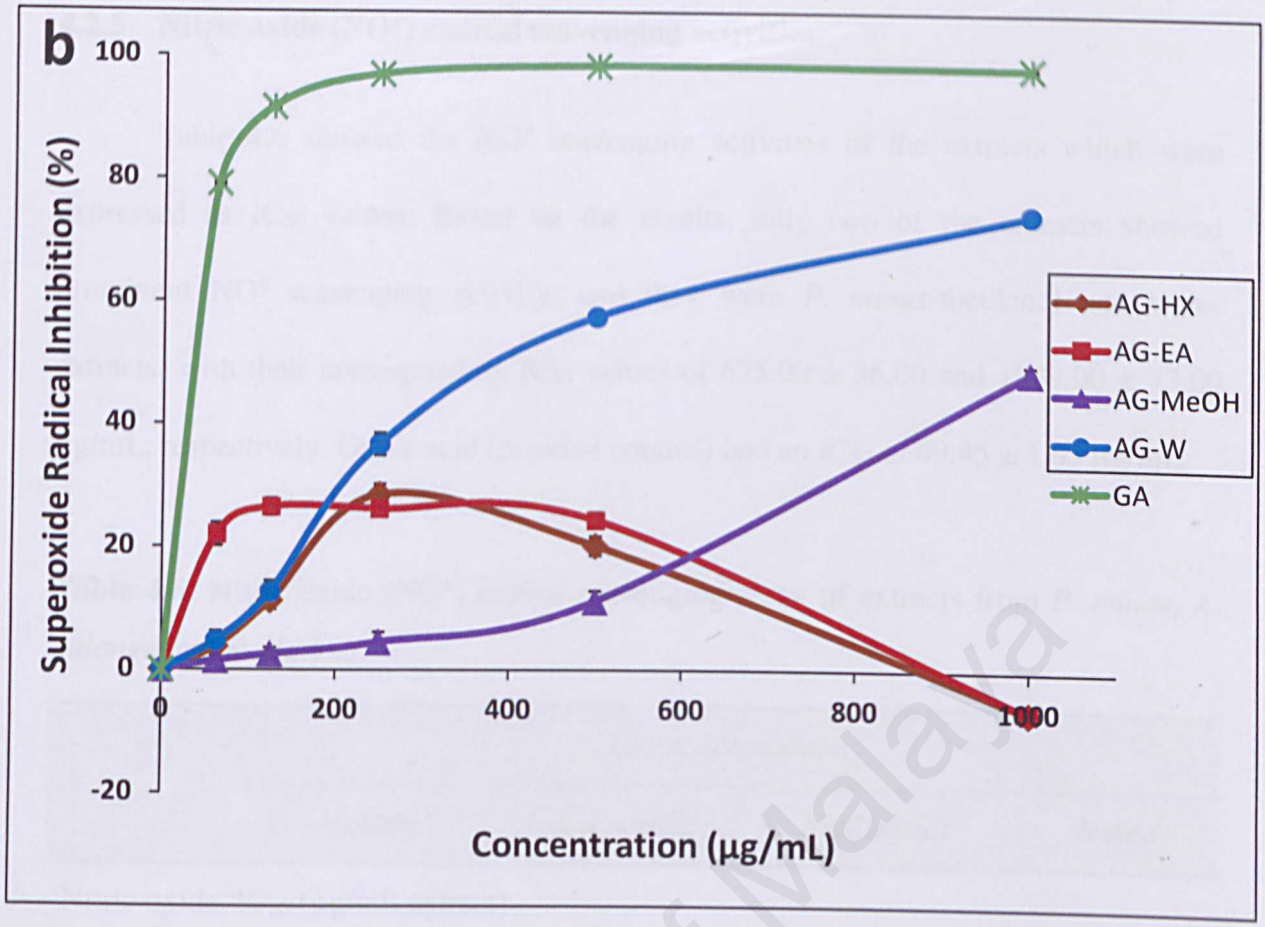


Figure 4.3(a-c): Superoxide anion ($O_2^{\bullet -}$) radical-scavenging activities of hexane, ethyl acetate, methanol and water extracts of *P. minus*, *A. galanga* and *E. elatior* (0-1000 µg/mL). The result were expressed as means percentage (%) inhibition of superoxide anion ($O_2^{\bullet -}$) ± SD (n=3). PM, *P. minus*; AG, *A. galanga*; EE, *E. elatior*; HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water; GA, gallic acid.



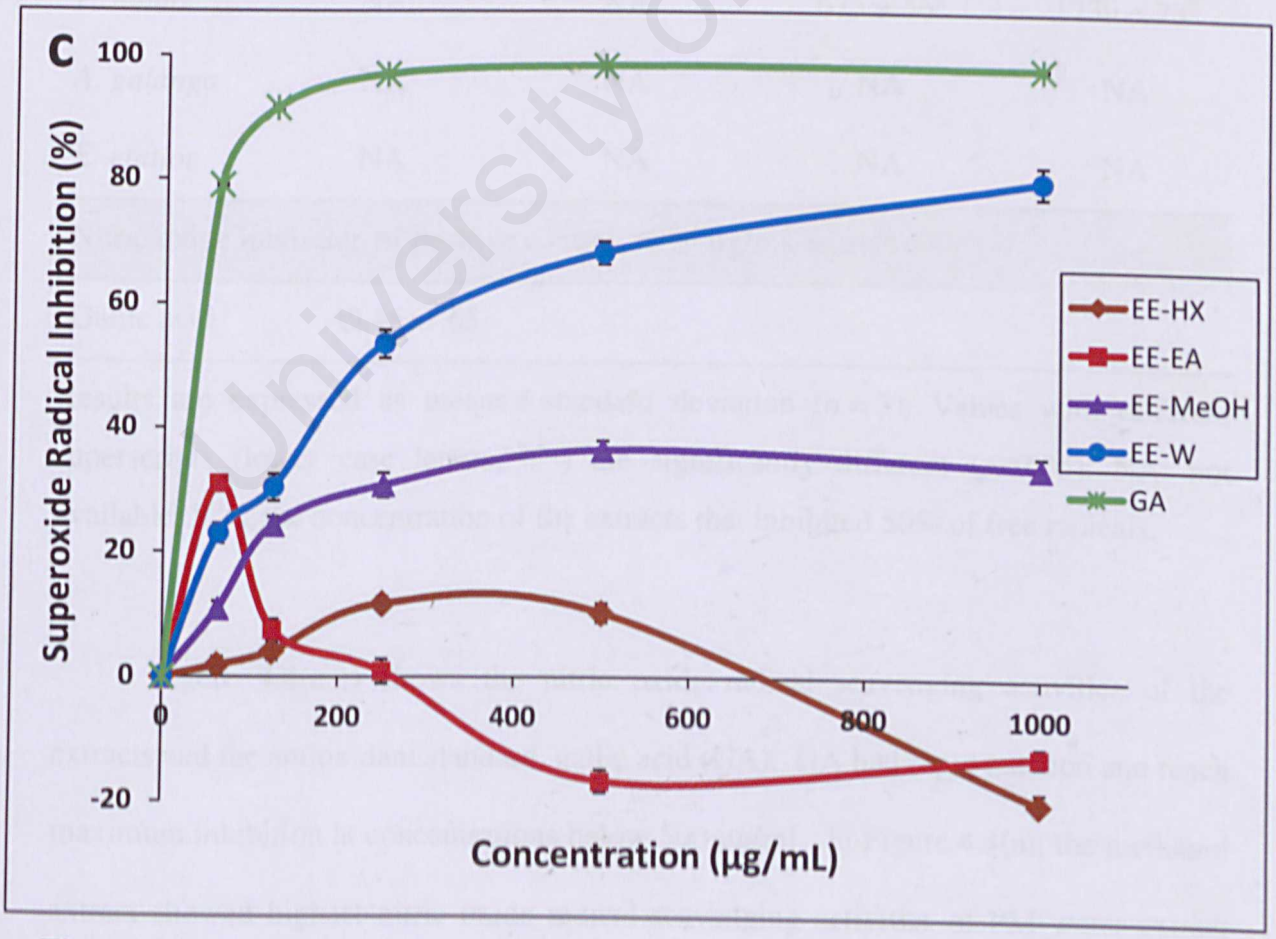


Figure 4.3(a-c), continued.

4.2.5 Nitric oxide (NO•) radical scavenging activities

Table 4.6 showed the NO• scavenging activities of the extracts which were expressed as IC_{50} values. Based on the results, only two of the extracts showed prominent NO• scavenging activity, and they were *P. minus*-methanol and water extracts, with their corresponding IC_{50} values of 675.00 ± 56.00 and 1239.00 ± 73.00 µg/mL, respectively. Gallic acid (positive control) had an IC_{50} of 49.45 ± 1.65 µg/mL.

Table 4.6: Nitric oxide (NO•) radical-scavenging assay of extracts from *P. minus*, *A. galanga* and *E. elatior*.

	Extraction solvent			
	Hexane	Ethyl acetate	Methanol	Water
Nitric oxide, IC_{50} (µg/mL extract)				
P. minus	NA	NA	675 ± 56^{a}	1239 ± 73^{b}
A. galanga	NA	NA	NA	NA
E. elatior	NA	NA	NA	NA
Nitric oxide inhibition of positive control, IC_{50} (µg/mL extract)				
Gallic acid	49.45±1.65			

Results are expressed as means ± standard deviation ($n = 3$). Values with different superscripts (lower case letters,$^{a,b,c...}$) are significantly different ($p<0.05$). NA: not available. IC_{50}, the concentration of the extracts that inhibited 50% of free radicals.

Figure 4.4(a-c) shows the nitric oxide radical scavenging activities of the extracts and the antioxidant standard, gallic acid (GA). GA had rapid reaction and reach maximum inhibition at concentrations below 500 µg/mL. In Figure 4.4(a), the methanol extract showed highest nitric oxide radical scavenging activities of PM water extract also close to the PM methanol extract. Both extracts increased in the percentage of

scavenging inhibition rapidly and able to scavenge 50 percent of nitric oxide radicals below concentration 2000 µg/mL of extract. In Figure 4.4(b), all extracts showed increasing activities as the concentration increased but not exceed 50 percent of radical scavenging activities below concentration 2000 µg/mL. The result in Figure 4.4(c) also showed the nitric oxides scavenge activities of methanol and water extracts were increased as the concentration increased. In addition, the hexane and ethyl acetate extracts do not have NO-scavenging activities due to no inhibition are observed at highest concentration of the extracts.

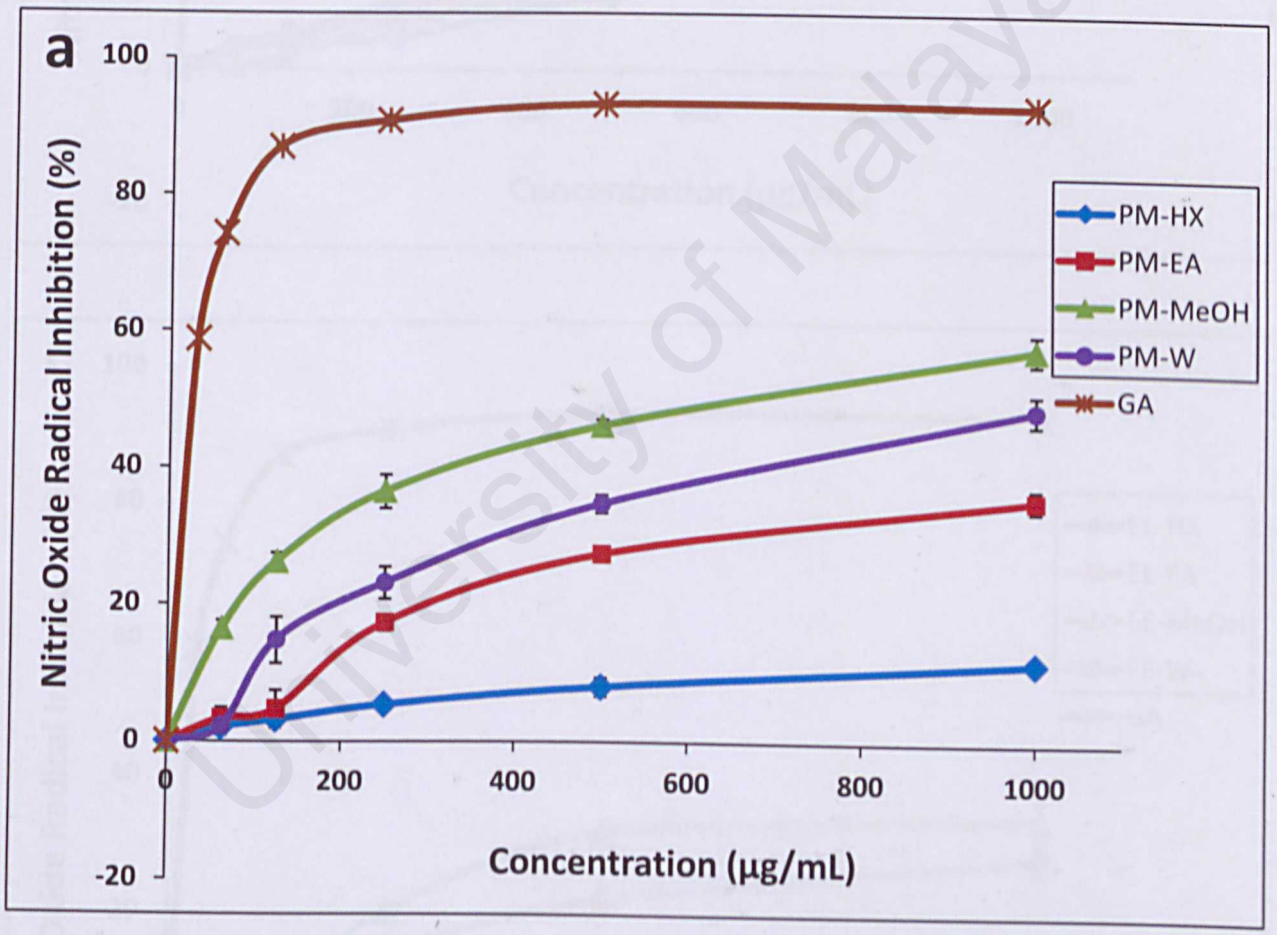
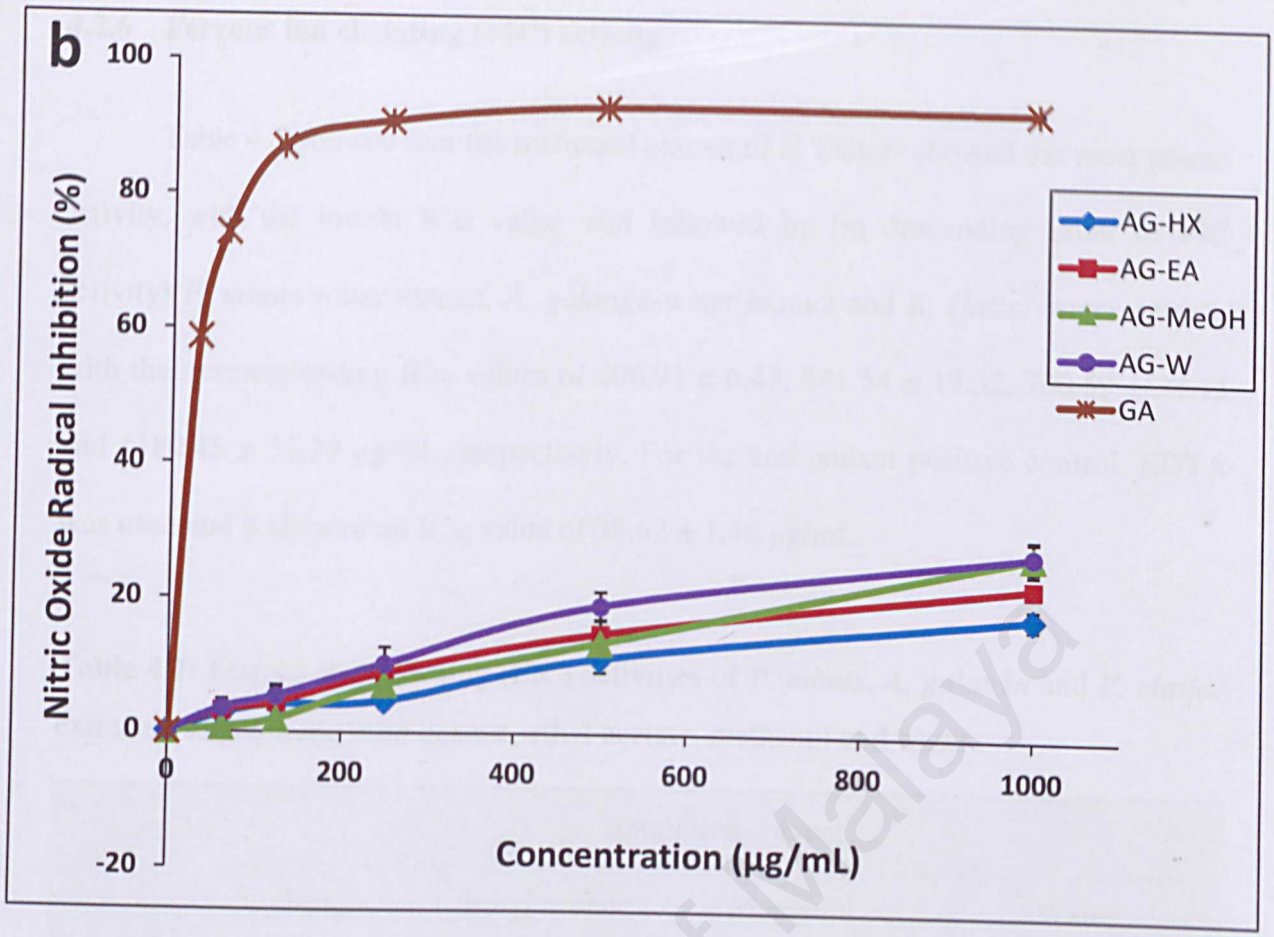


Figure 4.4(a-c): Nitric oxide ($NO^{\bullet}$) radical scavenging activities of extracts of *P. minus*, *A. galanga* and *E. elatior* (0-1000 µg/mL). The result were expressed as means percentage (%) of nitric oxide (NO) radicals inhibition ± SD (n=3). PM, *P. minus*; AG, *A. galanga*; EE, *E. elatior*; HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water; GA, gallic acid.



