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CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC PROPERTIES OF SELECTED FRUITS ENDEMIC TO BORNEO: INVESTIGATION ON *MANGIFERA PAJANG* AND *ARTOCARPUS ODORATISSIMUS*

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

July 2010

Abstract

Consumption of fruits and vegetables has been shown to reduce the risk of various types of cancer. Macro- and micro-nutrients as well as non-nutritive phytochemicals present in fruits and vegetables have been associated with this effect. This study was conducted to investigate the chemopreventive and chemotherapeutic potential of two types of fruits which are endemic to Borneo Island: *Mangifera pajang* (bambangan) and *Artocarpus odoratissimus* (tarap).

The first part of the project was to study the antioxidant potential of the crude extracts of the plants *in vitro*. The fruits were first separated into flesh, kernel and peel for *M. pajang* and flesh and seed for *A. odoratissmus*. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and FRAP (ferric reducing / antioxidant power) assay were employed for the antioxidant study. The result showed that the kernel of *M. pajang* extract displayed strongest antioxidant activity as assessed using both assays, followed by *M. pajang* peel, *A. odoratissmus* seed, *M. pajang* flesh and *A. odoratissmus* flesh.

The presence of selected phytochemicals in the plant extracts was determined in the next chapter. Polyphenols have been identified as major phytochemicals in the plant extracts, and in *M. pajang* kernel extract represents about 10% of its total weight. Gallic acid, coumaric acid, sinapic acid, caffeic acid, ferulic acid, chlorogenic acid, naringin, hesperidin, rutin, luteolin and diosmin have been identified as the key polyphenol phytochemicals present in the kernel of *M.*

pajang which might be responsible for the superior antioxidant properties as compared to other extracts.

Concern that the results for the chemical antioxidant assay do not necessarily reflect cellular activity led to the third part of the project; assessment of the cytoprotective activity of the crude extracts against oxidative stress induced by tert-butyl hydroperoxide (t-BHP). Only *M. pajang* kernel extract as well as the positive control (quercetin) displayed cytoprotective activity against this toxicant. It seems that non-cell based antioxidant assay does not necessarily reflect the activity in cell-based antioxidant assay. This is shown by lack of cytoprotective activity of both *M. pajang* peel and *A. odoratissimus* extracts despite their considerably high antioxidant activity in DPPH free radical scavenging and FRAP assay.

In order to study which proteins might be involved in the cytoprotection mechanism, western blotting method was employed to determine the expression of various cytoprotective proteins [i.e. quinone reductase (NQO1), glutathione peroxidase (GR), methionine sulfoxide reductase A (MSRA), heat shock protein 27 (HSP27) and heat shock protein 70 (HSP 70)]. Different cytoprotective mechanisms were observed by the kernel extracts and quercetin. In the present study, NQO1, GR, MSRA, HSP27 and HSP 70 have been shown to be involved in the cytoprotection activity of quercetin while only GR and MSRA were involved in the cytoprotection activity of *M. pajang* kernel extracts. Other cytoprotective proteins remain to be studied to fully understand the cytoprotection mechanism of both plant extract and quercetin.

Some chemopreventive agents have been shown to suppress cancer proliferation, induce apoptosis in cancer cells as well as inhibit angiogenesis and metastasis in pre-clinical and clinical trials. Thus, the last part of the project was to determine the anti-cancer potential of plant extracts in a variety of cancer cell lines (derived from breast, colon, liver and ovarian carcinoma). The results showed that the kernel extract of *M. pajang* displayed strong antiproliferative activity in breast cancer cell lines (MCF-7 and MDA-MB-231). The kernel extract induced cell cycle arrest in MCF-7 cells at the sub-G_1 (apoptosis) phase of the cell cycle in a time-dependent manner. For MDA-MB-231 cells, the kernel extract induced strong G_2-M arrest in cell cycle progression at 24 hours, resulting in substantial sub-G_1 (apoptosis) arrest after 48 and 72 hours of incubation. Staining with Annexin V-FITC and propidium iodide revealed that this apoptosis occurred early in both cell types, 36 hours for MCF-7 cells and 24 hours for MDA-MB-231cells, with 14.0% and 16.5% of the cells respectively undergoing apoptosis at these times. This apoptosis appeared to be dependent on caspases-2 and -3 in MCF-7 cells and on caspases-2, -3 and -9 in MDA-MB-231 cells.

As a conclusion, from the two plants (*M. pajang and A. odoratissimus*) studied, the extract of *M. pajang* kernel displayed diverse health benefit properties, antioxidant, chemoprevention and chemotherapeutic potential. *M. pajang* could be fully utilized for pharmaceutical, nutraceutical as well as food products. Further study (i.e. animal and clinical study, isolation of pure compounds, bioavailability study) are required to determine the efficacy in human population.

List of publications

1. Mohd Fadzelly Abu Bakar, Maryati Mohamed, Asmah Rahmat and Jeffrey Fry. 2009. Phytochemicals and antioxidant activity of different parts of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*). *Food Chemistry* 113 (2): 479-483

2. Mohd Fadzelly Abu Bakar, Maryati Mohamed, Asmah Rahmat, Steven Burr and Jeffrey Fry. 2010. Cytotoxicity and polyphenol diversity in selected parts of *Mangifera pajang* and *Artocarpus odoratissimus* fruits. *Nutrition and Food Science* 40 (1): 29-38

3. Mohd Fadzelly Abu Bakar, Maryati Mohamed, Asmah Rahmat, Steven Burr and Jeffrey Fry. 2010. Cytotoxicity, cell cycle arrest, and apoptosis in breast cancer cell lines exposed to an extract of the seed kernel of *Mangifera pajang* (bambangan). *Food and Chemical Toxicology* 48: 1688 - 1697

Acknowledgements

First and foremost, I would like to acknowledge the Malaysian government especially Ministry of Higher Education (MOHE) Malaysia for financing my study under the high profile scheme; 'Malaysia Nottingham Doctoral Program' (MNDP). I would like to extend my thanks to the ex-Director of the Institute for Tropical Biology and Conservation (ITBC), UMS, the honorable Prof. Datin Dr. Maryati Mohamed and the ex-Vice-Chancellor of UMS, the honorable Prof. Datuk. Dr. Mohd Noh Dalimin for their kind approval of my study. I would also like to convey my special thanks to the current Director of ITBC, Assoc. Prof. Dr. Abdul Hamid Ahmad and the current Vice-Chancellor of UMS, the honorable Prof. Datuk Seri Panglima Kamaruzzaman Ampon.

I am really grateful to my main supervisor, Assoc. Prof. Dr. Jeffrey R. Fry, for all the guidance, assistance and patience during my study at The University of Nottingham, UK and Universiti Malaysia Sabah, Malaysia. I am also indebted to my co-supervisors; Dr. Steven A. Burr, Prof. Datin Dr. Maryati Mohamed and Prof. Dr. Asmah Rahmat for their help throughout the project. I am also grateful to Dr. Abdah Md. Akim (UPM), Wan Nor I'zzah (UPM), Mr. Liaque Latif and Mr. Ahmad Hamed (The University of Nottingham, UK) for their help and technical assistant throughout the project. Special thanks to Ministry of Science, Technology and Innovation (MOSTI) Malaysia for granting research grant under eScienceFund scheme (SCF0008-BIO-2006).

The most important, my special love to my family; mother, father and my siblings (Adik, Ina and Ain) for their sincere prayers, love and continued support. Not forgotten are all my friends in Malaysia and United Kingdom, thank you for your support which has made this thesis possible.

Abbreviations

AAPH	2'-azobis(2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AEAC	Ascorbic acid equivalent antioxidant capacity
ANOVA	Analysis of variance
ARE	Antioxidant response element
ATCC	American Type Culture Collection
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
COX-2	Cyclooxygenase-2
Caov3	Human ovarian carcinoma cell line
DCPIP	2,6-dichlorophenolindophenol
DMBA	Dimethyl benzanthracene
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
EDTA	Ethylenediamine tetra acetic acid#
EGCG	Epigallocatechin-3-gallate
FBS	Foetal bovine serum
EC_{50}	Concentration of extract/compound producing 50% of effective effects
FGC4	Rat hepatoma cell line
FRAP	Ferric reducing/Antioxidant power
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Oxidized glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
Hepa1c1c7	Mouse hepatoma cell line
HepG2	Human Caucasian hepatoma carcinoma cell line
HPLC	High Performance Liquid Chromatography
HSP 27	Heat shock protein 27
HSP 70	Heat shock protein 70
HT-29	Human colon carcinoma cell line
IC_{50}	Concentration needed to produce 50% of cells inhibition
KDa	Kilo Dalton
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
MCF7	Hormone dependent breast carcinoma cell line
MDA-MB-231	Non-hormone dependent breast carcinoma cell line
MEME	Minimum essential medium eagle

MTT	3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	Nuclear factor κB
NO	Nitric oxide
NQO1	Quinone reductase
Nrf2	nuclear factor erythroid 2-related factor
OD	Optical density
ORAC	Oxygen radical absorbance capacity assay
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate poly acralamide gel electrophoresis
SOD	Superoxide dismutase
t-BHP	Tertiary-butyl hydroperoxide
TBHQ	tert-Butylhydroquinone
TBST	Tris-buffered saline tween
TEAC	Trolox equivalent antioxidant capacity
TNF	Tumour necrosis factor
TPTZ	2,4,6-Tris(2-pyridyl)-1,3,5-triazine

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General introduction

Cancer is currently one of the leading causes of death in the world (Adami and Hunter, 2002). Although intensive research has been conducted to reduce the occurrence, this disease is still increasing. In 2007, approximately 13% of deaths worldwide were caused by cancer (7.9 million deaths). According to World Health Organization (WHO, 2010) deaths from cancer are projected to almost double by the year 2030 (approximately 12 million deaths).

Current research in drug development and drug discovery, pharmacology and molecular biology, has introduced the term 'chemoprevention' or prevention of cancer. In reality, people would realize that even with effective chemotherapy, an increase in survival or cure rate would not be as successful as compared to the benefits of cancer prevention (Zelen, 1988). Cancer chemoprevention is defined as the use of any agent to inhibit, delay or reverse carcinogenesis. Since carcinogenesis is a multiple stage process and takes many years or decades to develop, there are several chances to intervene in this process (Tamimi et al., 2002) which could offer a more relevant approach to reduce the incidence of cancer.

In the present study, cancer chemoprevention and chemotherapeutic potential of natural products were explored. Focus was given to two types of fruits that were abundant in Borneo Island, namely *Mangifera pajang* (local name: bambangan) and *Artocarpus odoratissimus* (local name: tarap). Diverse pharmachological activity of plants in the family of *Mangifera* and *Artocarpus,* and given that both fruits are edible with no toxicity being noted at regular consumption; both fruits might have the potential for the development of anti-cancer materials with minor adverse effects.

In this chapter, emphases are given to the mechanism of cancer development and which part of cancer development to tackle. Roles of cytoprotective enzymes in cancer chemoprevention as well as the role of natural products as cancer chemotherapeutic are also discussed.

1.1 Mechanisms of cancer development

A multistage model of carcinogenesis was first postulated over 50 years ago following the sequential topical application of chemical agents possessing initiating and promoting activities in mouse skin. Since then, a similar multistage carcinogenic process has been found in many different tissues or organs in different animal models. The multistage model of chemical carcinogenesis divides carcinogenesis into at least three stages—initiation, promotion and progression (Pitot, 1993; Murakami et al., 1996). Initiation can

be caused by electrophilic carcinogens reacting with cellular DNA, leading to mutations in the genetic code (Counts and Goodman, 1994). Chemoprevention of cancer by phytochemicals aims to block one or more steps in the process of the carcinogenesis. Certain naturally occurring and synthetic substances are capable of interfering with the carcinogenesis process (Ho et al., 1994).

Mechanisms that have evolved to counter the potentially harmful effects of nascent electrophiles (or those activated by phase I enzyme action on procarcinogens) include those related to detoxifying enzymes or phase II enzymes, such as reductases (e.g., quinone reductase) and conjugating enzymes (e.g., glutathione-S-transferase) (Talalay, 2000). The detoxification process involves the conversion of electrophiles into inactive, more water-soluble, and readily excretable conjugates. Phase II enzymes also compete with phase I activating enzymes to limit the generation of electrophiles, thus reducing the risk of initiation (Wilkinson, 1997). Therefore, the maintenance of elevated levels of phase II enzymes in bodily tissues provides for cancer chemopreventive defence against highly reactive electrophiles.

Various synthetic organic compounds, such as naphthoflavone, tert-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA), have been reported to be potent chemopreventive agents because of their ability to induce phase II enzymes in mammalian cells (Wilkinson, 1997). BHT and BHA have also been reported to be carcinogenic, but this risk appears evident only at excessive dietary intakes and when biological defence mechanisms are compromised (Iverson, 1995). Many of the

non-nutritive food components, such as phenolics (De Long, 1986) and sulphur-containing compounds (Stoewsand, 1995), including glucose-inolates and their metabolites (Prochaska et al., 1992; Tawfiq et al., 1995), have also been shown to possess cancer chemopreventive properties. In the past few decades, laboratory, animal and epidemiological studies have indicated that ascorbic acid, α-tocopherol, β-carotene, retinol acid and tamoxifen and also certain steroidal anti-inflammatory agents can effectively inhibit carcinogenesis (Ho et al., 1994).

1.2 Chemoprevention by dietary phytochemicals: which part of cancer development to tackle?

The ultimate goal of cancer chemoprevention research is to identify the most effective agents and/or to develop efficient strategies for clinical trial and ultimately, application to human population (Surh, 1999). It is conceivable that in the future people might only need to take specially formulated pills that contain substances derived from edible plants to prevent cancer or delay its onset (Surh, 2003). In order to achieve better understanding of what is chemoprevention and which part of cancer development to tackle, the nature and stages of carcinogenesis must be established. From a standpoint of the experimental carcinogenesis in rodents, tumorigenesis can be classified into 3 distinct stages: initiation, promotion and progression (Figure 1-1) (Surh, 2003).

Initiation which occurs in days is an irreversible step that begins when normal cells are exposed to a carcinogen, and their genomic DNA undergoes damage that remains unrepaired or wrongly repaired. In case of chemically-induced carcinogenesis, the initiation process involves the uptake of exposed carcinogenic agent which leads to the transport and distribution of the carcinogen to organs and tissues where the interaction of reactive species with cellular DNA will lead to the alteration of DNA content which finally will cause genotoxic effect that lead to the DNA mutation (Surh, 1999). Meanwhile, promotion is a stage in which the cell with damaged genomic DNA expands to form an active proliferating multi-cellular premalignant tumour cell population. This process may take several years. Last but not least is the progression stage which may take less than a year is an irreversible processes which produces a new clone of tumour cells with increased proliferative activity, high invasiveness and metastatic potential (Surh, 1999).

Wattenberg (1985) classified chemopreventive agents into 2 groups: blocking agents and suppressing agents. This classification is based on the underlying mechanism by which they display crucial effects in a specific stage of different stages of carcinogenesis. Blocking agents are usually described as compounds that are able to block initiation stage during carcinogenesis either by blocking the formation of carcinogen from reactive metabolites of the main carcinogens, or by inhibiting interaction of the ultimate carcinogen with crucial cellular macro-molecules especially DNA, RNA and protein. Suppressing agents, on the other hand, are typically those compounds that can inhibit the

expression of malignant initiated cells, in either the promotion or the progression stages during carcinogenesis (Surh, 1999). Cancer chemopreventive agents can also retard the development and progression of pre-neoplastic cells into neoplastic cells. The actions of the blocking and suppressing agents are summarized in Figure 1-1.

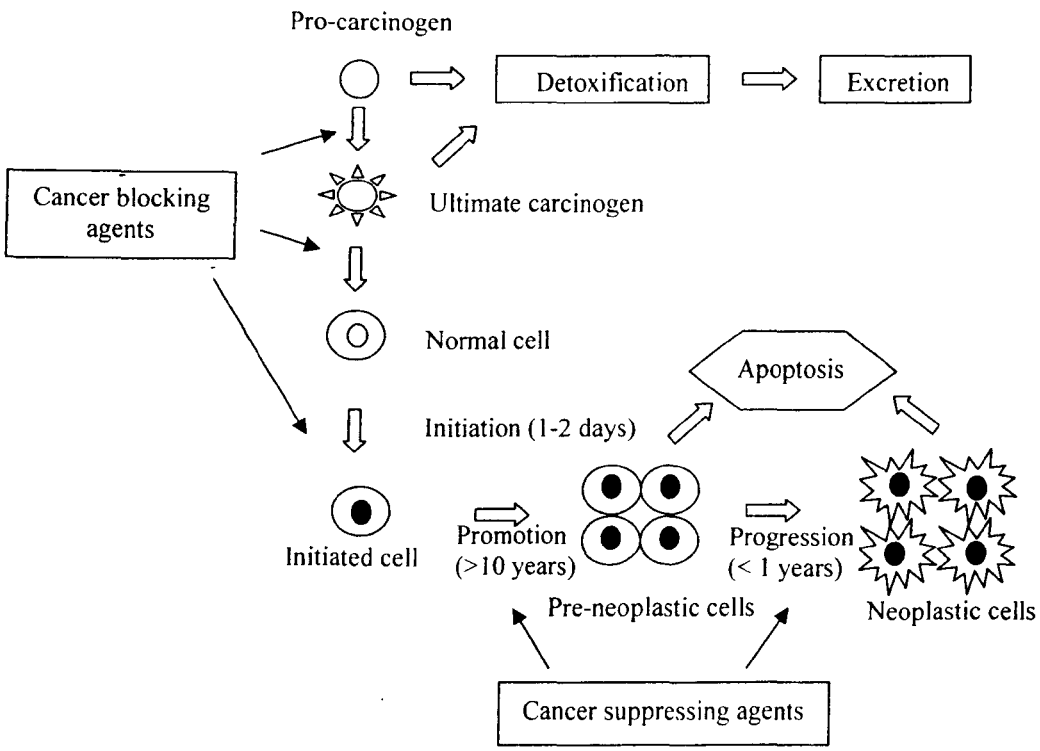


Figure 1-1: Novel targets for cancer chemoprevention by phytochemicals (Modified from Surh, 2003). Carcinogenesis is initiated with the transformation of the normal cell into a cancer cell (initiated). These cells undergo tumour promotion into preneoplastic cells, progress to neoplastic cells. Phytochemicals can interfere in any stage during carcinogenesis. Some chemopreventive phytochemicals prevent activation of pro-carcinogen to carcinogen or prevent carcinogens from reaching the crucial cellular molecules (such as DNA, RNA and protein). In addition, some phytochemicals can counter-attack the carcinogens, which lead to the detoxification and excretion of metabolites. These agents therefore block cancer initiation (blocking agents). While some other phytochemicals suppress the later steps (promotion and progression) of carcinogenesis (suppressing agents). Induction of apoptosis (cell death) in pre-neoplastic and neoplastic cells is crucial for the elimination of cancer cells.

1.3 Free radicals, antioxidants and cancer

A free radical is defined as 'any chemical species capable of independent existence and has one or more unpaired electrons' (e.g. superoxide, hydroxyl, metric oxide and peroxyl) (Halliwell and Gutteridge, 2007). Free radicals and radical oxygen species (non-radical molecules that can easily lead to intracellular free radical reaction (such as hydrogen peroxide, singlet oxygen, and hydrochloric acid) can be produced from several sources both externally (e.g. air pollution, ionizing radiation and cigarette smoke) and internally (e.g. cellular metabolism and cellular inflammation (Willcox et al., 2004). Imbalance between the production of free radicals and the body's defence mechanisms lead to oxidative damage.

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reaction (Velioglu et al., 1998). Antioxidant activity is fundamentally important in our life since many biological functions such as anti-mutagenicity, anti-carcinogenicity and anti-aging originate from this property (Cook and Samman, 1996). Antioxidant defence systems (which consist of some antioxidant enzymes such as superoxide dismutase, catalase and peroxidise as well as antioxidant biochemical such as glutathione) scavenge and minimize the formation of oxygen-derived species, but they are not 100% effective (Halliwell, 2009). This lack of complete protection will lead to extensive

damage to macromolecules such as DNA, RNA and proteins which further lead to tissue damage (Willcox et al., 2004). Hence, diet-derived antioxidants may be particularly important in diminishing cumulative oxidative damage.

Willcox and colleagues (2004) reported that there are 3 major ways a diet could contribute to body's antioxidant defence system: 1) by being a sources of antioxidant phytochemicals such as polyphenols in edible plants; 2) food that contain high level of minerals are important in the catalytic actions of antioxidant enzymes (e.g. Manganese and copper for the action of superoxide dismutase); and 3) some foods are rich with antioxidant vitamins (A, C and E) which could directly counter the attack of free radical action.

Endogenous antioxidant enzymes and/or exogenous antioxidant (phytochemicals, vitamins and minerals) act simultaneously to counter the attack of free radical and reactive oxygen species which could lead to the prevention of the initiation stage during carcinogenesis (Halliwell, 2009).

1.4 Phase II Detoxification Enzymes

Carcinogenesis is a complex and protected multistage process. The entire course can be initiated by a single event wherein a cellular macromolecule is damaged by an endogenous or exogenous agent. The strategies to protect the cells from the initiating events include decreasing activity of metabolic enzymes that are responsible for generating reactive

species (phase I enzyme) while increasing the activity and/or expression of phase II enzyme that can deactivate radicals and electrophiles known to intercede in normal cellular processes. Phase II enzymes which could be up-regulated by phytochemicals include quinone reductase (NQO1), glutathione reductase (GR) and methinone sulfoxide reductase A (MSRA). Thus, reduction of electrophilic quinones by NQO1 is an important detoxification pathway, which converts quinones to hydroquinones and reduces oxidative cycling (Talalay et al., 1988). GR is important to reduce glutathione disulfide (GSSG) to the sulfhydryl-form (GSH) which is an important cellular antioxidant (Meister, 1988). While MSRA reduces methionine sulfoxide (MetO) residues in proteins and free MetO to methionine (Met) which has been shown to play an important role in protecting cells against oxidative damage (Yermolaieva et al., 2004)

1.4.1 Quinone reductase (NQO1)

One important method for evaluating the anticarcinogenic potential of compounds is the assessment of their capability to induce the activity of phase II enzymes, such as quinone reductase (QR, NAD(P)H: quinone oxidoreductase EC 1.6.99.2), which are involved in the detoxification of xenobiotics. Quinones are among the most toxic products of oxidative metabolism of aromatic hydrocarbons. Reduction of electrophilic quinones by quinone reductase is an important pathway, which convert quinones to hydroquinones and reduces redox cycling (Benson et al., 1980; Kang and

Pezzuto, 2004; Prochaska et al., 1992; Prochaska and Santamaria, 1988; Talalay et al., 1995).

NQO1 elevation in *in vitro* and *in vivo* systems has been shown to correlate with induction of the other protective phase II enzymes and provides a reasonable biomarker for the potential chemoprotective effect of test agents against cancer initiation (Pezzuto, 1995). NQO1 is a convenient representative enzyme because it is widely distributed in mammalian tissues, easily measured and shows a large inducer response. Given the general agreement that phase II enzymes are primarily responsible for the detoxification of chemical carcinogens and other harmful oxidants, induction of NQO1 is therefore suggestive of potential cancer prevention at the tumour initiation stage (Prochaska and Santamaria, 1988).

Many fruit extracts and purified constituents of fruits have been shown to demonstrate induction of NQO1 activity *in vitro* (Bomser et al., 1996; Kraft et al., 2005; Kresty et al., 2001). Fruit-derived dietary antioxidants such as β-carotene, α-tocopherol and ascorbic acid, induced quinone reductase activity in cell-based assays. High consumption of fruits with high antioxidant vitamin levels may confer protection against cancer by the induction of specific detoxification enzymes (Wang and Higuchi, 1995).

1.4.2 Glutathione reductase (GR)

Glutathione (GSH) has been described for a long time as a defensive reagent against the action of toxic xenobiotics (drugs, pollutants, carcinogens). GSH is among the most efficient tool that cells can exploit in detoxification of drugs and xenobiotics in general. GSH in fact is both a nucleophile and a reductant, and can therefore react with electrophilic or oxidizing species before the latter interact with more critical cellular constituents such as nucleic acids and proteins. As a prototype antioxidant, GSH is involved in cell protection from the noxious effect of excess oxidant stress, both directly and as a cofactor of glutathione peroxidases (Pompella et al., 2003).

The primary function of human glutathione reductase (also known as GR) is to maintain the level of antioxidant glutathione to at least 99% in its reduced form (Meister, 1988). Thus, for every GSSG and NADPH, two reduced GSH molecules are gained, which again act as cellular antioxidants that can scavenge free radical and reactive oxygen species in the cell. The overall reaction is:

$$NADPH + GSSG + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

1.5 Methionine sulfoxide reductase A (MSRA)

Reactive oxygen species (ROS) are highly reactive and can damage biological macromolecules, including proteins, nucleic acids, and lipids. One of the amino acids that is most prone to oxidation is methionine, resulting in the formation of methionine sulfoxide. Oxidation of methionine to methionine sulfoxide (MetO) could, on the one hand, be an important component of signal transduction pathways and on the other hand, may lower the cellular antioxidant capacity, alter protein function, interfere with signal transduction, and damage proteins. The latter changes could lead to the accumulation and malfunction of various proteins and enhanced development of oxidative-stress related diseases such as cancer (Moskovitz, 2005).

However, this oxidation can be repaired by methionine sulfoxide reductase (MSRA), which catalyzes the NADPH and thioredoxin-independent reduction of free and protein-bound (MetO) to methionine. Methionine sulfoxide reductase A (MSRA) is a repair enzyme that reduces methionine sulfoxide residues in oxidatively damaged proteins to methionine residues in a stereospecific manner. These enzymes protect cells from oxidative stress and have been implicated in delaying the aging process and progression of neurodegenerative diseases (Kim and Gladyshev, 2007). Consistent high expression of MSRA enzyme in human T cells has been shown to prolong their survival under oxidative-stress induced conditions (Moskovits et al., 1998).

Since methionine residues are prone to oxidation by free radical and reactive oxygen species, MSRA could act by at least three different mechanisms in cellular metabolism: 1) as an antioxidant enzyme that scavenges free radical and reactive oxygen species by maintaining the interconversion cycle of methionine/methionine-protein residues between oxidized and reduced forms 2) as a repair enzyme by keeping critical methionine residues in their reduced form; and (3) as a regulator of critical enzyme activity through cyclic interconversion of specific methionine residues between oxidized and reduced forms (Moskovitz, 2005).

1.6 Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) are a group of proteins that can be found in all cells in all life forms, from human to bacteria. They can be induced under several circumstances such as heat, cold or oxygen, hence the name of 'stress protein'.

HSPs are also present in cells under normal conditions by simply "monitoring" the cell's proteins. They act as molecular chaperones, facilitating the synthesis and folding of proteins throughout the cell. In addition, heat shock proteins have been shown to participate in protein assembly, secretion, trafficking, and degradation, and the regulation of transcription factors and protein kinases (Parcellier et al., 2003). These activities are part of a cell's own repair system, called the "cellular stress response" or the "heat-shock response".

HSPs are classified according to their molecular weight and the ones that will be studied further in this project are HSP 27 and 70. HSPs are usually found in sub-cellular compartments such as cytoplasm, mitochondria, peroxisomes and endoplasmic reticulum where they function as chaperones in the synthesis and degradation of proteins.

HSPs are induced greatly when cells are exposed to stress (such as heat, cold and electrical shock). Exposure to such extreme conditions results in the alteration of conformation of proteins (e.g. misfolding) and HSPs (including HSP 27, HSP 70 and HSP 90) are required to repair these 'mistakes'. This process is very important to ensure the survival of cells. HSPs have been shown to reduce the intracellular level of reactive oxygen species and also abolish the burst of intracellular reactive oxygen species induced by carcinogens (Mehlen et al., 1996). Involvement of HSPs alone or in combination with other stress response pathways has the potential to reduce oxidative stress injury and accelerate the functional recovery of susceptible organs in living organisms including humans, and so have the potential to be novel targets for chemoprevention agents (Christians et al., 2002).

1.7 Involvement of apoptosis in cancer

Cancer or neoplasm refers to a group of diseases characterized by uncontrolled growth and spread of abnormal cells and if left untreated will result in death (Lafond, 1988). In other terms, cancer is characterized by genetic instability and long term un-regulated and un-controlled growth (Stass and Mixson, 1997). Without proper regulation, cancerous cells divide endlessly forming tumours. Malignancy occurs when cells break away from tumour and penetrate their surroundings. When they do not invade their surroundings, the tumour is called benign (Inoue et al., 1987).

Apoptosis is a physiological process of killing cells and is an important process means of eliminating tumours. Abnormalities in the induction of cell death control can contribute to various types of disease such as cancer, autoimmunity and degenerative disorders (Strasser et al., 2000). The apoptosis process is characterized by membrane blebbing (without loss of cell integrity), shrinkage of the cells and nuclear volume, chromatin condensation, DNA fragmentation and formation of membrane-bound vesicles (apoptotic bodies). Apoptosis is triggered by multiple independent pathways that are initiated either from triggering events within the cell or from outside the cell (Kaufmann and Earnshaw, 2000). The intrinsic pathway involves an increase of mitochondrial permeability and cytochrome c release whereas the extrinsic pathway is induced by the ligation of cell surface death receptor (Okada and Mak, 2004).