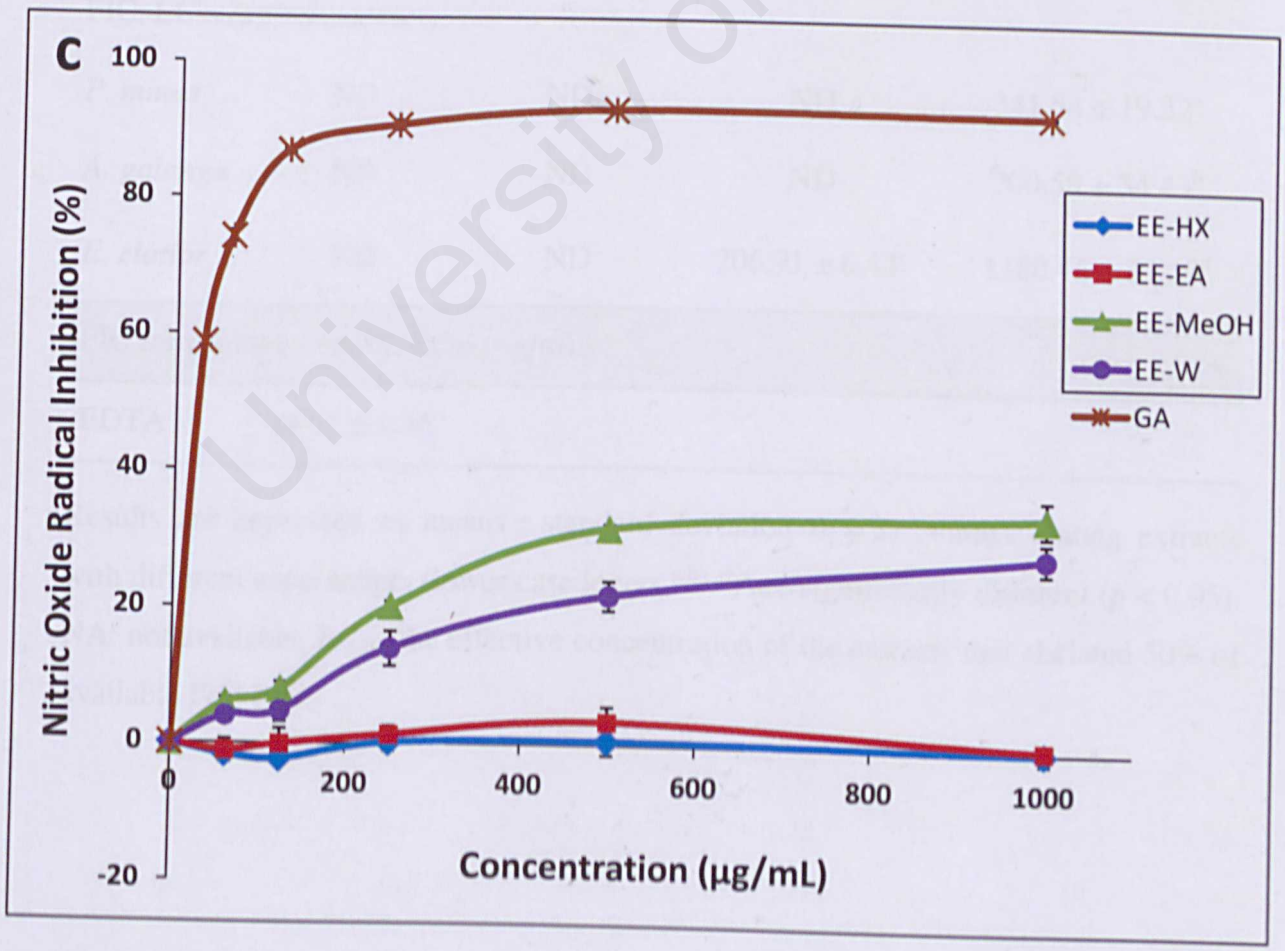


Figure 4.4(a-c), continued.

4.2.6 Ferrous ion chelating (FIC) activity

Table 4.7 showed that the methanol extract of *E. elatior* showed the most potent activity, with the lowest IC_{50} value, and followed by (in descending order of FIC activity) *P. minus*-water extract, *A. galanga*-water extract and *E. elatior*-water extract, with their corresponding IC_{50} values of 206.91 ± 6.42, 341.54 ± 19.32, 700.59 ± 34.43 and 1180.45 ± 31.39 µg/mL, respectively. For the antioxidant positive control, EDTA was used and it showed an IC_{50} value of 38.62 ± 1.46 µg/mL.

Table 4.7: Ferrous ion chelating (FIC) activities of *P. minus*, *A. galanga* and *E. elatior* extracted sequentially with hexane, ethyl acetate, methanol and water.

	Extraction solvent			
	Hexane	Ethyl acetate	Methanol	Water
FIC, EC_{50} (µg/mL extract)				
P. minus	ND	ND	ND	341.54 ± 19.32[a]
A. galanga	ND	ND	ND	700.59 ± 34.43[b]
E. elatior	ND	ND	206.91 ± 6.42[c]	1180.45 ± 31.39[d]
FIC for positive control, EC_{50} (µg/mL)				
EDTA	38.62 ± 1.46			

Results are expressed as means ± standard deviation ($n = 3$). Values among extracts with different superscripts (lower case letters,[a,b,c,d]) are significantly different ($p < 0.05$). NA: not available. EC_{50}, the effective concentration of the extracts that chelated 50% of available Fe^{2+} ion.

Figure 4.5(a-c) showed the ferrous ion chelating activities of the extracts with EDTA as the positive antioxidant standard. The extracts showed concentration-dependent FIC activity relationship. All of the extracts showed metal chelating capability. EDTA showed prominent FIC activity with a steep activity curve that reached the maximum FIC value at <125 µg/mL. At the maximum tested concentration of 2000 µg/mL, none of the extracts had their FIC activity below 10 percent. The EE-MeOH extract showed the highest FIC activity, with the lowest IC_{50} value which was 206.91 ± 6.42 µg extract/mL. This is followed by PM-water (341.54 ± 19.32 µg/mL), AG-water (700.59 ± 34.43 µg/mL) and EE-water (1180.45 ± 31.39 µg/mL) extracts.

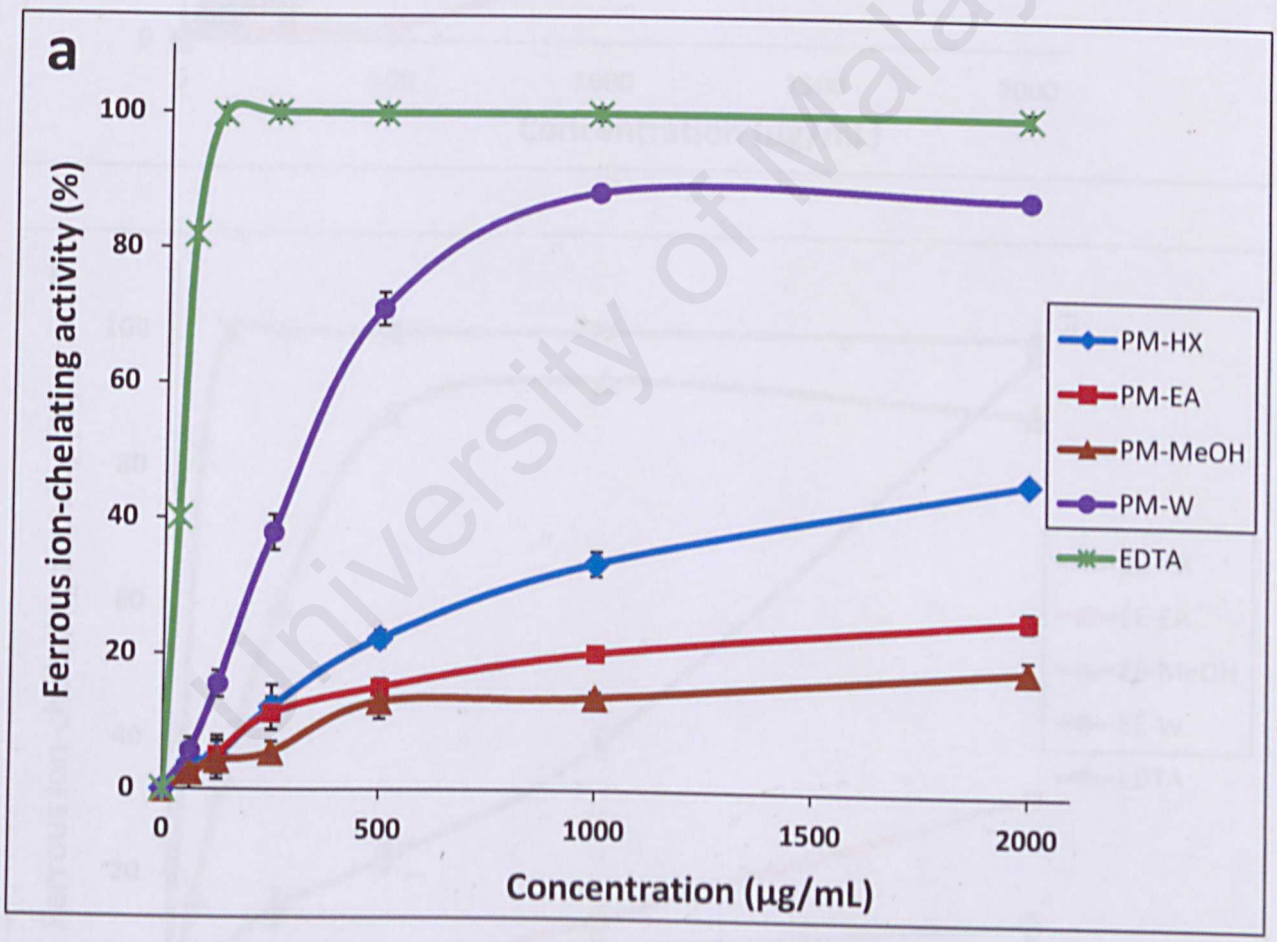
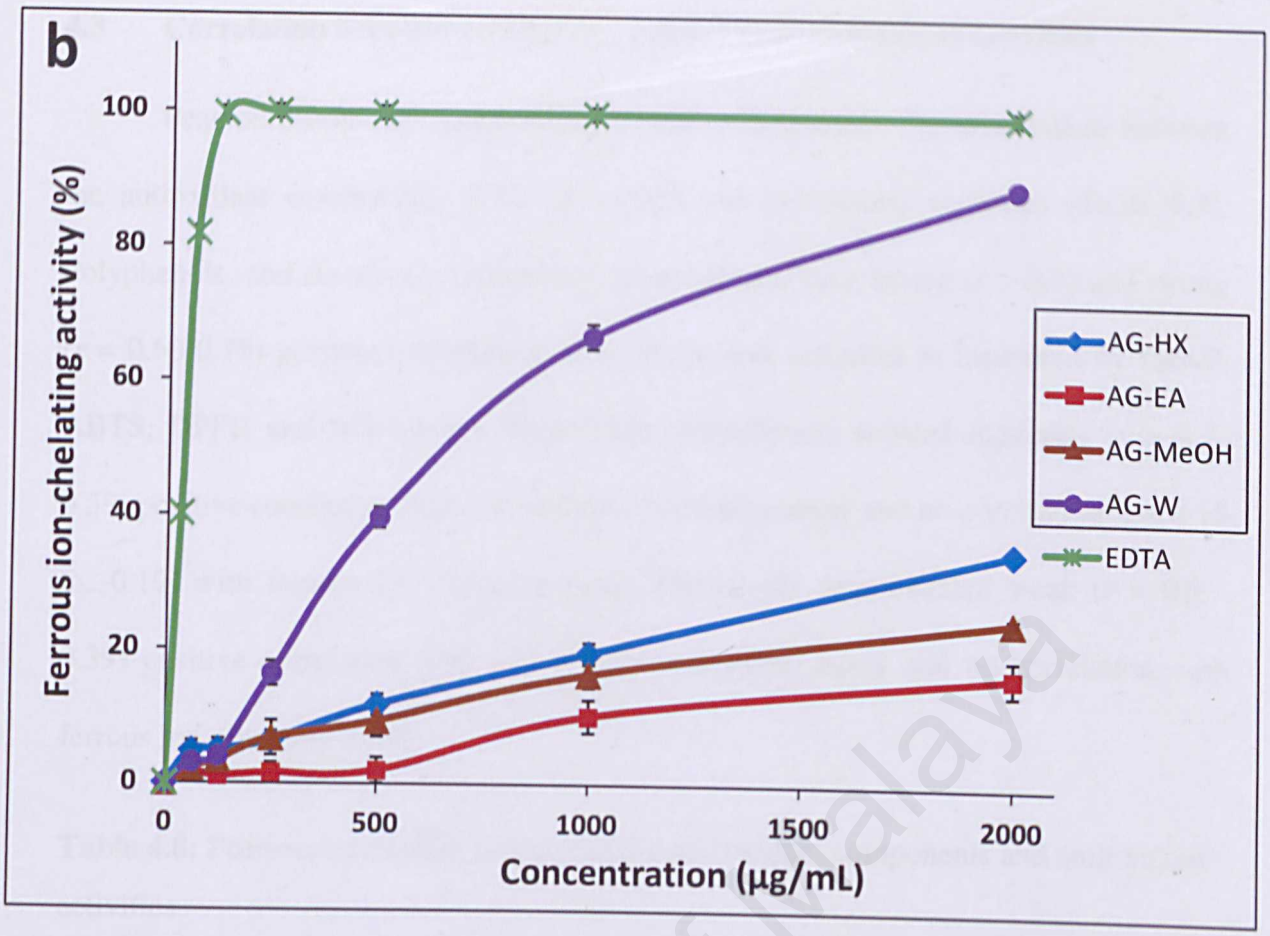


Figure 4.5(a-c): Ferrous ion-chelating activity of hexane, ethyl acetate, methanol and water extracts of *P. minus*, *A. galanga* and *E. elatior* plants in various concentration (0-2000 µg/mL). The result were expressed as means ± SD (n=3). PM, *P. minus*; AG, *A. galanga*; EE, *E. elatior*; HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water.



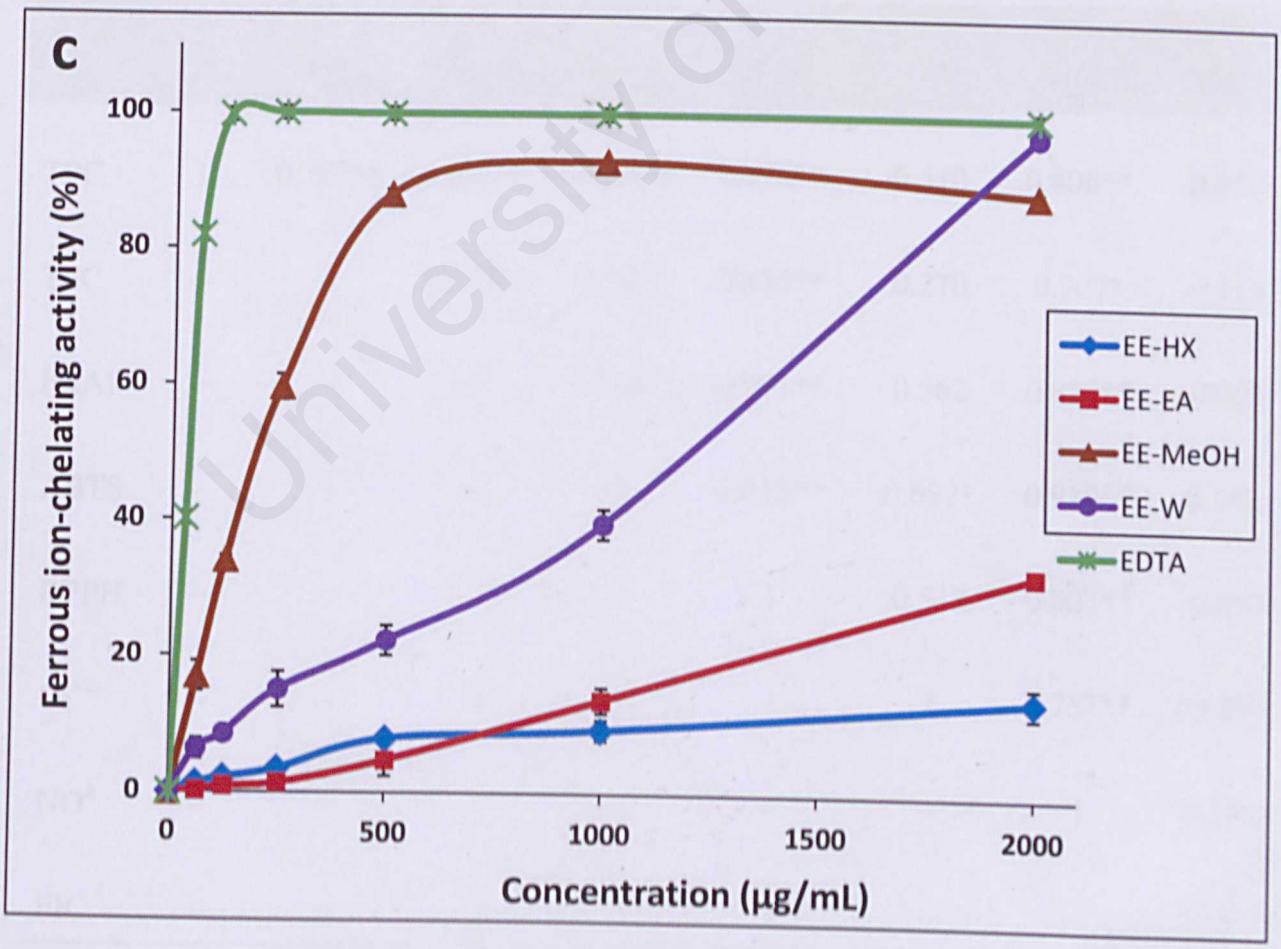


Figure 4.5(a-c), continued

4.3 Correlation between antioxidant content and antioxidant activities

Pearson correlation analyses were done to investigate the relationship between the antioxidant components (TPC and TFC) and antioxidant activities (Table 4.8). Polyphenols and flavonoids compounds demonstrated very strong ($r > 0.8$) and strong ($r = 0.60$-0.79) positive correlations with antioxidant activities as measured by FRAP, ABTS, DPPH and NO· assays. Meanwhile, polyphenols showed moderate ($r = 0.4$-0.59) positive correlation with $O_2^{\bullet-}$ radical-scavenging assay and no correlation ($r = 0.19$ to -0.19) with ferrous ion-chelating assay. Flavonoids demonstrated weak ($r = 0.2$ - 0.39) positive correlation with $O_2^{\bullet-}$ radical-scavenging assay and no correlation with ferrous ion-chelating assay.

Table 4.8: Pearson correlation analyses of the antioxidant components and antioxidant activities.

	TPC	TFC	FRAP	ABTS	DPPH	$O_2^{\bullet-}$	$NO^{\bullet}$	FIC
TPC	1	0.797**	0.993**	0.831**	0.978**	0.510	0.806**	-0.059
TFC	–	1	0.813**	0.720*	0.833**	0.270	0.707*	-0.113
FRAP	–	–	1	0.838**	0.991**	0.562	0.825**	-0.002
ABTS	–	–	–	1	0.815**	0.682*	0.910**	0.242
DPPH	–	–	–	–	1	0.548	0.807**	-0.053
$O_2^{\bullet-}$	–	–	–	–	–	1	0.757**	0.629*
$NO^{\bullet}$	–	–	–	–	–	–	1	0.281
FIC	–	–	–	–	–	–	–	1

** Correlation is highly significant ($p < 0.01$)
* Correlation is significant ($p < 0.05$)

4.4 Anti-proliferative activity analyses

MTT assay was performed to measure the anti-proliferative activity of the 12 extracts from the three plants. This was done using cancerous and normal colon cell lines (CCD841 and HCT116). The preliminary screening was done by incubating the cells for 48 hr using the extracts at concentrations ranging from 27-333 μg/mL. The results are as shown in Table 4.9 and Figure 4.6.

The MTT results (Table 4.9 and Figure 4.6) showed that the following 4 extracts showed promising anti-proliferative activity by inhibit >50% of the cells: PM-HX, PM-EA, AG-HX and AG-EA. Some extracts seems to promote the growth of the cancerous cells, such as PM-W and EE-HX extracts. Five of the extracts were able to inhibit the growth of CCD841 strongly when they are at the highest concentration tested: PM-HX, PM-EA, AG-HX, AG-EA and EE-W. In addition, some extracts were able to promote the growth of CCD841 cells, such as PM-MeOH, PM-W, AG-MeOH and EE-EA.

The objective of this study is to identify of extract(s) with anti-proliferative activity on cancerous colon cell line, with minimal toxicity to normal colon cell line CCD841. The AG-HX had prominent cytotoxicity towards CCD841, with only 7.49 ± 2.34% viability at 83 μg/mL. Thus, AG-HX was omitted, and only the following three extracts were subjected to further analyses: PM-HX, PM-EA and AG-EA extracts.

Table 4.9: The percentage of viability of CCD841 cells (normal colon cells) and HCT116 cell line (cancerous colon cells) treated with the 12 plant extracts.

Final ext. conc.[a] →	VIABILITY CCD841 (%)			VIABILITY HCT116 (%)		
	333 µg/mL	167 µg/mL	83 µg/mL	333 µg/mL	167 µg/mL	83 µg/mL
PM-HX	5.55 ± 0.36	57.37 ± 9.55	90.50 ± 5.58	3.32 ± 1.98	7.39 ± 1.34	22.16 ± 5.04
PM-MeOH	139.60 ± 6.08	125.76 ± 3.61	117.77 ± 7.10	86.43 ± 2.56	97.51 ± 8.44	99.45 ± 1.66
PM-W	160.13 ± 3.64	140.36 ± 6.73	128.19 ± 7.10	157.30 ± 8.48	134.83 ± 8.92	107.30 ± 2.38
AG-HX	7.51 ± 0.86	7.21 ± 1.08	7.49 ± 2.34	10.86 ± 5.07	12.36 ± 1.59	27.34 ± 4.54
AG-EA	4.65 ± 0.53	78.17 ± 8.30	97.16 ± 4.02	10.11 ± 5.87	14.83 ± 5.53	39.89 ± 2.38
AG-MeOH	125.17 ± 2.73	122.81 ± 3.44	110.66 ± 4.01	58.80 ± 1.65	86.67 ± 5.87	96.31 ± 4.51
AG-W	86.52 ± 3.97	93.85 ± 1.88	95.37 ± 5.41	81.39 ± 0.79	91.81 ± 4.59	97.12 ± 4.38
EE-HX	86.78 ± 8.46	89.95 ± 3.24	98.49 ± 7.02	135.46 ± 2.42	121.94 ± 3.00	108.58 ± 5.58
EE-EA	120.22 ± 2.20	104.52 ± 10.75	100.90 ± 3.01	115.32 ± 8.06	101.21 ± 7.46	100.97 ± 8.45
EE-MeOH	83.74 ± 4.36	91.49 ± 2.09	100.10 ± 3.29	119.75 ± 8.49	107.17 ± 6.64	103.26 ± 9.66
EE-W	27.44 ± 3.21	72.69 ± 4.02	99.31 ± 4.22	74.07 ± 7.22	88.81 ± 7.64	95.82 ± 8.79

Final ext. conc.[a] →	VIABILITY CCD841 (%)			VIABILITY HCT116 (%)		
	107 µg/mL	54 µg/mL	27 µg/mL	107 µg/mL	54 µg/mL	27 µg/mL
PM-EA*	26.94 ± 6.5	94.85 ± 4.24	98.35 ± 3.79	7.02 ± 1.69	9.7 ± 4.18	25.48 ± 4.65

Result are expressed as means ± standard deviation (n=3). *P. minus*, PM; *A. galanga*, AG; *E. elatior*, EE; HX, hexane; EA, ethyl acetate; MeOH, methanol, W, water. [a] = The PM-EA extract had lower solubility in DMSO, hence lower concentrations (27, 54 and 107 µg/mL) were used.

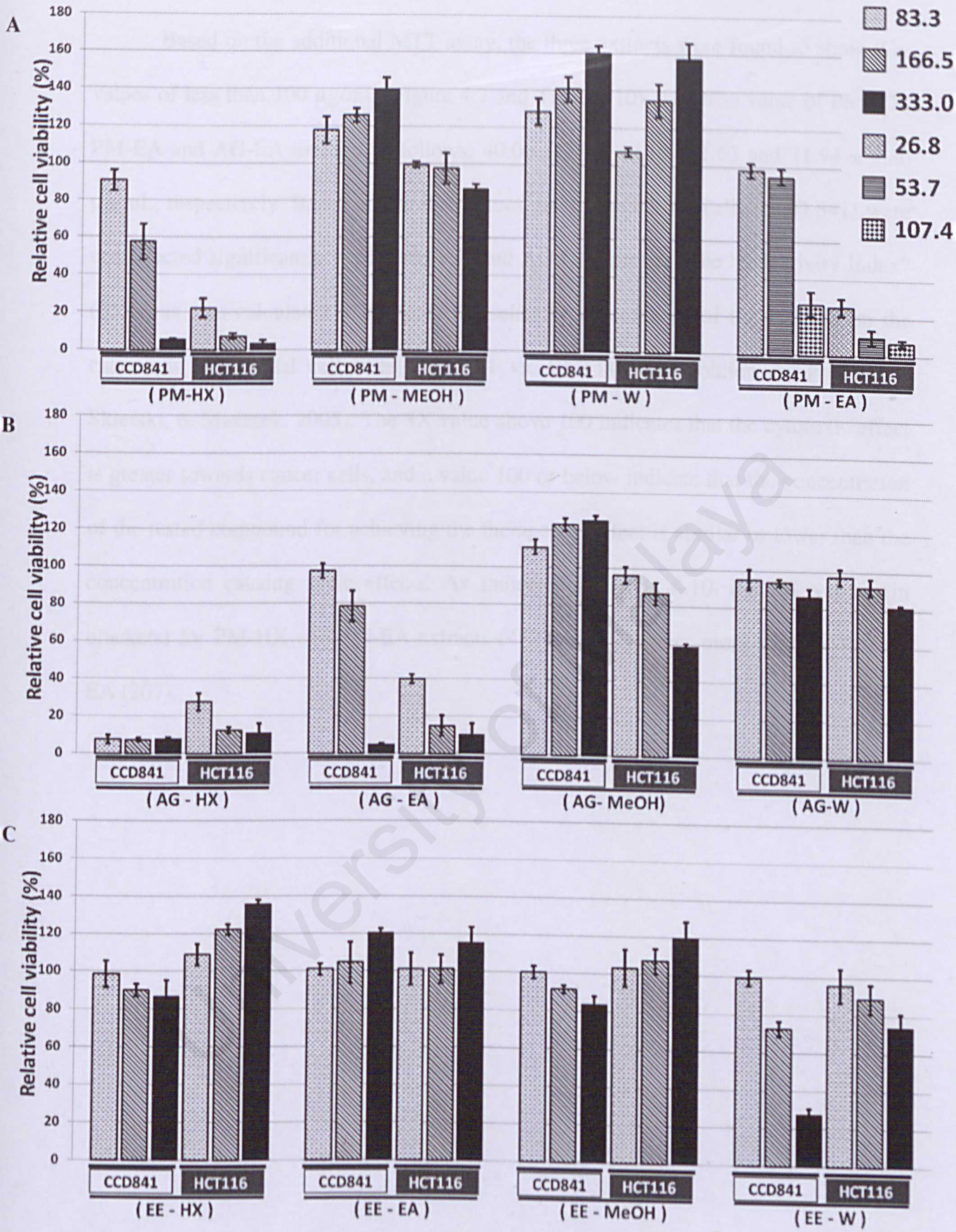


Fig 4.6: MTT anti-proliferative assay for (A) *P. minus*, (B) *A. galanga*, (C) *E. elatior* extracts using HCT116 (colon cancer) and CCD841 (normal colon). The legends indicate the concentration of the corresponding extracts (µg/mL). The relative cell viability is the ratio of viability of the treated to untreated cells x 100%. Values are means ± SD (n=3). PM-EA extract had lower solubility in DMSO, thus lower range of concentration (28.86, 53.72 and 107.44 µg/mL) were used

Based on the additional MTT assay, the three extracts were found to show IC_{50} values of less than 100 µg/mL (Figure 4.7 and Table 4.10). The IC_{50} value of PM-HX, PM-EA and AG-EA extracts as follows: 40.00 ± 2.04, 43.18 ± 1.63 and 71.94 ± 1.80 µg/mL, respectively. Based on the IC_{50} values, the normal colon cells (CCD 841) were not affected significantly by the PM-HX and AG-EA extracts. The "Selectivity Index" (SX) was also calculated to evaluate the selective cytotoxicity of the extracts on the cancerous vs. normal cell lines (CCD841 vs. HCT116) (Popiołkiewicz, Polkowski, Skierski, & Mazurek, 2005). The SX value above 100 indicates that the cytotoxic effect is greater towards cancer cells, and a value 100 or below indicate that the concentration of the tested compound for achieving the therapeutic effect is similar or lower than the concentration causing toxic effects. As indicated in Table 4.10, the SX values (in brackets) for PM-HX and AG-EA extracts (478 and 321), were more superior to PM-EA (207).

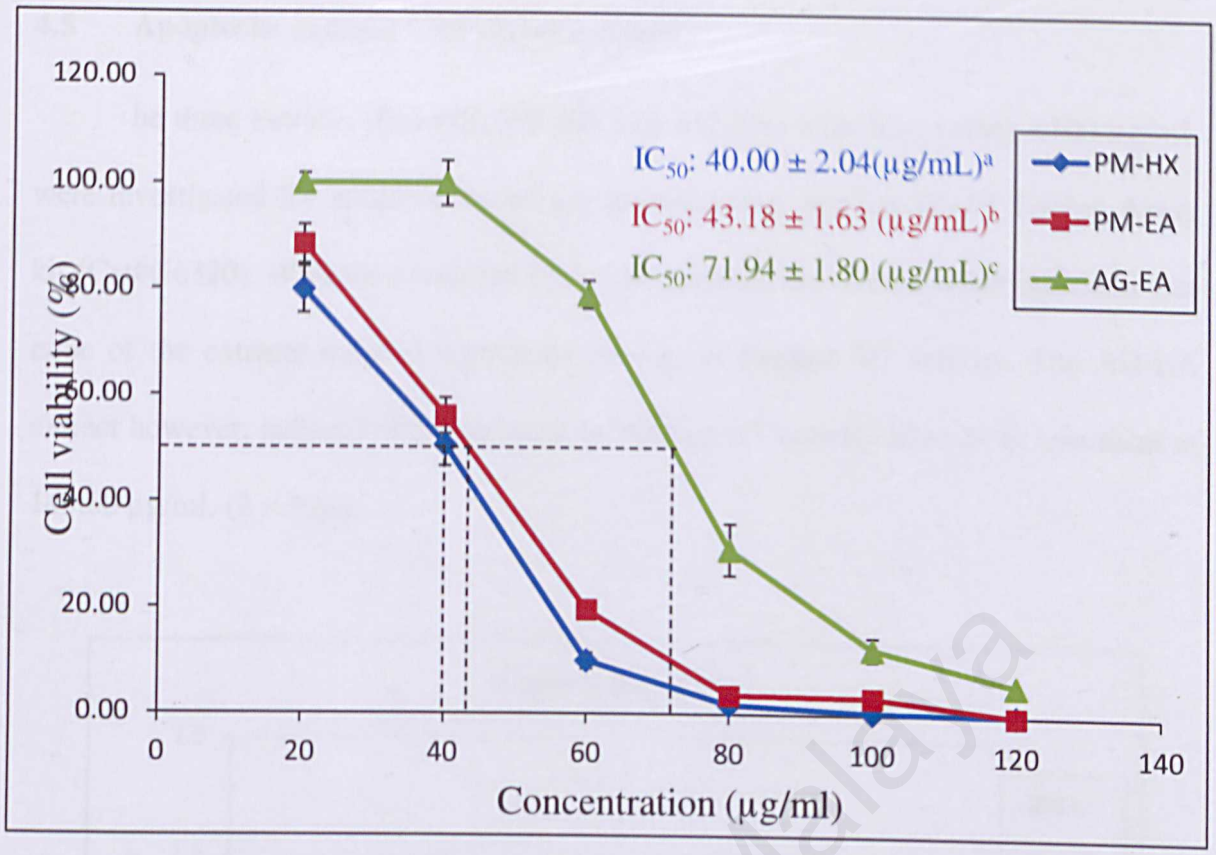


Figure 4.7: Antiproliferative activities of selected extracts on colon cancer cell lines (HCT116). Viability was determined on the basis of MTT assay. The values are expressed as the means±standard deviation (n=6). PM, *P. minus*; AG, *A. galanga*; HX, hexane; EA, ethyl acetate.

Table 4.10: The IC_{50} value of the selected extracts on HCT116 and CCD841 cells, and their selectivity index.

Extracts	HCT116 cell line, IC_{50} (µg/mL)	CCD841 cell line, IC_{50} (µg/mL)	Selectivity Index (SX)
PM-HX	40.00 ± 2.04	190.62 ± 15.71	478
PM-EA	43.18 ± 1.63	89.20 ± 5.11	207
AG-EA	71.94 ± 1.80	230.61 ± 12.28	321

4.5 Apoptosis: caspase – 3/7 activation assay

he three extracts (PM-HX, PM-EA and AG-EA) with IC_{50} values <100 µg/mL were investigated for apoptosis induction activity using ApoTox-Glo™ Triplex Assay kit (Cat#G6320). With the exception of AG-EA extract, the overall result indicated that none of the extracts induced significant change in caspase 3/7 activity. The AG-EA extract however, induced ~30% increase in caspase 3/7 activity after 24 hr treatment at 143.88 µg/mL (2 × IC_{50}).

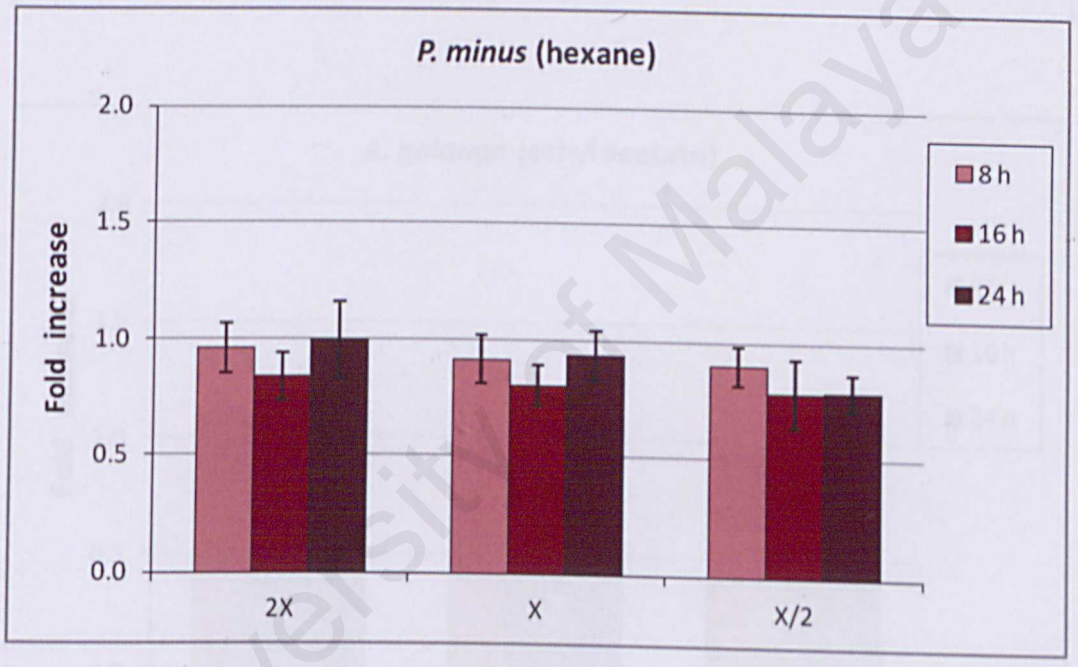
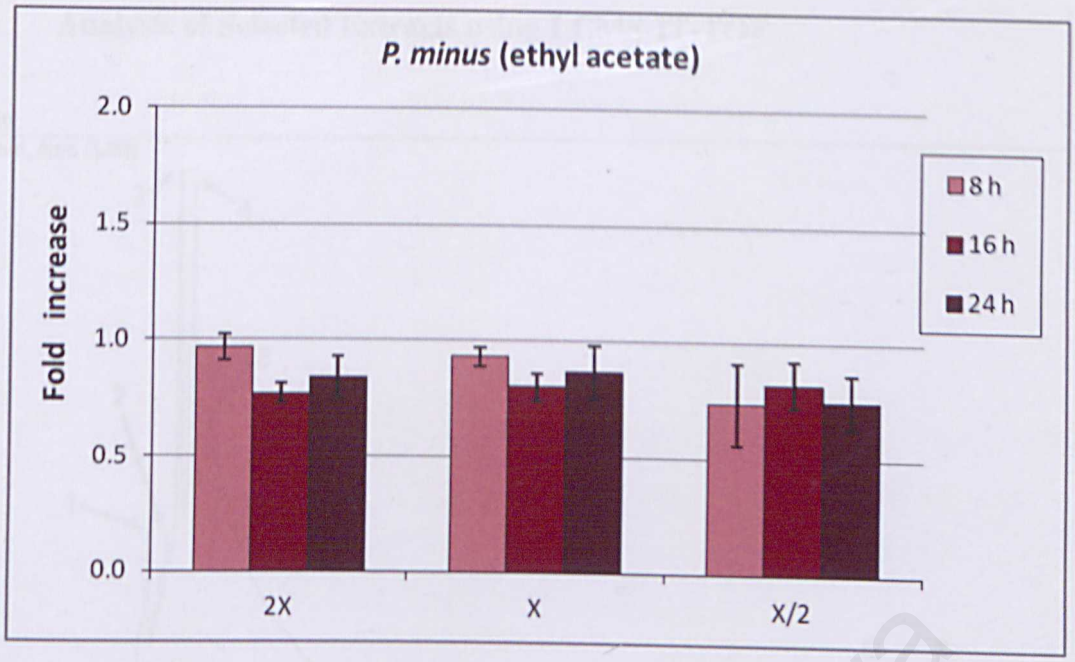


Figure 4.8: Caspase caspase 3/7 activity assay of HCT116 using Apotox-Glo Triplex kit. HCT116 cells were treated using PM-EA, PM-HX and AG-EA extracts at different IC50 values (2xIC_{50}, IC_{50}, ½ IC_{50}) and exposed for 3 different periods (8, 16 and 24 hours).