Apoptosis can be induced by several mechanisms (i.e. receptor mediated signals, removal of growth factor and cell damage) (Hampton et al., 1998). Each of these mechanisms has its own specific pathway and all have one thing in common, the 'caspases' or 'cysteine aspartate proteases' which act as a central component of apoptosis process. Caspases cleave a defined number of target proteins, and these changes lead to the special features of apoptotic body. Generally, caspases can be divided into 2 distinct groups, apoptotic initiators (caspase-2, -8 and-9) and apoptotic executioners (caspase-3, -6 and -7) (Wolf and Green, 1999).

Caspases are present as inactive pro-enzymes that are activated by proteolytic cleavage. The pro-enzymes are proteins of 30-50 kDa that contain three domains: an NH_2-terminal domain, a large subunit (~20kDa) and a small subunit (~10 kDa) (Thornberry and Lazebnik, 1998). Activation of caspase involves proteolytic processing between domains, followed by the large and small subunits to form heterodimers, the active form of caspases. Activated caspases can cleave procaspases in the caspase cascades and many intracellular proteins including lamins, PARP (poly ADP-ribose polymerase) and DFF45/ICAD. Cleavage of DFF45/ICAD is a critical step leading to the internucleosomal cleavage of DNA (DNA fragmentation), a characteristic hallmark of apoptosis (Thornberry and Lazebnik, 1998). Summary of the role of caspases are shown in Figure 1-2.

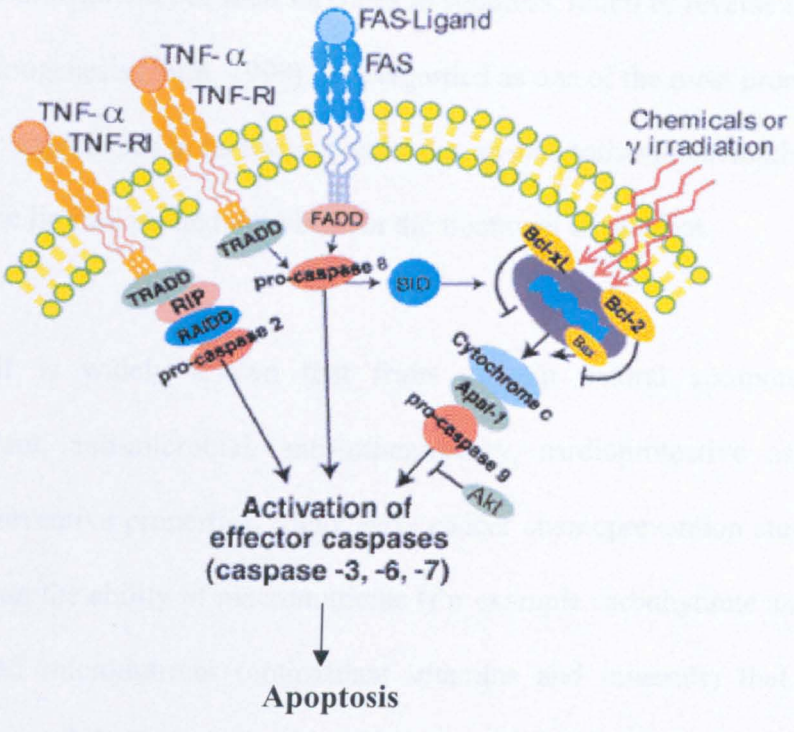


Figure 1-2. Caspases -2 could be activated by tumour necrosis factor (TNF). Meanwhile, caspases -8 could also be activated by number of ligands including tumour necrosis factor (TNF) and FAS. Caspases -9 could be activated by chemicals (i.e. chemotherapy) and radiation. The initiators caspases eventually could activate the executioner caspases (caspase -3, -6 and -7) which lead to the induction of apoptosis (Adapted and modified from Cortese, 2001).

1.8 Role of fruits and their phytochemicals in cancer chemoprevention

Chemoprevention is defined as the use of specific chemical substances (natural or synthetic) or their mixtures to suppress, retard or reverse the process of carcinogenesis (Surh, 1999). It is regarded as one of the most promising and realistic approaches in combating cancer as an alternative to chemotherapy that has some limitations and drawback in the treatment of patients.

It is widely known that fruits contain natural compounds with antioxidant, anti-microbial, anti-inflammatory, cardioprotective as well as chemopreventive properties. Many early cancer chemoprevention studies have focused on the ability of macronutrients (for example carbohydrate and dietary fibre) and micronutrient (antioxidant vitamins and minerals) that exist in vegetables and fruits to reduce the risk of cancer (Surh, 2003, Kinghorn et al., 2004). However, plants contain other non-nutritive phytochemicals that might also be useful in cancer chemoprevention. The examples of dietary phytochemicals that have been shown to display cancer chemopreventive properties and their possible mechanisms of actions as well as their dietary sources are shown in Table 1-1.

Phytochemicals/ Sources	Possible mechanism of action
Resveratrol/ Grapes	Induction of phase II drug-metabolizing enzymes (Jang et al., 1997)
Curcumin/ Turmeric	Inhibition of chemically induced carcinogenesis during initiation, promotion and progression of colon cancer (Kawamori et al., 1999)
indole-3-carbinol/ cabbage	Inhibition of chemically induced carcinogenesis during initiation, promotion and progression of breast cancer; Induction of phase II drug-metabolizing enzymes (Grubbs et al., 1995)
Proanthocyanidin/ Cranberry	Inhibition of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity (promotion stage) (Kandill et al., 2002)
Lycopene/ Tomato	Protection against oxidative DNA damage (Matos et al., 2000) and delayed the onset and reduced spontaneous mammary tumor growth *in vivo* (Nagasawa et al., 1995)
Sulforaphane/ Broccoli	Inhibition of phase I enzymes and induction of phase II drug-metabolizing enzymes (Clarke et al., 2008)

Table 1-1: Dietary phytochemicals that have been shown to display cancer chemopreventive properties and their possible mechanisms of actions as well as their dietary sources

1.9 *Mangifera pajang*

Mangifera pajang or bambangan (in Malay) is a species of plant in the mango group, found in Borneo Island (Malaysia- Sabah and Sarawak, Brunei, and Indonesia-Kalimantan). The taxonomy of the plant is as follows:

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Anacardiaceae

Genus: Mangifera

Species: pajang

The fruit of bambangan weighs about 0.5 - 1 kg or more, and is the largest known in the *Mangifera* genus. It has a rough, potato-brown skin which distinguishes it from the other *Mangifera* fruits. When ripe, the flesh is bright yellow in colour, sweet-sour and juicy, although rather fibrous, with a somewhat strong turpentine aroma (Wong and Siew, 1994). The flesh of *M. pajang* is usually eaten and the peel and kernel of the fruit are discarded.

This tree is rare within its natural habitat type. The only area where it is common is on the west coast plains of Sabah. It is found both wild and cultivated in Dayak gardens in the north-east and west of Kalimantan, but it is very rarely found in the south. It has been cultivated outside its natural range.

(Fah, 1987; World Conservation Monitoring Centre, 1998). Photographs of the fruits are shown in Figure 1-3. A recent study showed that mango species contained numerous health-important phytochemicals and displayed health benefits properties. Kernel from *Mangifera indica* L. contained a high amount of gallic acid and ellagic acid which might provide a novel source of natural antioxidants (Soong and Barlow, 2006).

Previous study showed that flavonoids extracted from *Mangifera indica* (normal mango) fruits induced free radical-scavenging enzymes and reduced lipid peroxidation in hypercholecterolaemic rats (Anila and Vijayalakshmi, 2003). Mango peel extracts displayed high amount of polyphenols, anthocyanins and carotenoids and the extracts exhibited good antioxidant activity in different non-cell based antioxidant assays (Ajila et al., 2007). Mango peel extract also has been shown to protect erythrocytes against oxidative stress and may impart health benefits and could be used as nutraceutical products (Ajila and Rao, 2007).

(a)

(b)

(c)

Figure 1-3: Fruit of *Mangifera pajang*. (a) Picture of fruits on a tree; (b) Picture of whole fruit; (c) Cross-sectional picture of the fruit.

1.10 *Artocarpus odoratissimus*

Artocarpus odoratissimus, also called tarap (in Malaysia), is a tree related to jackfruit, cempedak and breadfruit, and is native to Borneo (especially in Sabah, Sarawak and Brunei). Photographs of the fruits are shown in Figure 1-4. The fruit is considered to be delicious; soft flavoured which can be appreciated from the first bite. The fruit is considered superior in flavour to both jackfruit and cempedak. This versatile tree has served the needs of the rural communities well providing food, nutrition and many other uses to the people of the region. The pulp of the fruit has delightful fragrant aroma, sweet, nutritious and has been used in many food preparations. The people also value the seeds. Roasted seeds have a firm texture, nutty, not too oily and a taste reminiscent of chestnut (Serudin and Tinggal, 1992). The taxonomy of the plant is as follows:

Kingdom:	Plantae
Phylum:	Magnoliophyta
Class:	Magnoliopsida
Order:	Morales
Family:	Moraceae
Genus:	*Artocarpus*
Species:	*odoratissimus*

The genus of *Artocarpus* is a rich source of phenolic secondary metabolites. Fruit of *Artocarpus nibilis* (the only endemic species of *Artocarpus* in Sri Lanka) contains geranylated phenolic compounds which display strong antioxidant activity against DPPH free radical (Jayasinghe et al., 2006). The seeds of jackfruit or *Artocarpus heterophyllus* have a highly nutritional value (i.e. protein, fat, fibre, oil and minerals) (Ajayi, 2007). The crude extracts of the root bark, leaves, fruits and seeds of *Artocarpus heterophyllus* also exhibit a broad spectrum of antibacterial activity (Khan et al., 2003)

(a)

(b)

(c)

Figure 1-4: Fruit of *Artocarpus odoratissimus*. (a) Picture of a tree; (b) Picture of whole fruit; (c) Picture of a replica of the fruit.

1.11 Research Intent

As the prevalence of cancer is increasing, more research on alternative prevention or treatment such as plants with cancer chemoprevention and chemotherapeutic properties need to be further explored. Both plants from the genus of *Mangifera* and *Artocarpus* have been shown to display diverse health benefit properties including cancer chemopreventive and chemoptherapeutic activity. Hence, both *M. pajang* and *A. odoratissimus* which are endemic to Borneo Island and under-utilised fruits could display health benefits properties.

1.12 Objectives of the present study

The main objective of this study was to determine the potential cancer chemopreventive activities of *M. pajang* and *A. odoratissimus* extracts in multiple stages of carcinogenesis.

Specific objectives:

i. To determine the phytochemical components present in the extracts of *M. pajang* and *A. odoratissimus.*

ii. To determine the antioxidant activity of *M. pajang* and *A. odoratissmus* extracts using a non-cell based antioxidant assay.

iii. To determine the cytoprotective action of the extracts against oxidative damage induced cytotoxicity in hepatocytes (HepG2 cells).

iv. To determine the cytotoxicity activity of the extracts against selected cancer cell lines and determine their possible mechanism(s) of action.

1.13 Hypotheses

i. Diverse phytochemical components would be present in the extracts of *M. pajang* and *A. odoratissimus*.

ii. Extracts of *M. pajang* and *A. odoratissimus* would display antioxidant activity in non-cell based antioxidant assays and a cell based antioxidant assay.

iii. Extracts of *M. pajang* and *A. odoratissimus* would prevent the oxidative damage caused by toxicants.

iv. Extracts of *M. pajang* and *A. odoratissimus* would display anticancer properties in a selected range of cancer cell lines. The possible mechanisms of anticancer action may involve the activation of caspases, cell cycle arrest and apoptosis.

CHAPTER 2

Antioxidant and phytochemical properties of different parts of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*)

CONTENTS

CHAPTER 2

Antioxidant and phytochemical properties of different parts of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*)

2.1 Introduction

Fruits and vegetables have been associated with prevention of degenerative diseases, such as cancer and cardiovascular diseases (Liu, 2003) and the presence of wide range of phytochemicals such as phenolics, thiols, carotenoids, anthocyanins and tocopherol in the fruits and vegetables have been suggested to exert chemopreventive (Dragsted et al., 1993), and cardioprotective effects (Vita, 2005) and to protect the human body against oxidative damage by free radical (Halliwell, 1997).

In recent years, under-utilised fruit species endemic to specific locations have been popular for intensive investigation. *Euterpe oleraceae* or Acai is a large palm tree indigenous to the Amazon River and the fruit has been shown to display outstanding antioxidant properties (Schauss et al., 2006) and is becoming a popular target of health-conscious consumers and the nutraceutical industry application. Netzel et al. (2007) investigated seven native fruits of Australia in terms of their antioxidant activities and phytochemical components and the results showed that some fruits displayed a high level of total phenolic content as well as antioxidant properties.

To fully utilize local fruits for the development of functional foods with health-benefits properties, we undertook a systematic study on the phytochemicals and bioactivity of selected fruits native to Borneo Island (especially found in Sabah, Malaysia). If fruits of *M. pajang* (flesh, kernel and peel) and *A. odoratissimus* (flesh and seed) show potential to improve health when consumed, their commercialization and utilization should proceed. Therefore, the objective of this study was to evaluate the phytochemical content and antioxidant activity of the fruits and their by-products. Antioxidant activity, total phenolic content, flavonoid content and anthocyanin content of the fruits and their by-products (i.e kernel/seed and peel) were quantified using colorimetric techniques while the polyphenol profile was determined using high performance liquid chromatography.

2.2 Materials and methods

2.2.1 Plant materials and sample preparation

The fruits of *M. pajang* and *A. odoratissimus* were collected from Sabah, Malaysia on July – August 2007. The herbarium voucher specimens were identified by Johnny Gisil (Botanist) and deposited in BORNEENSIS, Universiti Malaysia Sabah, Malaysia. The fruits were cleaned and separated into flesh, kernel and peel for *M. pajang* and flesh and seed for *A. odoratissmus*. The peel of *A. odoratissimus* was not included since the texture is hard and impossible to cut them into small pieces. The small cut pieces were freeze-dried in a freeze dryer (Virtis, USA). The freeze-dried samples were ground into a fine powder using dry grinder. The ground samples were sieved to get uniform particle size, then kept in an air-tight container and stored in a freezer (-20°C) until further analysis.

2.2.2 Extraction

Sample (0.1 g) was extracted for 2 h with 5 ml of 80% methanol at room temperature on an orbital shaker set at 200 rpm (Velioglu et al., 1998). The mixture was centrifuged at 1400 x g for 20 min and the supernatant was decanted into a 15 ml vial. The pellet was re-extracted under identical conditions. Supernatant was combined and used for total antioxidant activity, total phenolic content, total flavonoid content, and total anthocyanin content.

2.2.3 DPPH free radical scavenging assay

The scavenging activity of the extracts was estimated by using 1, 1 - diphenyl-2-pycrylhydrazyl (DPPH) (Sigma, USA) as a model of free radical, the method was adapted from Magalhaes et al. (2006) with modification. An aliquot (300 µl) of samples or control (80% methanol) were mixed with 3.0 ml of 500 µM (DPPH) in absolute ethanol (Merck, Germany). The mixture was shaken vigorously and left to stand at room temperature for 30 minutes in the dark. The mixture was measured spectrophotometrically (using spectrophotometer: SECOMAM CE, France) at 517 nm and the free radical scavenging activity calculated as follows:

Scavenging effect (%) =

{1-(absorbance of sample / absorbance of control)} x100

A standard of ascorbic acid (Sigma, USA) was run using several concentrations ranging from 0.05 to 0.25 mg/ml. A standard curve was then prepared by plotting the percentage (%) of free radical scavenging activity of ascorbic acid (Sigma, UK) versus its concentration. The final result was expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of sample (mg AEAC/g).

2.2.4 FRAP (Ferric reducing/antioxidant power) assay

This procedure was conducted according to Benzie and Strain (1996) with modification. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma, USA) solution and 20 mM $FeCl_3.6H_2O$ (Sigma, USA) in a 10:1:1 ratio prior to use and heated to 37°C in water bath. A total of 3.0 ml FRAP reagent was added to a test tube and a blank reading was be taken at 593 nm using spectrophotometer. A total of 100 µl of selected plant extracts and 300 µl of distilled water were added to the cuvette. After addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 90 minutes of incubation at 37°C in a water bath. The changes in absorbance after 90 minutes from initial blank reading were compared with standard curve. Standards of known Fe (II) (Sigma, UK) concentrations were run using several concentrations ranging from 100 to 1000 µM. A standard curve was then prepared by plotting the FRAP values of each standard versus its concentration. The final result was expressed as the concentration of antioxidant having a ferric reducing ability in 1 gram of sample (µM/g).

2.2.5 Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent (Merck, Germany) as adapted from Velioglu et al. (1998) with slight modifications. Extract (300 µl) was mixed with 2.25 ml of Folin-Ciocalteu

reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 minutes, after which 2.25 ml of sodium carbonate (60 g/l) solution was added to the mixture. After 90 minutes at room temperature, absorbance was measured at 725 nm using a spectrophotometer. A standard of gallic acid in the concentration range 0 – 10 µg/ml was treated identically. Results were expressed as mg gallic acid equivalents in 1 g of dried sample (mg GAE/g).

2.2.6 Determination of total flavonoid content

Total flavonoid content was determined using the colorimetric method described by Dewanto et al. (2002) with slight modification. Briefly, 0.5 ml of the extract was mixed with 2.25 ml of distilled water in a test tube followed by addition of 0.15 ml of 5% $NaNO_2$ (Sigma, USA) solution. After 6 min, 0.3 ml of a 10% $AlCl_3.6H_20$ (Sigma, USA) solution was added and allowed to stand for another 5 minutes before 1.0 ml of 1 M NaOH (Sigma, USA) was added. The mixture was mixed well by vortex. The absorbance was measured immediately at 510 nm using a spectrophotometer. A standard of rutin (Sigma, USA) in the concentration range 0 – 10 µg/ml was treated identically. Results were expressed as mg rutin equivalents in 1 g of dried sample (mg RE/g).

2.2.7 Determination of total anthocyanin content

Total anthocyanin content was measured using a spectrophotometric pH differential protocol described by Giusti and Wrolstad (2001) and Wolfe et al.

(2003) and with slight modification. Briefly, 0.5 ml of the extract was mixed thoroughly with 3.5 ml of 0.025 M potassium chloride buffer (pH 1). This mixture was mixed by vortex and allowed to stand for 15 minutes. The absorbance was then measured at 515 and 700 nm against a blank of distilled water. The extract was then combined similarly with 0.025 M sodium acetate buffer pH 4.5, and the absorbance was measured at the same wavelength after being allowed to stand for 15 minutes. The total anthocyanin content was calculated using the following equation:

Total anthocyanin content (mg/100g of dried sample) = A x MW x DF x 1000 / (ε x C)

where A is absorbance = $(A_{515} - A_{700})_{pH\ 1.0} - (A_{515} - A_{700})_{pH\ 4.5}$; MW is the molecular weight for cyanidin-3-glucoside = 449.2; DF is the dilution factor of the samples, ε is the molar absorbtivity of cyanidin-3-glucoside = 26900; and C is the concentration of the buffer in mg/ml. Results were expressed as mg of cyanidin-3-glucoside equivalents in 100 g of dried sample (mg c-3-gE/100g dried sample)

2.2.8 *Determination of Polyphenols*

2.2.8.1 HPLC apparatus

The separation of phenolic acids (caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, gallic acid), flavanones (naringin, hesperidin), flavonols (quercetin, myrecetin, kaempferol, rutin) and flavones (sinensetin, luteolin and

diosmin) was performed on a HPLC series 1100 (Hewlett Packard, Germany) equipped with G1322A degasser, G1311A quaternary pump, G1313A autosampler and G1315A diode array detector system. The column used was a LiChrospher® 100RP18e, 5 µm, (250 x 4.0mm internal diameter) (Agilent, USA)

2.2.8.2 Chemicals

The standards of phenolic acids (caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, gallic acid), flavanones (naringin, hesperidin), flavonols (quercetin, myrecetin, kaempferol, rutin) and flavones (sinensetin, luteolin and diosmin) were purchased from Sigma-Aldrich (USA). All solvents were from the highest purity (i.e HPLC grade; > 99.0%).

2.2.8.3 Analysis of phenolic acids and flavonoids by HPLC

Determination of phenolic acids and flavonoids compositions were conducted according to Schieber et al. (2001) and Wang et al. (2007) with slight modification. Briefly, 0.1 g of sample was mixed with 2 ml of methanol-DMSO (v:v; 50:50) in a centrifuge tube and kept in the dark for 24 hours. The mixture was then centrifuged at 4000 rpm for 20 min at 4°C. The supernatants were filtered through a 0.45 µm membrane filter prior to injection into the HPLC apparatus. The mobile phase was composed of (A) 2% acetic acid (aqueous) and (B) 0.5% acetic acid (aqueous)-acetonitrile (50:50; v:v), and the gradient elution was performed as follows: 0 min, 95:5; 10 min, 90:10; 40 min,

60:40; 55 min, 45:55; 60 min, 20:80; and 65 min, 0:100. The mobile phase was filtered under vacuum through a 0.45 μm membrane filter before use. The flow rate was 1 ml/min. UV absorbance (for phenolic acid,s flavanones and flavonols) was measured at 280 nm, and for flavones at 340 nm. The operating temperature was maintained at room temperature.

2.2.9 Statistical analysis

All experiments were carried out in 3 independent experiments and presented as mean ± standard error of mean (S.E.M) using Prism version 15.0. The data were statistically analysed by one-way ANOVA using Tukey's post-hoc. The level of statistical significance was set at $p < 0.05$. Pearson correlation was used to determine the correlation between the antioxidant activity and the phytochemical contents.

2. 3 Results

2.3.1 Scavenging activity on 2,2-diphenyl-2-picrylhydrazyl radical

One of the mechanisms to investigate antioxidant activity is to study the scavenging effect on radical. In the present study, investigation of total antioxidant capacity was measured as the cumulative capacity of the compounds present in the sample to scavenge free radicals, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) reaction (Magalhaes et al., 2006). The presence of antioxidant in the sample leads to the disappearance of DPPH radical chromogens which can be detected by spectrophotometrically at 517 nm.

For *M. pajang* fruit, the kernel extract of the fruit displayed the highest scavenging effect with 23.23 mg AEAC/g, followed by the peel and flesh extracts with the values of 20.32 and 9.94 mg AEAC/g, respectively. These values were significantly ($p<0.05$) less than the kernel. Meanwhile, for *A. odoratissimus*, the seed extract showed a statistically significant ($p<0.05$) higher scavenging effect compared to the flesh, with values of 13.69 and 2.44 mg AEAC/g, respectively (Table 2-1).

Samples	DPPH Free Radical Scavenging[1]	FRAP[2] (μM)
M. pajang		
Flesh	9.94 ± 0.22	150.00 ± 1.44
Peel	20.32 ± 0.10	343.17 ± 7.25*
Kernel	23.23 ± 0.03	3130.00 ± 35.47
A. odoratissimus		
Flesh	2.44 ± 0.15	116.67 ± 5.43
Seed	13.69 ± 0.59	208.67 ± 1.17*

Table 2-1. Antioxidant properties of *M. pajang* and *A. odoratissimus*.

Values are presented in mean ± S.E.M (n = 3).

All values are significantly different at $p < 0.05$.

*The FRAP values for *M. pajang* peel and *A. odoratissimus* seed extracts are statistically not significant.

[1]DPPH free radical scavenging activity was expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) in 1 g of dry sample.

[2]FRAP was expressed as μM ferric reduction to ferrous in 1 g of dry sample.

2.3.2 Ferric reduction based on FRAP assay

Although the mechanisms of action of DPPH and FRAP are different, i.e. scavenging of DPPH cation radicals in the DPPH assay and reduction of ferric ions in the FRAP assay, the results of these two assays were significantly correlated in all samples tested.

FRAP assay is an assay that directly measures antioxidants or reductants in a sample. FRAP assay measures the reducing ability of antioxidant that react with ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex and produces a coloured ferrous tripyridyltriazine (Fe^{2+}–TPTZ) (Benzie and Strain, 1996). In the FRAP assay, results were expressed as the combined concentration of all electron-donating reductants which occured in the samples in a variety of sample plants.

The reducing ability of the tested extracts was in the order of *M. pajang* kernel > *M. pajang* peel > *A. odoratissmus* seed > *M. pajang* flesh > *A. odoratissmus* flesh; the same trend shown by the DPPH free radical scavenging assay. There were significant differences ($p < 0.05$) among the samples except for *M. pajang* flesh and *A. odoratissimus* seed. *M. pajang* kernel exhibited superior reducing capacity, approximately 9 and 20 times higher compared to the peel and flesh, respectively. Meanwhile, the reducing capacity of *A. odoratissmus* seed extract was almost doubled compared to the flesh (Table 2-1).

2.3.3 Total phenolic content

Folin Ciocalteu reagent was used to determine total polyphenol content in sample extracts. Folin-Ciocalteu reagent is a yellow acidic solution containing complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. This reagent oxidizes phenolates, resulting in the production of complex molybdenum-tungsten blue (Singleton and Rossie, 1965) which can be detected spectrophotometrically at 725 nm.

The results showed that for *M. pajang*, the phenolic content was in the order kernel > peel > flesh with the values of 103.30, 22.93 and 5.96 mg GAE/g, respectively. There were significant differences ($p < 0.05$) among the samples tested (Table 2-2). Meanwhile, the seed of *A. odoratissimus* contained a higher amount of total phenolic (14.67 mg GAE/g) compared to the flesh (4.39 mg GAE/g) ($p<0.05$) (Table 2-2). The Pearson correlation analysis revealed that the reducing ability of the extracts (FRAP assay) was strongly correlated with the phenolic content ($r = 0.985$; $p < 0.01$) (Appendix: Table A).

Samples	Total phenolic[1]	Total flavonoid[2]	Total anthocyanin[3]
M. pajang			
Flesh	5.96 ± 0.34	0.07 ± 0.00	1.47 ± 0.28
Peel	22.93 ± 0.36	7.50 ± 0.09	28.29 ± 0.70
Kernel	103.30 ± 0.63	10.98 ± 0.16	N.D.
A. odoratissimus			
Flesh	4.39 ± 0.20	1.08 ± 0.03	11.02 ± 0.38
Seed	14.67 ± 0.17	3.65 ±0.04	3.80 ± 0.34

Table 2-2. Total phenolic, total flavonoid and total anthocyanin content.
Values are presented in mean ± S.E.M (n = 3).
All values are significantly different with each other at $p < 0.05$.
[1]Total phenolic was expressed as mg gallic acid equivalent in 1 g of dry sample.
[2]Total flavonoid was expresed as mg rutin equivalent in 1 g of dry sample.
[3]Total monomeric anthocyanin was expressed as mg cyanidine-3-glucoside equivalent in 100 g of dry sample.
N.D. = Not detected.

2.3.4 Total flavonoid content

Large amounts of natural phenolic compounds are found in teas, fruits, and vegetables (Ho et al., 1992). Flavonoids are the most common and widely distributed group of plant phenolic compounds that are characterized by a benzo-*y*-pyrone structure, which is ubiquitous in fruits and vegetables. Total flavonoid can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride which can be monitored spectrophotometrically at 510 nm. For *M. pajang*, the kernel contained the highest flavonoid content (10.98 mg GAE/g), followed by the peel (7.50 mg GAE/g), and the flesh (0.07 mg GAE/g) ($p<0.05$). The seed of *A. odoratissmus* contained higher total flavonoid content compared to the flesh ($p<0.05$) (Table 2-2). The Pearson correlation analysis revealed that the reducing ability of the extracts (FRAP assay) was strongly correlated with the flavonoid content ($r = 0.663$; $p < 0.01$) (Appendix: Table A).

2.3.5 Total anthocyanin content

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra. The pH-differential method is based on the difference between the coloured oxonium form (which predominates at pH 1.0) and the colourless hemiketal

form (at pH 4.5), and permits accurate and rapid measurement of the total monomeric anthocyanin (Giusti and Wrolstad, 2001).

The anthocyanin content was relatively low in all samples tested. For *M. pajang*, anthocyanin was only detected in the flesh (1.47 mg c-3-gE/100g) and the peel (28.29 mg c-3-gE/100g). The flesh of *A. odoratissimus* contained higher total anthocyanin content compared to the seed ($p<0.05$) (Table 2-2).

2.3.6 Polyphenols composition

2.3.6.1 Phenolic acids composition

Phenolic acids were found to be dominant in the kernel and peel of *M. pajang*. Table 2-3 shows that out of six phenolic acid tested, chlorogenic, caffeic and p-coumaric acids were found to be the most abundant in all samples tested. Gallic acid was only detected in the kernel and peel of *M. pajang* with the values of 236.6 ± 28.31 and 30.72 ± 5.83 μg/g dw, respectively. The fruit parts listed on the basis of p-coumaric acid content (in order from the highest to lowest) were M. *pajang* kernel (301.10 ± 19.12 μg/g dw), *M. pajang* peel (199.00 ± 17.53 μg/g dry weight), *A. odoratissimus* seed (35.33 ± 1.56 μg/g dw), *A. odoratissimus* flesh (31.00 ± 2.07 μg/g dw) and *M. pajang* flesh (29.46 ± 3.68 μg/g dw). Sinapic acid was only detected in the kernel and peel of *M. pajang* with the values of 20.06 ± 1.00 and 0.67 ± 0.31 μg/g dw, respectively. For caffeic acid content, *M pajang* kernel and peel were found to be much higher than others, showing 150.00 ± 13.48 and 440.50 ± 21.90 μg/g dw, respectively; while other parts ranged from 26.75 ± 0.55 to 56.39 ± 4.60 μg/g dw. The level of ferulic acid varied widely from not detected to 5334.00 ± 513.90 μg/g dw. Chlorogenic acid was found in minor quantity in all samples tested, and the level ranged from 14.10 ± 0.94 to 5.81 ± 0.26 μg/g dw.

Samples	gallic	p-coumaric	sinapic	caffeic	ferulic	chlorogenic
M. pajang						
Kernel	236.6 ± 28.31^{a}	301.10 ± 19.12^{a}	20.06 ± 1.00^{a}	150.00 ± 13.48^{a}	5334.00 ± 513.90^{a}	14.10 ± 0.94^{a}
Peel	30.72 ± 5.83^{b}	199.00 ± 17.53^{b}	0.67 ± 0.31^{b}	440.50 ± 21.90^{b}	784.20 ± 144.10^{b}	$8.15 \pm 0.14^{b,e}$
Flesh	N.D.	29.46 ± 3.68^{c}	N.D.	26.75 ± 0.55^{c}	N.D.	5.81 ± 0.26^{c}
A. odoratissimus						
Seed	N.D.	35.33 ± 1.56^{c}	N.D.	43.94 ± 3.06^{c}	444.40 ± 23.13^{b}	$7.99 \pm 0.11^{c,d,e}$
Flesh	N.D.	31.00 ± 2.07^{c}	N.D.	56.39 ± 4.60^{c}	N.D.	8.73 ± 0.40^{e}

Table 2-3: Phenolic acids composition (μg/g dry weight) of the fruits.

Composition is presented as μg/g dry weight, with mean ± S.E.M (n = 3) which with different letters are significantly different at $p < 0.05$.

For example, gallic acid content in the kernel of *M. pajang* (^{a}) is statistically significant different from the peel of *M. pajang* (^{b}).

Meanwhile, p-coumaric content in the flesh of *M. pajang* (^{c}) is statistically not different from the seed (^{c}) and flesh (^{c}) of *A. odoratissimus*.

N.D. – Not detected.

2.3.6.2 *Flavanones composition*

Table 2-4 shows the amount of flavanones (naringin and hesperidin) present in different part of the fruits. The highest content of naringin was found in the kernel of *M. pajang* with the values of 294.50 ± 98.20 mg/g dw ($p<0.05$). All other fruit samples tested had a naringin content of 1.5 mg/g or less. Essentially an identical distribution of hesperidin was determined with the exception that it could not be detected in the flesh of *A. odoratissimus*.

Samples	naringin	hesperidin
M. pajang		
Kernel	294.50 ± 98.20[a]	221.60 ± 24.91[a]
Peel	1.51 ± 0.23[b]	1.01 ± 0.07[b]
Flesh	1.45 ± 0.27[b]	0.93 ± 0.17[b]
A. odoratissimus		
Seed	1.40 ± 0.35[b]	1.64 ± 0.02[b]
Flesh	0.24 ± 0.04[b]	N.D.

Table 2-4: Flavanones compositions (mg/g dry weight) of the fruits.
Composition is presented as μg/g dry weight, with mean ± S.E.M (n = 3) which with different letters are significantly different at $p < 0.05$.
For example, naringin content in the kernel of *M. pajang* ([a]) is statistically significant different from the peel of *M. pajang* ([b]).
Meanwhile, hesperidin content in the peel of *M. pajang* ([b]) is statistically not different from flesh of *M. pajang* ([b]) and seed of of *A. odoratissimus* ([b]).
N.D. – Not detected.

2.3.6.3 *Flavonols composition*

The content of three flavonoids – quercetin, kaempferol and rutin in different parts of the fruits was also examined. The results in Table 2-5 show that flavonols are abundant in the peel of *M. pajang* by which all flavonols tested being present in the sample. Rutin was detected only in the kernel and peel of *M. pajang* with the values of 721.10 ± 34.13 and 132.10 ± 19.21 μg/g dw, respectively ($p<0.05$). The level of kaempferol in the seed of *A. odoratissimus* was the highest (2462.00 ± 70.88 μg/g dw), followed by the peel and flesh of *M. pajang* with the values of 200.40 ± 16.41 and 182.70 ± 6.22 μg/g dw ($p<0.05$). No kaempferol was detected in the kernel and flesh of *M. pajang* and *A. odoratissmus*, respectively. For quercetin content, *M. pajang* flesh was the highest (165.10 ± 6.08 μg/g dw), followed by the peel of *M. pajang* and the flesh of *A. odoratissimus* with the values of 81.85 ± 21.17 and 8.81 ± 0.66 μg/g dw, respectively. No quercetin was detected in the kernel and seed of *M. pajang* and *A. odoratissimus*.

Samples	quercetin	kaempferol	rutin
M. pajang			
Kernel	N.D.	N.D.	721.10 ± 34.13^{a}
Peel	81.85 ± 21.17^{b}	200.40 ± 16.41^{b}	132.10 ± 19.21^{b}
Flesh	165.10 ± 6.08^{c}	182.70 ± 6.22^{b}	N.D.
A. odoratissimus			
Seed	N.D.	2462.00 ± 70.88^{a}	N.D.
Flesh	8.81 ± 0.66^{a}	N.D.	N.D.

Table 2-5: Flavonols compositions of the fruits.
Composition is presented as μg/g dry weight, with mean ± S.E.M (n = 3) which with different letters are significantly different at $p < 0.05$.
For example, quercetin content in the peel of *M. pajang* (^{b}) is statistically significant different from the flesh of *M. pajang* (^{c}).
Meanwhile, kaempferol content in the peel of *M. pajang* (^{b}) is statistically not different from flesh of *M. pajang* (^{b}).
N.D. – Not detected.

2.3.6.4 Flavones composition

Levels of the two flavanone compounds - luteolin and diosmin were also quantified in different parts of the fruits as shown in Table 2-6. Luteolin was detected only in the peel and flesh of *M. pajang* with values of 253.50 ± 1.15 and 292.10 ± 7.27 μg/g dw, respectively. Luteolin was not detected in the kernel of *M. pajang* or in either part of *A. odoratissimus*. On the other hand, diosmin was only detected in by-products of both fruits, i.e. kernel and peel of *M. pajang* and seed of *A. odoratissimus* with the values of 2386.00 ± 325.10, 198.50 ± 2.56 and 288.90 ± 9.02 μg/g dw, respectively.

Samples	luteolin	diosmin
M. pajang		
Kernel	N.D.	2386.00 ± 325.10^{a}
Peel	253.50 ± 1.15^{a}	198.50 ± 2.56^{b}
Flesh	292.10 ± 7.27^{b}	N.D.
A. odoratissimus		
Seed	N.D.	288.90 ± 9.02^{b}
Flesh	N.D.	N.D.

Table 2-6: Flavones compositions of the fruits.

Composition is presented as μg/g dry weight, with mean ± S.E.M (n = 3) which with different letters are significantly different at $p < 0.05$.

For example, luteolin content in the peel of *M. pajang* (^{a}) is statistically significant different from the flesh of *M. pajang* (^{b}).

Meanwhile, diosmin content in the peel of *M. pajang* (^{b}) is statistically not different from seed of *A. odoratissimus* (^{b}).

N.D. – Not detected.

2.4 Discussion

Block et al. (1992) reviewed almost 200 epidemiological studies that evaluated the relationship between intake of fruits and vegetables and cancer of the lung, colon, breast, cervix, oesophagus, oral cavity, stomach, bladder, pancreas and ovary. Strong epidemiological evidence suggests that regular consumption of fruits and vegetables can reduce cancer risk. The past several decades seen an explosion of research focused on the role played by antioxidant phytochemicals in the prevention and treatment of human cancer.

In the present study, the antioxidant potential of different parts of *M. pajang* (kernel, peel and flesh) and *A. odoratissimus* (seed and flesh) were studied. Two assays were used to determine the antioxidant capacity of each fruit extract that responded to the different antioxidant mechanisms, namely DPPH radical scavenging assay and FRAP assay. Generally, all extracts displayed the same trend of antioxidant activity. For *M. pajang* fruits, the kernel extract displayed highest antioxidant capacity, followed by the peel and flesh of the fruit extracts. As for *A. odoratissimus*, the seed displayed a higher antioxidant capacity as compared to the flesh. In each case, the by-products (i.e. kernel and seed) displayed higher antioxidant activity compared to the flesh.

The results are in agreement with earlier literature. The total antioxidant activity of the seeds from avocado, jackfruit, longan, mango and tamarind is always higher than the edible portions (Soong and Barlow, 2004). Extracts

from peels of apple (Wolfe et al., 2003) or mango (Ajila et al., 2007) exhibit good antioxidant activity both *in vitro* and *in vivo* antioxidant assays. Ajila and Rao (2008) reported that the mango peel extract showed protection against membrane protein degradation caused by hydrogen peroxide and suggested that this effect was due to the antioxidant and phytochemical pool in the crude extract. Secondary plant metabolites (i.e. phenols, phenolic glycosides, flavonoids, anthocyanins and aromatic amino acids) which are concentrated in the peel, seed / kernel and bark appear to function primarily in disease resistance and as a defence system against predators, harmful microorganisms and pathogens (Croft, 1998), resulting in the high phytochemicals and antioxidant properties.

Free radical chain reaction is a phenomenon associated with direct attacks of free radical on genetic components, including DNA, protein and membrane lipid (Yu et al., 2004). This damage has been associated in many human degenerative diseases including cancer (Ju et al., 2004). Damage of DNA may take many forms, ranging from specifically oxidized purine and pyrimidine bases to DNA lesions such as strand breaks, sister chromated exchanges and the formation of micronuclei. This effect could then trigger the initiation process of carcinogenesis (Lopaczynski and Zeisel, 2001). Inhibition of free radical and reactive oxygen species by phytochemicals may lead to the prevention of cancer via inhibition of those metabolites in mammalian systems.

The phytochemicals which might be responsible for the free radical scavenging activity in this experiment are the phenolic and flavonoid

constituents. Pearson correlation analysis across the five plant extracts studied revealed that the DPPH free radical scavenging activity was strongly correlated with the total flavonoid content ($r=0.815$; $p<0.01$) and moderately correlated with total phenolic content ($r=0.558$; $p<0.01$) (Appendix: Table A). However, no correlation was observed between scavenging activity and the total anthocyanins content. The antioxidant properties of phenolic compounds was contributed by the reactive phenol moiety (hydroxyl group on aromatic ring) which have the capability to scavenge free radicals via hydrogen or electron transfer (Shahidi and Wanasundara, 1992).

Mango (*M. indica*) seed kernel powder displayed outstanding antioxidant properties compared to other fruits seeds and the antioxidant activity was strongly correlated with the total polyphenol content (Soong and Barlow, 2004), as well as the content of phytosterols (i.e. campesterol, β-sitosterol, stigmasterol) and tocopherols (Soong and Barlow, 2006). Maisuthisakul et al. (2007) reported that the antiradical activity of seeds of berries was higher compared to the flesh and higher than other selected plants endemic to Thailand. There was also a significant correlation between the antiradical activity and total phenolic and flavonoid contents.

The results for total phenolic content of mango (*M. indica*) kernel by Soong and Barlow (2004) were comparable to *M. pajang* kernel with the values of 117 mg GAE/g for mango kernel versus 103.3 mg GAE/g for *M. pajang* kernel. However, the total phenolic for jackfruit (*A. heterophyllus)* seed was lower compared to *A. odoratissmus* seed with the values of 27.7

GAEmg/g and higher compared to *A. odoratissmus* flesh with the value of 0.9 GAEmg/g (Soong & Barlow, 2004).

The reducing ability of the tested extracts was in the order of *M. pajang* kernel > *M. pajang* peel > *A. odoratissmus* seed > *M. pajang* flesh > *A. odoratissmus* flesh; the same trend showed in the DPPH free radical scavenging assay. There were significant differences ($p < 0.05$) among the samples except for *M. pajang* flesh and *A. odoratissimus* seed. *M. pajang* kernel exhibited superior reducing capacity, approximately 9 and 20 times higher compared to the peel and flesh, respectively. Meanwhile, the reducing capacity of *A. odoratissmus* seed extract was almost doubled compared to the flesh.

The reducing ability of the extracts was strongly correlated with the contents of phenolics ($r=0.985$; $p<0.01$) and flavonoids ($r=0.663$; $p<0.01$) (Appendix: Table A). This result was in agreement with Soong and Barlow (2004) and Othman et al. (2007) who found a strong correlation between total phenolic content and FRAP assay in the extracts of cocoa powder. Rice-Evans et al. (1997) reported that phenolic compounds exhibited redox properties, (i.e. act as reducing agents, hydrogen donators and singlet oxygen quenchers), and that the redox potential of phenolic phytochemicals plays a crucial role in determining the antioxidant properties (Rice-Evans et al., 1997).

A recent study has shown that mango kernel crude extract and oil increased the oxidative stability of sunflower oil incubated at ambient temperature and sunflower oil during frying. Moreover, both extract and oil improved the stability and quality characteristics of fresh and stored potato chips (Abdalla et al., 2007b). This study demonstrated the ability of mango kernel crude extract to act as antioxidant in food system by preventing the oxidation of sunflower oil as well as fried-products.

Gallic, ellagic, coumarin, vanillin, cinammic, ferulic and caffeic acids have been identified as the key phenolic compounds in mango kernel and longan seed (Abdalla et al., 2007a; Soong and Barlow, 2006) and those compounds might contribute to the total phenolic pool in *M. pajang* kernel and *A. odoratissimus* seed. In addition, the content of non-flavonoid compound is approximately 90% from the total polyphenol present in the *M. pajang* kernel, showing that the major compounds of the polyphenols are phenolic acids (i.e. gallic acid, ellagic acid and ferulic acid).

Flavonoids are important for human health because of their powerful pharmacological activities as radical scavengers (Hertog et al., 1993). Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidative activity, free radical scavenging capacity, coronary heart disease prevention, and anticancer activity, while some flavonoids also exhibit potential against human immunodeficiency virus functions (Yao et al., 2004).

The anthocyanin content was relatively low in all samples tested. For *M. pajang*, anthocyanin were only detected in the flesh (1.47 mg c-3-gE/100g) and the peel (28.29 mg c-3-gE/100g). The flesh of *A. odoratissimus* contained higher total anthocyanin content compared to the seed ($p<0.05$) (Table 2). Anthocyanin pigments are responsible for the attractive red to purple to blue colours of many fruits and vegetables. Anthocyanin are relatively unstable and often undergo degradative reactions during processing and storage (Giusti and Wrolstad, 2001).

Anthocyanin content in mango and apple peels were reported to be in the ranges of 203 – 565 and 2.1 – 26.8 mg cyanidin-3-glucoside equivalent in 100 g of sample, respectively, depending on the variety (Ajila et al., 2007; Wolfe et al., 2003)

The genus of *Mangifera* (Anacardiaceae) and *Artocarpus* (Moraceae) are a rich source of secondary metabolites including polyphenols. The seed and flesh of mango (*M. indica*) contained high total phenolic content and displayed high antioxidant activity compared to other tropical fruits (Soong and Barlow, 2004). Several phenolic acids such as gallic acid, *m*-digallic acid, *m*-trigallic acid and ellagic acid have been detected in the fruit of *M. indica* (El Ansari et al., 1969). This was confirmed by Saeed et al. (1976) who also demonstrated an absence of detectable gallic acid and m-digallic acid concomitant with the absence of condensed tannins, flavonols, chlorogenic acid and caffeic acids in the pulp of several varieties of mango (Saeed et al., 1976). Prabha and Patwardhan (1986) reported that gallic acid is the substrate of polyphenol oxidase in the fruit pulp; meanwhile ellagic acid is the predominant substrate in

mango peel. During mango fruit development, the total polyphenol are higher in the peel and pulp than in the flesh at all stages of maturation (Lakshminarayana et al., 1970). All phenolic acids tested were detected in the kernel and peel of *M. pajang* while there were an absence of gallic, sinapic, and ferulic acid in the flesh of the fruit. Schieber et al. (2000) reported no evidence for the presence of ellagic acid and gallic acid dimers or trimers in the mango puree, although they were detected by El Ansari et al. (1969). Phenolic acids (such as ferulic and *p*-coumaric acids) have been shown to display antioxidant properties and act as chemoprevention against colon cancer cell line (HT-29) (Ferguson et al., 2005).

On the other hand, flesh and seed from jackfruit (*Artocarpus heterophyllus*) contained a very low amount of total phenolic and displayed low antioxidant activity compared to other tropical fruits (Soong and Barlow, 2004). Gallic and sinapic acids were not detected in the flesh nor seed of *A. odoratissimus* while ferulic acid was only detected in the seed of *A. odoratissimus*. A summary of the phytochemicals results is presented in Table 2-7.

The kernel of *M. pajang* is regarded as safe since it is eaten with the flesh as pickle by local community. Thus, a nutraceutical might be developed based on the natural products or the purified extracts being incorporated with food products to give therapeutic effects (Soong and Barlow, 2004). The kernel could be a valuable natural resource of polyphenols. The seed of *A.*

odoratissimus might also be useful as nutraceutical as the roasted or fried seed are already eaten by the local community.

The kernel from *M. pajang* could be commercialized as food and nutraceutical products. The kernel of mango (*M. indica*) has been transformed into processed flour which can be used to substitute normal wheat flour in the making of biscuits (Arogba, 1999). The kernel was also regarded safe as no toxic effects were shown by rats fed with diet containing mango kernel crude extract (Rukmini and Vijayaraghavan, 1984). However, clinical study is needed to determine the toxicity in a short and long term consumption in human.

2.5 Conclusion

In conclusion, our investigation on selected fruits endemic to Borneo Island indicates that both fruits and their by-products are rich sources of antioxidants, with a high level of phytochemicals (phenolics and flavonoids) content. Further elucidation of their antioxidant and cytoprotection properties as well as their cytotoxic potential are investigated in the next chapters.

Samples	Total phenolic	Total flavonoid	Total anthocyanins	gallic	p-coumaric	sinapic	caffeic	
M. pajang								
Kernel	5.96 ± 0.34	0.07 ± 0.00	1.47 ± 0.28	236.6 ± 28.31	301.10 ± 19.12	20.06 ± 1.00	150.00 ± 13.48	
Peel	22.93 ± 0.36	7.50 ± 0.09	28.29 ± 0.70	30.72 ± 5.83	199.00 ± 17.53	0.67 ± 0.31	440.50 ± 21.90	
Flesh	103.30 ± 0.63	10.98 ± 0.16	N.D.	N.D.	29.46 ± 3.68	N.D.	26.75 ± 0.55	
A. odoratissimus								
Seed	4.39 ± 0.20	1.08 ± 0.03	11.02 ± 0.38	N.D.	35.33 ± 1.56	N.D.	43.94 ± 3.06	
Flesh	14.67 ± 0.17	3.65 ±0.04	3.80 ± 0.34	N.D.	31.00 ± 2.07	N.D.	56.39 ± 4.60	
ferulic	chlorogenic	naringin	hesperidin	quercetin	kaempferol	rutin	luteolin	diosmin
5334.00 ± 513.90	14.10 ± 0.94	294.50 ± 98.20	221.60 ± 24.91	N.D.	N.D.	721.10 ± 34.13	N.D.	2386.00 ± 325.10
784.20 ± 144.10	8.15 ± 0.14	1.51 ± 0.23	1.01 ± 0.07	81.85 ± 21.17	200.40 ± 16.41	132.10 ± 19.21	253.50 ± 1.15	198.50 ± 2.56
N.D.	5.81 ± 0.26	1.45 ± 0.27	0.93 ± 0.17	165.10 ± 6.08	182.70 ± 6.22	N.D.	292.10 ± 7.27	N.D.
444.40 ± 23.13	7.99 ± 0.11	1.40 ± 0.35	1.64 ± 0.02	N.D.	2462.00 ± 70.88	N.D.	N.D.	288.90 ± 9.02
N.D.	8.73 ± 0.40	0.24 ± 0.04	N.D.	8.81 ± 0.66	N.D.	N.D.	N.D.	N.D.

Table 2-7: Summary of the phytochemicals which present in different parts of the fruits. Please refer to Table 2-2 until 2-6 for the details of the statistical analysis.

CHAPTER 3

Cytoprotection activity of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*)

CONTENTS

CHAPTER 3

Cytoprotection activity of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*)

3.1 Introduction

Many research has been conducted in the quest of a cure for cancer, but it's still regarded as a challenge for human health worldwide. The number of mortality caused by cancer is estimated to double in the next 5 decades (Mann et al., 2005). Conventional chemical cancer therapy or so called 'chemotherapy' has been reported to cause short- and long-term side-effects such as emesis, nausea, stomatitis, neuropathy, fatigue and cognitive dysfunction (Partridge et al., 2001), thus compromising the quality of life of cancer patients. Moreover, the increasing trend of cancer resistance against chemotherapy treatment makes it difficult to deal with this disease. Thus, cancer prevention (also called 'chemoprevention') has become an important approach to control cancer.

Cancer chemoprevention is defined as prevention of the occurrence of cancer via intake of one or more compounds which are either blocking agents

or suppressing agents. Blocking agents act by preventing carcinogenic compounds from targeting the critical sites (i.e. DNA, RNA) in the cells, while suppressing agents come into play after attack by cancer producing compounds (Wattenberg, 1996).

Fruits and vegetables have been shown to contain phytochemicals which possess cancer chemopreventive properties via balancing between the carcinogen-activating phase I enzymes and phase II detoxification enzymes (Prestera et al., 1993). Targeting the induction of phase II detoxification enzymes, such as quinone reductase, glutathione peroxidase, glutathione reductase, superoxide dismutase and methionine sulfoxide reductase A by dietary anticarcinogens, are likely to play a protective role against oxidative damage caused by free radicals (Hashimoto et al., 2002).

In this study, the plant extracts used in Chapter 2 and quercetin as a positive control were studied for their cytoprotective potential against tert-buthyl hyroperoxide (t-BHP) induced oxidative damage in a mammalian cell line (HepG2). However, the extraction method was changed for this particular experiment and subsequent experiments. Instead of using methanol which has a high toxicity in human, ethanol was used as a substitute. Extraction using ethanol has also been shown to extract major fraction of polyphenol and the extract can also being considered as polyphenol-rich extract (Ruzaidi et al., 2008; Othman et al., 2007). Three detoxifying enzymes were chosen as potential biomarkers for the biological cytoprotective and detoxification

enzymes. Quinone reductase is a flavoprotein which is found in cytosol that catalyzes two-electron reduction of quinones into stable hydroquinones, by which the development of cytotoxic semiquinone radicals (that are naturally oxidized to produce carcinogenic molecules) can be stopped (Talalay et al. 1988). Glutathione reductase can recycle oxidized glutathione (GSSG) back to the bioactive reduced form (GSH) and maintain the level of glutathione in the cells (as discussion in 1.4.2). While methionine sulfoxide reductase A is crucial to repair the easily oxidized methionine residue (methionine residue is oxidized to methionine sulfoxide derivative) back to its natural form (Moskovitz et al., 2001). In addition to that, the heat shock proteins, namely HSP 27 and HSP 70 were also studied. Previous studies have demonstrated that up-regulation of heat shock protein has been shown to play an important role in defending cells from harmful xenobiotics or harmful conditions, thus ensuring cell survival (Urani et al., 2005; De Maio, 1999).

3.2 Materials and methods

3.2.1 Chemicals

Fungizone and gentamicin were purchased from E.R. Squibb and Sons Ltd. (Hounslow, UK). Minimum Essential Medium Eagle, foetal bovine serum, 0.25% trypsin–EDTA solution, Dulbecco's phosphate buffered saline, L-glutamine, phenylmethylsulfonyl fluoride and protease inhibitor cocktail were purchased from Sigma–Aldrich Company Ltd. (Poole, UK). Tissue-culture treated Costar plastic 24-well plates were obtained from Corning Inc., NY, USA. All other chemicals and solvents used were of the highest pure grade available. Details of primary and secondary antibodies used for western blotting will be given later in this chapter.

3.2.2 Plant materials and sample preparation

As described in chapter 2 (2.3.1)

3.2.3 Extraction

Fruit powder was extracted with ethanol in the ratio of 1:5. One hundred gram of powdered freeze dry sample was soaked in 500 ml of ethanol three times over night. Each infusion was filtered and the three filtrates were combined and concentrated using a rotary evaporator. The concentrated

extracts were diluted using dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml.

3.2.4 Culture of HepG2 cells

Human Caucasian hepatocyte carcinoma HepG2 cells (ATCC) were maintained in 175 cm^2 Nunclon culture flasks using 50 ml of Minimum Essential Medium Eagle supplemented with 10% foetal bovine serum, 2mM L-glutamine, 1% non-essential amino acid solution, 2 µg/ml fungizone and 0.05 mg/ml gentamicin at 37 °C with 5% CO_2. Cell's passage number used in this experiment is between 10 to 40.

3.2.5 Treatment

The cells were grown for approximately 72 hours prior to treatment in order to obtain a 70 – 80% confluent monolayer. All cells were plated out onto 24-well sterile plates with 0.4×10^6 cells per well. After allowing for attachment of the cells for 24 hours, the cells were treated with extracts (diluted in medium to concentrations ranging from 0 – 100 µg/ml) for 24 hours. Collection of cell lysates and assessment of cell viability were carried out simultaneously. The results presented are the mean ± S.E.M. of at least three independent experiments.

3.2.6 Cell viability assay

Cytotoxicity of the extracts was determined by using neutral red uptake assay following 24 hours incubation with the test extract or quercetin (as positive control). This assay is based on the fact that this supravital dye is taken up and accumulated only in the lysosomes of living cells (Borenfreund and Puerner, 1985). The absorbance of the solubilised neutral red (present in the viable cells) is taken as a measure of the number of living cells.

Briefly, a stock solution of neutral red was diluted in tissue culture medium giving a final concentration of 20 µg/ml. Following incubation of the cells with the plant extracts (or quercetin) at different concentrations (0 – 50 µg/ml), cells were incubated with diluted neutral red solution (400 µl/well) for 1 h at 37 °C. Following a single wash using phosphate buffered saline (PBS; 400 µl/well) 400 µl of neutral red fixative solution (water:ethanol:glacial acetic acid 50:50:1) was added into each well and the plate shaken on an orbital shaker for 10 minutes to solubilise the cell-retained dye. Aliquots of desorbed neutral red were transferred to a 96-well plate (100 µl/well). The optical density was read at 540 nm using an Ascent micro-plate reader (Thermo Electron Corporation, UK). A cell-free blank was used for background correction of the absorbance. The viability of the cells was expressed as a percentage of control values after subtracting contribution from the 'no cell blank'.

3.2.7 Cytoprotection assay against tert-butylhydroperoxide (t-BHP) in HepG2

The cytoprotection effect of the plant extracts against t-BHP-induced cytotoxicity was also evaluated by using the neutral red uptake assay. For the experiment, 0.4 x 10^6 cells/well were seeded onto 24-well plates and allowed to adhere for 24 hours. After that, the medium was discarded and preincubated with plant extracts (diluted in complete growth medium) at non-toxic concentration (based on experiment 3.2.6) as well as the positive control (quercetin) and 0.5% v/v DMSO (plant extract solvent) for 24 hours. After this time, the medium was replaced with 400 µl of 0.325 mM t-BHP (diluted in medium containing all supplements including 2% serum) and the incubation was allowed to continue for another 5 hours. Following the t-BHP challenge, the medium was discarded and the monoloyer cells were washed once with PBS (400 µl/well) and the cell viability was determined using neutral red uptake assay as described in 3.2.6. At this concentration, 0.325 mM t-BHP has been shown to induce up to 90% cell cytotoxicity in the HepG2 cell line.

3.2.7.1 Calculation of cytoprotection index

To measure the effectiveness of different compounds/plant extracts as cytoprotectants in this assay, the following equation was used:

$$\text{Percentage (\%) of protection} = 100 - [(Y / X) \times 100]$$

Where Y = Difference in absorbance between the presence and absence of the putative cytoprotectants at each concentration (i.e. 0, 3.125, 6.25, 12.5, 25 and 50 µg/ml)

X = Absorbance at maximum damage produced by t-BHP in this experiment in the absence of putative cytoprotectants.