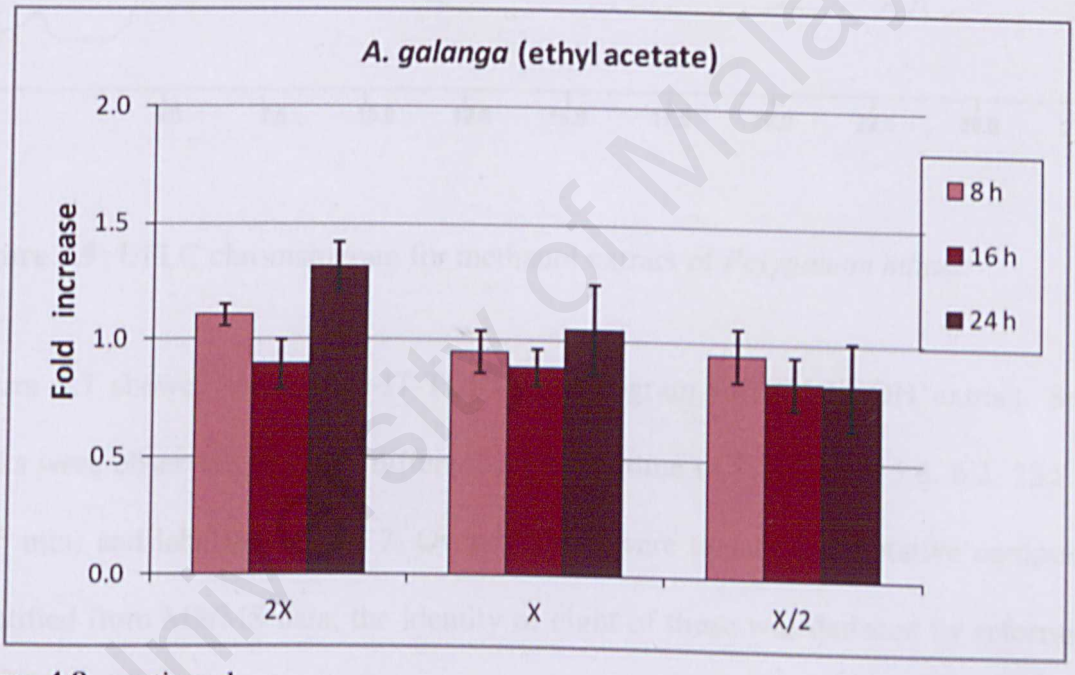


Figure 4.8, continued.

4.6 Analysis of Selected Extracts using LCMS-IT-TOF

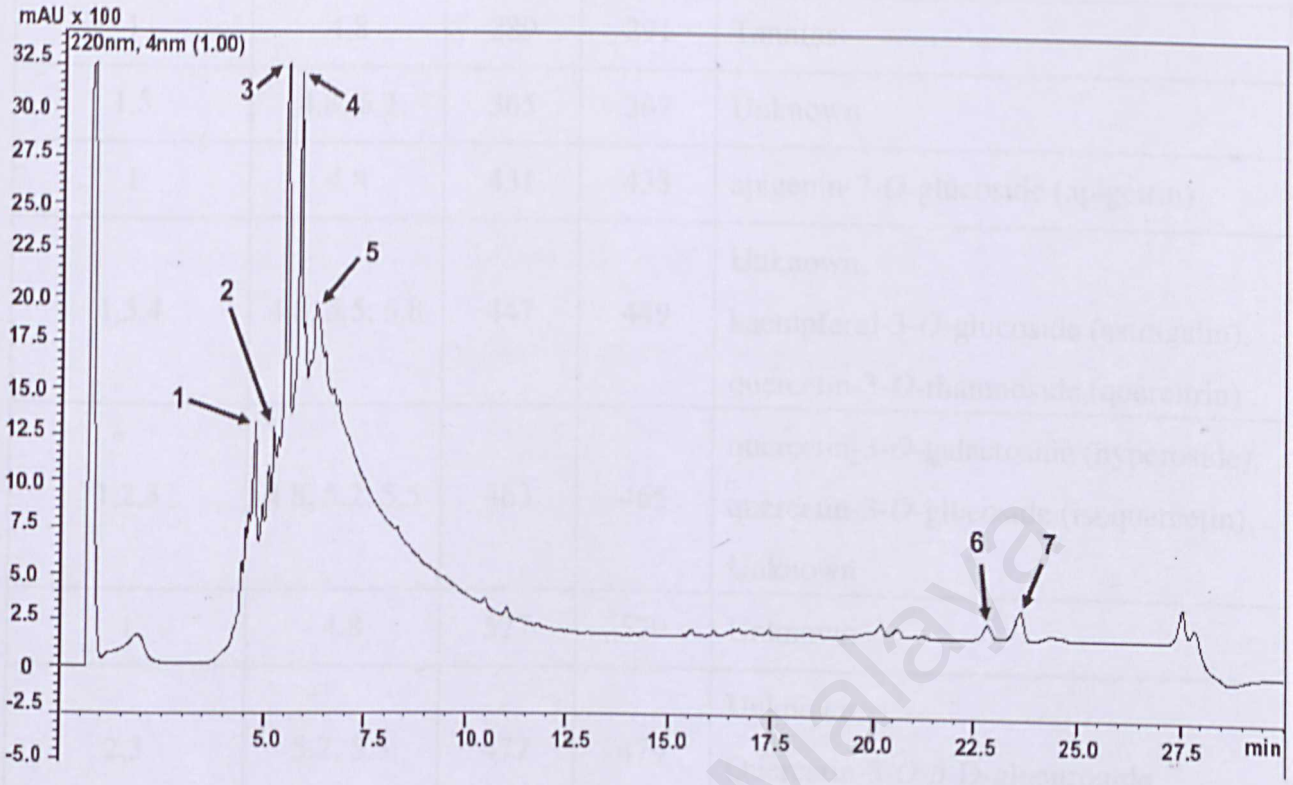


Figure 4.9: UFLC chromatogram for methanol extract of *Polygonum minus*.

Figure 4.7 showed the LCMS-IT-TOF chromatogram for PM-MeOH extract. Seven peaks were observed, at seven different retention time (4.8, 5.2, 5.5, 5.8, 6.2, 22.8 and 23.6 min) and labelled as 1 to 7. Overall, there were twenty one putative compounds identified from MS/MS data; the identity of eight of those was deduced by referring to the MS data from the literature (Huang, Cai, Xing, Corke, & Sun, 2008; Noor Hashim et al., 2012). The spectroscopic data is summarized in Table 4.11. The compounds identified were flavonoids. They were six flavonols, a flavone (apigenin) and tannins.

Chromatogram peak (s)	Retention time (min)	m/z $[M-H]^-$	m/z $[M-H]^+$	Proposed compound
1	4.8	289	291	Tannins
1,5	4.8, 6.2	365	367	Unknown
1	4.8	431	433	apigenin-7-*O*-glucoside (apigetrin)
1,3,4	4.8, 5.5, 5.8	447	449	Unknown, kaempferol-3-*O*-glucoside (astragalin), quercetin-3-*O*-rhamnoside (quercitrin)
1,2,3	4.8, 5.2, 5.5	463	465	quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercetin), Unknown
1	4.8	577	579	Unknown
2,3	5.2, 5.5	477	479	Unknown, Quercetin-3-*O*-*β*-D-glucuronide (miquelianin)
3	5.5	285	287	Unknown
4	5.8	301	303	Quercetin
4	5.8	381	-	Unknown
4	5.8	461	463	Unknown
5	6.2	285	287	Unknown
6	22.8	885	887	Unknown
7	23.6	870	872	Unknown

Table 4.11: MS data of known and unknown compounds from methanol extract of *P. minus* and their proposed identities.

CHAPTER FIVE

DISCUSSION

5.1 Extraction processes to obtain bioactive compounds

When investigating the nutritional and medicinal benefits of plants, the solvent system adopted for the extraction of bioactive phytochemicals is one of the most important factors needing careful attention, to ensure the desired bioactive constituents are recovered effectively for their isolation and bioactivity study. Effective extraction procedure should be inexpensive, safe, efficient, reproducible and able to optimally preserve the active constituents that were originally present in the plants matrix. Before proceeding with the extraction step, plant materials are washed with distilled water and dried in the oven at 40 °C, followed by grinding it into powder form. This could rupture the plants' tissue and maximize the surface area that come into contact with the extraction solvents, to enhance the mass transfer or recovery of active medicinal compounds from the sample.

Solvent extraction is the most common procedure used to obtain plants active phytochemicals. Generally, the yield of extraction vary, and is highly dependent on these parameters: the type of solvents and their polarities, temperature, extraction time, sample-to-solvent ratio, pH, the number of extraction steps, as well as the chemical structure or composition of plant materials (Dai & Mumper, 2010; Thoo et al., 2013). The solubility of active components is determined by their biochemical properties and the polarity of the solvent used. Plant active constituents could vary, from simple to highly complex substances which could be found in varying amounts. For example, phenolic compounds obtainable from plants are diverse, from the simplest phenolic

compound such as phenolic acid, to the complex one such as tannins. Considering such fact, the selection of the right solvent(s) may affect the quantities and rate of extraction of the active constituent(s) from the plant material. Ethyl acetate was reported be efficient in extracting samples containing high polyphenols, proanthocyanidins and flavonoids (Pekić, Kovač, Alonso, & Revilla, 1998). The selection of solvent may also depend on the specific nature of the bioactive compound being aimed. For instance, methanol is more efficient in extracting lower molecular weight polyphenols, however, the use of aqueous acetone is better for extraction of higher molecular weight flavanols (Dai & Mumper, 2010). This could be explained where the intermolecular force of small carbon chains are easily dissociated with methanol molecules; while longer carbon chains have a higher strength of covalent bond between the molecules. The extraction of hydrophilic constituents may use polar solvents such as methanol, ethanol or water, while dichloromethane may be used to extract more lipophilic constituents. In some cases, hexane is used to remove chlorophyll (Jeyaseelan, Tharmila, Sathiyaseelan, & Niranjan, 2012).

Solid-to-solvent ratio also plays a significant role in the extraction. High solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by the solvent. Besides, higher solvent volume means that the compounds have higher opportunities to interact with the solvent as an equilibrium is reached.

The recovery of active constituents from plants through extraction procedure must also consider a careful selection of the extraction temperature and time. The use of elevated the temperature during extraction could lower the viscosity and surface tension of the extraction solvent, allowing the solvent to reach the sample matrices more effectively and improving the extraction efficiency. However, some active compounds

are easily hydrolyzed and oxidized, if the extraction time is too long or the extraction is done at high temperatures. For example, anthocyanin showed a rapid degradation at temperature >70 °C (Havlíková & Míková, 1985). Therefore, the selection of proper extraction temperature and time are of importance to maintain the stability of active compounds. In this study, a moderate temperature of 40 °C was used and the total extraction time was 24 hour (8 hr x 3 extractions).

In order to obtain a wider spectrum of bioactive compounds, this study employed the sequential use of four extraction solvents with different polarities, namely hexane, ethyl acetate, methanol, and water. Hexane is useful for extraction of non-polar constituents, such as tocopherols (Lagouri & Boskou, 2009) and carotenoids (Herrero, Martín-Álvarez, Señoráns, Cifuentes, & Ibáñez, 2005), while ethyl acetate is useful to extract semi-polar constituents such as alkaloids and volatile oils (Kim & Pratt, 1992). In contrast, methanol and water are able to extract the polar constituents and most polar constituents, respectively. These include phenolic and hydroxyl-phenolic compounds with acids, alcohols, sugars or glycosides as the side groups (Kim & Pratt, 1992). This study extraction temperature of 40 °C that was used in this study, and the extraction time of 8 hours which repeated three times. The extraction yield and antioxidant components in *P. minus*, *A. galanga* and *E. elatior* extracted with the four solvents are shown in Table 4.1.

For *P. minus*, the methanol extract showed the highest yield of 15.41% and this was followed by water (7.09%), ethyl acetate (1.85%) and hexane (1.56%). Previous studies showed that *P. minus* is rich with secondary metabolites such as flavonoids, aldehydes, terpenoids, gerniol, geranial and phenolic compounds; nearly fifty-three compounds, which were isolated from *P. minus* leaves (Narasimhulu & Mohamed, 2014; Vikram, Chiruvella, & Ripain, Ilfah Husna Abdullah Arifullah, 2014). Among the

A. galanga extracts, the methanol extract had the highest yield at 24.01%, followed by (in decreasing order of yield) hexane, water and ethyl acetate extracts. Previous studies reported that *A. galanga* is rich with phenolic acids, flavonoids, carbohydrates and essential oils (Chudiwal et al., 2010; Kaushik et al., 2011). For *E. elatior*, the hexane extract had showed the highest yield of 12.18%. Previous studies reported the inflorescence of *E. elatior* are rich in phenolics, flavonoids, anthocyanins, terpenoids, saponins, tannins, carbohydrates and essential oils (Chan et al., 2011b; Lachumy et al., 2010; Wijekoon, Bhat, & Karim, 2011). The sum of yields for the extracted materials in each of *P. minus, A. galanga* and *E. elatior* using the four solvents (apolar to polar) were 25.91%, 33.66% and 28.31%, respectively. Methanol extracted the highest yield for *P. minus* and *A. galanga*. Methanol has been reported to extract the highest bioactive compounds (Ismail, Bagalkotkar, Iqbal, & Adamu, 2012). However, the highest yield for *E. elatior* was extracted by hexane. This suggests that the polarity and the amount of phytochemicals among these plants are not similar, and thus explaining the differences in the yields among them using differing solvents. The chemical structure of bioactive compound influences its polarity and solubility in the solvents in accordance with the previous reports (Thoo et al., 2013; Yusri, Chan, Iqbal, & Ismail, 2012) and thus influencing the yields.

5.2 Analysis of antioxidant components: total phenolic and total flavonoid contents

5.2.1 Total phenolic content (TPC) analyses

The total phenolic content was determined by the Folin-Ciocalteu assay and the results were expressed as μg of gallic acid equivalent (GAE) per mg of extract. The Folin-Ciocalteu procedure is a colorimetric method that is frequently used to estimate phenolic contents of biological materials due to its simplicity, although it has some limitations when there are interfering substances, such as sugars, vitamins, aromatic amines, sulphur dioxide, inorganic ions and ascorbic acid (Everette, Bryant, Green, Abbey, Wangila, & Walker, 2010; Prior et al., 2005). Also, the Folin-Ciocalteu procedure is not completely accurate for TPC measurement, as it did not provide a full picture of the quality or quantity of the phenolic constituents in the extracts (Katsube et al., 2004). However, this method had been extensively used by researchers worldwide and has been reported to be merely an appropriate method to estimate total phenolic content (Rudnicki, de Oliveira, Veiga Pereira, Reginatto, Dal-Pizzol, & Moreira, 2007). The Folin-Ciocalteu reagent is believed to be composed of heteropoly-phos-photungstates/ molybdates [possibly $(PMoW_{11}O_{40})^{4-}$](Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez, 2013). The reaction takes place at pH~10 after the addition of sodium carbonate. Under basic condition, a phenolic proton are dissociated to form phenolate ion, which is capable of reducing the Folin-Ciocalteu reagent. The intensity of the blue coloured complex will increase when there is more of hydrogen donating groups due to the presence of phenolic compounds and this relationship is used to deduce the total phenolic content in the sample tested (Kaur, Arora, & Singh, 2008).

Phenolics account for the majority of antioxidant activity in plants. Plant phenolics are taken as antioxidants due to its free radical scavenging activities, and thus it is an important nutritional component from the diet for humans. The antioxidant properties of phenolic compounds are mainly due to their redox potential, which allow them to act as reducing agents, hydrogen donator, metal chelators and singlet oxygen quenchers (Rice-Evans, Miller, & Paganga, 1996). By preventing imbalances in the amount of free radicals, plant phenolics may be able to prevent cellular damage or DNA damage, and this eventually could prevent cancer or chronic disease development. The health promoting property of plant phenolics has attracted great attention of the public media, as well as scientists, and therefore, it was reasonable to determine the total phenolic content in the plant extracts.

The TPC values of a particular plant could vary among different studies due to the differences in extraction procedure. In addition, the phenolic compositions of plant tissues are reported to be varying considerably with seasonal, genetic and agronomic factors (Hilton & Palmer-Jones, 1973). Soluble phenolics are present in higher concentrations in the outer tissues (epidermal and sub-epidermal layers) of fruits and grains than in the inner tissues (mesocarp and pulp) (Antolovich, Prenzler, Robards, & Ryan, 2000). Total polyphenol content and antioxidant activity were found to be different for different parts (leaf, phloem, bark, cork, needle) of trees (pine, birch, spruce, aspen) (Kähkönen et al., 1999). For example, Ismail *et al.* (2012) reported total phenolic contents of *Baccaurea motleyana* was higher in the fruits, as compared with the peels.