An example of this calculation is presented in Table 3-1. Percentage (%) of damage and cytoprotection was calculated for each concentration and from this, a cytoprotective index (expressed as an EC_{50} – the concentration that reduces toxicity by 50%) was calculated by the method of Alexander et al. (1999).

Concentrations of extracts (μg/ml)	$A_{-tBHP} - A_{+tBHP}$	Percentage of damage	Percentage of cytoprotection
0	0.551-0.078 = 0.473	(0.473/0.473) x 100 = 100.00	100 – 100 = 0
3.125	0.55-0.090 = 0.460	(0.46/0.473) x 100 = 97.25	100-97.25 = 2.75
6.25	0.592-0.223 = 0.369	(0.369/0.473) x 100 = 78.01	100 – 78.01 = 21.99
12.5	0.575-0.431 = 0.144	(0.144/0.473) x 100 = 30.44	100 – 30.44 = 69.56
25	0.655-0.567 = 0.088	(0.088/0.473) x 100 = 18.60	100 – 18.60 = 81.6
50	0.625-0.609 = 0.016	(0.016/0.473) x 100 = 3.38	100 – 3.38 = 96.62

From the above results, cytoprotection index (EC_{50}) was calculated based on the equation below:

$$EC_{50} = D - \frac{(A-50)\times(D-C)}{(A-B)}$$ (Alexander et al., 1999)

D = concentration (μg/ml) above the 50% cell viability
A = percentage (%) of cell viability value near the 50% (higher value)
C = concentration (μg/ml) below the 50% cell viability
B = percentage (%) of cell viability lower the 50% (lower value)

Example:
$$EC_{50} = 12.5 - \frac{(69.56-50)\times(12.5-6.25)}{(69.56-21.99)}$$

$$= 9.93$$

Table 3-1: Example of the calculation of the cytoprotection index.

3.2.8 Preparation of cell lysates

Following 24 hours of incubation with plant extracts, positive control (quercetin) or solvent (0.5% v/v DMSO), the culture medium was discarded. Following a single wash using phosphate buffered saline (PBS; 400 µl/well), lysis buffer (cold PBS containing 1% v/v Triton X114, 500µM phenylmethylsulfonylfluoride and 1% v/v protease inhibitor cocktail) was added to each well (400µl/well) (Kelland et al., 1999). The plates were left on ice for 30 minutes, the cell lysates collected in labelled Eppendorf tubes and centrifuged (12000 rpm for 5 minutes). The supernatants were then kept at -80 °C and thawed prior to use for protein determination and western blotting assay.

3.2.9 Protein determination by Bradford method

The total protein content of the cell lysates was determined using the assay developed by Bradford (1976). This assay is based on the binding of the amino group of the amino acids in the protein to Coomassie Brilliant Blue. The binding of the protein with the dye leads to a change in colour of the solution which can be detected colorimetrically. The colour of the solution is proportional to the amount of the protein present in the samples (Bradford, 1976).

Bradford reagent was prepared by dissolving 100 mg of Brilliant Blue G in 50 ml of ethanol and 100 ml of orthophosphoric acid and made up to 1

litre with distilled water. For the experiment, briefly, 200µl of Braford reagent was added into each well of a 96-well microplate. A series of standard solutions (bovine serum albumin) (0 – 300 µg/ml in 0.5M NaOH) were added into each well (20 µl /well). Similarly, the diluted cell lysate samples (1:10) were added into each well. Following the incubation of the mixture for 5 min, the absorbance was read using an Ascent® multiscan plate reader with dual wavelength measurement (570 nm as test and 405 nm as the reference for background correction). The software of the instrument was used to construct the standard curve from which the sample concentrations were calculated using curve fit modification of quadratic polynomial. All samples were run in triplicate.

3.2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

3.2.10.1 Sample preparation for western blotting

The cell lysates were diluted using 6 x solubilisation buffer (containing 12% SDS, 30% glycerol, dithiothreitol 0.09g/ml, 4µl/ml of 2.5% bromophenol blue solution and 0.125M tris HCL dissolved in distilled water). The samples were then used directly for western blotting or stored at -20°C until further used.

3.2.10.2 SDS-PAGE

The samples (cell lysates containing 6 x solubilisation buffers) were denatured on a heat block for 5 min at 95°C prior to use. Equal amounts of protein (10 μg) of each sample were loaded on to a precast polyacrylamide gel [10-20%, 12 well, 1mm Tris glycine gel (Lonza, USA)]. Protein molecular weight marker (ColorBurst™ marker, Sigma, UK) was also loaded (5 μl) on to the precast gel to estimate the sample molecular weights, to monitor the progress of an electrophoretic run and to confirm that an electroblot is complete. Finally, the samples were separated by electrophoresis (50 min; 200 V; Bio-Rad Protean, Hercules, USA) using electrophoresis buffer (see Appendix for the composition).

3.2.10.3 Transfer of resolved proteins onto nitrocellulose membrane

Upon the completion of the SDS-PAGE, the gel was transferred onto nitrocellulose membrane by assembling the gel, nitrocellulose membrane (Hybond ECL, Amersham Bioscience UK, Ltd), sponge pads and filter papers in the transfer cassette arranged as illustrated in Figure 3-1. The cassette was then fitted to the transfer tank with ice pack-cold transfer buffer (see Appendix for the composition), and transfer effected using a constant voltage of 100V for 90 minutes.

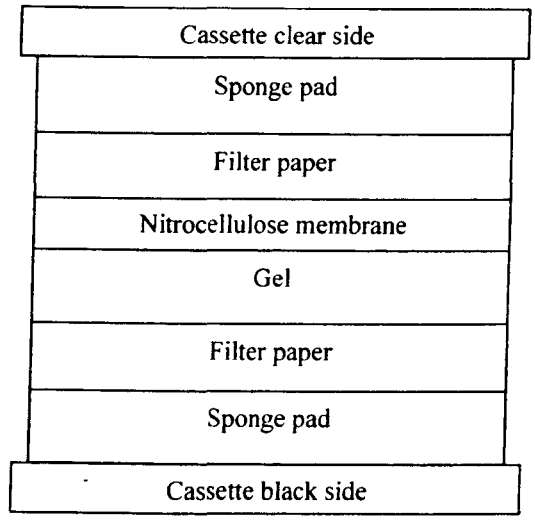


Figure 3-1: Assembly of the transfer cassette components

The nitrocellulose membrane was then checked for a successful protein transfer by staining with Ponceau Red staining. The blot was then cut into smaller strips using the appropriate molecular weight marker as a guide, depending on which protein was to be measured. Finally, the smaller strips were washed with distilled water, followed by TBST (Tris-Buffered Saline Tween 20) 3 times for 10 minutes.

3.2.10.4 SNAP i.d.™ Protein Detection System

Following electro-transfer onto a nitrocellulose membrane, the membrane was cut accordingly and probed with the appropriate primary antibody indicated in Table 3-2 and the appropriate fluorescent-labelled secondary antibody in Table 3-3 using the SNAP i.d protein detection system (Milipore).

Briefly, the membranes were placed in a SNAP i.d. Protein Detection Chamber. The membranes were blocked with blocking buffer (1.5% fish skin gelatin in TBST) for 5 minutes and quickly drained out using a vacuum to ensure even distribution of the reagents. The blocking procedure was crucial to minimize the non-specific binding of the antibody. The membranes were subsequently incubated with primary antibody appropriate to each protein (Table 3-2) for 10 minutes. After 10 minutes, the primary antibody was filtered and the membranes washed 3 times with TBST. Later, secondary antibodies (Table 3-3) were added and incubated for 10 minutes before another washing took place. Finally, the membranes were washed again with TBST (3 times) and the blots were then ready to be scanned.

3.2.10.5 Scanning of the blots

The blots were detected and quantified by an Odyssey® infrared fluorescent imaging system (LI-COR) and analyzed using Odyssey® V3 software.

Primary antibodies	Specification	Supplier	dilution
β-actin	Mouse monoclonal (A-5441)	Sigma-Aldrich, UK	1:30,000
Quinone reductase (NQO1)	Goat polyclonal (ab2346)	Abcam, UK	1:600
Glutathione reductase (GR)	Rabbit polyclonal (ab16801)	Abcam, UK	1:600
Methionine sulfoxide reductase A (MSRA)	Rabbit polyclonal (ab 16803)	Abcam, UK	1:600
HSP 27	Rabbit polyclonal (SPA-803)	Stressgen	1:600
HSP 70	Mouse monolclonal (C92F3A-5)	Stressgen	1:600

Table 3-2: Details of primary antibodies

Secondary antibodies	Protein	Band colour	Dilution
Polyclonal goat-anti mouse IgG (926-322010), LI-COR IRDye (LI-COR, Biosciences, UK)	B-actin HSP 70	Green	1:1000
Polyclonal Goat-anti Rabbit IgG (926-32211), LI-COR IRDye (LI-COR, Biosciences, UK)	Glutathione reductase (GR); Methionine sulfoxide reductase A (MSRA); HSP 27	Red	1:1000
Polyclonal Donkey-anti Goat IgG (926-32214), Li-COR IRDye (LI-COR, Biosciences, UK)	Quinone reductase (NQO1)	Green	1:1000

Table 3-3: Details of secondary antibodies.

3.2.11 Statistical analysis

All experiments were carried out in 3 independent experiments and presented as mean ± standard error of mean (S.E.M) using Prism version 15.0. The data were statistically analysed by one-way ANOVA using Dunnett's post-hoc. The level of statistical significance was set at $p < 0.05$.

3.3 Results

3.3.1 The effects of plant extracts on the viability of HepG2

The cytotoxicity of the plant extracts and quercetin alone against the viability of HepG2 cell line was evaluated (Figures 3-2, 3-3 and 3-4). The cells were incubated for 24 hours with different concentrations of the plant extracts or quercetin and the viability of the cells was determined by neutral red uptake assay. The results showed that no cytotoxicity was observed after incubation of the plant extracts and quercetin with the HepG2 cell line at the concentration tested (0-50 μg/ml). Thus, non-toxic concentrations ranging from 0 – 50 μg/ml for both plant extracts and quercetin were chosen for further cytoprotection study.

3.3.2 Toxicant (t-BHP) induced oxidative damage in HepG2 cell line

Incubation of the HepG2 monolayer for 5 hours with t-BHP alone at a concentration of 0.325 mM has been shown to induce cytotoxicity up to 88% (Table 3-4). Thus concentration of t-BHP at 0.325 mM was chosen to investigate the cytoprotection potential of plant extracts against oxidative damage caused by this compound.

t-BHP concentration (mM)	Cell viability (%)
0	98.3 ± 2.5
0.325	12.8 ± 5.6

Table 3-4: Cytotoxic activity of t-BHP
Results were shown as mean ± S.E.M of 3 independent experiments

3.3.3 Cytoprotective activity of plant extracts and quercetin

The cytoprotective potential of the plant extracts against the cell death induced by t-BHP was evaluated in HepG2 cell line. At the beginning of the experiment, quercetin (as positive control) was used to evaluate this assay. As shown in Figure 3-2, incubation of the cells with quercetin for 24 hours prior to the incubation with 0.325 mM t-BHP for 5 hours, resulted in a dose-dependent cytoprotective activity. No cytotoxicity was observed with the incubation of HepG2 with quercetin alone at different concentrations (0 – 50 μg/ml) (Figure 3-2).

Figure 3-3 displays the concentration-response graph of the cytoprotective activity of quercetin against *t*-BHP induced cytotoxicity in HepG2 cells. Effective concentration (EC_{50}) was calculated according to 3.2.7.1: "Calculation of cytoprotection index". The results show that the quercetin displays cytoprotective activity against t-BHP induced oxidative damage with a cytoprotection index value of 5.34 ± 0.66 μg/ml. This value is equivalent to the EC_{50} value of this experiment.

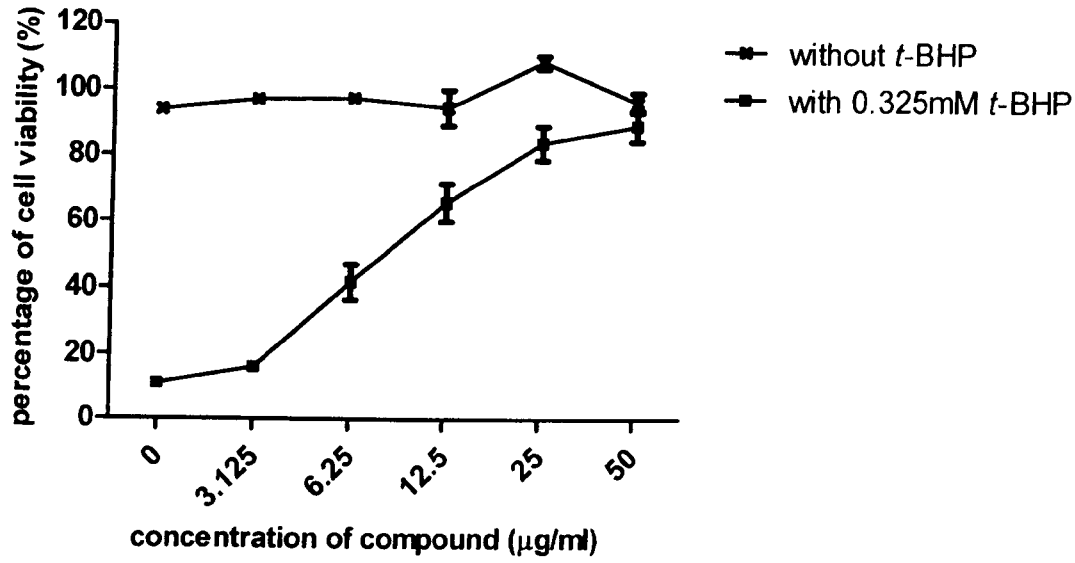


Figure 3-2: The effects of quercetin (µg/ml) on HepG2 cell viability in the absence or presence of t-BHP. Incubation of the quercetin with HepG2 cells (for 24 hours) has been shown to increase the cell viability against *t*-BHP induced oxidative damage at increasing concentration. No cytotoxicity was observed in the cells after incubation with the compound alone for 24 hours in the concentration range 0 – 50 µg/ml.

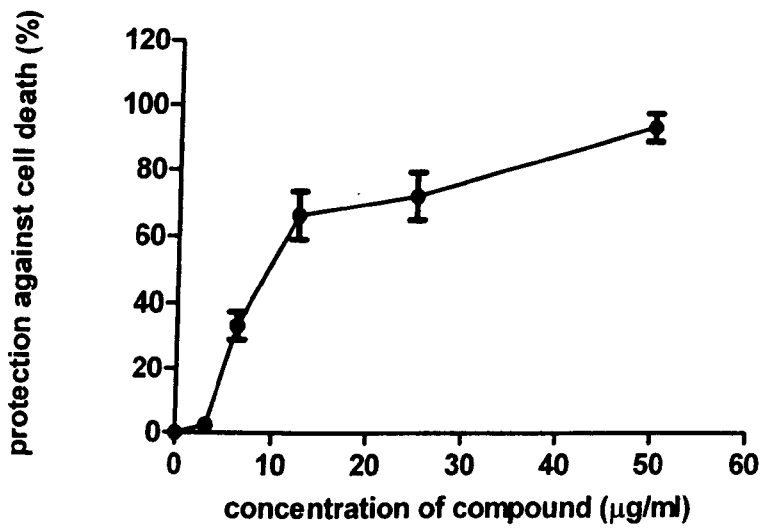


Figure 3-3: Concentration-response effect of the cytoprotective activity of quercetin against t-BHP induced cytotoxicity in HepG2 cells. The data presented in Figure 3-2 have been recalculated to indicate % cytoprotection, from which EC_{50} values can be generated.

Among the plant extracts, only *M. pajang* kernel extract displayed cytoprotective activity against t-BHP induced oxidative damage. As shown in Figure 3-4, *M. pajang* kernel extract protected HepG2 cells against t-BHP induced-cell death in a concentration dependent manner. As shown in Figure 3-5, *M. pajang* kernel extract displayed a higher cytoprotective index (lower EC_{50} value) than quercetin with a value of 1.21 ± 0.13 μg/ml. Meanwhile, no cytoprotective activity was shown by *M. pajang* peel and flesh extracts nor the extracts of *A. odoratissimus* flesh and seed extracts (data not shown).

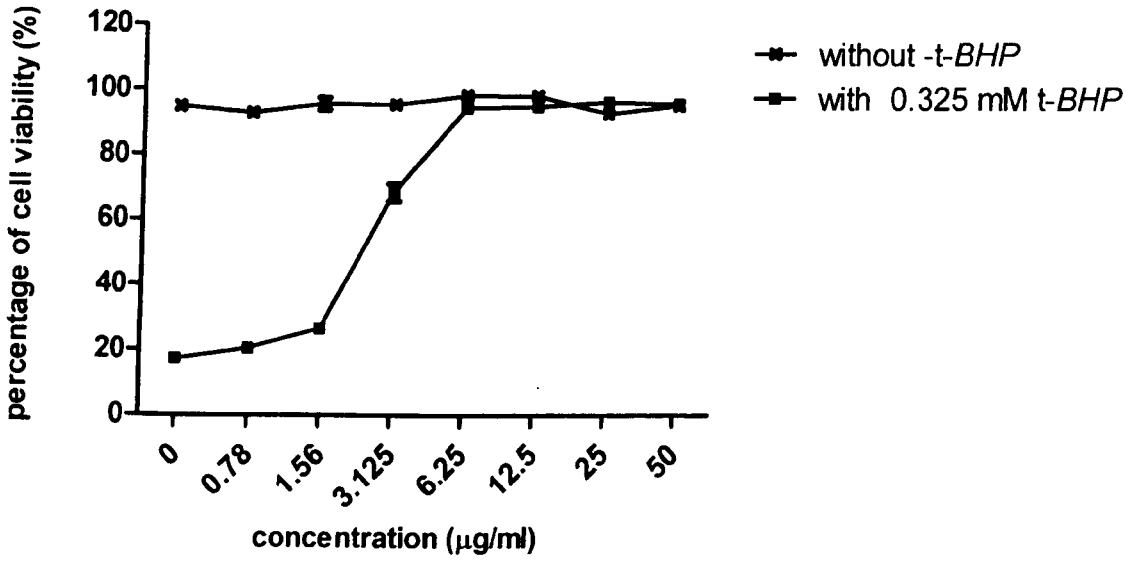


Figure 3-4: The effects of *M. pajang* kernel extract (μg/ml) on HepG2 cell viability in the absence or presence of t-BHP. Incubation of the kernel extract with HepG2 cells (for 24 hours) has been shown to increase the cell viability against t-BHP induced oxidative damage at increasing concentrations. No cytotoxicity was observed in the cells after incubation with the compound alone for 24 hours in the concentration range 0 – 50 μg/ml.

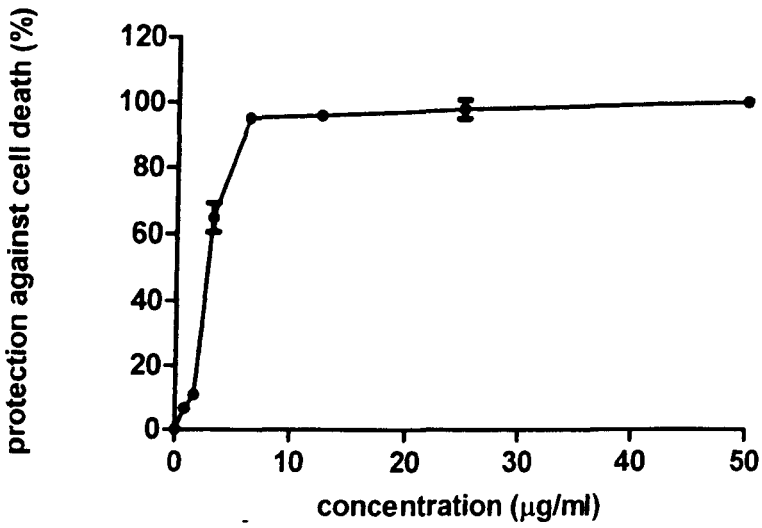


Figure 3-5: Concentration-response effect of the cytoprotective activity of *M. pajang* kernel extract against t-BHP induced cytotoxicity in HepG2 cells. The data presented in Figure 3-4 have been recalculated to indicate % cytoprotection, from which EC_{50} values can be generated

3.3.4 Proteins expression using western blot assay

Since the kernel of *M. pajang* extract and quercetin displayed cytoprotective activity, further study on their possible mechanism of cytoprotection action was conducted. In the present study, the level of the expression of the cytoprotective-related proteins was measured by western blotting. HepG2 cell line was exposed to *M. pajang* kernel extract for 24 hours at increasing concentrations as follows: 0 µg/ml (control), 0.78 µg/ml, 1.563 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25.0 µg/ml and 50.0 µg/ml; as well as quercetin (positive control) for 24 hours at increasing concentrations as follows: 0 µg/ml (control), 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25.0 µg/ml and 50.0 µg/ml. The controls were treated with DMSO (0.05%). No t-BHP was added into the cell culture after the extracts treatment. The cell lysates were collected and analyzed for the induction or suppression of the expression of targeted protein. All results were normalized to β-actin to eliminate variation in protein loading, thus each protein band could be compared accordingly.

3.3.4.1 Expression of NQO1

The effects of the expression levels of quinone reductase (NQO1) protein following the incubation with different concentrations of quercetin for 24 hours were tested in HepG2 cells. Control (solvent used to dissolve quercetin, DMSO at concentration of 0.05%) was used as a control. NQO1 levels were normalized to β-actin as a housekeeping protein in HepG2 cells.

As shown in Figure 3-6, the induction of NQO1 in the cells treated with quercetin occurred in a concentration-dependent manner. The quantification of the protein bands showed that at concentrations of 3.125 – 6.25 μg/ml, quercetin induced a trend of increased expression, which was not statistically significant ($p>0.05$) (Figure 3-7). A significant and steady increase in NQO1 expression was observed for concentrations ranging from 12.5 to 50 μg/ml ($p<0.05$). The largest inductions were noted at quercetin concentrations of 25 and 50 μg/ml by which the induction had almost doubled compared to the control. This result suggested the role of NQO1 as a potent cytoprotective protein involved in the mechanism to protect cells from oxidative damage.

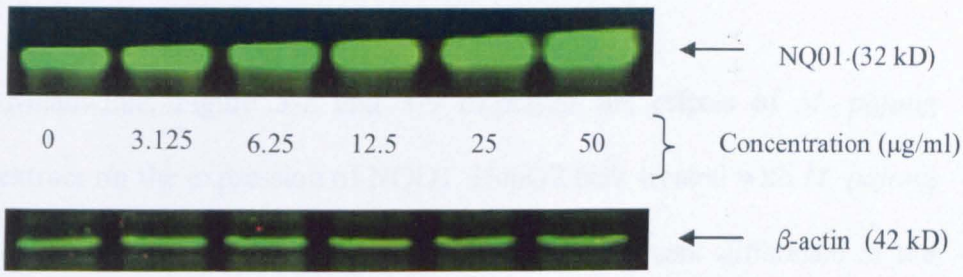


Figure 3-6: Expression levels of NQO1 protein in quercetin-treated HepG2 cells. Cultured HepG2 cells were treated with increasing concentration of quercetin (0 – 50 µg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 800 nm (green band). Blots shown are representative of three independent experiments.

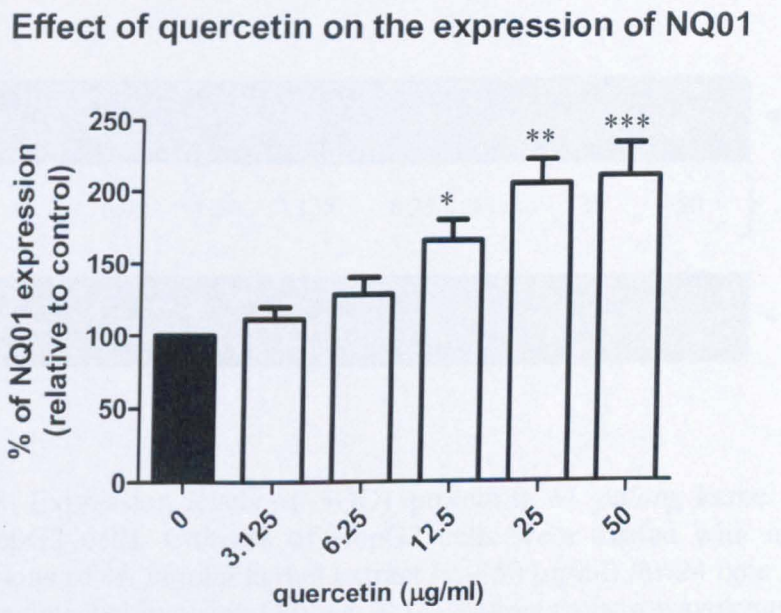


Figure 3-7: The effect of quercetin treatment on the expression of quinone reductase (NQO1) in HepG2 cells. The monolayer cells were exposed to quercetin at increasing concentrations. All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean of three independent determinations. Significant results are denoted by $p<0.05$ = *; $p< 0.01$ = ** and $p<0.001$ = *** (One way ANOVA followed by Dunnett's tests)

Meanwhile, Figure 3-8 and 3-9 displayed the effects of *M. pajang* kernel extract on the expression of NQO1. HepG2 cells treated with *M. pajang* kernel extract (0 – 50 μg/ml) did not show any significant difference in the expression of NQO1 relative to the control ($p > 0.05$), suggesting that NQO1 may not be involved in the cytoprotective mechanism to protect the cells from t-BHP induced oxidative damage.

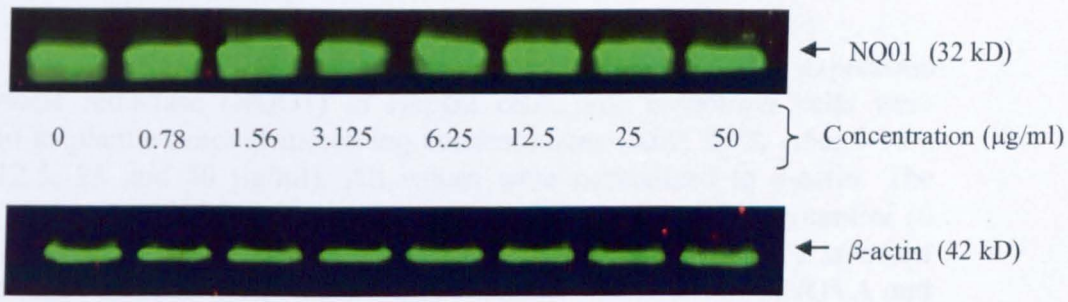


Figure 3-8: Expression levels of NQO1 protein in *M. pajang* kernel extract - treated HepG2 cells. Cultures of HepG2 cells were treated with increasing concentrations of *M. pajang* kernel extract (0 – 50 μg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 800 nm (green band). Blots shown are representative of three independent experiments.

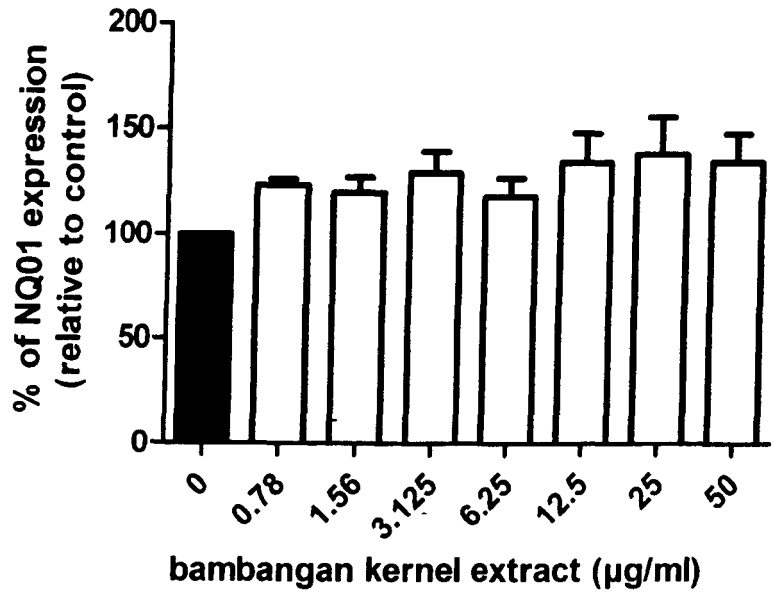


Figure 3-9: The effect of *M. pajang* kernel extract treatment on the expression of quinone reductase (NQO1) in HepG2 cells. The monolayer cells were exposed to plant extract at increasing concentrations (0.00, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 µg/ml). All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean of three independent determinations. The One-way-ANOVA and the Dunnett's tests showed no significant statistical values of NQO1 expression following the *M. pajang* kernel extract treatment compared to the control.