In this study, TPC values of the 12 extracts from *P. minus*, *A. galanga* and *E. elatior* are presented in Table 4.1. The methanol extract of *P. minus* exhibited the highest TPC value at 174.00 ± 0.31 mg GAE/g, while the *P. minus*-water extract

showed the second highest phenolics at 73.90 ± 0.23 mg GAE/g. Ethyl acetate extract of *P. minus* TPC value was at 37.18 ± 0.23 mg GAE/g, while the hexane extract had the lowest TPC value at 2.45 ± 0.08 mg GAE/g. The result suggests *P. minus* leaves is rich with phenolic compounds. Although phenolics could be detected in all of the *P. minus* extracts, it is particularly high in the water (very polar) and methanol (polar) extracts. This could be due to the phenolic constituents in *P. minus* which contain one or more hydrophilic functional groups such as hydroxyl, carboxyl and amino groups. Previous reports indicated that several of phenolic compounds have been isolated from *P. minus* leaves and this included apigenin, coumaric acid, kaempferol, rutin, quercetin, rhamnetin, gallic acid and tannins (Noor Hashim et al., 2012; Vikram et al., 2014), and these may account for the prominent TPC value in *P. minus*. Tannins have been reported to be prominently found in the methanol extract of *Polygonum* species (Huang et al., 2008). The TPC value for PM-MeOH in this study is higher than that reported by Huang et al. (2008), which used freeze dried leaf/ stem of whole plants materials of 4 different *Polygonum* species. Other studies have reported the following TPC values for *P. minus*: ethanol and water extracts, 207 ± 0.011 and 55.5 0.0021 mg GAE/g extract respectively (Qader, Abdulla, Chua, Najim, Zain, & Hamdan, 2011); water extract, 55.1 ± 0.9 mg GAE/g extract (Wan-Ibrahim, Sidik, & Kuppusamy, 2010) and acetone extract, 12.9 ± 2.8 mg GAE/g extract (Sulaiman, Sajak, Ooi, Seow, & Supriatno, 2011).

The TPC values for *A. galanga* extracts ranged from 9.36 to 20.87 mg GAE/g extract. The relative TPC value values (in descending order) for *A. galanga* extracts were: AG-EA>AG-MeOH>AG-W>AG-HX. Other studies have reported the following TPC values (mg GAE/g extract) for *A. galanga*: water extract, 7.98 ± 0.08 (Wongsa, Chaiwarit, & Zamaludien, 2012); methanol extract, 2.14 ± 0.20 (Wong, Lim, & Omar, 2009) and 60% ethanol, 58.25 ± 1.89 (Lu, Yuan, Zeng, & Chen, 2011). A variety of

phenolic compounds have been isolated from the rhizome of *A. galanga*, such as chavicol analogues (Janssen & Scheffer, 1985; Matsuda, Morikawa, Managi, & Yoshikawa, 2003), *p*-hydroxycinnamaldehyde and [di-(*p*-hydroxy-*cis*-styryl)] methane (Barik, Kundu, & Dey, 1987), cinnamic and coumaric acid derivatives (Barik et al., 1987; Morikawa et al., 2005; Wongsa et al., 2012). These may account the phenolics content in *A. galanga*.

The TPC values for *E. elatior* ranged from 59.89 to 2.29 mg GAE/g extract. The relative TPC value values (in descending order) for *E. elatior* extracts: EE-MeOH>EE-EA>EE-W>EE-HX. Other studies have reported the following TPC values (mg GAE/g extract) for *E. elatior*: MeOH extract, 25.0 ± 2.2 mg GAE/g extract (Chan, Lim, & Tan, 2011); water extract, 53.4 ± 4.2 mg GAE/g extract; 70% acetone, 89.3 ± 1.1 mg GAE/g extract (Sulaiman et al., 2011). Caffeoylquinic acids were reported to be the major phenolic in *E. elatior* leaves (Chan et al., 2009) and quercetin are the main contributor of phenolic content in the inflorescence (Andarwulan et al., 2010); they may possibly contributing to the observed polyphenol content. No association was found between yield and TPC value, which agrees with previous report (Chandini, Ganesan, & Bhaskar, 2008).

5.2.2 Total flavonoid content (TFC) analyses

Flavonoids are widely distributed in plants and they are commonly found highly concentrated in the skin of fruits and the leaf epidermis. They play many physiological functions, such as acting as chemical messengers, physiological regulators, cell cycle inhibitors, protection against UV radiation, colourful pigmentation and disease resistance (Aggarwal & Shishodia, 2006; Liu, 2004). Flavonoids are important antioxidant constituents of plants because their hydroxyl groups contribute to the radical-scavenging ability. Flavonoids exhibit various range of biochemical activities including antioxidant, antibacterial, antithrombotic, antiallergic, anti-inflammatory, vasodilatory actions Low Density Lipoprotein (LDL)-inhibiting and cardioprotective activities (Cook & Samman, 1996; Jin, Zhang, Yan, Guo, & Niu, 2012). Flavonoids can be subclassified as flavones, flavonols, flavanones, isoflavones, and anthocyanidins. These subclasses are differing in term of the presence of certain functional group (ketone or hydroxyl group), numbers of functional group (single or multiple) and the presence of salt ion. The basic structure of a flavonoid contains a diphenylpropane skeleton (Figure 5.1), comprising of two benzene rings (ring A and B) which is linked by a three carbon chain that forms a closed pyran ring (heterocyclic ring containing oxygen, the C ring) with benzenic A ring. Therefore, their structure is also referred to as C6-C3-C6.

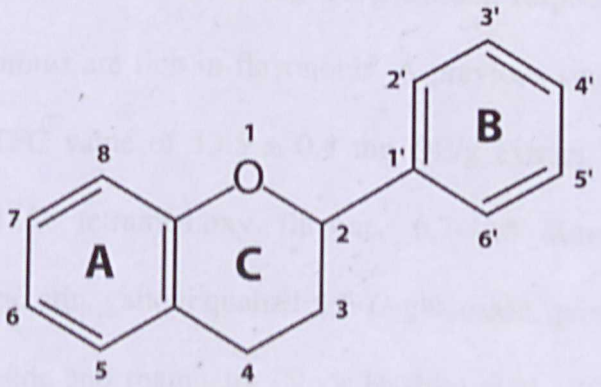


Figure 5.1: Basic chemical structure of flavonoid

The biochemical structure of a flavonoid including the numbers, positions and type of substitutions influence ROS/RNS-radical scavenging and metal chelating activities. The B-ring hydroxyl configuration is the most important determinant of radical scavenging potential. Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (Heim, Tagliaferro, & Bobilya, 2002). The increase of number of hydroxyl group and the decrease glycosidic moieties also affect the radical scavenging potential of a flavonoid (Heim et al., 2002).

The total flavonoid content (TFC) for all of the plant extracts was determined by the aluminium chloride colorimetric assay. The results were expressed as μg of quercetin equivalent (QE) per mg extract. The assay involved the formation of acid stable complexes of aluminium chloride with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Kalita, Barman, Pal, & Kalita, 2013). Aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids (Kalita et al., 2013).

Flavonoids were detected in all extracts (Table 4.1), and the values ranged from 2.12 ± 0.16 to 53.19 ± 1.23 mg QE/g extract. *P. minus* exhibited a relatively significant amount of flavonoid in ethyl acetate, methanol and water extracts, which were 47.22±1.23, 53.19±1.23 and 43.65±0.62 mg QE/g extract, respectively. This indicates that the leaves of *P. minus* are rich in flavonoids. A previous study reported *P. minus* water extract had a TFC value of 13.5 ± 0.4 mg QE/g extract. The flavonoids 6,7-methylenedioxy-5,3',4',5' tetramethoxy flavone, 6,7-4',5'-dimethylenedioxy-3,5,3'-trimethoxyflavone, quercetin, galloyl quercetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside and rhamnetin (Noor Hashim et al., 2012; Urones, Marcos,

Pérez, & Barcala, 1990) have been isolated from *P. minus* and these may in part account for the TFC values observed in this study.

The TFC values for *E. elatior* ranged from 5.50 to 18.98 mg QE/g extract, and these are higher than the following values that were previously reported: water and MeOH extracts, 1.825 ± 0.032 and 7.628 ± 0.445 mg QE/g extract respectively (Wijekoon et al., 2011); 70% acetone, 70% methanol and water extracts, 1.7 ± 0.3, 1.8 ± 0.2 and 1.5 ± 0.1 mg QE/g extract, respectively (Sulaiman et al., 2011). The inflorescences of *E. elatior* contained flavonoids such as kaempferol and quercetin (Miean & Mohamed, 2001). The flavonoids kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside and quercetin 3-rhamnoside have been isolated from the leaves of *E. elatior* (Williams & Harborne, 1977). For *A. galanga* extracts, ethyl acetate extract gave the highest TFC value. Lu *et al.* (2011) reported that 60% ethanolic extract of *A. galanga* had the strongest antioxidant capacity and contained a significant amount of galangin (4333.5 µg/g). Galangoflavonoid, a flavones glycoside have also been isolated from *A. galanga* (Jaju, Indurwade, Sakarkar, Fuloria, & Basu, 2009).

5.3 *In vitro* antioxidant assays

5.3.1 Ferric Reducing Antioxidant Power (FRAP) activities

The FRAP assay is a rapid, reproducible, and easy to perform assay in measuring the ferric reducing ability of the plant extracts (Benzie & Strain, 1996). Furthermore, it has been frequently applied to measure the antioxidant activity of dietary polyphenols and flavonoids (Pulido, Bravo, & Saura-Calixto, 2000). The ferric tripyridyltriazine (Fe^{3+}-TPTZ) reagent could form an intense blue Fe^{2+}-TPTZ complex in the presence of antioxidant or reducing agents, with a λ_{max} at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The intensity of the colour is proportional to the amount of antioxidant presence/content. The ferric reducing activity for the extracts was deduced by quantifying the amounts of Fe^{2+} using $FeSO_4.7H_2O$ standard curve. Results were expressed as µmol ferrous iron (II) equivalent per gram of sample.

Table 4.2 shows the FRAP values of the plant extracts along with the positive controls (quercetin, gallic acid, ascorbic acid and trolox). The FRAP values for all of the extracts ranged from 14.21 ± 2.65 to 1728.33 ± 1.66 µmol Fe^{2+}/g extract. In each of the plants, the methanol extract consistently showed the highest FRAP value compared to other extracts. This probably means the ferric reducing components are mostly polar. In contrast, the hexane extracts gave three of the lowest FRAP values. The strength of ferric reducing capacities could be categorized into four groups: low antioxidant power (<100 µmol Fe^{2+}/g), medium (100–250 µmol Fe^{2+}/g), high (250–625 µmol Fe^{2+}/g), extremely high (>625 µmol Fe^{2+}/g) (Borneo, León, Aguirre, Ribotta, & Cantero, 2009). Based on such classification, PM-MeOH and PM-W could be categorized as having 'extremely high' antioxidant power. The *E. elatior* methanol and *P. minus* ethyl acetate extracts had 'high' antioxidant power, while *A. galanga* extracts had 'moderate' antioxidant power: *A. galanga* ethyl acetate and methanol extracts; *E. elatior* ethyl

acetate and water extracts. The hexane extracts for the 3 plants and the water extract of *A. galanga* showed FRAP values that were less than 100 μmol Fe^{2+}/g and thus putting them under 'low' antioxidant power category.

The following previous studies have reported lower FRAP values for *P. minus* extracts: 781.32 ± 4.2 μmol Fe^{2+}/g extract (PM-MeOH); 68.3 ± 3.3 and 842 ± 21.3 μmol Fe^{2+}/g extract (PM-W) (Mohd Ghazali, Al-Naqeb, Krishnan Selvarajan, Hazizul Hasan, & Adam, 2014; Wan-Ibrahim et al., 2010). The FRAP values for AG-MeOH and AG-W extracts in this study are higher than the values reported in the following previous studies: 82.21 ± 2.92 μmol Fe^{2+}/g extract and 17.98 ± 0.94 μmol Fe^{2+}/g extract (Li et al., 2013). The FRAP values for EE-MeOH and EE-W extracts in this study are also higher compared to the same values previous previously: 9 ± 1 and 26 ± 1 μmol Fe^{2+}/g fresh weight respectively (Wijekoon et al., 2011).

The FRAP values of all extracts were significantly lower than antioxidant positive controls. The high FRAP values for positive controls are likely due to them existing in a highly purified state, unlike the plant extracts which were presented in complex matrices. The FRAP assay does have some limitations, where it cannot be used to determine reducing capacities of carotenoids and antioxidants with oxidizable functional group, such as thiols (–SH, which can be found in protein) or those which react with Fe (II) (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002; Ou et al., 2002; Somogyi, Rosta, Pusztai, Tulassay, & Nagy, 2007).

5.3.2 $ABTS^{\bullet+}$ radical scavenging activities

ABTS assay involved the use of radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$). The ABTS solution is made from the oxidation of ABTS by potassium persulfate (Re et al., 1999). The resulting solution has a blue/green characteristic color, due to the presence of $ABTS^{\bullet+}$ chromophore, and this could react with electron/hydrogen-donating antioxidants and causing the bleaching of the ABTS solution (Liu et al., 2009) that could be monitored spectrophotometrically. The $ABTS^{\bullet+}$ radicals were generated in the ABTS/$K_2S_2O_8$ system:

$$S_2O_8^{2-} + ABTS \rightarrow SO_4^{2-} + SO_4^{-\bullet} + ABTS^{+}$$

where the scission of the peroxodisulfate could take place after the electron transfer. In the presence of excess ABTS, the sulphate radical will react according to the following:

$$SO_4^{-} + 2ABTS \rightarrow SO_4^{2-} + 2ABTS^{+}$$

The whole reaction could be represented by the following equation:

$$S_2O_8^{2-} + 2ABTS \rightarrow 2SO_4^{2-} + 2ABTS^{+}$$

$ABTS^{\bullet+}$ radicals are more reactive than DPPH radicals and the reactions with $ABTS^{\bullet+}$ radicals in the assay involve electron transfer process (Kaviarasan, Naik, Gangabhagirathi, Anuradha, & Priyadarsini, 2007). The reaction is pH-independent. The decreasing amount of $ABTS^{\bullet+}$ is linearly correlated with the increasing amount of antioxidant, and this assay is also suited for hydrophilic and lipophilic antioxidants (Charles, 2013).

Figure 4.1(a-c) shows the ABTS-scavenging activities of the extracts, along with the positive controls (trolox, quercertin, gallic acid and vitamin C). The positive controls showed rapid scavenging activity and reach maximum inhibition below 200 µg/ml,

except for ascorbic acid. All extracts showed concentration-dependent radical-scavenging activity relationship. In Figure 4.1(a), the inhibition activities of PM-MeOH and PM-W extracts increased rapidly below 200 μg/mL and they achieved the maximum radical inhibition at 2000 μg/mL. The IC_{50} values for PM-MeOH and PM-W extracts were 159.62 ± 25.14 and 198.36 ± 26.44 μg/mL respectively. In Figure 4.1(b), all of *A. galanga* extracts showed activities closely near to each other, however, all of them did not reach 50% radical inhibition at the maximum concentration of 2000 μg/ml. In Figure 4.1(c), the EE-MeOH and EE-EA extracts showed increasing activity which did not plateaued until 2000 μg/mL, and their IC_{50} values were 1308.27 ± 186.27 and 1621.69 ± 5.60 μg/mL, respectively. The hexane extract of *P. minus* and *E. elatior* are less reactive, showing less than 20% radical inhibition at the highest concentration studied.

The ABTS-scavenging activities of the plant extracts were also expressed as trolox equivalent antioxidant capacity (TEAC) and the results are shown in Table 4.3. The TEAC values for the extracts ranged from 25.43 ± 1.77 to 226.25 ± 7.36 μmol trolox equivalents (TE) per gram of extract. All of the extracts had their antioxidant capacities lower than the positive controls. The PM-MeOH extract exhibited the highest $ABTS^{\bullet+}$ radical-scavenging activity and the TEAC value was 226.25 ± 7.36 μmol TE/g extract. It was however 7 times lower than ascorbic acid. The relative strength of $ABTS^{\bullet+}$ radical-scavenging activity for all of the extracts, in descending order is as follows: PM-W > EE-MeOH > PM-EA > AG-W > AG-EA > EE-W > AG-MeOH > AG-H > EE-EA > EE-H > PM-H. The methanol and water extracts of *P. minus* showed higher ABTS-scavenging capacities compared to other extracts. This may be due to the presence of high polyphenols in both of the extracts. It has been reported that plant extracts rich in polyphenols also had high $ABTS^{\bullet+}$-scavenging activity (Wang, Chang,

Inbaraj, & Chen, 2010). To our knowledge, the evaluation of $ABTS^{\bullet+}$-scavenging activity by *P. minus* extract has never been reported. In this study, the TEAC values for *A. galanga* extracts were found to be higher than the following previously reported values: 25.4 µmol TE/g extract (MeOH extract) (Surveswaran, Cai, Corke, & Sun, 2007); 45.45 µmol TE/g (80% MeOH extract) (Gan et al., 2010) and 21.75 µmol TE/g (water extract) (Li et al., 2013). However, Lu et al. (2011) reported a higher TEAC value of 593.90 µmol TE/g extract for 60% ethanol extract of *A. galanga*. The difference could have been due to the differences in extraction procedure and plant sources. A lower TEAC value of 1.40 ± 0.05 µmol TE/g for *E. elatior* extract had previously been reported (Andarwulan et al., 2010).