3.3.4.2 Expression of GR

The effect of quercetin on the expression levels of glutathione reductase (GR) protein were also tested in HepG2 following the treatment of these cells with quercetin of increasing concentrations (0 – 50 µg/ml) for 24 hours. Interestingly, the results obtained from this experiment showed the same pattern of results which were observed with NQO1 protein expression (Figure 3-11). The analysis of the GR protein bands (Figure 3-10) showed that at concentrations of 3.125 – 6.25 µg/ml induced a slight, but statistically not significant, increase in GR. A significant increase in GR protein expression was observed for the concentrations range from 12.5 to 50 µg/ml ($p<0.05$), with a slight drop at a concentration of 25 µg/ml (Figure 3-11).

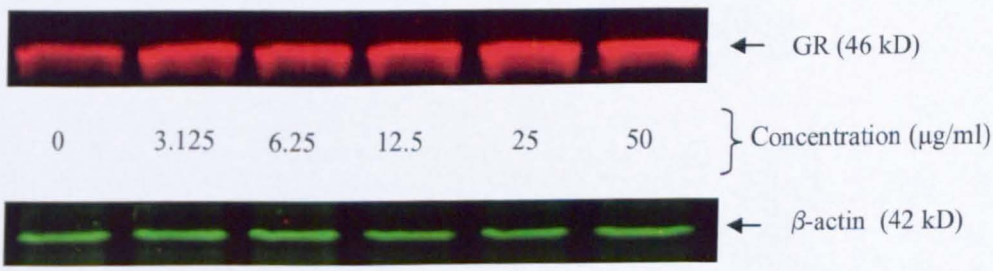


Figure 3-10: Expression levels of GR protein in quercetin-treated HepG2 cells. Cultures of HepG2 cells were treated with increasing concentrations of quercetin (0 – 50 µg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 700 nm (GR; red band) and 800 nm (β-actin; green band). Blots shown are representative of three independent experiments.

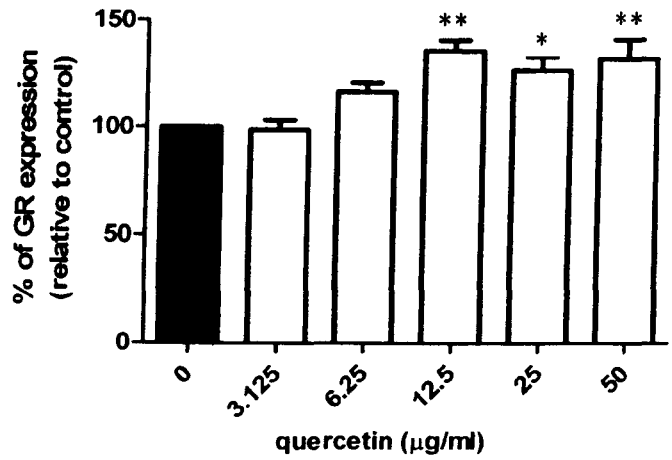


Figure 3-11: The effect of quercetin treatment on the expression of glutathione reductase (GR) in HepG2 cells. The monolayer cells were exposed to quercetin at increasing concentrations. All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent determinations. Significant results are denoted by $p<0.05$ = * and $p< 0.01$ = ** (One way ANOVA followed by Dunnett's tests).

Meanwhile, the effects of 24 hours treatment of cultured HepG2 cell line with *M. pajang* kernel extract on GR protein expression is shown in Figure 3-12. Analysis of band intensities obtained for GR protein expression (Figure 3-12), showed that *M. pajang* kernel extract treatment produced an induction of GR protein expression. At concentrations of 0.78 – 1.56 µg/ml, *M. pajang* kernel showed a slight induction of GR which was not statistically significant ($p > 0.05$). A significant induction was shown at concentrations from 3.125 – 50 µg/ml ($p < 0.05$) (Figure 3-13).

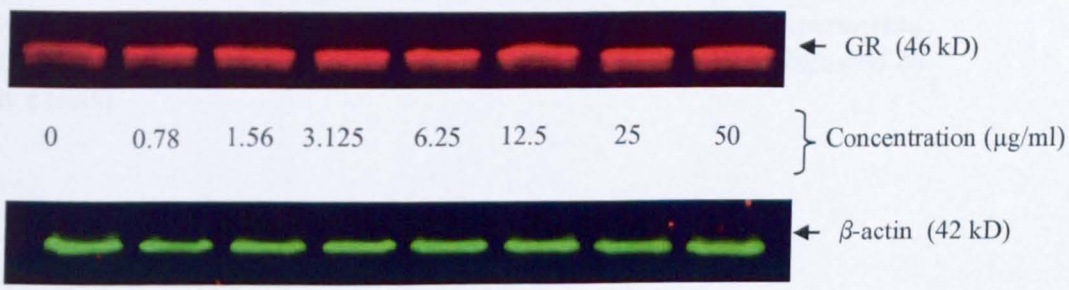


Figure 3-12: Expression levels of GR protein in *M. pajang* kernel extract - treated HepG2 cells. Cultured of HepG2 cells were treated with increasing concentrations of *M. pajang* kernel extract (0 – 50 µg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 700 nm (GR; red band) and 800 nm (β-actin; green band). Blots shown are representative of three independent experiments.

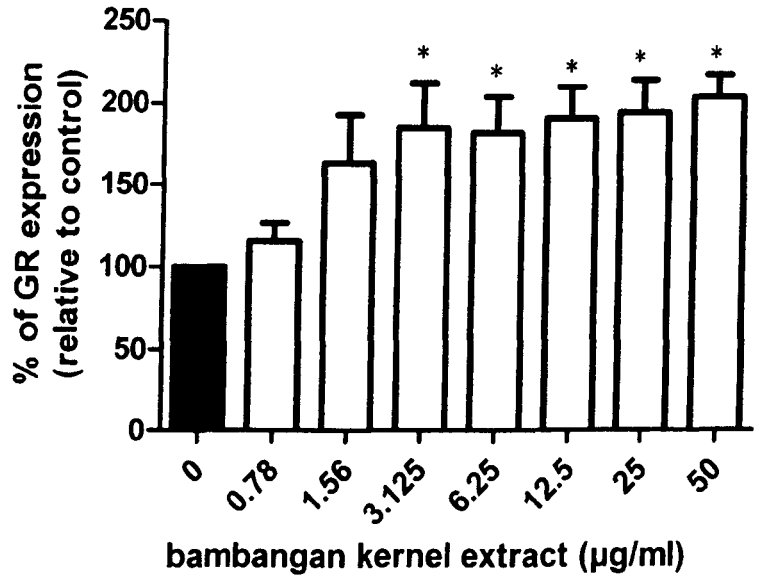


Figure 3-13: The effect of *M. pajang* kernel extract treatment on the expression of glutathione reductase (GR) in HepG2 cells. The monolayer cells were exposed to plant extracts at increasing concentrations. All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent experiments. Significant results are denoted by $p<0.05$ = * (One way ANOVA followed by Dunnett's tests).

3.3.4.3 Expression of MSRA

The effect of 24-hours treatment of cultured HepG2 cells with quercetin on the expression of MSRA was also evaluated as shown in Figure 3-14. Analysis of the band intensities obtained for MSRA (Figure 3-14), as optimized to that of β-actin bands, revealed that quercetin treatment produced a significant concentration-dependent increase in MSRA protein expression. Quercetin at a concentration of 6.25 μg/ml, produced a significant ($p < 0.05$) 50% increase in MSRA protein expression. The MSRA protein expression increased as quercetin concentrations increased (12.5, 25 and 50 μg/ml) recording a statistically significant induction with values of 170. 1 ± 11.8 %, 196.5 ± 14.64 % and 254.8 ± 15.3 % as compared to the control, respectively (Figure 3-15).

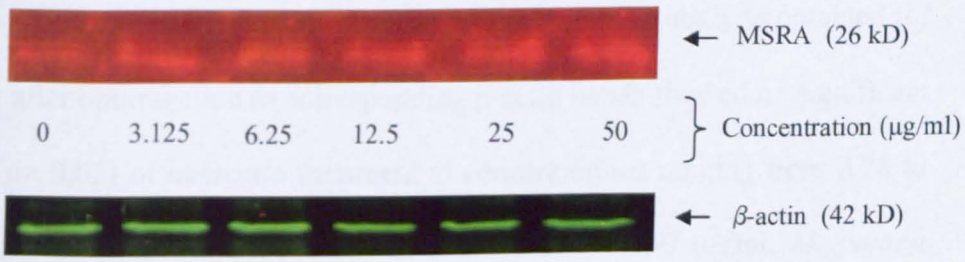


Figure 3-14: Expression levels of MSRA protein in quercetin-treated HepG2 cells. Cultures of HepG2 cells were treated with increasing concentrations of quercetin (0 – 50 μg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 700 nm (MSRA; red band) and 800 nm (β-actin; green band). Blots shown are representative of three independent experiments.

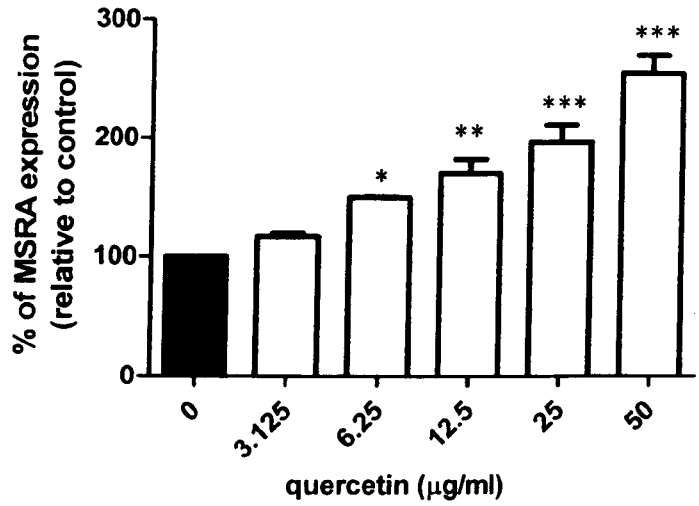


Figure 3-15: The effect of quercetin treatment on the expression of methionine sulfoxide reductase A (MSRA) in HepG2 cells. The monolayer cells were exposed to quercetin at increasing concentrations. All values were normalized to β-actin. The results were presented as relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent experiments. Significant results are denoted by $p<0.05$ = *; $p< 0.01$ = ** and $p<0.001$ = *** (One way ANOVA followed by Dunnett's tests)

Meanwhile, the effect of *M. pajang* kernel extract on the expression of MSRA in HepG2 is shown in Figure 3-16. Band intensity analysis obtained for MSRA after optimization to corresponding β-actin bands showed no significant effect ($p> 0.05$) of quercetin treatment at concentrations ranging from 0.78 to 6.25 µg/ml. However, at concentrations from 12.5 to 50 µg/ml, *M. pajang* kernel extract produced a significant induction of MSRA with the values of 147.3 ± 2.5, 145.8 ± 12.1 and 151.8 ± 8.2 % as compared to the control, respectively ($p < 0.05$) (Figure 3-17).

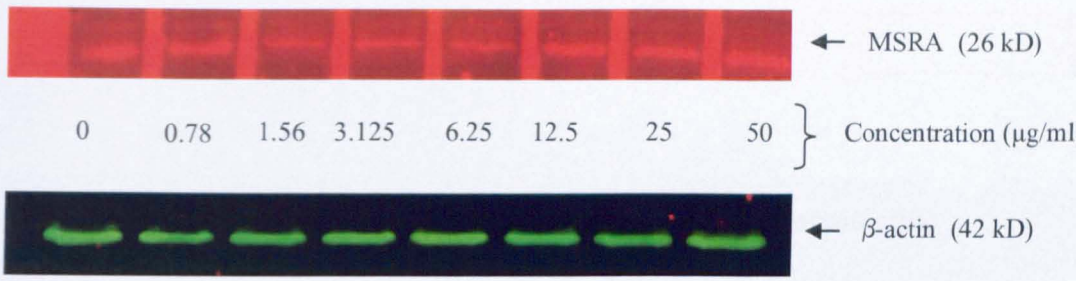


Figure 3-16: Expression levels of MSRA protein in *M. pajang* kernel extract - treated HepG2 cells. Cultured of HepG2 cells were treated with increasing concentrations of *M. pajang* kernel extract (0 – 50 µg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 700 nm (MSRA; red band) and 800 nm (*β*-actin; green band). Blots shown are representative of three independent experiments.

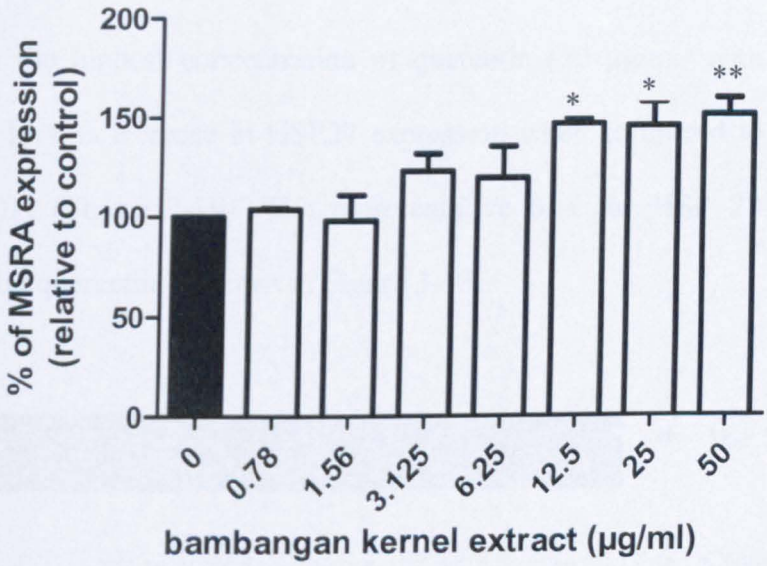


Figure 3-17: The effect of *M. pajang* kernel extract treatment on the expression of methionine sulfoxide reductase A (MSRA) in HepG2 cells. The monolayer cells were exposed to plant extract at increasing concentrations. All values were normalized to *β*-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent experiments. Significant results are denoted by $p<0.05$ = * and $p< 0.01$ = ** (One way ANOVA followed by Dunnett's tests).

3.3.4.4 Expression of HSP27

In order to investigate the possibility of multiple cytoprotective mechanisms for quercetin and *M. pajang* kernel extract in HepG2 cells, the expression of heat shock protein 27 (HSP 27) was also studied. The cells treated for 24 hours with quercetin (0 – 50.0 μg/ml) exhibited a concentration-dependent increase in HSP27 protein expression (Figure 3-18). Notably, the increase in HSP27 expression is between 1.3 – 3 fold as the concentration increases, when compared to the control. The most remarkable induction was noted in the highest concentration of quercetin (50 μg/ml) with a value of 302.2 ± 19.4 % increase in HSP27 expression when compared to the control ($p < 0.001$) (Figure 3-19). The representative blot for HSP 27 expression induced by quercetin is shown in Figure 3-18.

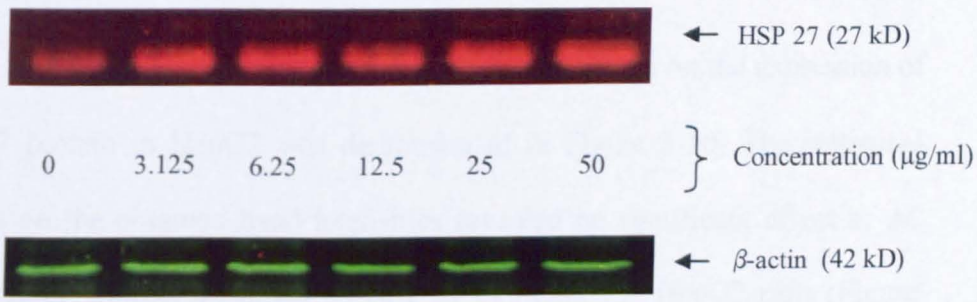


Figure 3-18: Expression levels of Heat Shock Protein 27 (HSP 27) in quercetin -treated HepG2 cells. Cultures of HepG2 cells was treated with increasing concentration of quercetin (0 – 50 μg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 700 nm (HSP 27; red band) and 800 nm (β-actin; green band). Blots shown are representative of three independent experiments.

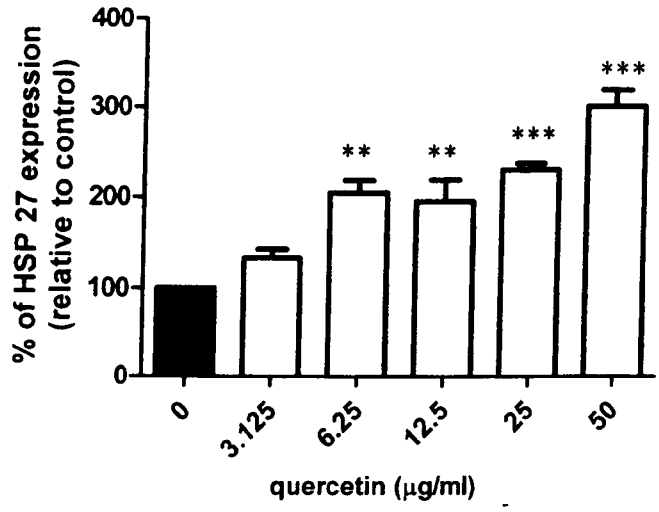


Figure 3-19: The effect of quercetin treatment on the expression of Heat Shock Protein 27 (HSP 27) in HepG2 cells. The monolayer cells were exposed to quercetin at increasing concentrations. All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent determinations. Significant results are denoted by $p<0.01$ = ** and $p< 0.001$ = *** (One way ANOVA followed by Dunnett's tests)

Meanwhile, the effects of *M. pajang* kernel extract on the expression of HSP 27 protein in HepG2 was demonstrated in Figure 3-20. The statistical analysis on the obtained band intensities revealed no significant effect of *M. pajang* kernel extract treatment on HSP 27 expression in HepG2 cells (Figure 3-21). The representative blot for HSP 27 expression induced by *M. pajang* is shown in Figure 3-20.

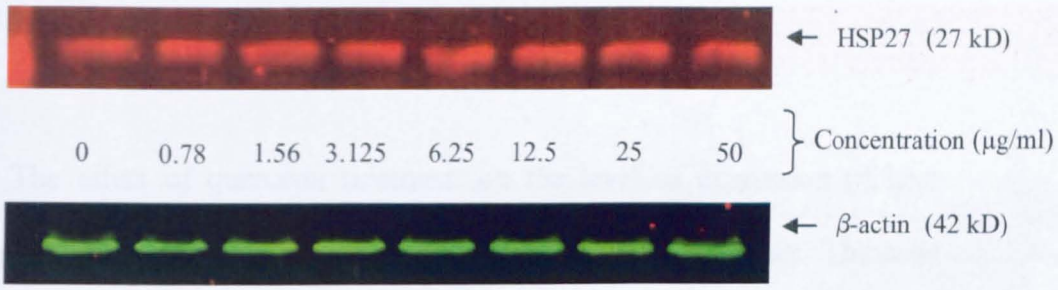


Figure 3-20: Expression levels of Heat Shock Protein 27 (HSP 27) protein in *M. pajang* kernel extract -treated HepG2 cells. Cultured of HepG2 cells were treated with increasing concentration of *M. pajang* kernel extract (0 – 50 µg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 700 nm (HSP27; red band) and 800 nm (β-actin; green band). Blots shown are representative of three independent experiments.

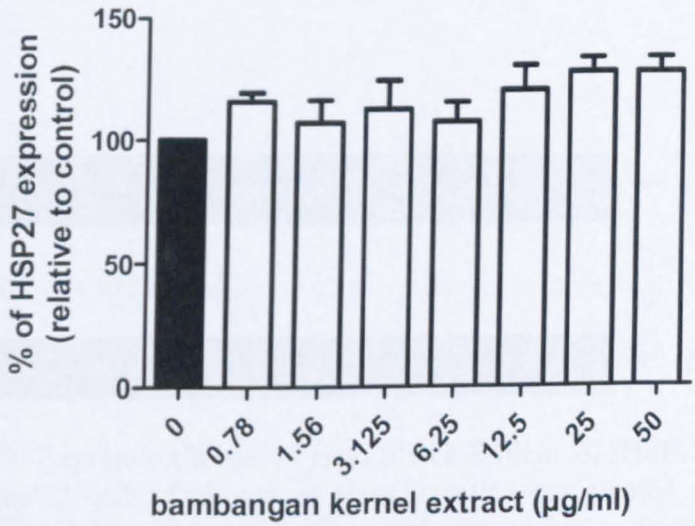


Figure 3-21: The effect of *M. pajang* kernel extract treatment on the expression of Heat Shock Protein 27 (HSP 27) in HepG2 cells. The monolayer cells were exposed to plant extract at increasing concentrations. All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent experiments. The One-way-ANOVA and the Dunnett's tests showed no significant statistical values of HSP27 expression following the *M. pajang* kernel extract treatment as compared to the control.

3.3.4.5 Expression of HSP70

The effect of quercetin treatment on the level of expression of heat shock protein 70 (HSP70) protein was also studied in HepG2 cells. The cells were treated with quercetin (0 – 50 μg/ml) for 24 hours. The results showed that the treatment of HepG2 cells with increasing concentrations of quercetin produced a significant concentration-dependent increase in the expression of HSP70 protein as shown in Figure 3-22. Quercetin at concentrations from 12.5 to 50.0 μg/ml elicited a 1.3 – 1.6 fold induction of HSP70 protein expression compared to the control ($p < 0.01$) (Figure 3-23).

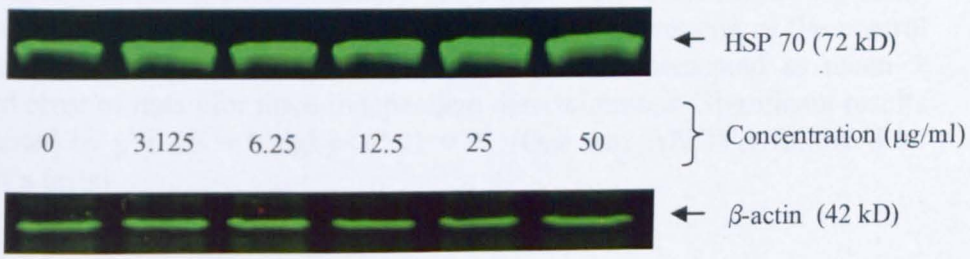


Figure 3-22: Expression levels of Heat Shock Protein 70 (HSP 70) in quercetin -treated HepG2 cells. Cultured of HepG2 cells were treated with increasing concentrations of quercetin (0 – 50 μg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 800 nm (green band). Blots shown are representative of three independent experiments.

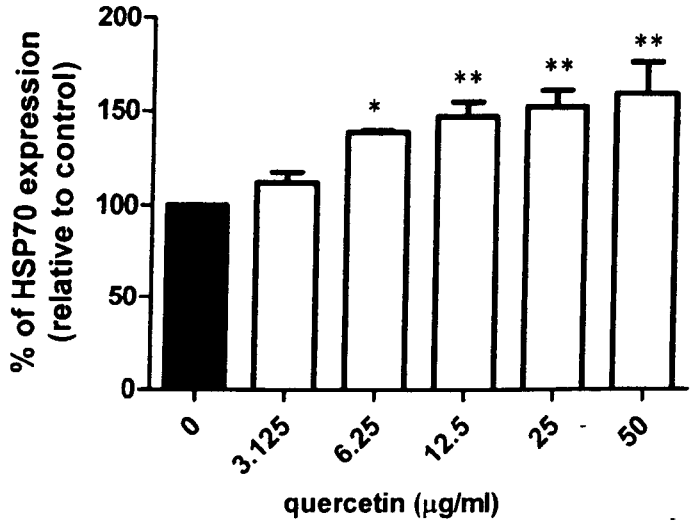


Figure 3-23: The effect of quercetin treatment on the expression of Heat Shock Protein 70 (HSP 70) in HepG2 cells. The monolayer cells were exposed to quercetin at increasing concentrations. All values were normalized to β-actin. The results were presented relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent determinations. Significant results are denoted by $p<0.05$ = * and $p< 0.01$ = ** (One way ANOVA followed by Dunnett's tests)

Meanwhile, for *M. pajang* kernel extract, HSP70 expression exhibited no statistically significant changes with respect to control although there was some suggestion of a slight increase upon analysis of the protein band obtained (Figure 3-24 and Figure 3-25)

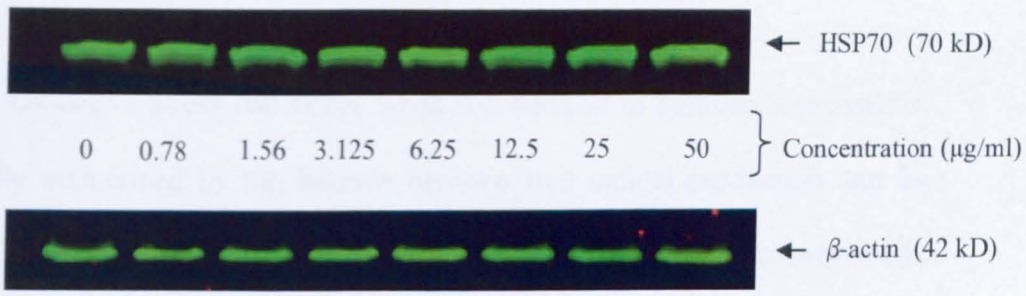


Figure 3-24: Expression levels of Heat Shock Protein 70 (HSP 70) protein in *M. pajang* kernel extract -treated HepG2 cells. Culture of HepG2 cells were treated with increasing concentrations of *M. pajang* kernel extract (0 – 50 µg/ml) for 24 hours. Protein bands were detected by using Odyssey® IR-scanner using a wavelength of 800 nm (green band). Blots shown are representative of three independent experiments.

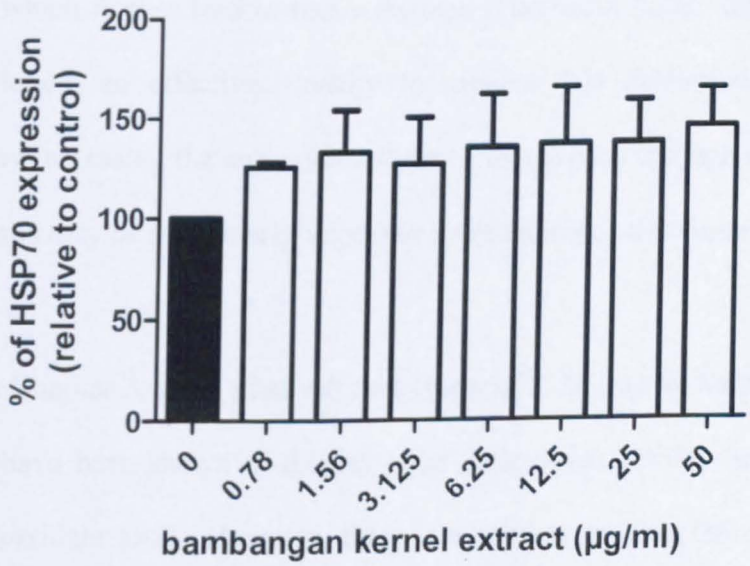


Figure 3-25: The effect of *M. pajang* kernel extract treatment on the expression of Heat Shock Protein 70 (HSP 70) in HepG2 cells. The monolayer cells were exposed to plant extract at increasing concentrations. All values were normalized to β-actin. The results were presented as relative to the percentage expression of the control (0 µg/ml containing 0.05% DMSO). The values are presented as mean ± standard error of mean for three independent experiments. One-way ANOVA and Dunnett's tests showed no significant statistical values of HSP70 expression following the *M. pajang* kernel extract treatment compared to the control.

3.4 Discussion

Oxidative stress can occur when the balance in cellular homeostasis, normally maintained by the balance between free radical production and its detoxification by cellular antioxidant, is jeopardized. The failure to correct this imbalance may lead to the tissue injury observed in many diseases, including cancer (Wattenberg, 1985; Willcox et al., 2004). Antioxidant defence systems (naturally occurring antioxidant molecules and enzymes) scavenge and minimize the formation of free radicals, but they are not 100% effective which will lead to extensive damage to macromolecules such as DNA, RNA and proteins which further lead to tissue damage (Halliwell, 2009; Willcox et al., 2004). Hence, an effective strategy to prevent this deleterious oxidative damage by increasing the antioxidant defence mechanism through diet-derived antioxidants may be particularly important in diminishing this damage.

In Chapter 2, some plant extracts (especially *M. pajang* kernel and peel extracts) have been shown to display good antioxidant activity in a non-cell based antioxidant assay. However, there is a need to confirm their activity at the cellular level since the non-cell based antioxidant assay did not measure the antioxidant activity with regard to on-going cell function. Therefore, the work presented in this chapter is designed to investigate the antioxidant activity of plant extracts or compounds at a cellular level. The HepG2 cell line was selected for the development of a cellular antioxidant assay for plant extracts or

compounds against oxidative damage caused by tert-butyl hydroperoxide (t-BHP). The possible mechanism of cytoprotection activity has also been determined, by studying the changes in expression of selected proteins believed to exert cytoprotective functions.

Why HepG2 cell line? Although HepG2 is a cell line derived from hepatocellular carcinoma, the cells have been shown to possess inducible phase I and phase II enzymes, and hence are able to activate or detoxify xenobiotics, thus reflecting the metabolism of xenobiotics in the human body and so are comparable and sometimes better than other metabolically incompetent cells used in cellular antioxidant assays (Mersch-Sundermann et al., 2004). This cell line also retains many of the specialized functions normally lost by primary hepatocytes in culture such as secretion of the major plasma proteins (Knowles et al., 1980). HepG2 cells have also been shown to express a wide range of enzymes including phase II detoxification enzymes such as quinone reductase (NQO1), glutathione-S-transferase (GST), glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase, (reviewed by Knasmuller et al., 1998; Lee et al., 2002). For these reasons, the HepG2 cell line was chosen for the cytoprotection assay.

In this experiment, tert-butyl hydroperoxide (t-BHP) was used as toxicant to the HepG2 cells. This compound has been shown to act as a potent inducer of reactive oxygen species, capable of inducing cell injury in different *in vitro* and *in vivo* systems (Mersch-Sundermann et al., 2004). t-BHP has been

shown to induce oxidative damage which leads to the GSH depletion, lipid peroxidation, ROS generation and decreases the antioxidant enzymes (i.e. glutathione peroxidise and glutathione reductase) activity in HepG2 cells prior to cell death (Goya et al., 2007). Therefore, t-BHP has been widely used to study the effects of antioxidant phytochemicals in HepG2 cells (Goya et al., 2007; Harper et al., 1999; Lima et al., 2007). In the present study, the treatment of the HepG2 cell line with t-BHP at a concentration of 0.325 mM produced a decrease in cell viability up to 88% as revealed by the cytotoxicity assay as compared to non-t-BHP treated control. This concentration has been optimized and used in our lab to run the cellular antioxidant assay.

For this work, it is crucial to determine the non-toxic concentration of the plant extracts or compounds. This step is to make sure that only the non-toxic concentrations were chosen for the next experiments; thus eliminating the possible induction of cytoprotective proteins caused by the cytotoxic effects of those plant extracts or compounds. The results showed that all plant extracts and compounds did not show any cytotoxic effects (after 24 hours of treatment) at concentrations of less than 50 μg/ml (Figures 3-2 and 3-5). Thus, this concentration range was used for further experiments. As for quercetin, previous studies reported that short-term exposure of HepG2 cells to quercetin at concentrations as high as 100 μg/ml did not show any signs of cytotoxicity as assessed by different cell viability assays (MTT and neutral red uptake assay) (Alia et al., 2006; Lima et al., 2006).

In the present study, the potential cellular antioxidant and cytoprotective effects of *M. pajang* kernel, peel and flesh extracts as well as *A. odoratissimus* seed and flesh extracts were tested against t-BHP induced toxicity in HepG2 cells. The results showed that out of all plant extracts, only *M. pajang* kernel extract displayed protection against oxidative damage caused by t-BHP, with a cytoprotective index value of 1.21 ± 0.13 μg/ml.

Meanwhile, the positive control (quercetin) also showed protective effects against t-BHP induced cell death. The cytoprotective activity of the positive control however showed lower anti-t-BHP activity as shown by a higher cytoprotective index value of 5.34 ± 0.66 μg/ml. The cytoprotective activity of quercetin in HepG2 is in agreement with previous studies (Alia et al., 2006; Lima et al., 2006). Beside quercetin, luteolin and phenolic acids (caffeic and rosmarinic acids) have also been shown to display cytoprotective activity against t-BHP induced oxidative damage in HepG2 cells (Lima et al., 2006)

The cytoprotective activity of *M. pajang* kernel extract in this experiment is in agreement with results obtained in the non-cell based antioxidant assay (Chapter 2). However, this experiment also suggested that plant extracts or compounds that displayed good antioxidant activity in the non-cell based antioxidant assay, do not necessary reflect the antioxidant activity in the cellular antioxidant assay (*M. pajang* peel extract and *A.*

odoratissimus seed extract display no cytoprotective activity at the concentrations tested, even though they are effective antioxidants in non-cellular antioxidant assays).

To elucidate the possible cytoprotective mechanism of *M. pajang* kernel extract and quercetin, western blotting technique was employed to investigate the expression of cytoprotective proteins (i.e. NQO1, GR, MSRA, HSP 27 and HSP 70), which might be involved in the protection of HepG2. Induction of cytoprotection proteins or endogenous antioxidant mechanism (i.e NQO1, GR, MSRA) have been considered as an ultimate goal in the strategy of chemoprevention and cytoprotection action (Wang and Hoguchi, 1995; Valerio et al., 2001; Moon et al., 2006). Activation of cytoprotection system may resolve the oxidative-damage that are already in progress, and to ensure long term protection against subsequent challenges (Dinkova-Kostova and Talalay, 2008). This assay was designed to investigate whether the antioxidant substances could modulate the expression of cytoprotection proteins, and may offer protection of the cells from injury caused oxidative damage.

First cytoprotective protein of interest is quinone reductase or NQO1. NQO1 is considered as one of the important cytoprotective antioxidant enzymes which helps in the detoxification of quinones, and so limits the formation of damaging semiquinone reactive intermediates which can further generate reactive oxygen species by oxidative cycling (Talalay and Kostova-Dinkova, 2008). Induction of this enzyme and other phase II detoxification

enzymes, that protect cells against oxidative stress caused by free radicals, has been considered as an effective strategy for cancer chemoprevention. This enzyme has also been used as a biomarker for the discovery of chemopreventive phytochemicals from natural resources (Bensasson et al., 2008; Cuendet et al., 2006; Dinkova-Kostova et al., 2004; Fahey et al., 2004; Talalay, 2000; Talalay and Dinkova-Kostova, 2004).

In the present study, the positive control (quercetin) displayed gradual induction of NQO1 protein expression in a non-toxic concentration-dependent response, although the statistical analysis revealed that the significant effect of NQO1 protein expression commenced at 12.5 μg/ml ($p<0.05$), followed by quercetin concentrations of 25 and 50 μg/ml, recording 2.2 and 2.4 fold increases in NQO1 protein expression over control ($p<0.01$ and $p<0.001$) respectively. This result is in agreement with previous studies which showed that quercetin induced NQO1 activity and protein expression in some mammalian cell lines (Hashimoto et al., 2002; Valerio et al., 2001).

However, as for *M. pajang* kernel extract, although this extract displayed better cytoprotective activity against t-BHT induced cell damage (*M. pajang* kernel extract displayed a lower cytoprotective index compared to quercetin), no significant induction of NQO1 protein expression was observed. This result suggested the role of other cytoprotective proteins which might be involved in the cytoprotection activity of the kernel extract.

The second enzyme of interest is glutathione reductase or GR. In the present study, both quercetin and *M. pajang* kernel extract significantly induced the expression of GR in HepG2 cells. This effect indeed is very crucial for the survival and cytoprotection of HepG2 against oxidative damage caused by t-BHP.

As reviewed by Lima et al. (2006) and Martin et al. (2001) t-BHP could be metabolized in the hepatocytes by glutathione peroxidase, generating oxidized glutathione (GSSG). Glutathione reductase plays a crucial role in maintaining the level of reduced glutathione (GSH) by converting the GSSG back to GSH at the expense of NADPH. Depletion of GSH and NADPH oxidation will lead to loss of cell viability (eventually leading to cell death) mediated by the disturbance of calcium homeostasis. A similar study by Lima et al. (2006) reported that five phenolic phytochemicals have been shown to display cytoprotective activity against t-BHP induced oxidative damage in HepG2 cells and that the mechanism involved in the cytoprotection activity is via protection against t-BHP induced GSH depletion. In addition, polyphenolic compounds have been shown to act as indirect antioxidants via modulating the activity of antioxidant enzymes, detoxifying and repairing enzymes as well as enzymes which involved in the elimination of xenobiotics (Ferguson, 2001; Ferguson et al., 2004) (i.e t-BHP in this present experiment).

A third enzyme of interest is methionine sulfoxide reductase A (MSRA). As reviewed previously (Chapter 1 - 1.5), MSRA is essential to

repair the formation of methionine sulfoxide (MetO) back to methionine after oxidation caused by free radicals. At the same time, MSRA could act in antioxidant defence mechanisms via scavenging reactive oxygen species; facilitating the cyclic interconversion of methionine residues between oxidized and reduced forms. In the present study, quercetin displayed a significant gradual induction of MSRA protein expression in a concentration-dependent manner. Statistical analysis showed that the increment started from concentration as low as 6.25 µg/ml ($p<0.05$). The induction of MSRA continued to rise up to 1.9 ($p< 0.01$), 2.1 ($p<0.001$) and 2.7 ($p<0.001$) fold more than the control. The concentration dependence of MSRA induction appeared to match that for cytoprotection ability. As for *M. pajang* kernel extract, the expression of MSRA was also observed to be concentration-dependent. However, significant results were only observed at higher concentrations ranging from 12.5 – 50 µg/ml. These findings appear to be the first report of induction of MSRA by plant phytochemicals. As such, these results offer a new approach to understanding the mechanisms of cytoprotection potential by phytochemicals in mammalian cells.

Previous studies showed that bacteria and yeast lacking MSRA genes are susceptible to oxidative stress and have lower survival rates. Meanwhile, high levels of oxidized methionine (MetO) are observed in yeast lacking MSRA genes which leads to the oxidative stress-induced cell damage (Moskovits et al., 1998, Moskovits et al., 2005). Moskovits et al. (2001) reported that, as compared to mice lacking of MSRA genes, wild type mice

(which display MSRA genes) (i) exhibit lower sensitivity to oxidative stress; (ii) have a longer lifespan under both normal and hyperoxic conditions; (iii) reduced aging process; (iv) accumulate less oxidized protein under oxidative stress; and (v) have induced the expression of thioredoxin (also an antioxidant protein acting as an intracellular thiols reductant) under stressed condition.

With regard to heat shock proteins or HSPs, the present study also evaluated the effect of quercetin and *M. pajang* kernel extract on the expression of two of these proteins (HSP 27 and HSP 70). These HSPs are widely conserved through evolution, suggesting the role in the survival of cells (De Maio, 1999). In the present study, the results show that quercetin induced the expression of both HSP 27 and HSP70 in a non-toxic-concentration-dependent manner. This phenomenon is probably as a consequence of mild proteotoxicity by quercetin which activates a cascade of events responsible for the expression of HSP 27 and HSP 70, which are aimed at repairing or degrading damaged protein (Urani et al., 2005). However, this study revealed that *M. pajang* kernel extract did not show any statistically significant change in either HSP27 or HSP70 expression in HepG2 cells over the range of concentrations tested ($p>0.05$). These results suggest that the cytoprotective activity of *M. pajang* kernel extract is not mediated by HSPs.

The expression of HSPs is a universal response to mild stress (i.e. heat shock, cold or chemical stress) which plays an important role in protecting cells to ensure their survival. These proteins are also considered as 'life guards'

for the survival of cell, where they act as chaperones for cellular proteins when the cells are under stress (Jaattela, 1999). Marimoto and Santoro (1998) suggested that 'a little stress is good' which refers to the fact that transient exposure to elevated temperatures or other forms of chemical stress as a cross-protective effect against normally lethal exposure to different forms of stress.

The expression of HSPs has also been reported to protect against cell death, by decreasing the intracellular reactive oxygen species, raising the total glutathione level and provide resistance against toxicants (e.g. hydrogen peroxide) (Mehlen et al., 1996). However, in the present experiment, the sort of response (with regards to quercetin) needs further investigation aimed at understanding how HSPs are involved in the defence mechanism.

3.5 Conclusion

Out of all the extracts tested, only *M. pajang* kernel extract displayed cytoptotective activity against t-BHP induced oxidative damage, as was also found for the positive control, quercetin. Taken together, the findings of the present study have revealed the possible involvement of NQO1, GR, MSRA, HSP27 and HSP70 in the cytoprotection activity of quercetin in HepG2 cells. Meanwhile, for *M. pajang* kernel extract, only GR and MSRA appeared to be involved in the cytoprotection activity of this extract in HepG2 cells. Further study is needed to investigate other cytoprotective proteins which might be involved in the cytoprotection mechanism.

CHAPTER 4

Anti-cancer properties of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*)

CONTENTS

CHAPTER 4

Anticancer properties of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*)

4.1 Introduction

Cancer is the second leading cause of mortality worldwide. Although much research has been conducted to search for the new treatment and prevention, still the number of cancer cases worldwide increases. Thus, there is a continuous effort to search for new chemotherapeutic drugs by screening the enormous pool of synthetic and natural resources (Mukherjee et al., 2001). Over the centuries, no fewer than 3,000 plant species have been used for chemotherapeutic and chemoprevention (Lewis and Elvin-Lewis, 1977). The World Health Organization (WHO) estimated that approximately 80% of the world's populations still depend on complementary and traditional medicine for the prevention and treatment of diseases, for which plant extracts are used most of the time (WHO, 2002).

Fruits and vegetables have been shown to contain a diverse source of phytochemicals such as carotenoids, tocopherols and polyphenols compounds which possess chemopreventive properties at multiple stages of carcinogenesis

(Dragsted et al., 1993; Liu, 2003, Naczk and Shahidi, 2006). Studies have suggested that these phytochemicals, especially polyphenols, display high antioxidant properties, which helps to reduce the risk of degenerative diseases, such as cancer and cardiovascular diseases (Ames et al., 1993). The anticancer mechanisms of those polyphenols are well established (Kim et al., 2006; Mertens-Talcott and Percival, 2005). The complex mechanism of the phytochemicals implies that the cancer chemopreventive properties of fruits, vegetables and their products are likely to arise from the combination of certain molecules present in them (Liu, 2003).

Our recent study (Chapter 2) showed that the kernel of *M. pajang* contains a high phenolic phytochemicals (103.3 mg gallic acid equivalent/g of dry weight) and displays high antioxidant properties compared to other fruit parts. While some plant extracts display potential antioxidant properties, they also have been shown to display cytotoxic effects in a wide range of cancer cell lines (Boivin et al., 2009; Ju et al., 2004) and therefore, it is postulated that *M. pajang* kernel extract will display cytotoxic properties in selected cancer cell lines. The objectives of this present study were to determine the anti-cancer potential of the fruit part extracts against selected cancer cell lines and investigate their possible mechanism(s) of action.

4.2 Materials and methods

4.2.1 Chemicals

RPMI 1640, foetal bovine serum (FBS), penicillin-streptomycin and accutase were obtained from PAA (Austria). MTT kit was purchased from Roche (USA). Annexin V-FITC - Propidium Iodide Apoptosis Detection Kit was purchased from Sigma (USA). All other chemicals and solvents used were of the highest pure grade available. Cell culture plasticware was from BD Falcon (USA).

4.2.2 Sample Collection

As described in Chapter 2 (2.3.1)

4.2.3 Extraction

As described in Chapter 3 (3.2.3)

4.2.4 Cytotoxicity assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The HepG2 (human liver cancer-derived), HT-29 (human colon cancer-derived), Caov3 (human ovarian cancer-derived), MCF-7 (hormone-dependent breast cancer) and MDA-MB 231 (non-hormone dependent breast cancer) cell lines were grown in RPMI 1640 culture medium, supplemented with 10% fetal bovine serum and 100 IU/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C under 5% CO_2 in a humidified atmosphere. All cell lines were bought from ATCC, USA. The viability of cells was determined by

staining with trypan blue. Exponentially growing cells were harvested and counted using a haemocytometer. The medium for the cell lines was used to dilute the cells to a concentration of $1x10^5$ cells/ml. From this cell suspension, 100 µl was pipetted into each well of a 96-well microtiter plate and incubated for 24 hours. The old medium was tapped out and sample extracts (diluted in medium) in ranges of 10, 20, 40, 60, 80 and 100 µg/ml were added to the plate. The plate was incubated for a further 72 hours. Then, 10 µl of MTT reagent (Roche, USA) was added to each well and the plate was incubated for 4 more hours. Subsequently, 100 µl of solubilization solution (Roche, USA) was added to each well. The cells were then left to incubate overnight. Finally, the absorbance was read using a microplate reader (at wavelength of 550 nm).

$$\% \text{ cytotoxicity} = \frac{\text{optical density of sample}}{\text{optical density of control}} \times 100\%$$

The inhibition concentration (IC_{50}), which is the concentration of extract able to inhibit cell proliferation by 50%, was calculated graphically for each cell proliferation curve.

4.2.5 Cell cycle analysis

Cells at a concentration of 1 x 10^5 cells/ml were incubated with the extracts at IC_{50} value for 24, 48 and 72 hours. All adherent and floating cells were harvested and transferred to a sterile centrifuge tube (~1 x 10^6 cells). The cells were then washed with cold phosphate buffer saline (PBS) (10 mM sodium phosphate pH 7.2, 150 mM sodium chloride) and resuspended in 0.5 ml of cold PBS. Ice-cold 70% ethanol (5 ml) was added to the cell suspension

and incubated at -20°C for 2 hours. The cells were then washed with cold PBS twice. Five hundred µl of propidium iodide/RNAse (10 µg/ml propidium iodide containing 1 mg/ml RNAse) solution was added to the cells and incubated in the dark for 30 minutes at room temperature. The cells were then analysed by flow cytometer (CyAn^{TM} ADP; Dako Cytomation, USA) within 3 hours of staining; 10,000 cells were collected for analysis. The results were analysed using Summit 4.3 software.

4.2.6 Annexin V-FITC Early Late Apoptosis study

Cells at a concentration of 1 x 10^5 cells/ml were incubated with the extracts at IC_{50} value for 12, 24, 36 and 48 hours. All adherant and floating cells were harvested and washed twice with PBS before being transferred to a sterile centrifuge tube (~1 x 10^6 cells). The cell pellet was then suspended in binding buffer (100 mM HEPES/NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM $CaCl_2$) at a concentration of 1 x 10^6 cells per ml. A sample (500 µl) of this cell suspension was then transferred to a 5 ml culture tube. Annexin V-FITC conjugate (5 µl) and propidium iodide (10 µl) were added to the cell suspension. The cells were gently vortexed and incubated for exactly 10 minutes at room temperature in the dark. The fluorescence of the cells was then immediately determined using flow cytometer (CyAn^{TM} ADP; Dako Cytomation, USA) and the results were analyzed using Summit 4.3 software.

4.2.7 Caspases activity assay

ApoTarget caspase colorimetric protease assay sample kit (Catalog Number KHZ10001, BioSource International) was used to determine the activity of caspases -2, -3, -6, -8 and -9. Cells (~3 x 10^6 cells) were collected and transferred into sterile test tubes and lysed using the cell lysis buffer supplied. Fifty microliters of the lysate were aliquoted into wells of a 96-well microplate. Fifty microliters of reaction buffer containing 10 mM DTT were then added to the sample wells. Five microliters of the 4 mM VDVAD-pna (substrate for caspase-2), DEVD-pna (substrate for caspase- 3), VEID-pna (substrate for caspase-6), IETD-pna (substrate for caspase-8), LEHD-pna (substrate for caspase-9), were added to the appropiate wells and the plate was then incubated at 37 °C for 2 hours. The plate was then read in a microplate reader (at a wavelength of 405 nm) and the absorbance from treated samples was compared with untreated control to allow the determination of any fold increase in caspase activity.

4.2.8 Statistical Analysis

All experiments were carried out with 3 independent experiments and values presented as mean ± standard error of mean (S.E.M.) using Prism 5. Flow cytometry data (cell cycle and annexin-V FITC) were analysed using student's T-test (unpaired). Meanwhile, the caspases were statistically analysed by one-way ANOVA using Dunnett's post-hoc test. All analysis applying a significance level of $p < 0.05$.

4.3 Results

4.3.1 Cytotoxic effects of M. pajang and A.odoratissimus crude extracts

Study of cellular proliferation is crucial for biological research, especially in cancer research. The MTT assay is one of the methods widely used to study the effects of natural products on cell proliferation, viability and toxicity. This assay is based on the reduction of a tetrazolium salt to purple formazan crystal by metabolically active cells (Mosmann, 1983). The absorbance of the solubilised formazan crystals is taken as a reflection of the number of living cells.

The results showed that the crude kernel extract of *M. pajang* displayed a broad spectrum of inhibition of cancer cell proliferation in all cancer cell lines tested (Table 4-1). The peel of *M. pajang* inhibited the proliferation of liver and ovarian cancer lines with IC_{50} values of 36.5 and 55.0 μg/ml. No IC_{50} values were detected by *M. pajang* flesh, and the seed and flesh of *A. odoratissimus* in all cancer cell lines tested as proliferation was inhibited less than 50% at the highest concentration tested (100 μg/ml).

M. pajang kernel extract displayed the strongest inhibition on breast cancer cell lines, as compared to other cell lines. As illustrated in Figure 4-1, incubation of *M. pajang* kernel extracts with breast cancer cell lines (MCF-7 and MDA-MB-231) inhibited cell proliferation in a concentration-dependent manner (from 10 – 40 μg/ml), with little further change in effect in the

concentration range of 40 – 100 μg/ml. From figure 4-1, the IC_{50} values (amount of the extract (μg/ml) to inhibit cell proliferation by 50%) were determined and for subsequent experiments to examine effects of the extracts on cell cycle distribution and apoptosis, these IC_{50} values were used (MCF-7: 23.0 μg/ml and MDA-MB-231: 30.5 μg/ml).

Samples	HepG2	HT-29	Caov3	MCF-7	MDA-MB-231
M. pajang					
Flesh	>100	>100.	>100	>100	>100
Peel	36.5 ± 1.2	>100	55.0 ± 3.2	>100	>100
Kernel	34.5 ± 2.3	63.0 ± 3.2	92.0 ± 4.5	23.0 ± 1.6	30.5 ± 2.1
A. odoratissimus	>100	>100	>100	>100	>100
Flesh	>100	>100	>100	>100	>100
Seed					

Table 4-1: IC_{50} values (μg/ml) of *M. pajang* and *A. odoratissimus* crude extracts.
Values are presented in mean ± S.E.M of three independent experiments.
>100 : IC_{50} value greater than the top concentration of 100 μg/ml tested.

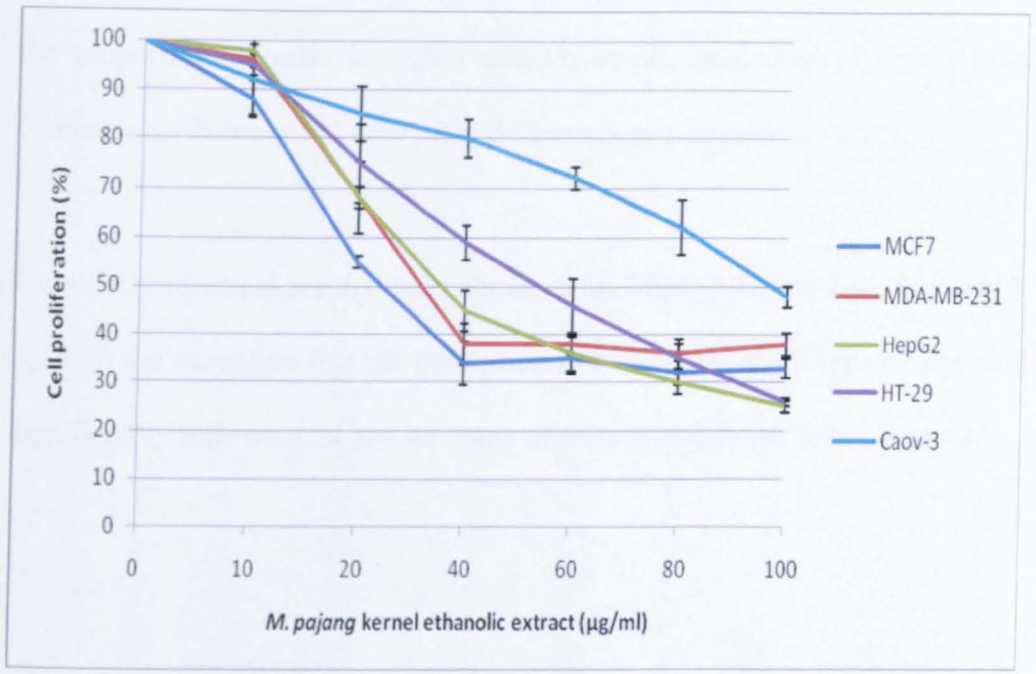


Figure 4-1: The effect of the *M. pajang* kernel extract on MCF-7, MDA-MB-231, HepG2, HT-29 and Caov-3 cell proliferation. Cells (1 x 10^4 cells/well) were incubated at different concentrations of *M. pajang* kernel ethanolic extract (0–100 µg/ml of cell growth media) for 72 hours. Cell proliferation was evaluated as the ability to reduce MTT to blue formazan crystals. Results are presented as mean ± standard error of mean for 3 independent experiments.

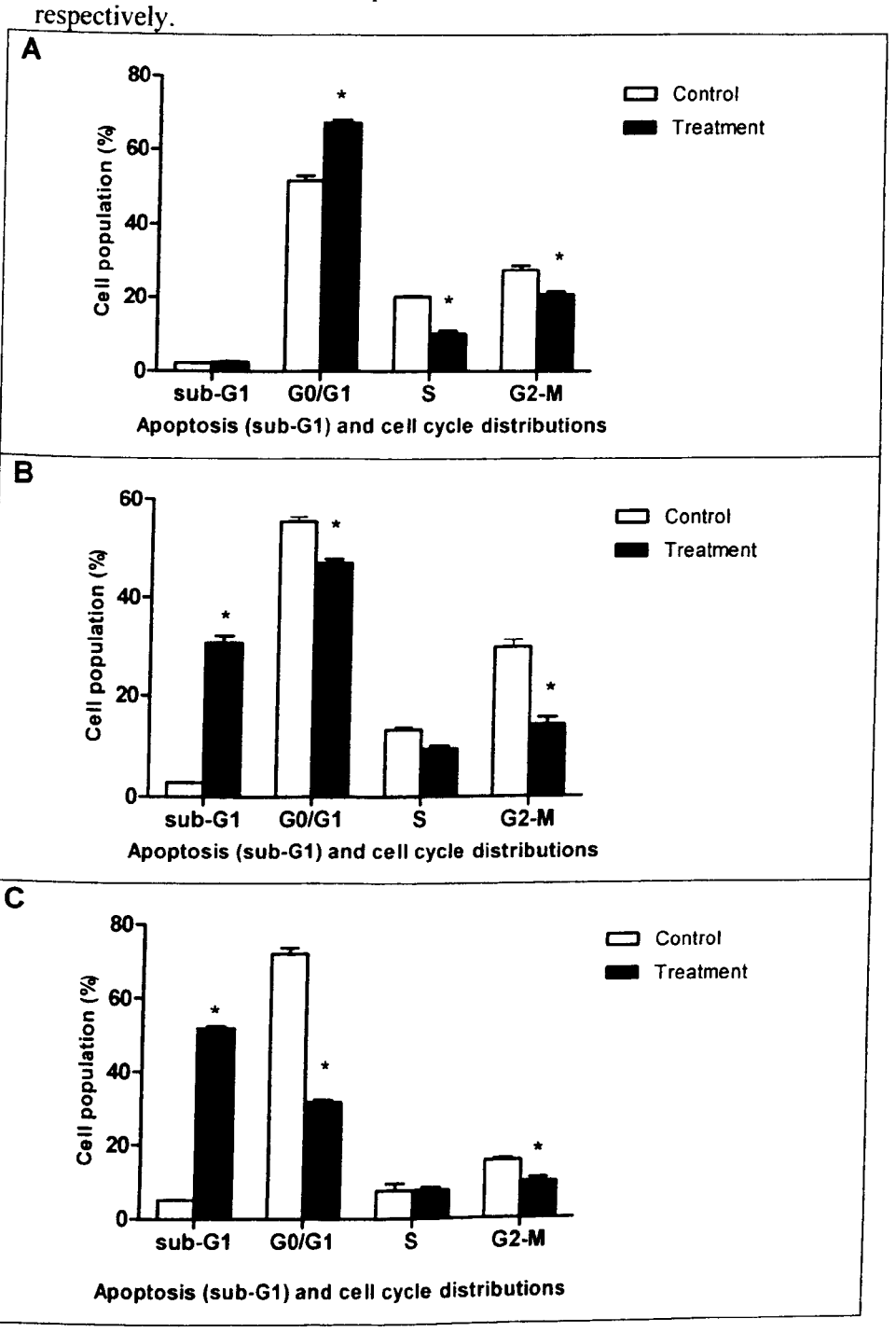
4.3.2 Effects of M. pajang kernel extract on cell cycle distribution

Apoptosis (cells with broken DNA) and cell cycle distribution in MCF-7 and MDA-MB-231 cells were studied after exposure to *M. pajang* kernel extract at IC_{50} concentration for 24, 48 and 72 hours (Figure 4-2 and Figure 4-3). For MCF-7, a significant arrest at G_0/G_1 was observed at 24 hours of treatment ($p<0.05$), whilst the number of cells in S and G_2-M phases were reduced significantly ($p<0.05$); apoptosis (sub-G_1 phase) was not observed during this period of time. Apoptosis also occur during G_0/G_1 arrest besides

sub-G_1 for the first 24 hours of the experiment. After 48 and 72 hours of treatment, the apoptosis phase has significantly increased up to 30.7 % and 51.8 % compared to their respective controls (2.9 % and 5.2 %) ($p<0.05$). Also, the proportion of cells in G_0/G_1 and G_2-M phases decreased significantly compared to their control after 48 and 72 hours of treatment ($p<0.05$).