5.3.3 DPPH• radical scavenging activities

DPPH• scavenging assay is among the most commonly used assay to evaluate plant antioxidant activity. The 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) is a stable free radical which has an unpaired valence electron (Eklund et al., 2005). The DPPH• radicals could be reduced to DPPH-H in the presence of hydrogen-donating antioxidant substances. The purple coloured DPPH• solution changes into light yellow in the presence of antioxidants or when reacting with a radical species (R•), causing a decrease in the solution absorbance and this change could be monitored spectrophotometrically (Bastos et al., 2007; Dvorakova, Moreira, Dostalek, Skulilova, Guido, & Barros, 2008).

$$DPPH\bullet + AH \longrightarrow DPPH\text{–}H + A\bullet$$

$$DPPH\bullet + R\bullet \longrightarrow DPPH\text{–}R$$

The reaction between phenols (Ar–OH) and DPPH• proceeds through both the direct hydrogen atom transfer (HAT) and the sequential proton loss electron transfer (SPLET) mechanisms (Foti, Daquino, & Geraci, 2004; Musialik & Litwinienko, 2005):

$$\text{(HAT) } Ar\text{–}OH + DPPH\bullet \longrightarrow Ar\text{–}O\bullet + DPPH\text{–}H$$

$$\text{(SPLET) } Ar\text{–}OH \rightleftharpoons Ar\text{–}O^- + H+$$

$$Ar\text{–}O^- + DPPH\bullet \longrightarrow Ar\text{–}O\bullet + DPPH^-$$

$$DPPH^- + H^+ \longrightarrow DPPH\text{–}H$$

Generally, higher activity of DPPH scavenging is correlated to a higher antioxidant activity (Liu, Qiu, Ding, & Yao, 2008). The advantage of this method is its compatibility with broad range of solvents of differing polarities (Cheng, Moore, & Yu, 2006), thus both lipophilic and hydrophilic antioxidant constituents could be evaluated

without the use of stabilizing agents. This assay deviates from natural biological conditions due to the use of artificial DPPH radical and methanol as the solvent (Cao, Sofic, & Prior, 1997). However, due to its simplicity and convenience in screening many samples at once, as well its inexpensiveness, has made it a popular choice for *in vitro* antioxidant assay (Frankel, 1993; Frankel & Meyer, 2000).

Figure 4.2(a-c) showed the DPPH radical scavenging activities of the extracts, as well as the antioxidant standards, quercetin, gallic acid, ascorbic acid and trolox. Most of the extracts showed a concentration-dependent activity relationship. Positive controls showed rapid activity curve, and reached the maximum inhibition at concentrations below 200 µg/mL. In Figure 4.2(a), PM-MeOH and PM-W scavenging activity increased rapidly and reach a plateau at ~ 500 µg/mL. The IC_{50} values for both PM-MeOH and PM-W extracts were 125.51 ± 8.14 and 210.16 ± 2.34 µg/mL, respectively. These values imply higher activity than that reported by Mohd Ghazali et al., (2014), which were 540.0 ± 10.8 µg/mL for PM-MeOH and 472.5 ± 15.2 µg/mL for PM-W extracts. This suggests that the antioxidants in the methanol and water extract are strong radical-scavengers because they have potent activities. However, in this study, the PM-EA extract showed a lower activity, with an IC_{50} value of 602.82 ± 19.62 µg/mL, compared to the same value as reported by Mohd Ghazali et al., (2014), which was 120.3 ± 2.7 µg/mL. The sequential extraction might have caused some components to be "captured" by the hexane extract, and thus, causing the ethyl acetate extract to have a lower DPPH activity. PM-MeOH, PM-EA and PM-W extracts gave among the highest TPC and TFC values. High correlation coefficient (r = 0.969) between TPC and the DPPH radical scavenging assay of some medicinal plants have been reported (Katsube et al., 2004). In Figure 4.2(b), all *A. galanga* extracts showed relatively lower scavenging activity and they generally did not reach 25% DPPH• inhibition. A previous

study reported *A. galanga* MeOH extract had an IC_{50} value of 2300 ± 200 µg/mL (Wong et al., 2009). For *E. elatior*, only EE-MeOH extract showed significant activity with an IC_{50} value of 766.77 ± 77.33 µg/ml dry extract, compared to 9140 µg/mL as reported by Lachumy *et al.*, (2010). The DPPH• scavenging activity could also be expressed as trolox equivalent antioxidant capacity (TEAC), and this is shown in Table 4.4. The TEAC values ranged from 4.74 ± 1.92 to 1276 ± 12.26 µmol TE/g extract. Five of the extracts showed prominent TEAC values and their relative activities (in descending order) were: PM-MeOH>PM-W>PM-EA>EE-MeOH>AG-MeOH. The results indicated that *P. minus* had the most prominent DPPH• scavenging activity.

5.3.4 Superoxide anion ($O_2^{\bullet-}$) radical scavenging activities

In this present study, the superoxide anion radical scavenging activity of the extracts was assayed through PMS-NADH-NBT system. Superoxide anion was generated by the oxidation of NADH in PMS/NADH coupling reaction and followed by the reduction of nitroblue tetrazolium (NBT) (Oliveira, Coelho, Baltasar, Pereira, & Baptista, 2009). In the presence of superoxide-scavenging antioxidant, a decrease in absorbance at 570 nm is resulted and this property was used to measure the antioxidant activity of the plant extracts.

Superoxide is biologically toxic and produced in human body continuously through normal cellular metabolism. Immune system deployed superoxide to destroy invading pathogens. This radical is generated in vivo by auto-oxidation reaction of enzymes and, by electron transfer of non-enzymatic molecules. It is a precursor to the highly reactive free radical such as hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), peroxynitrite ($OONO^-$) and singlet oxygen, which induce oxidative damage in proteins, lipids, and DNA and cause changes in the redox environment on a cellular level (Mandade, Sreenivas, & Choudhury, 2011). In vivo $O_2^{\bullet-}$ can be eliminated enzymatically by superoxide dismutase (SOD). There are several enzymatic (superoxide dismutase) and non-enzymatic $O_2^{\bullet-}$ scavengers that have been found in plants (Alscher, 2002; Hagerman et al., 1998; Scandalios, 1993).

The $O_2^{\bullet-}$ scavenging capacities for all of the extracts, along with the positive control (gallic acid) are shown in Table 4.5. Gallic acid gave an IC_{50} value of 25.60 µg/mL. To the best of our knowledge, there has not been any other studies reporting the $O_2^{\bullet-}$ scavenging activity of *P. minus* and *E. elatior* extracts. The PM-W extract gave the lowest IC_{50} value (103.77 ± 6.68 µg/mL) among all of the extracts, and this corresponds to the highest superoxide scavenging. The activity of other extracts, in descending order

were as follows: PM-MeOH (195.64 ± 18.30 µg/mL), EE-W (236.40 ± 24.77 µg/mL), AG-W (422.85 ± 73.11 µg/mL) and AG-MeOH (950.93 ± 14.70 µg/mL). The remaining extracts showed lower activities, and were not able to reach 50% $O_2^{\bullet-}$ inhibition at the maximum concentration tested (1000 µg/mL). Previous studies reported that the rhizome of *A. galanga* showed quite good scavenging superoxide radical activity, where the following IC_{50} values were reported: 7033 µg/mL and 10509 µg/mL for ethanol and acetone extracts, respectively (Divakaran, Hema, Nair, & Nair, 2013).

The superoxide scavenging activities of the extracts are also shown in Figure 4.3(a-d). Most of the extracts showed a concentration-dependent activity relationship, either in a positive or negative activity relationship. Gallic acid showed rapid increase in scavenging activity, and the activity plateaued at ~ 250 µg/mL. In Figure 4.3(a), PM-W extract showed the strongest scavenging effect compared to other extracts, with the steep curve of activity, not far behind gallic acid. The PM-W extract reached the maximum $O_2^{\bullet-}$ inhibition at approximately 500 µg/mL. The PM-MeOH extract had its inhibition activity plateaued at about 1000 µg/mL. However, the remaining PM-EA and PM-HX extracts had almost negligible activities in scavenging $O_2^{\bullet-}$. In Figure 4.3(b), the $O_2^{\bullet-}$ scavenging activity of AG-W extract increased in a dose-dependent manner. It did not show a levelling off of activity, and gave a 74% of inhibition at 1000 µg/ml. Previous study reported that a 50% ethanol extract of *A. galanga* rhizome showed 63% $O_2^{\bullet-}$ inhibition at 1000 µg/mL (Juntachote & Berghofer, 2005). Interestingly, AG-MeOH extract showed a lower $O_2^{\bullet-}$ inhibition in the lower range of concentrations (below 500 µg/mL), but the inhibition increased rapidly thereafter. On the contrary, AG-EA and AG-HX extracts exhibited a different pattern of inhibition, showing negative superoxide anion scavenging activities when their concentrations went above 400 µg/mL. In Figure 4.3(c), EE-W showed excellent superoxide scavenging activities as its

concentration increased. EE-MeOH also exhibited dose-dependent inhibition, however, the activity plateaued at about 200 µg/mL and did not reach 50% $O_2^{\bullet-}$ inhibition at the maximum concentration tested. In addition, EE-EA and EE-HX extracts showed negative inhibitions, similar to that seen in AG-EA and AG-HX extracts. The negative inhibition of ethyl acetate and hexane extracts suggests the pro-oxidant effect of the extracts. The same situation was been observed in a recent study on *Lignosus tigris* (Yap et al., 2014), where pro-oxidant behaviour was seen when the extract concentrations increased. A few antioxidants have been identified to have dualistic behavior of antioxidant and pro-oxidant, and this included ascorbate (vitamin C), α-tocopherol (vitamin E), carotenoids and flavonoids. Vitamin C is able to generate superoxide anions and hydroxyl radicals in the presence of Fe^{3+} (Rietjens et al., 2002). In addition, flavonoids also may behave as pro-oxidant due to the effect of number and location of hydroxyl group in flavonoid structure, the interference of transition metal ions and the increase in concentration of flavonoids (Magnani, Gaydou, & Hubaud, 2000; Procházková, Boušová, & Wilhelmová, 2011). In this study, it is generally observed that the polar extracts (MeOH and water extracts) showed higher superoxide-scavenging activities. The various polar compounds in the extracts may contain flavonoids with high number of hydroxyl groups in ring B, hydroxyl group substituted at C-3, the presence of saturated C2-C3 bond and the absence of a carbonyl group at C4 position of the flavonoid structure as these increase the superoxide anion radicals scavenging activity (Cos et al., 1998; Hu et al., 1995).

Some of the extracts showed pro-oxidant behaviour. The pro-oxidant constituent in the plant extracts may be able to bind to various cell biomolecules irreversibly, by covalent bond with sulfhydryl groups or other essential groups, generating secondary free radicals (Metodiewa, Jaiswal, Cenas, Dickancaité, & Segura-Aguilar, 1999). The

oxidation of polyphenols also leads to the formation of H_2O_2, O_2 and a complex mixture of quinones/semi-quinones, all of which are potentially cytotoxic (Halliwell, 2008). Halliwell (2008) however mentioned that there is no clear evidence that antioxidants showing pro-oxidant behaviour may behave in a similar manner *in vivo*. However, pro-oxidant may bring beneficial effect *in vivo*, where it can initiate mild oxidative stress that is linked to an increase in the antioxidant defense levels and activation of xenobiotic-metabolising enzymes, thus leading to an overall cytoprotective environment (Halliwell, 2008).

5.3.5 Nitric oxide ($NO^{\bullet}$) radical scavenging activities

Nitric oxide is an essential molecules mediator for both physiological and pathological processes in the human body. It exhibits broad range of beneficial bioregulatory function in organisms, including acting as cell signalling molecules in vasodilation and neurotransmission, regulating hormone secretion, mediating in thrombosis, destruction of pathogenic microbes and killing of tumour cells (Hummel, Fischer, Martin, Schafer, & Buettner, 2006; Kang, Yokozawa, Kim, & Park, 2006; Kumaran & Karunakaran, 2006b). However, $NO^{\bullet}$ also has undesired biological effects. It can act as pro-oxidant at high concentrations and it could generate extremely reactive peroxynitrite (ONOO-), which could intoxicate living cells and damage biomolecules (Hummel et al., 2006). In this assay, sodium nitroprusside (SNP), an inorganic complex in phosphate buffered saline (at physiological pH) decomposed into NO^{+}, and in the presence of light irradiation, $NO^{\bullet}$ radicals are released (Mirkov et al., 2004). $NO^{\bullet}$ scavenger (such as antioxidants) and oxygen molecules can compete to reduce the $NO^{\bullet}$ production (Sreejayan & Rao, 1997). Nitrite is formed by the spontaneous oxidation of the residual $NO^{\bullet}$ with oxygen, and the Griess diazotization reaction was then used for nitrite measurement. Griess diazotization happened when sulphanilic acid react with nitrite to form diazonium salt and azo dye agent is added to develop distinguished pink colour.

In Table 4.6, only two extracts showed robust $NO^{\bullet}$ scavenging activity, which were PM-MeOH and PM-W extracts, with their IC_{50} values of 675 ± 56 µg/mL and 1239 ± 73 µg/mL, respectively. The remaining extracts were unable to reach 50% $NO^{\bullet}$ inhibition at the maximum concentration of the extracts. Meanwhile, gallic acid gave an IC_{50} value of 49.45±1.65 µg/mL.

The nitric oxide scavenging activities of extracts are shown in Figure 4.4(a-d). The extracts showed dose-dependent activity relationship. Gallic acid showed a rapid increase in scavenging activity and the activity plateaued at about 250 μg/mL. In Figure 4.4(a), PM-MeOH extract showed the highest scavenging activity, followed by PM-W, PM-EA and PM-HX extract. In this study, it was earlier observed that PM-MeOH extract has the highest TPC and TFC value among all extracts. Previous study also reported that tannins (polyphenols) and alkaloids were the active component in NO•-scavenging activities in traditional crude drugs (Yokozawa, Chen, & Tanaka, 2000). The galloyl groups in polyphenol (especially in tannins) play important role in NO•-scavenging activities (Nakagawa & Yokozawa, 2002). Tannins are polyphenol which can be found commonly in methanol extract of *Polygonum* species (Huang et al., 2008). In Figure 4.4(b), all extracts from *A. galanga* showed moderate scavenging activity that were close to each other, however all of them were unable to reach 50% NO• inhibition at the maximum concentration tested. In Figure 4.4(c), the EE-MeOH and EE-W extracts showed some increase in NO• inhibition as their concentrations increased, however they did not reach 50% NO• inhibition at the maximum concentration tested. On the other hand, EE-HX and EE-EA extract showed weak of inhibition, failing to reach even 5% of NO• inhibition even the concentration maximum concentration of the extracts. To the best of knowledge, characterisation of NO•-scavenging activity using Greiss reagent has never been done or reported for the three plants used in this study.

5.3.6 Ferrous ion-chelating (FIC) activities

Ferrous irons (Fe^{2+}) are essential in biological systems, for processes such as enzyme activity and oxygen transportation. Ferrous irons are the strongest pro-oxidant among the various species of transition metal ions (Halliwell & Gutteridge, 1984), due to its high reactivity. Ferrous ions produce highly reactive hydroxyl radicals when reacting with hydrogen peroxide and such reactive species could induce oxidative damage (Nićiforović et al., 2010; Oh, Jo, Cho, Kim, & Han, 2013). The hydroxyl radicals can also induce lipid peroxidation and this leads to food spoilage, and this problem could be minimized by ferrous ion chelating and deactivation.

Ferrous ion chelating ability was measured following Decker and Welch (1990). Ferrozine, a metal chelator could form a complex with Fe^{2+} ion. The Fe^{2+}–ferrozine complex is red in colour. The presence of other metal chelators (including antioxidants) will disrupt the complex formation. The bleaching or decolourisation of Fe^{2+}–ferrozine complex allows the measurement of the coexisting metal chelator ability (Kumaran & Karunakaran, 2006a). Metal chelating ability is a significant property that can be used to reduce the concentrations of metal catalyst, an agent that is known to significantly promote lipid peroxidation. Metal chelators are effective antioxidant when they bind to metal ions with σ-bond (Kumaran & Karunakaran, 2006a). This stabilizes the oxidized metal ion from involving in the redox chain reaction that can occur in the cells. Some polyphenols have been reported to exhibit antioxidant activity by chelating of metal ions (Zhao et al., 2008).

Figure 4.5(a-c) shows the FIC activities of the extracts with EDTA being used as a positive control. EDTA is a commonly known metal ion chelating agent. It binds strongly to metal cations through its two amines and four carboxylates as found in its

chemical structure. EDTA exhibited an excellent FIC acctivity and it showed maximum Fe^{2+} inhibition at <200 µg/mL. Most of the extracts showed concentration-dependent FIC activity relationship. All of the extracts showed metal chelating capacities, and none had FIC activity <10 % at 2000 µg/mL. EE-MeOH extract showed the highest FIC activity which was comparable to EDTA. EE-MeOH had the lowest EC_{50} value, which was 206.91 ± 6.42 µg extract/mL (Table 4.7). The remaining extracts with significant FIC activity had the following activity (in descending order), based on their EC_{50} values: PM-W (341.54 ± 19.32 µg/mL), AG-W (700.59 ± 34.43 µg/mL) and EE-W (1180.45 ± 31.39 µg/mL). The result suggests that the phenolic compounds in the extracts may contain two or more of the following functional groups: -OH, -SH, -COOH, $-PO_3H_2$, C=O, $-NR_2$, -S- and -O- in a favorable structure-function configuration (Ak & Gülçin, 2008; Nićiforović et al., 2010), which could contribute to the availability of sites for metal complexation.

The inflorescence of EE-MeOH from *E. elatior* showed very strong FIC activity, despite the extract having moderate TPC and TFC values. This suggests that it has components that could strongly bind to metal ion(s). The EE-MeOH extract in this study also showed a very potent EC_{50} value of 206.91 µg/mL, compared previous studies, which reported EC_{50} values of >3000 µg/mL for the inflorescence, leaves and rhizomes extracts of *E. elatior* (Chan, Lim, & Omar, 2007; Chan et al., 2008). The PM-W extract in this study also showed a more potent FIC activity, with a lower EC_{50} value of 341.54 ± 19.32 µg/ml, compared to 5400 µg/mL as reported by Chan et al. (2014). Previous studies using the rhizome of *A. galanga* also reported weak FIC activities (Chan et al., 2008; Juntachote & Berghofer, 2005; Wong, Lim, & Omar, 2009), with EC_{50} values of more than 1 mg/mL. This also contrasts with the result found in this study, where the AG-W extract showed higher FIC activity.