Essentially identical results were obtained for MDA-MB-231 cells (Figure 4-3), with the exception that the proportion of cells in the G_2-M phase increased significantly following 24 and 48 hours of treatment with the crude extracts.

Figure 4-2: Cell cycle analysis of MCF-7 cancer cells treated with *M. pajang* kernel ethanolic extract at IC_{50} value.
The distribution of cells undergoing apoptosis and in various phases of the cell cycle was determined in MCF-7 cells following treatment with *M. pajang* kernel extract for 24 hours (A), 48 hours (B) and 72 hours (C) in comparison to their respective control. The values are presented as mean ± standard error of mean for three determinations, and, where indicated by *, showed a significant difference ($P<0.05$) relative to their respective control.
Representative flow cytometric scans of untreated MCF-7 cells and those treated with *M. pajang* kernel ethanolic extract for 24, 48, and 72 hours are presented in figures D-G respectively. Sectors 1-4 represent the cells in sub G_1, G_0/G_1, S, and G_2-M phases respectively.



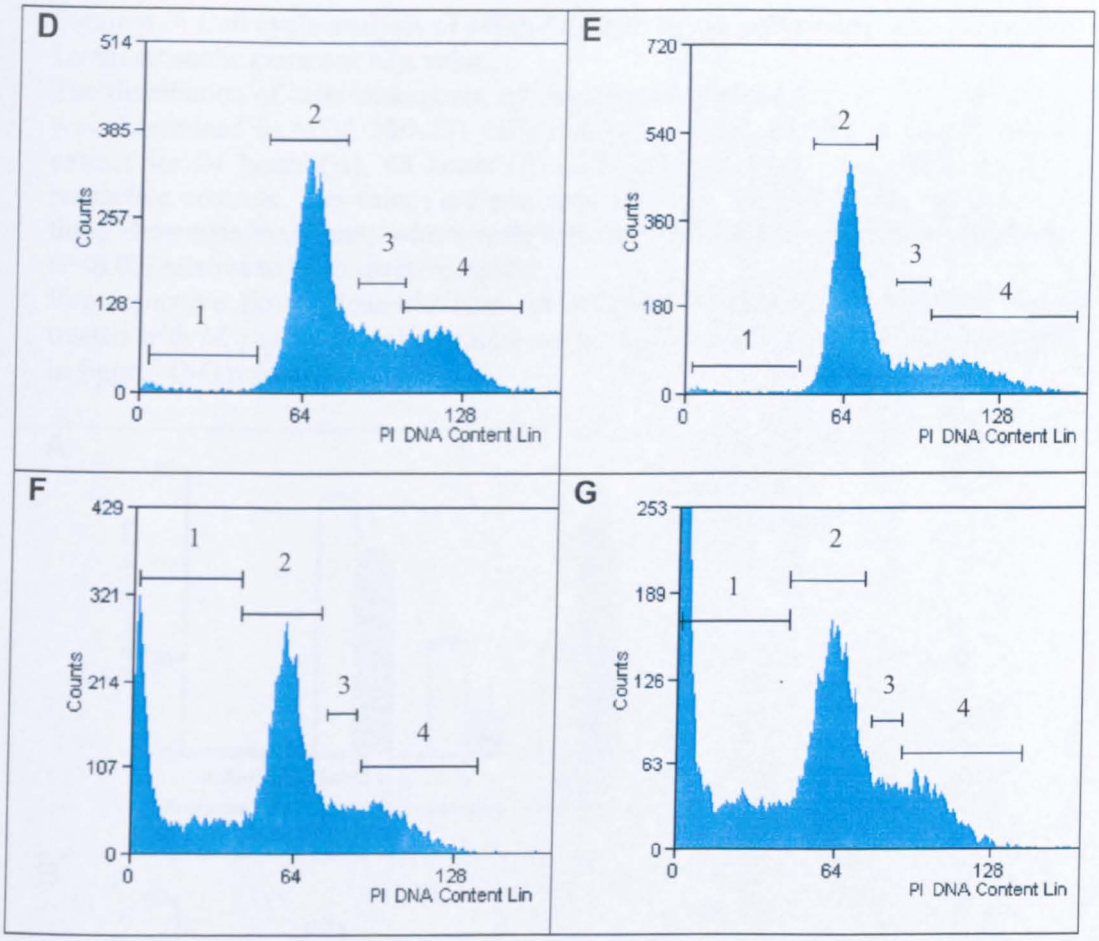

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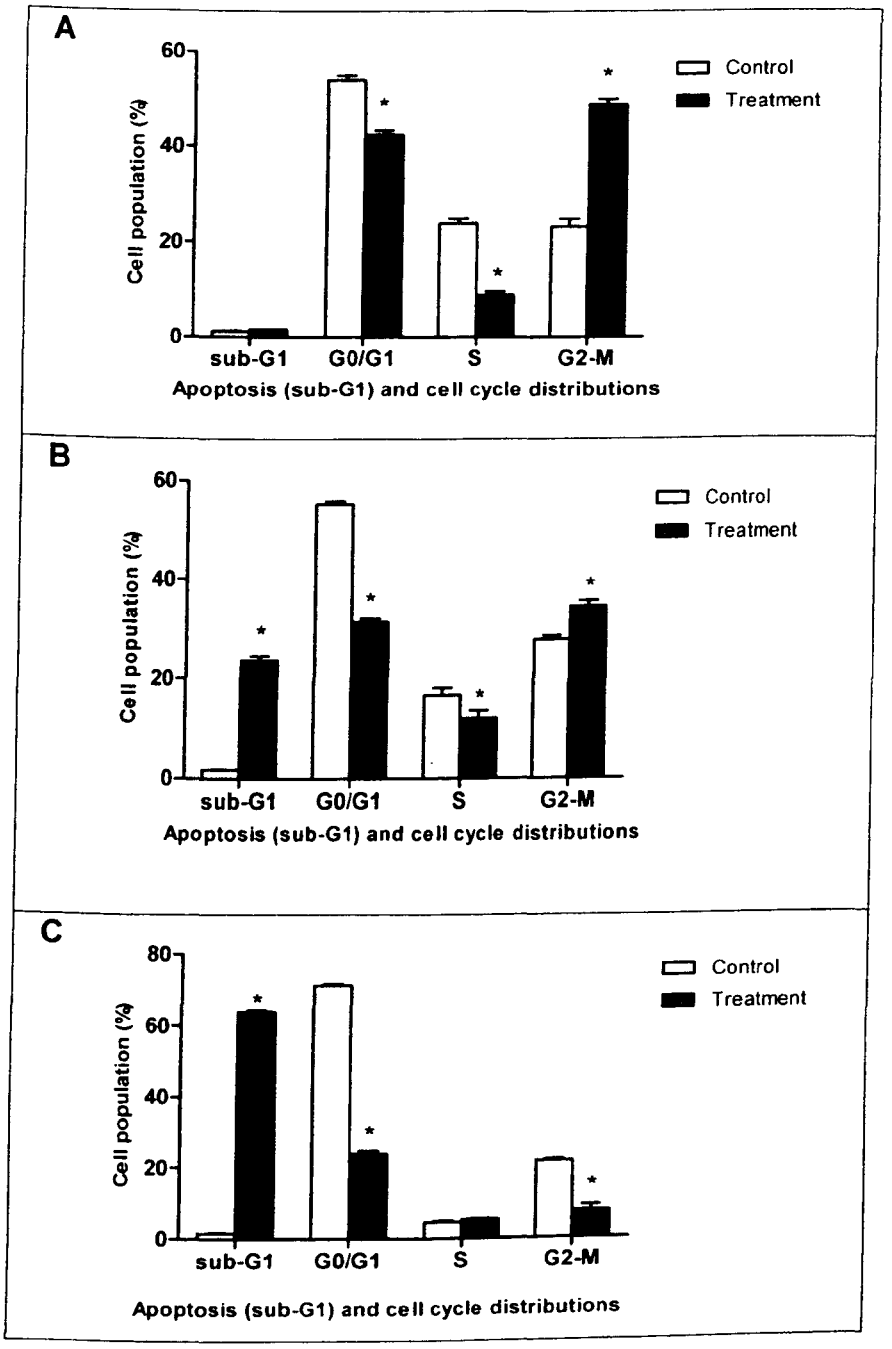
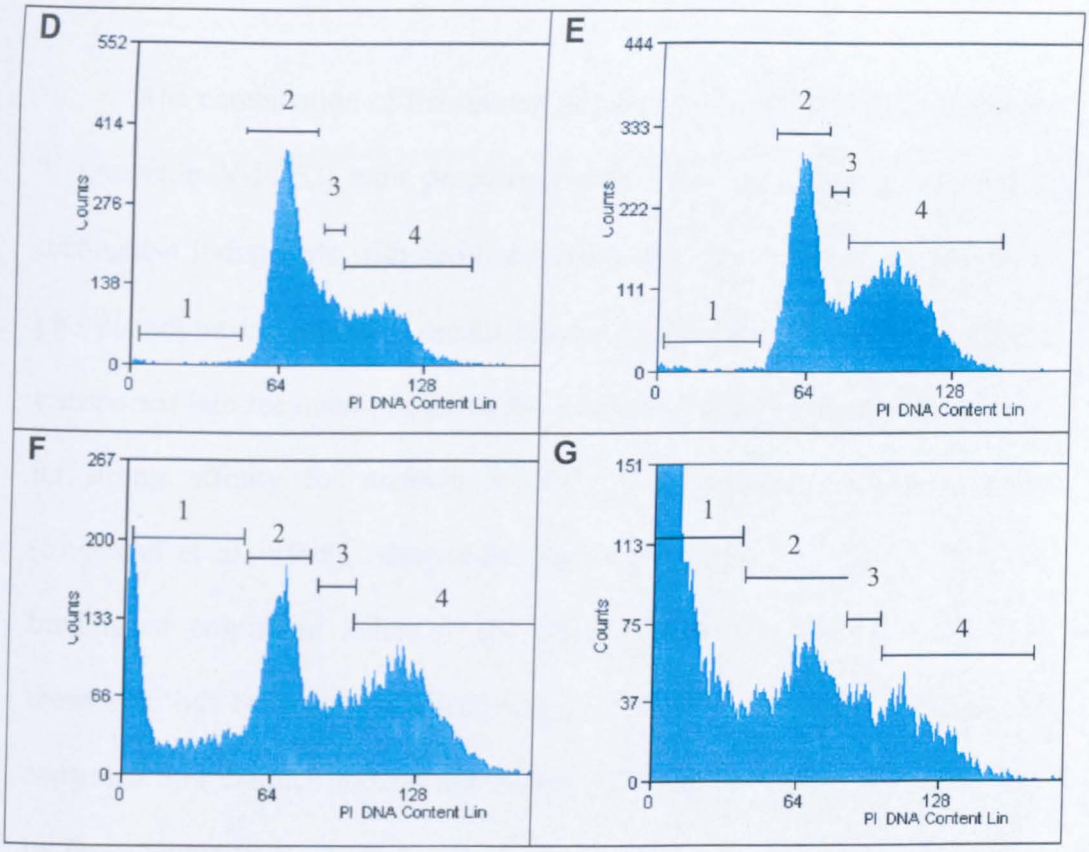
Figure 4-3: Cell cycle analysis of MDA-MB-231 cancer cells treated with *M. pajang* kernel ethanolic extract at IC_{50} value.
The distribution of cells undergoing apoptosis and in various phases of the cell cycle was determined in MDA-MB-231 cells following treatment with *M. pajang* kernel extract for 24 hours (A), 48 hours (B) and 72 hours (C) in comparison to their respective controls. The values are presented as mean ± standard error of mean for three determinations, and, where indicated by *, showed a significant difference ($P<0.05$) relative to the respective control.
Representative flow cytometric scans of untreated MDA-MB-231 cells and those treated with *M. pajang* kernel ethanolic extract for 24, 48, and 72 hours are presented in figures D-G respectively.

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4.3.3 Effects of M. pajang kernel extract on early and late apoptosis

The combination of fluorescein isothiocyanate (FITC)-labelled annexin V (Annexin V-FITC) with propidium iodide has been intensively used to distinguish living cells with early and late apoptosis. During early apoptosis, phosphatidylserine, which is usually located in the inner membrane of cells, is transported into the outer portion of the membrane, and this can be detected by its strong affinity for annexin V-FITC, a phospholipid binding protein (Engeland et al., 1998). Meanwhile, the dead cells can be detected by the binding of propidium iodide to the cellular DNA in cells where the cell membrane has been compromised. Because of these properties, cells can be separated into distinct populations (intact, early apoptosis and late apoptosis) by flow cytometry.

For MCF-7, no significant difference in the proportion of cells that were intact or undergoing either early or late apoptosis could be detected in cells treated with *M. pajang* kernel extract for 12 and 24 hours of treatment when compared to the controls ($p>0.05$) (Figure 4-4). However, following 36 hours of treatment, the proportion of cells in the live cell group declined concomitantly with an increase in the proportion of cells in early and late apoptosis with values of 50.2 %, 14.0 % and 25.3%, respectively ($p<0.05$). At the end of 48 hours of treatment, the total apoptosis increased up to 73.9 % (comprised of early apoptosis: 12.8% and late apoptosis: 61.1%) (Figure 4-4). Similarly, no significant apoptosis event occurred during 12 hours exposure of

M. pajang kernel extract in MDA-MB-231 cells (Figure 4-6). However, treatment of the cells for 24 hours resulted in a strong shift from live cells to early and late apoptotic cell populations with the value of 13.4 % and 11.3 %, respectively ($p<0.05$). Total apoptosis increased following 36 and 48 hours of treatment to levels of 80.2 % (early apoptosis: 16.5 %; late apoptosis: 63.7 %) and 89.3% (early apoptosis: 9.0 %; late apoptosis: 80.3%) respectively (Figure 4-6). Figure 4-5 and Figure 4-7 show representative flow cytometric scans of MCF-7 and MDA-MB-231 cells after the incubation of cells with Annexin-V FITC positive and propidium iodide, respectively.

Figure 4-4: Apoptosis study of MCF-7 cells exposed to *M. pajang* kernel ethanolic extract at IC_{50} value.
The distribution of cells undergoing early and late apoptosis, together with those live cells not in apoptosis (intact cell) and the total extent of apoptosis, was determined in MCF-7 cells following treatment with *M. pajang* kernel extract for 12 hours (A), 24 hours (B), 36 hours (C), and 48 hours (D) in comparison to their respective controls, using Annexin-V FITC and propidium iodide flow cytometric analysis. The values are presented as mean ± standard error of mean for three determinations, and, where indicated by an asterisk, showed a significant difference ($P<0.05$) relative to the respective control.

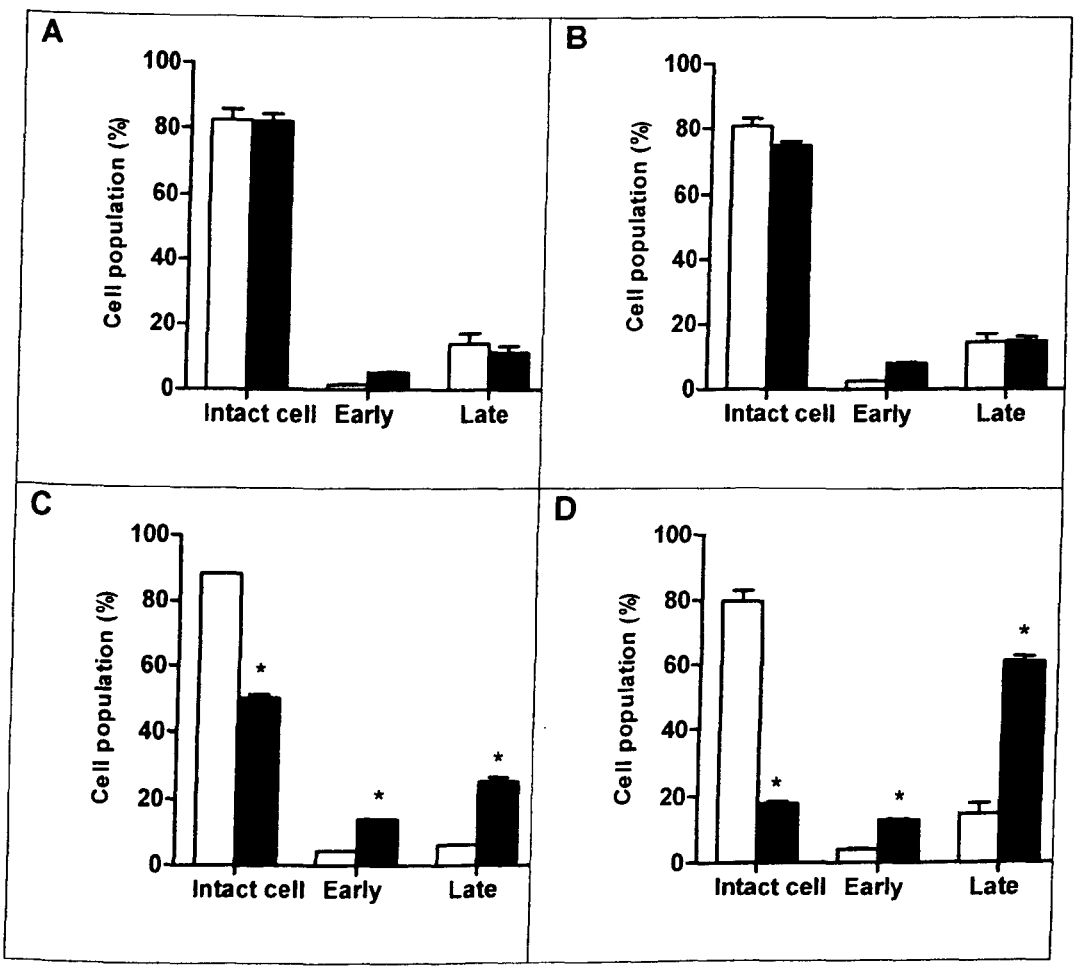


Figure 4-5: Representative flow cytometric scans of untreated MCF-7 cells and those treated with *M. pajang* kernel ethanolic extract for 12, 24, 36, and 48 hours are presented in figures E-I respectively. Note that in the control group (E), most of the intact (live) cells are categorized into a double negative group (lower left quadrant, LL). Early apoptosis is categorized into Annexin-V FITC positive and propidium iodide negative group (lower right quadrant, LR) and late apoptosis is categorized into double positive group (upper right quadrant, UR).

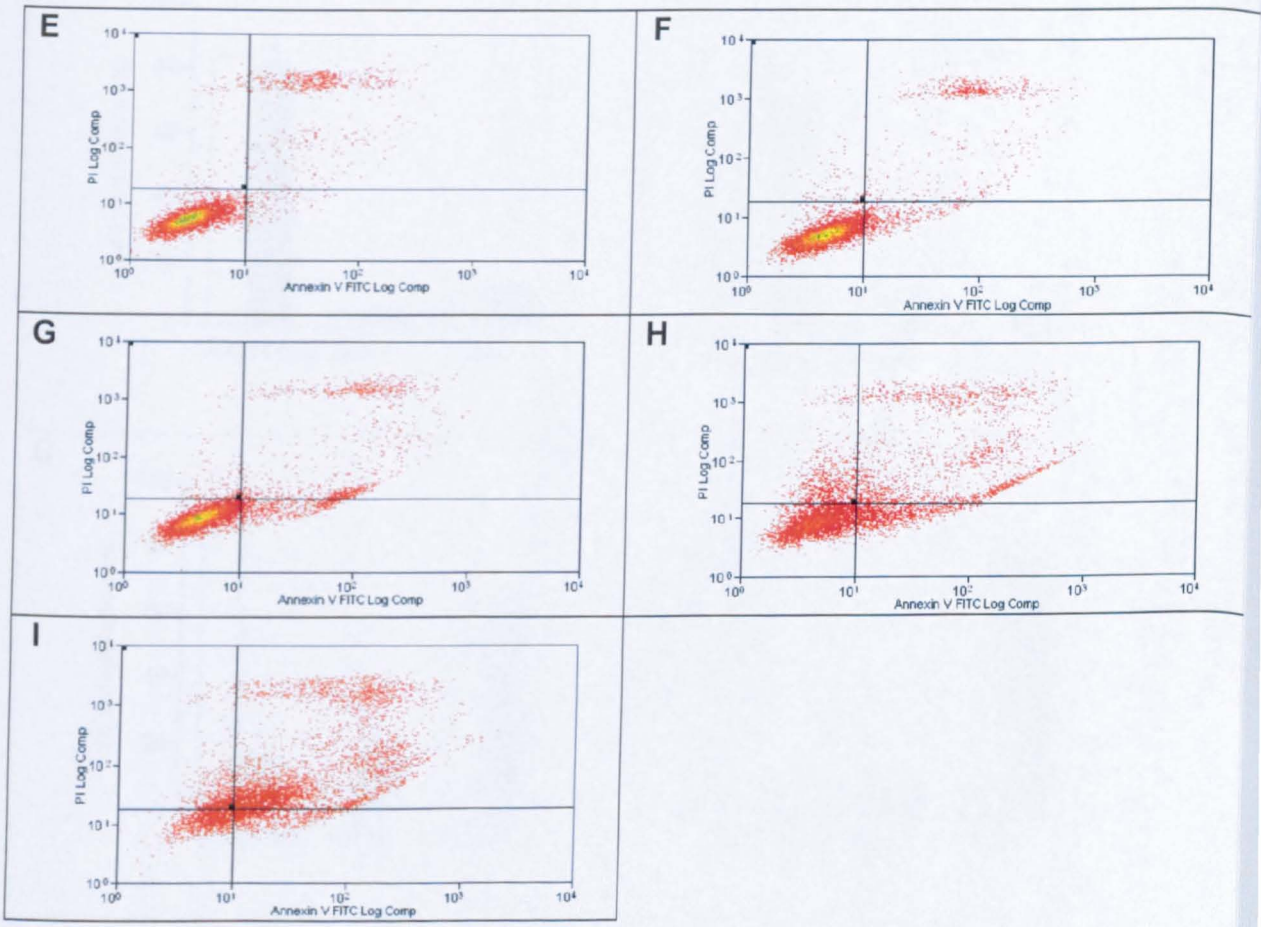


Figure 4-6: Apoptosis study of MDA-MB-231 cells exposed to *M. pajang* kernel ethanolic extract at IC_{50} value.
The distribution of cells undergoing early and late apoptosis, together with those live cells not in apoptosis (intact cell) and the total extent of apoptosis, was determined in MDA-MB-231 cells following treatment with *M. pajang* kernel extract for 12 hours (A), 24 hours (B), 36 hours (C), and 48 hours (D) in comparison to their respective controls, using Annexin-V FITC and propidium iodide flow cytometric analysis. The values are presented as mean ± standard error of mean for three determinations, and, where indicated by an asterisk, show a significant difference ($P<0.05$) relative to their respective controls.

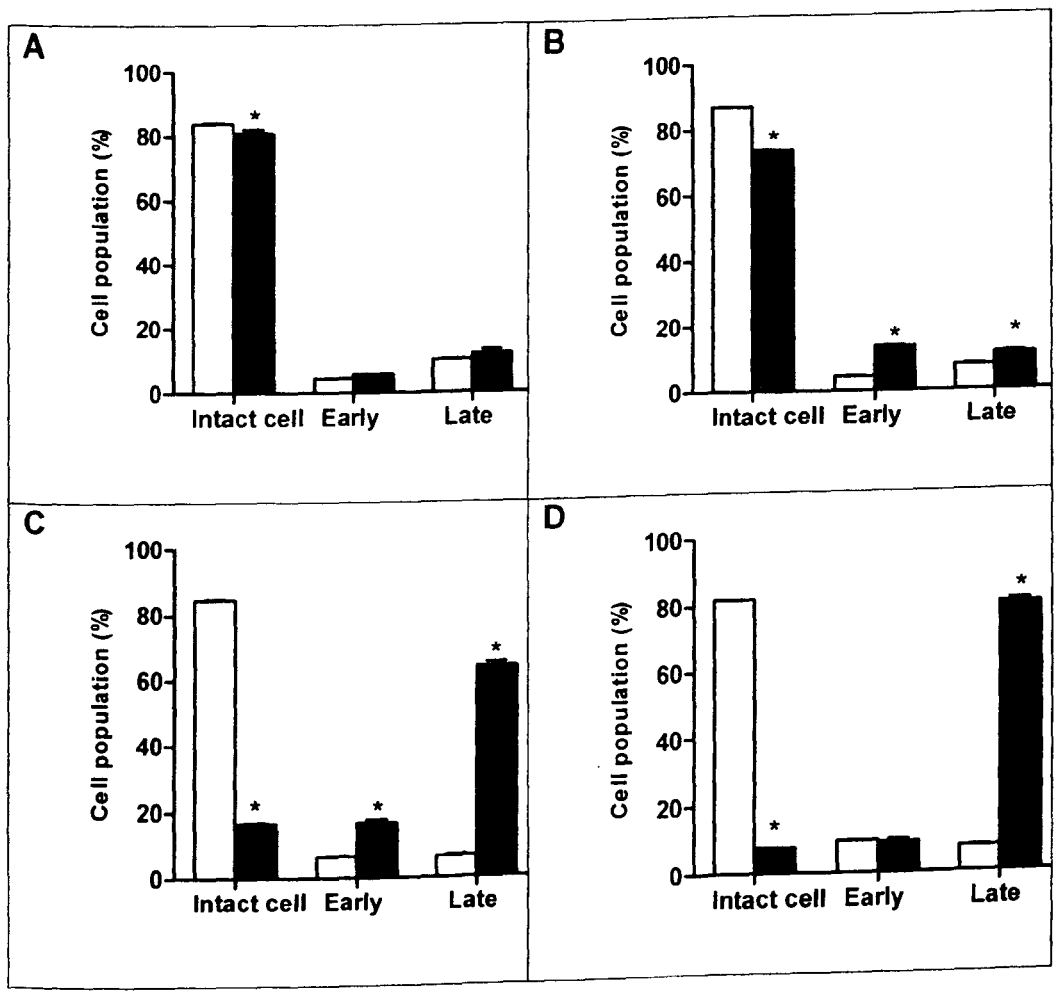
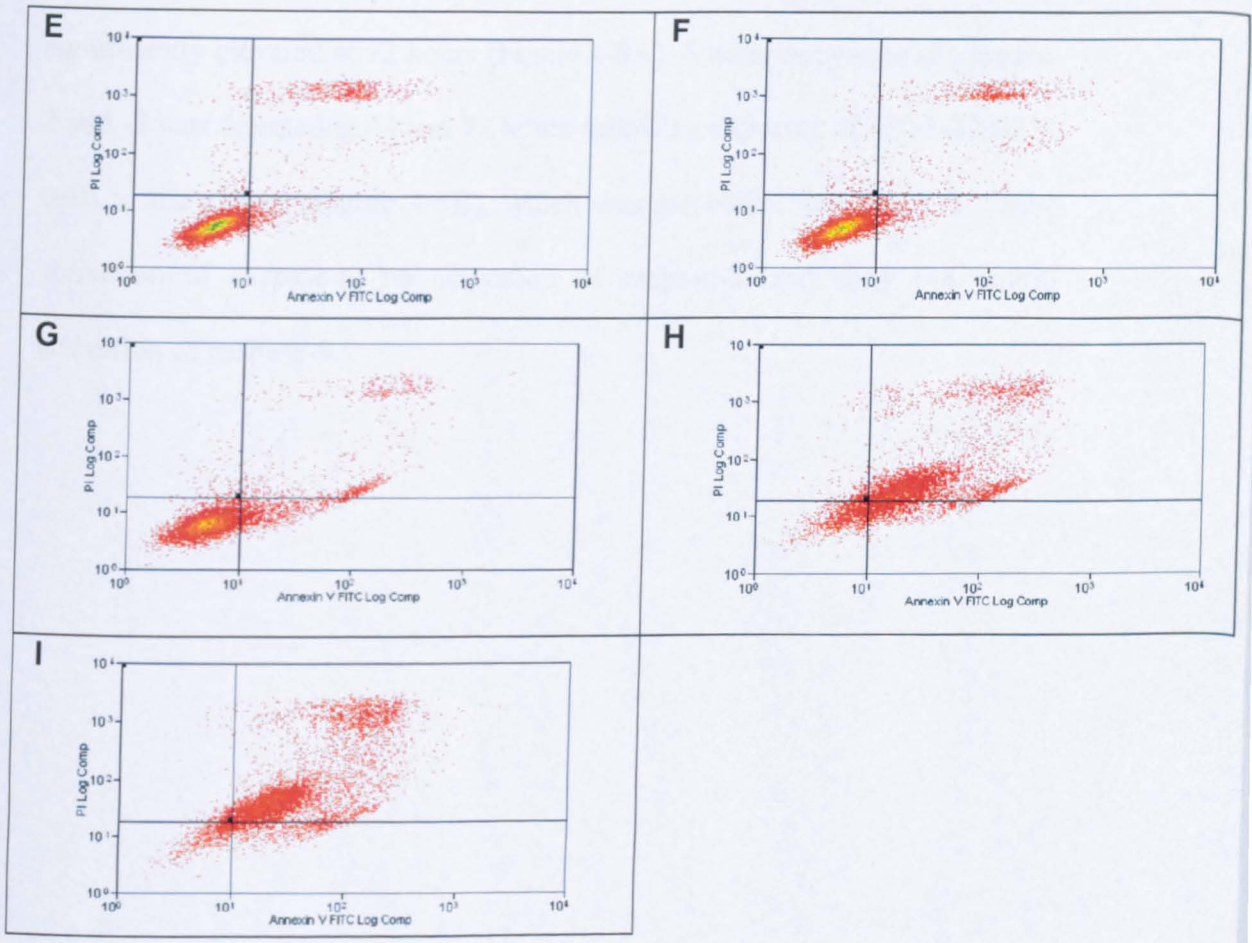