5.4 Correlation between antioxidant content and antioxidant activities

Pearson correlation analyses were done to investigate the relationship between the antioxidant components and antioxidant activities. Polyphenols and flavonoids demonstrated very strong ($r > 0.8$) and strong ($r = 0.60$-0.79) positive correlations with antioxidant activities as observed through FRAP, ABTS, DPPH and NO· assays. This suggests that polyphenols and flavonoids in the PM, AG and EE extracts contributed to the role of hydrogen/proton donors and could act as reducing agents to scavenge free radicals. This observation is concordant with renowned activity of polyphenol and flavonoids as antioxidant agents. Previous studies have reported strong positive correlation between phenolics content and antioxidant assays such as FRAP, ABTS and DPPH involving *P. minus* and *E. elatior* plants (Andarwulan et al., 2010; Maizura, Aminah, & Wan Aida, 2011; Sulaiman et al., 2011; Wan-Ibrahim et al., 2010). Meanwhile, polyphenols showed moderate ($r = 0.4$-0.59) positive correlation with $O_2^{\bullet-}$ radical-scavenging assay, but no correlation ($r = 0.19$ to -0.19) was observed with ferrous ion-chelating assay. Flavonoids demonstrated weak ($r = 0.2$ - 0.39) positive correlation with $O_2^{\bullet-}$ radical-scavenging assay and no correlation with ferrous ion-chelating assay. A number of previous studies have reported that there was no correlation between polyphenol content with ferrous ion-chelating activities (Silva, Sobrinho, Castro, Lima, & Amorim, 2011; Zhao et al., 2008).

5.5 Anti-proliferative activity of the plant extracts

The anti-proliferative activity was done using MTT assay for 48 hours, using each of the 12 plant extracts at various concentrations indicated in Table 4.9 and Figure 4.6. Four extracts were able to stimulate the proliferation of CCD841 cells in a dose dependent manner (Table 4.9 and Figure 4.6), and they were (in descending order of growth stimulation): PM-W>PM-MeOH>AG-MeOH>EE-EA. These extracts contain compounds that could potentially be used to promote the healing of colon cells and act as wound healing agent. In contrast, four extracts were not cytotoxic and were able to stimulate HCT116 proliferation (Table 4.9 and Figure 4.6), in a dose dependent manner, and they were (in descending order of growth stimulation): PM-W>EE-HX>EE-MeOH>EE-EA. These extracts contain compounds that may promote progression of colon cancer. The following extracts showed marked cytotoxicity towards CCD841 cells, as they caused >50% reduction in cell viability at the highest concentration tested: PM-HX, PM-EA, AG-HX, AG-EA and EE-W. Three extracts showed moderate cytotoxicity on HCT116 cells, as they did not cause >50% reduction in cell viability at the highest concentration tested, and these extracts were (with the corresponding percentage of HCT116 viability): PM-MeOH (86.43 ± 2.56%), AG-MeOH (58.80 ± 1.65%) and EE-W (74.77 ± 7.22%). Four extracts showed prominent anti-proliferative activity towards HCT116 cell line, as they caused >50% reduction in cell viability. The four extracts were: PM-HX, PM-EA, AG-EA and AG-HX.

The anti-proliferative activity of PM-HX, PM-EA and AG-EA extracts on HCT116 was investigated further by determining their IC_{50} values, since these extracts were more cytotoxic towards HCT116 compared to CCD841 as shown in Figure 4.7. The results indicated that PM-HX, PM-EA and AG-EA extracts showed prominent anti-proliferative activity on HCT116 cells in a dose dependent manner, and their relative

cytotoxicity (in descending order) were: PM-HX>PM-EA>AG-EA. The IC_{50} values for the three extracts on CCD841 were markedly higher than HCT116, indicating their selective cytotoxicity towards colon cancer cells (Table 4.10). A parameter, Selectivity Index (SX) was calculated in this assay. It is an objective indicator to evaluate the selectivity of the tested extracts cytotoxicity action on cancerous and non-cancerous cells (Popiołkiewicz et al., 2005). The SX value above 100 indicates that the cytotoxic effect is greater towards cancer cells and the value 100 or below means that concentration of substance for achieving therapeutic effect is similar or lower than the concentration causing toxic effects. As shown in table 4.10, all of the extracts showed SX values of more than 100, and this indicated their selective cytotoxicity. The results suggest that the three extracts have promising hope to provide sources of novel compound(s) with selective cytotoxicity on colon cancer cells, with minimal side effects to the normal colon cells. PM-HX had the highest SX value (478), suggesting that the extract was 378% more cytotoxic to HCT116 than CCD841.

A previous study reported that the water and ethanolic extracts of *P. minus* did not show any cytotoxicity towards normal lung cell line (Hs888Lu) at 500 µg/mL (Qader et al., 2011), providing further evidence of the selective cytotoxicity of the polar extract from *P. minus*. A recent study reported that *P. minus* exhibited potent anti-proliferative activity on the following cell lines, with corresponding IC_{50} values: HepG2 (ethyl acetate extract, 32.25± 3.72 µg/mL); HeLa (ethyl acetate extract, 63.09 ± 6.70 µg/mL); WRL 68 (petroleum ether extract, 56.23 ± 3.2 µg/mL); HCT116 (methanol extract, 86.3±3.5 µg/mL) (Mohd Ghazali et al., 2014). In this study, the IC_{50} value of ethyl acetate extract on HCT116 cells was lower, compared to that reported by Mohd Ghazali *et al.* (2014), suggesting that sequential extraction employed in this study resulted to PM-EA extract with a higher anti-proliferative activity. Regarding the

previous studies of *A. galanga*, the petroleum ether and chloroform extracts were cytotoxic to these cells with the respective IC_{50} values: human breast cancer cells, MDA-MB-231 (2.87 µg/ml and 6.19 µg/mL); normal lung cells, MRC-5 (2.86 µg/mL and 11.87 µg/mL) (Chew, Abidin, & Wahab, 2012). In addition, the human hepatocellular carcinoma SMMC-7721 and breast cancer MDA-MB-231 cell lines were moderately inhibited (IC_{50} >100 µg/mL) by ethanol and methanol extracts (Chew et al., 2012; Lu, Zhao, & Jiang, 2013). 1'S-1'-acetoxychavicol acetate (ACA) has been isolated from the rhizome of *A. galanga*. ACA showed cytotoxic activity on MCF7 and lung cancer COR L23 cell lines, with the following IC_{50} values: 23.9µM and 7.8µM, respectively (Chudiwal et al., 2010). ACA may be the possible component that is responsible for the anti-proliferative activity of AG-EA extract as observed in this study.

5.6 Apoptosis: caspase – 3/7 activation

Caspase 3/7 is belongs to the cysteine-aspartate proteases family, that play major roles in triggering caspase-dependent apoptosis and the pair is often used as an indicator of programmed cell death (PCD) or popularly known as apoptosis. Higher levels of caspase 3/7 are usually observed during apoptosis. Apoptosis is an energy-dependant process which involves activation of specific caspases which require a complex cascade of biochemical signaling events and eventually resulted to cell death. The apoptotic cell exhibits several structural and biochemical modifications including protein cleavage, DNA fragmentation, membrane disruption and caspase activation (McNamara et al., 2014). The *Promega ApoTox Glo Triplex assay* kit relies on the on the properties of a proprietary thermostable luciferase, which is formulated to generate a stable "glow-type" luminescent signal. The presence of caspase 3/7 during apoptosis causes the cleavage of the luminogenic substrate in the reaction mixture, which contains the DEVD recognition sequence. The catalysis resulted to the release of aminoluciferin, and the luciferase reaction produces luminogenic light that could be measured by a luminometer. Luminescence signal is then proportional to the amount of caspase activity.

With the exception of AG-EA extract, the overall results showed that caspase 3/7 activity was similar between the treated and untreated HCT116 cells, at 2x IC_{50}, IC_{50} and ½ IC_{50} concentrations of each PM-HX, PM-EA and AG-EA extracts. For AG-EA extract, an increase of ~30% in caspase 3/7 activity (24 hr treatment, 2xIC_{50} concentration) was detected. The three extracts indeed showed selective and potent cytotoxicity towards HCT116. Therefore, with the exception of AG-EA extract, it is possible that the other two extracts might have caused cell death by mechanism other than apoptosis, most likely by caspase independent cell death (CICD) processes, among

which include autophagy, paraptosis, mitotic catastrophe, apoptosis-like and necrosis-like PCD (Chen, Chi, Wang, Chien, & Lin, 2009).

Apoptosis-like PCD is characterized by less-compact chromatin condensation, while necrosis-like PCD has no chromatin condensation (Bröker, Kruyt, & Giaccone, 2005). CICD can also be mediated by non-caspase proteases such as calpains, endonucleases; cathepsins B, D, and L; and granzymes A and B (Leist & Jäättelä, 2009). Furthermore, CICD can be directed by several cellular organelles, including mitochondria, lysosomes, and the endoplasmic reticulum (ER), which act independently or collaborate with each other (Bröker et al., 2005).

For AG-EA extract, the caspase 3/7 activity induction could have been attributed to the presence of 1's-1'-acetoxychavicol (ACA), which have been isolated from Alpinia spp. previously. ACA had been reported to induce apoptosis in cancer cell lines such as breast adenocarcinoma (MCF-7 & MDA-MB-231), oral squamous carcinoma (HSC-2 & HSC-4), hepatocyte carcinoma (HepG2), epidermoid cervical carcinoma (CaSki), lung carcinoma (COR-L23) and human myeloma leukemic cell lines (Awang et al., 2010; Campbell, Prince, Landry, Kha, & Kleiner, 2007; Ito et al., 2005; Lee & Houghton, 2005).

5.7 Analyses of *P. minus* methanolic extract through LCMS-IT-TOF

The methanol extract of *P. minus* showed significant antioxidant activity and was subjected to LCMS-IT-TOF analysis (Figure 4.9). Twenty one distinctive m/z values were detected (Table 4.11) and the identity of eight of the components was proposed based on published MS data (Huang et al., 2008; Noor Hashim et al., 2012). The proposed compounds were flavonoids, comprising of six flavonols (quercetin and kaempferol), a flavone (apigenin) and tannins. These compounds have been reported to exhibit antioxidant and free radical scavenging activities (Burda & Oleszek, 2001; Cai, Sun, Xing, Luo, & Corke, 2006; Conforti, Statti, Tundis, Menichini, & Houghton, 2002; Fiorentino, Abrosca, Pacifico, Golino, & Oriano, 2007; Huang et al., 2008; Peng et al., 2003). Flavonoids have been reported to be important bioactive constituents of *Polygonum* species (Huang et al., 2008). Quercetin-3-*O*-rhamnoside or also known as quercitrin, are common flavonoid constituent found in the *Polygonum* species (Huang et al., 2008). Noor Hashim *et al.* (2012) reported quercetin and quercetin-3-*O*-rhamnoside presence in *P. minus*, and significant antioxidant activities were observed with their DPPH IC_{50} value less than 20 µg/mL. The compounds detected in this study could explain the potent antioxidant activity displayed by PM-MeOH extract. It is reasonable to assume that the phenolic compounds identified in PM-MeOH extract may possibly be acting synergistically and contributing to the potent antioxidant activities of *P. minus* extracts.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

P. minus, *E. elatior* and *A. galanga* extracts contained polyphenolic and flavonoid components and these components showed potent antioxidant activities, which include metal chelating activity (FIC) as well as radical-scavenging abilities against $ABTS^{\bullet+}$, DPPH•, superoxide anion and nitric oxide radicals. The LCMS-IT-TOF analysis revealed PM-MeOH extract contained eight flavonoids with renowned antioxidant activities. This study demonstrated the promising potential of local plants as sources of natural antioxidants. Further work is warranted to identify and isolate the antioxidant components, and to study their effects, particularly through in *vivo* investigations, to understand their health promoting benefits.

PM-HX, PM-EA and AG-EA extracts showed potent cytotoxicity against colon cancer cell line HCT116, with minimal toxicity against normal colon cell line CCD841. These extracts may provide sources of novel compounds that could selectively target colon cancer cells, and thus further analysis is warranted to investigate the cytotoxic modes of action exerted by the components of the extracts.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication:

1) Mohamad Zakkirun Abdullah, Johari Mohd Ali. *Polygonum minus* Huds., *Alpinia galanga* (L.) Willd and *Etlingera elatior* (Jack) R.M.Smith: sequential extraction using four solvents of varying polarity and evaluation of the extracts antioxidant and anti-proliferative activities (Journal of the Science of Food and Agriculture – submitted)

Conference:

1) International Conference on Natural Products 2013 (ICNP2013), Shah Alam Convention Centre, Malaysia; 4-6 March 2013.

APPENDIX

Appendix A: LCMS-IT-TOF Data

Level#	: 0
Sample Name	: 2c
Sample ID	: Zakkirun
ISTD Amount	: (Level1 Conc.)
Sample Amount	: 1
Dilution Factor	: 1
Tray#	: 1
Vial#	: 42
Injection Volume	: 25
Data File	: Zakkirun 2c.lcd
Method File	: Derep Method 0-100 30 min.lcm
Original Method	: C:\OLD-Lab Solution\Data\Cenar\Methods\Derep Method 0-100 30 min.lcm
Report Format	: Derep Report.lcr
Tuning File	: C:\OLD-Lab Solution\Data\Cenar\Autotuning\2013-09-25-LSL-mza.lct
Processed by	: Admin
Modified Date	: 11/7/2013 11:59:49 AM

Spectrum Index

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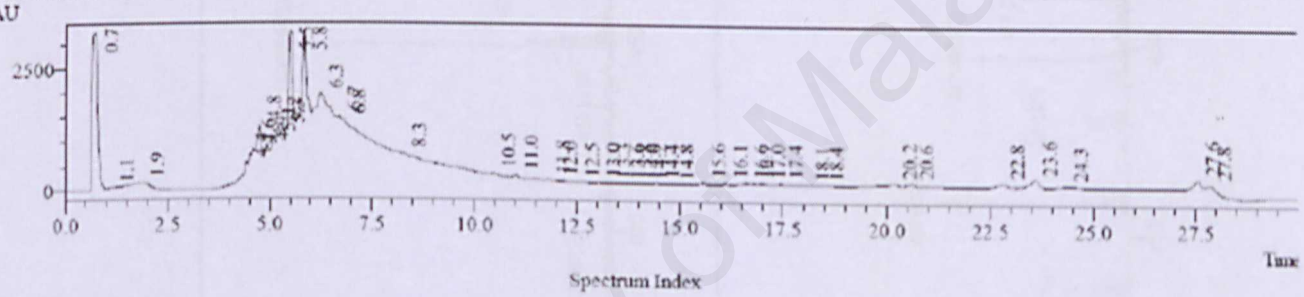
mAU



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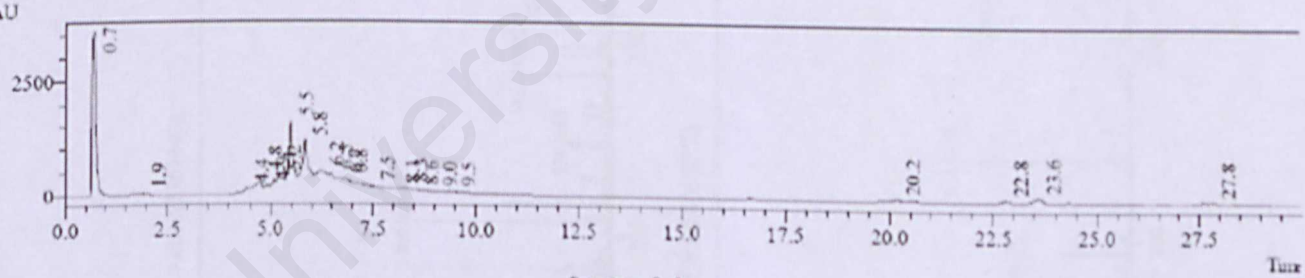

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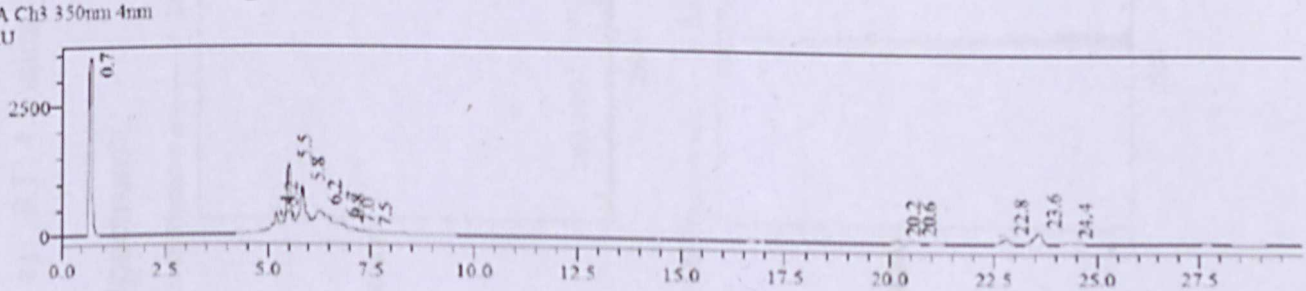

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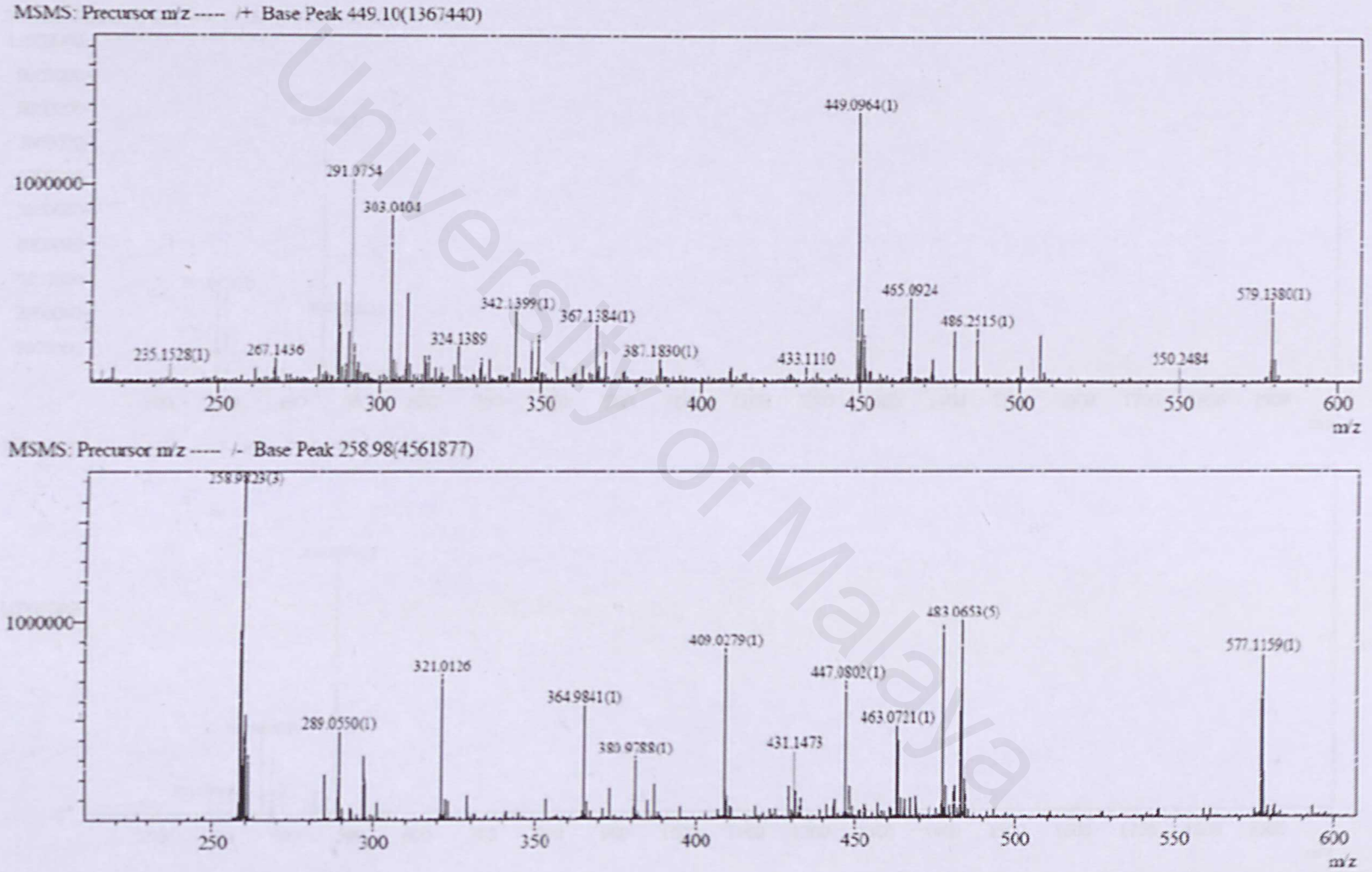

b) RT: 5.5 mins

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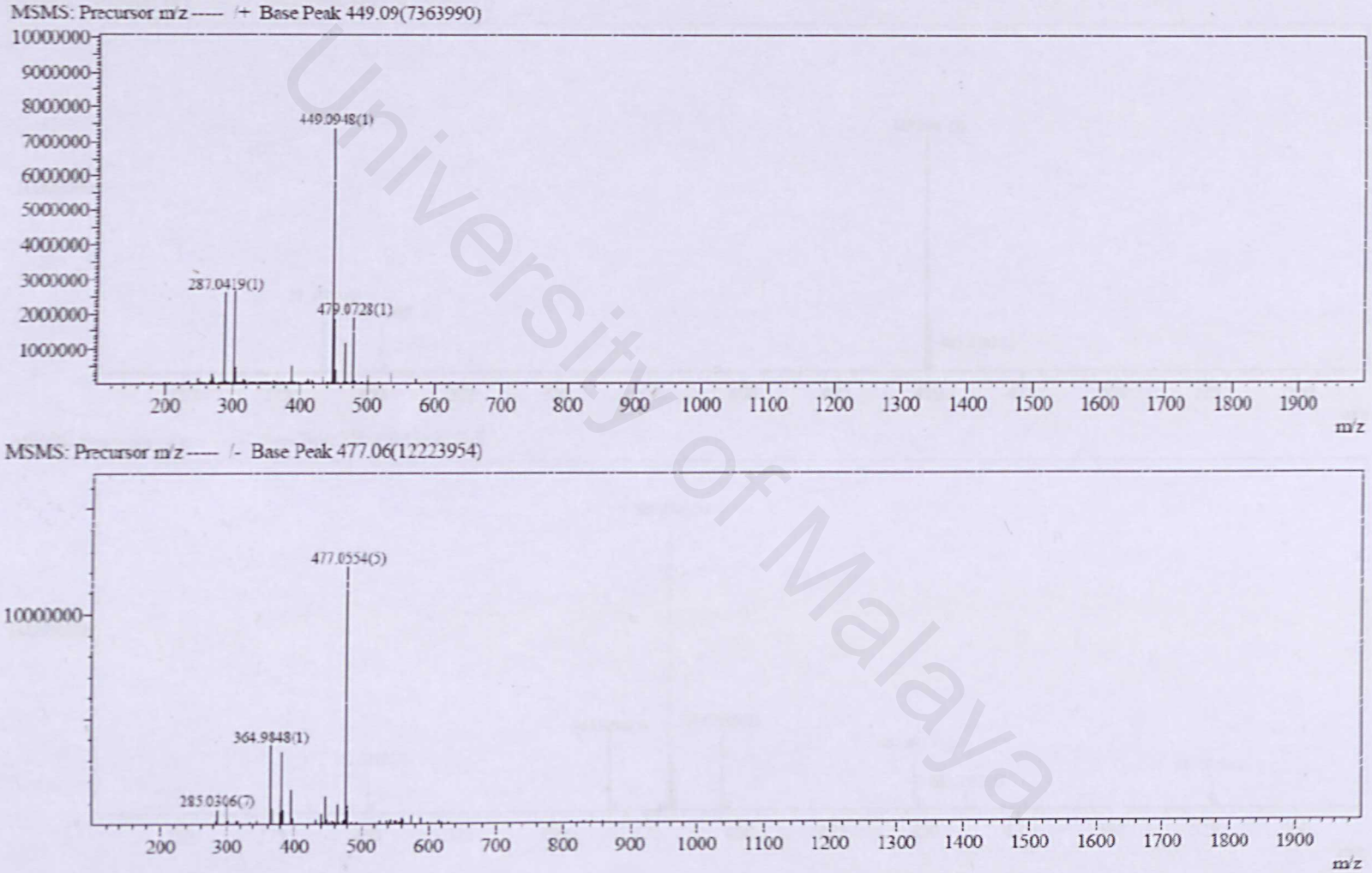

c) RT: 5.8 mins

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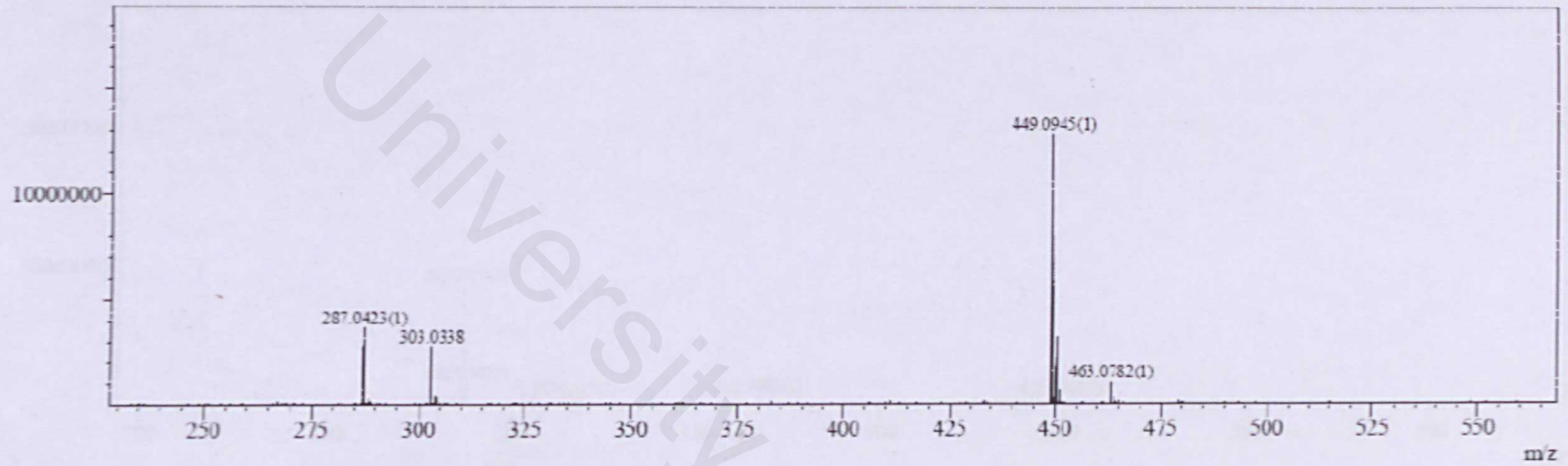
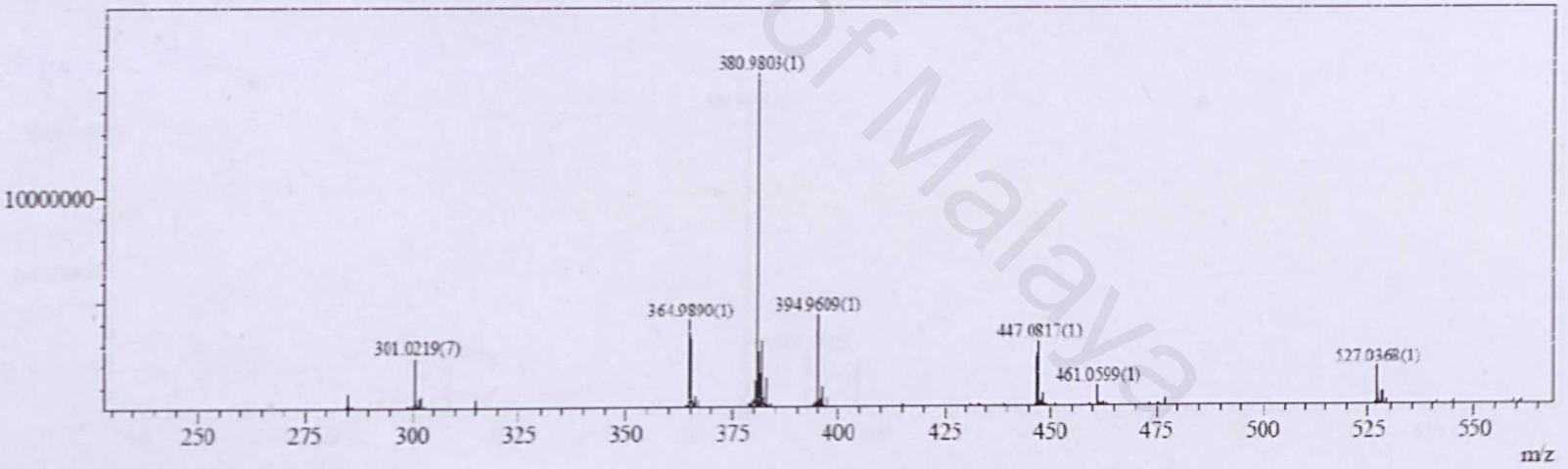
MSMS: Precursor m/z ----- /+ Base Peak 449.09(12849647)



MSMS: Precursor m/z ----- /- Base Peak 380.98(15815317)



d) RT: 6.2 mins

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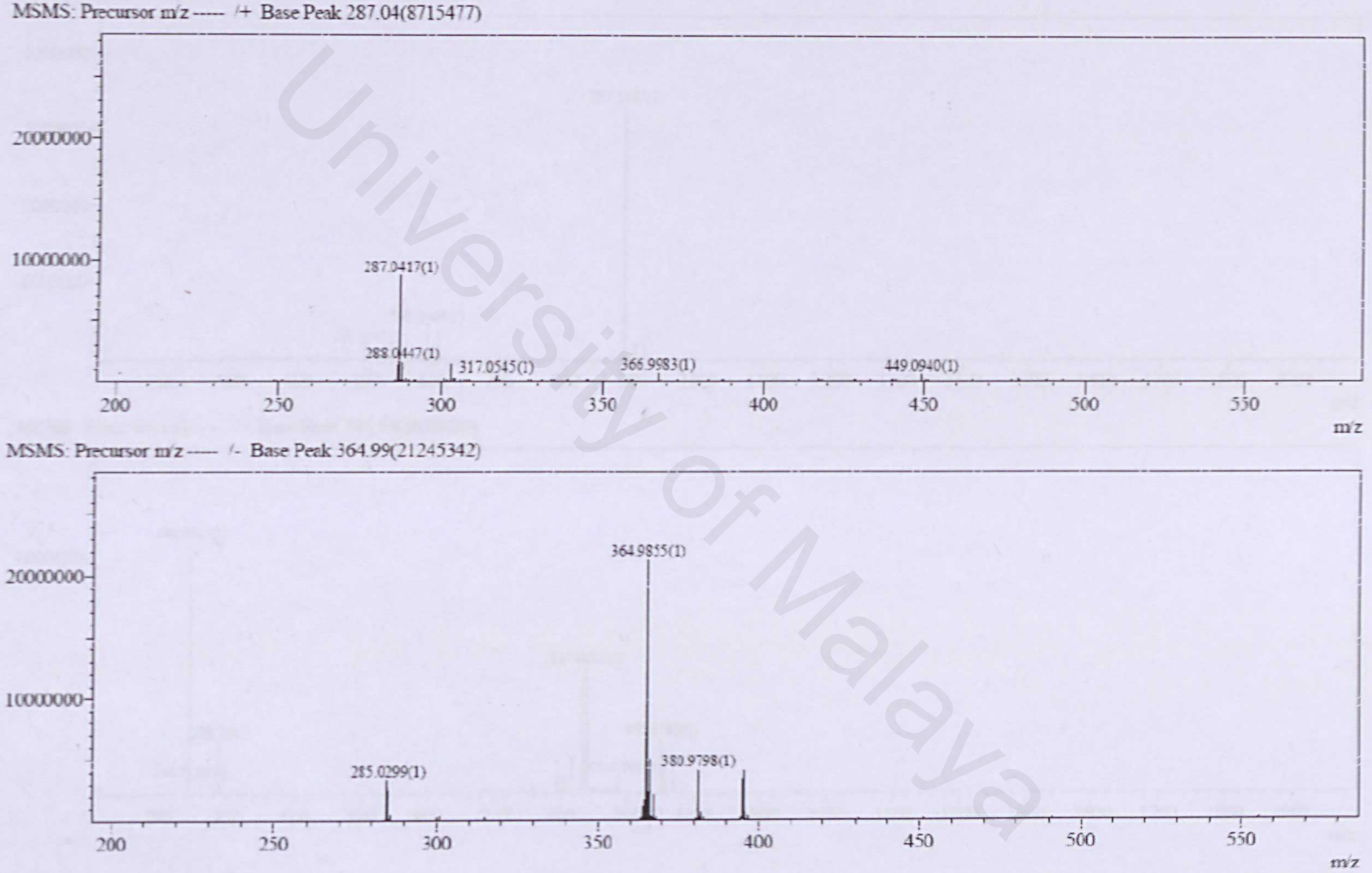

e) RT: 22.8 mins

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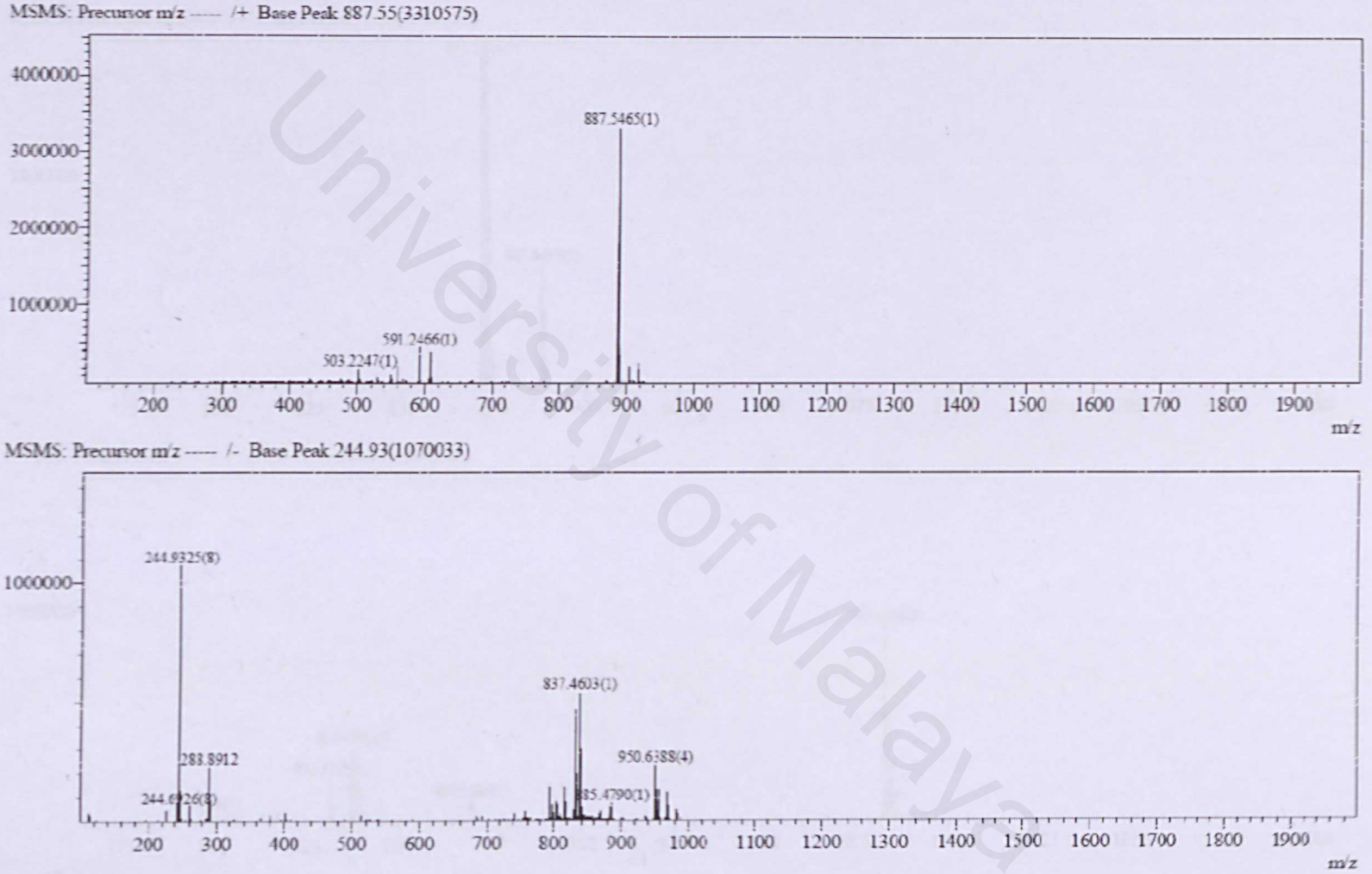

f) RT: 23.6 mins

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