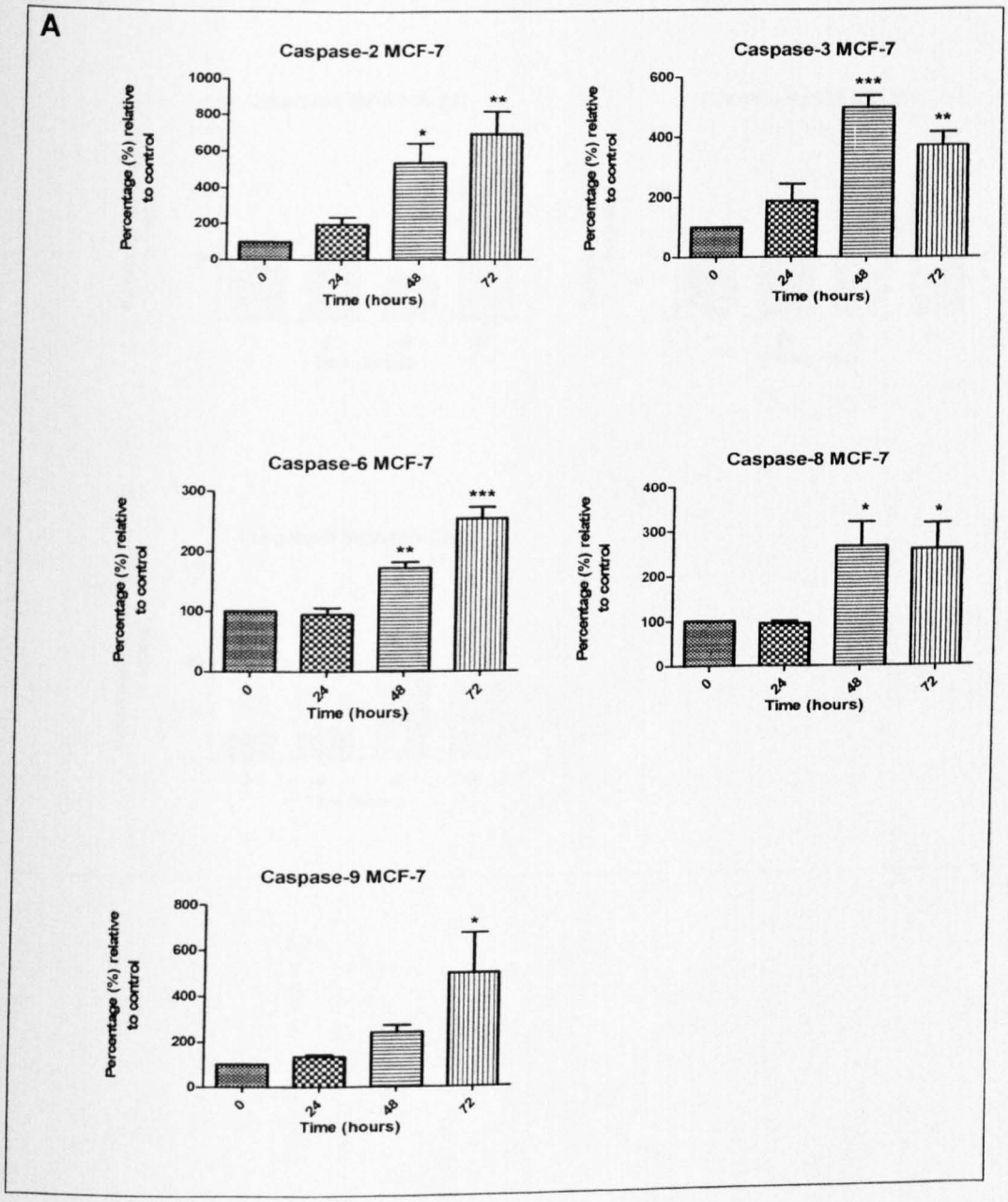
Figure 4-7: Representative flow cytometric scans of untreated MDA-MB-231 cells and those treated with *M. pajang* kernel ethanolic extract for 12, 24, 36, and 48 hours are presented in figures E-I respectively. Note that in the control group (E), most of the intact (live) cells are categorized into a double negative group (lower left quadrant, LL). Early apoptosis is categorized into Annexin-V FITC positive and propidium iodide negative group (lower right quadrant, LR) and late apoptosis is categorized into double positive group (upper right quadrant, UR).

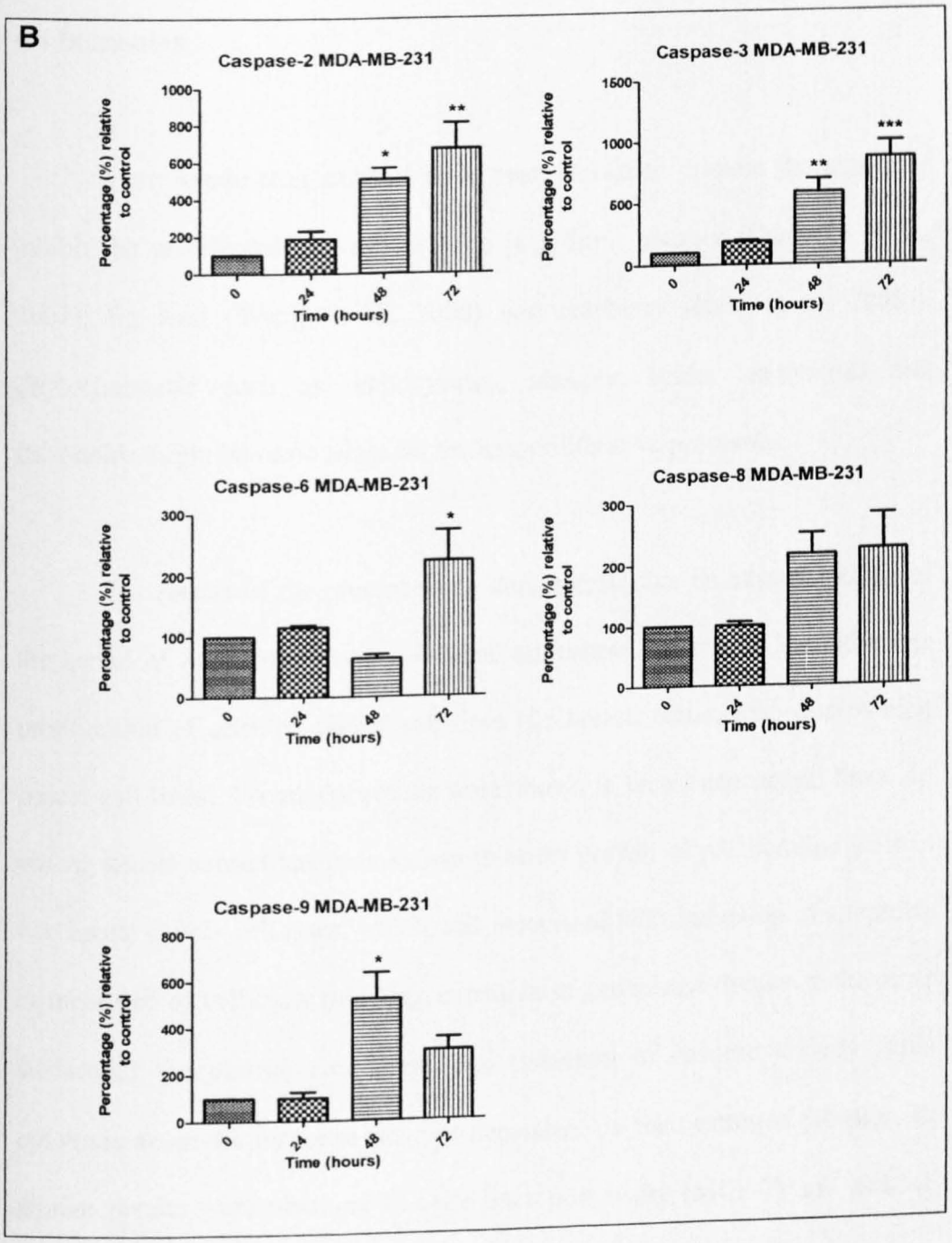


4.3.4 Effects of M. pajang kernel extract on caspases activity

The time course of effect of *M. pajang* kernel extract on activity of the initiator caspases 2, 8 and 9 as well as the executioner caspases 3 and 6 is shown in Figure 4-8. The kernel extract was found to significantly increase the activity of caspase-2, -3, -6, and -8 in MCF-7 cells following exposure to the extract for 48 and 72 hours, whereas the activity of caspase-9 was only significantly elevated at 72 hours (Figure 4-8A). Similar activation of caspase-2 and -3 was detected at 48 and 72 hours following exposure of MDA-MB-231 cells to the extract (Figure 4-8B), which was associated with late (72 hours) activation of caspase-6, no activation of caspase-8, and early (48 hours) activation of caspase-9.

Figure 4-8: Activation of caspases by *M. pajang* kernel extract.
MCF-7 cells (A) or MDA-MB-231 cells (B) were incubated with *M. pajang* kernel extract at IC_{50} value for 24, 48 or 72 hours, after which the activity of various caspases was determined as described in the 'Materials and Methods' section. Data are presented in mean ± S.E.M (n = 3). Where indicated, values for the treated group are significantly different to the untreated control group at the same time point at *P<0.05, **P<0.01, and ***P<0.001.

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Time (hours)
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Percentage (%) relative to control
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Time (hours)
Caspase-9 MDA-MB-231
Percentage (%) relative to control
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600
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200
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*
0
24
48
72
Time (hours)

4.4 Discussion

Many crude fruit extracts have been shown to possess the ability to inhibit the proliferation of cancer cells [e.g. from oranges (Camarda et al., 2007); fig fruit (Wang et al., 2008) and cranberry (Neto et al. 2008)]. Phytochemicals such as anthocyanins, phenolic acids, carotenoids and flavonoids might be responsible for the antiproliferative properties.

The results of the present study demonstrate that an ethanol extract of the kernel of *M. pajang* displayed broad anticancer activity by inhibiting the proliferation of selected cancer cell lines (i.e breast, colon, liver and ovarian cancer cell lines). Prominent results were shown in breast cancer cell lines. *M. pajang* kernel extract has been shown to arrest growth of proliferating cells in two breast cancer cell lines, which was associated with induction of apoptosis as measured by cell cycle profiling, extrusion of phosphatidylserine to the outer surface of the plasma membrane, and induction of caspase activity. This cytotoxic apoptotic response was not dependent on the oestrogen receptor, as similar results were obtained in cells lines possessing (MCF-7) and lacking (MDA-MB-231) this receptor.

In the present study, the inhibition of both breast cancer cell lines can be partially explained by the presence of phenolic phytochemicals (i.e. phenolic acids and flavonoids) in the kernel of *M. pajang* extract. Alternatively, particular phenolic phytochemicals might act additively,

synergistically, and/or antagonistically with other compounds to display the antiproliferative activity (Yang et al., 2009). *M. pajang* kernel extract has been demonstrated to contain a high total phenolic content (~10% of total dry weight) and modest flavonoid content (10.98 mg/g of total dry weight) while no anthocyanins were detected in the kernel of *M. pajang*. *M. pajang* kernel extract also displayed high antioxidant properties, compared to the flesh and peel of the fruit (refer to Chapter 2). Liu (2004) has suggested that the cancer chemopreventive properties possessed by fruits and vegetables are the outcome of additive and synergistic actions between the complex mixture of phytochemicals present in whole foods. For example, pomegranate juice has been shown to display more potent antiproliferative activity in different human colon cancer cell lines when compared to its isolated bioactive compounds such as punicalagin and ellagic acid (Seeram *et al.*, 2005); suggesting synergistic actions of phytochemical components present in the crude extract.

In Chapter 2, it was demonstrated that gallic acid, coumaric acid, sinapic acid, caffeic acid, ferulic acid, chlorogenic acid, naringin, hesperidin, rutin and diosmin were identified as the key polyphenol phytochemicals present in the kernel of *M. pajang* and that these compounds might contribute to the cytotoxicity activity against selected cancer cell lines. Many of these are known to inhibit the growth of breast cancer cells (Indap et al., 2008; Manthey and Guthrie, 2002; Noratto et al., 2009), and so it is likely that at least some of the growth inhibitory effect of the extract can be ascribed to these phenolic compounds. In addition, similar extracts of a related *Mangifera* species, *M.*

indica (mango), have been demonstrated to contain a xanthine glycoside, mangiferin, in addition to other phenolic compounds, which is a highly active cytotoxic agent (reviewed in Masibo and He, 2008). It is possible that the fruit of *M. pajang* also contains mangiferin, although this awaits confirmation, and that this also contributes to the growth inhibitory actions of the kernel extract.

Other types of fruits such as olive fruits and their derivatives (phenolic acids) have been reported to induce cytotoxicity and increased apoptosis rate in HT-29 human colon cancer cells (Juan et al., 2006). Ellagic acid (a naturally occurring bioactive phenolic in fruit) displays a selective cytotoxicity against selected cancer cell lines without any toxic effect in a normal human lung cell line (Losso *et al.* 2008). Furthermore, Kuntz et al. (1999) reported that all 30 flavonoids tested displayed promising antiproliferative activity, with IC_{50} values ranging from 39.7 ± 2.3 μM (for baicalein) to 203.6 ± 15.5 μM (for diosmin) in human colon cancer cell lines (Caco2 and HT-29). The kernel of *M. pajang* is regarded as safe since it is eaten with the flesh as a pickle by the indigenous community. Furthermore, Rukmini and Vijayaraghavan, (1984), reported that rats fed with diet containing mango (*Mangifera indica*) kernel fat extract displayed no sign of toxicity at a relatively high concentration (10% of mango kernel fat).

Under the chosen experimental conditions, the *M. pajang* kernel extract did not completely inhibit cell proliferation, with 30-40% of both breast cell types being resistant to the effects of the extract in the concentration range 40-100 μg/ml. Similar findings have been reported for other plant extracts and its

isolated phytochemicals (Conforti et al., 2008; Kim et al., 2007; Sandhya and Mishra, 2006), and it is likely that this effect can, in part, be explained by the time dependence of inhibition of proliferation (Sandhya and Mishra, 2006); this aspect was not explored further.

Some flavonoids have been shown to induce cytotoxicity in cancer cell lines. Structure–activity relationships for the cytotoxic activity are apparent from a study by Pouget et al. (2001). The unsubstituted flavanones appeared to be a weak inhibitor of MCF-7 cells growth as were flavanones with hydroxyl-group-like compounds. However, the substitution by a methoxy group at position 7 and/or 5 in the structure of flavonones increased the antiproliferative activity. Five prenylated flavanones isolated from the leaves of *Monotes engleri* have been shown to display cytotoxic activity against several human cancer cell lines (Seo et al., 1997). Mammary tumorigenesis was shown to be delayed following supplementation of naringin in the diet compared with the control group (So et al., 1996) supporting the efficacy of flavanones potential in the prevention and treatment of cancer.

Schieber et al. (2000) reported the presence of seven different quercetin and five kaempferol glycosides in the puree of mango (*M. indica*). The same pattern of the content for both quercetin and kaempferol was observed in the flesh of *M pajang*. Citrus flavonoids have been shown to be effective inhibitors of human breast cancer cell proliferation *in vitro*, especially when paired with quercetin, which is widely distributed in other foods (So et al., 1996). Quercetin has also been showed to induce mild cytotoxic activity against

selected cancer cell lines (Kawaii et al., 1999). Luteolin has been reported to induce strong antiproliferative properties against lung, skin, blood and stomach cancer cells compared to other polypohenols tested (Kawaii et al., 1999). However, flavones have been reported to induce weak antiproliferative properties against MCF-7 breast cancer cells except for 7,8-dihydroxyflavone which is highly active (Pouget et al., 2001).

To identify whether the growth inhibitory effect of *M. pajang* kernel extract was caused by specific perturbation of cell cycle-related events, the DNA contents of both MCF-7 and MDA-MB-231 cells were measured by means of flow cytometric analysis. In MCF-7 cells, this analysis demonstrated that *M. pajang* kernel extract caused an accumulation of cells in the G_0/G_1 phase and thus inhibited the transition of cells into S phase, followed by increases in the proportion of cells in sub-G_1 phase. The increase of cells in sub-G_1 was taken to reflect the induction of apoptosis. This condition might be due by the inhibition of DNA replication (Hartwell and Kastan, 1994) caused by the inability of the cells to replicate damaged DNA caused by the plant extract. These sites of action in the cell cycle are similar to those identified for phenolics (and related chemicals) present in the extract. Thus, naringin and hesperetin (the aglycone of hesperidin) have been reported to cause G_1 arrest, whilst gallic and ferulic acids have been reported to cause G_2-M arrest (Agarwal et al., 2006; Janicke et al., 2005; Kim et al., 2008; Lee et al., 2008). This result suggested the use of *M. pajang* kernel extract to inhibit the growth of breast cancer cells.

On the other hand, more pronounced effects on the cell cycle distributions were observed in MDA-MB-231 cells treated with *M. pajang* kernel extract. After 24 hours treatment, *M. pajang* kernel extract significantly decreased G_0/G_1 and S phases up to 42.5% and 8.9% as compared to their control 54.0% and 23.9%, respectively, concomitant with an increase in the proportion of G_2-M phase cells by 48.93% compared to the control (23.2%) ($p<0.05$). The same trend was observed after 48 hours of treatment in conjunction with increased in sub-G_1 phase by up to 23.7% compared to the control (1.74%) ($p<0.05$). After 72 hours of incubation period, sub-G_1 phase has increased dramatically up to 64.0% compared to the control ($p<0.05$). This result clearly showed the transient accumulation of the treated cells at G_2-M phase before shifting to apoptosis in the cell cycle. Previous studies have shown that G_2-M cell-cycle arrest may occur as a result of DNA damage or incomplete replication that causes the cells to be arrested at G_2 checkpoint and prevent entry into mitosis (Jangi et al., 2006; Poon et al., 1997). G_2/M checkpoints include the checks for damaged DNA, unreplicated DNA, and those to ensure that the genome is replicated once and only once per cell cycle. If cells pass these checkpoints, they follow normal transition to the M phase. However, if any of these checkpoints fail, mitotic entry is prevented by specific G_2/M checkpoint events. A similar mechanism may occur with the kernel extract used in the present studies. Part of the mechanism by which the plant extract blocks cells at the G_2 checkpoint involves inhibition of Cdc2, the cyclin-dependent kinase required to enter mitosis. Cdc2 is inhibited and the

binding of Cdc2 to Cyclin B1 is required for its activity, and repression of the cyclin B1 gene by p53 also contributes to blocking entry into mitosis (Taylor and Stark, 2001).

Some fruits / fruit parts extracts have been shown to display different cell cycle distribution characteristics. Fig fruit latex extract has been shown to increase the apoptosis and G_0/G_1 phases in human glioblastoma and hepatocellular carcinoma while decreasing the number of cells in S and G_2/M phases (Wang et al., 2008). Certain polyphenols (i.e. flavone, luteolin, and the structurally similar daidzein) have been shown to arrest the cell cycle at G_0/G_1 (Matsukawa et al., 1995). On the other hand, daidzein (a member of the isoflavone subclass of flavonoids found in some fruits, nuts, soybeans, and soy based products) has been shown to induce cell cycle arrest at G_2-M via reduction of cyclin D, CDK2, CDK1 and CDK4 expression and increased the expression of cyclins A and B (Choi and Kim, 2008; Matsukawa et al., 1995).

A recent study also showed that most naturally occurring flavonoids induced diverse cytotoxic mechanism and exert their cell cycle growth inhibitory or killing activity by different mechanisms in different cells. Some flavonoids induced G_0/G_1 and G_2-M cell cycle arrest in human cancer cells, whilst others inhibited the cell cycle at S phase in various human cancer cells (Choi et al., 1999). In this study, it was noted that the polyphenols pool present in the extract of *M. pajang* kernel only affected the G_0/G_1 and S phase in MCF-7 with no significant arrest at other cell cycle events. Meanwhile, In the MDA-

MB-231 cells, *M. pajang* reduced the S phase and induced strong G_2-M cell cycle arrest, which lead to the induction of apoptosis.

The induction of apoptosis indicated by changes in the cell cycle was confirmed by analysis of two independent markers of the apoptotic cascade, the externalisation of phosphatidylserine (using Annexin V-FITC), and the induction of caspases. For MCF-7 cells, exposure to the extract led to induction of activity of all the caspases studied (-2, -3, -6, -8, -9), whilst exposure of MDA-MB-231 cells led to induction of caspase-2, -3, -6, and -9. Caspase-2 and -9 are early biomarkers of apoptosis, which act as downstream targets of cytochrome c release from mitochondria, which go on to activate caspase-3, activity of which is responsible for the DNA fragmentation and morphological changes associated with apoptosis (Gupta et al. 2009). In agreement with these findings, many of the polyphenols identified in the kernel extract, as well as mangiferin, have been demonstrated to induce apoptosis in various cell types (Agarwal et al., 2006; Park et al., 2008; Roseghini et al., 2009; Tang et al., 2004). Some fruit extracts have been shown to induce apoptosis in selected cancer cell lines via activation of caspase. Fruit extract of *Gleditsia sinensis* was found to enhance significantly the activity of caspase 3 which led to the induction of apoptosis (Chui et al., 2005). Olive fruit extract has been shown to increase caspase-3-like activity to 6-fold that of control cells (Juan et al., 2006) which led to the induction of apoptosis. The growth-inhibitory actions of the ethanol extract of *M. pajang* kernel demonstrated with cells derived from human breast cancer cell lines (this study) and human hepatic, ovarian, and

colon cancer cell lines suggest that this extract has potential as an anti-tumour agent, for which further, *in vivo*, studies are warranted.

4.5 Conclusion

We demonstrated that *M. pajang* kernel crude extract has promising anticancer properties in various type of cancer cells especially breast cancer cells. Phenolic acids and flavonoids are all likely contributors to the observed anticancer properties and may act in a complementary fashion to limit cancer cell progression.

CHAPTER 5

General discussion and conclusion

CONTENTS

5.1 Introduction

Mangifera pajang (bambangan) and *Artocarpus odoratissimus* (tarap) are fruit trees endemic to Borneo Island (Malaysia, Indonesia and Brunei). These fruits have been used locally as food. The flesh of *M. pajang* fruit is eaten fresh by indigenous people and also cooked with fish/chicken/meat for a distinctive 'mangory' and sour flavour. The flesh of the fruit is also used to make juice, and together with the grated kernel is used to make pickle which is delicious eaten with the local staple food (rice). Meanwhile, the fruit of *A. odoratissimus* is usually eaten fresh or fried (like banana fritter), the seed sometimes being roasted. To our knowledge, although these fruits are abundant in Borneo Island, the scientific data pertaining to the health benefits of these fruits are lacking. Thus, since 2006, with funding provided by Ministry of Science, Technology and Innovation of Malaysia, as well as expertise and facilities from University of Nottingham, UK, Universiti Malaysia Sabah and Universiti Putra Malaysia, intensive investigations have been conducted to study the potential health benefits of *M. pajang* and *A. odoratissimus* and their by-products (i.e. seed, peel, and kernel).

Ingestion of fruits and vegetables are widely known to be associated with a decreased risk of cancer (Steinmetz and Potter, 1991). A large number of potentially chemopreventive and chemotherapeutic agents could be found in

these natural food sources. In addition, fruits in the genus of *Mangifera* and *Artocarpus* have been shown to contain antioxidant phytochemicals and display cytoprotective and anticancer properties (Ajila et al., 2007; Anila and Vijayalakshmi, 2003; Soong and Barlow, 2006; Jayasinghe et al., 2006; Kim et al., 2010; Masibo and He, 2008; Percival et al., 2006). Thus, the cancer chemoprevention and chemotherapeutic potential of both *M. pajang* and *A. odoratissimus* are becoming the areas of interest.

5.2 Polyphenol compositions and antioxidant properties

As a first study, a simple antioxidant assessment was conducted on extracts of the fruits. The fruit of *M. pajang* was separated into flesh, peel and kernel, and the fruit of *A. odoratissimus* was separated into seed and flesh and the antioxidant assessment of alcoholic extracts conducted by a free radical scavenging assay (using DPPH) and a ferric reduction (FRAP) assay. For the *M. pajang* extracts, the results showed that the kernel extract of the fruit displayed the highest DPPH scavenging effect, followed by the peel and flesh extracts. The reducing ability of the tested extracts was in the order of kernel > peel > flesh, the same trend as shown in the DPPH free radical scavenging assay. Meanwhile, as for *A. odoratissimus*, the DPPH free radical scavenging activity as well as the reducing ability was higher in the seed extract, as compared to the flesh extract. A recent study has shown that the kernel of mango (*M. indica*) contain a high content of total phenolics, and also displayed effective antioxidant properties (Maisuthisakul and Gordon, 2009).

The kernel and peel of *M. indica* have also been found to exert better antioxidant activity as compared to the commercial antioxidant compound BHA. This antioxidant result was also supported by Kim et al. (2010) who found that the ethanolic extract of *M. indica* peel displayed better antioxidant activity compared to the flesh. The antioxidant assessment in the present study showed that the kernel extract of *M. pajang* displayed potent free radical scavenging activity as well as reducing activity, as compared to other fruit parts extracts.

The next part of the work was determination of selected compounds which might be present in the plant extracts. Total phenolic, total flavonoid and total anthocyanin contents were determined via spectrophotometric assays. For total phenolic content, results showed a similar trend to the antioxidant activity, in that the total phenolic content was in the order: *M. pajang* kernel > *M. pajang* peel > *A. odoraissimus* seed > *M. pajang* flesh > *A. odoratissimus* flesh. Total flavonoid was also highest in *M. pajang* kernel, followed by *M. pajang* peel > *A. odoraissimus* seed > *A. odoratissimus* flesh > *M. pajang* flesh. However, for anthocyanins relatively low levels were detected in the fruits while no anthocyanin was detected in the kernel of *M. pajang*. The presence of these phenolic phytochemicals might explain the antioxidant properties of the plant extracts. Phenolic phytochemicals have been shown to exert their antioxidant properties via reactivity of the phenol moiety, and have the ability to scavenge free radicals, via hydrogen donation or electron donation (Shahidi and Wanasundara, 1992).

The high level of total phenolics in the plant extracts (especially for *M. pajang* kernel where total phenolics represented approximately 10% of its total weight) led us to the next experiment, investigation of selected polyphenols which might present in the fruit parts by using high performance liquid chromatography (HPLC). HPLC profiling of polyphenol phytochemicals was conducted using available standards which are normally found in the genus of *Mangifera* and *Artocarpus*. Although many individual phenolic compounds that have antioxidant activity in fruits cannot be identified and measured by HPLC methods (Ferreira et al., 2002), this method at least could identify selected phytochemicals which might be present in the extracts. The present experiment revealed the presence of at least 10 phenolic phytochemicals in the kernel of *M. pajang*. These comprised phenolic acids (gallic, p-coumaric, sinapic, caffeic, ferulic and chlorogenic), flavonols (rutin), flavones (diosmin) and flavanones (naringin and hesperidin), many of which are recognized as potent antioxidants with demonstrable health benefit properties (Rice-Evans et al., 1997).

5.3 Cytoprotective activity

Although some plant extracts have been shown to exert high antioxidant activity (with regards to *M. pajang* kernel and peel extracts) in non-cellular antioxidant assay (i.e. DPPH free radical scavenging and FRAP assay), there is a need to verify their antioxidant properties in a biological systems.

Therefore, in the present study, the potential cellular antioxidant and cytoprotective effects of *M pajang* kernel, peel and flesh extracts as well as *A. odoratissimus* seed and flesh extracts were tested against oxidative stress toxicity induced by t-BHP in the HepG2 (hepatocyte) cell line. In this study, HepG2 cells were pre-incubated with the plant extracts or compounds for 24 hours, after which t-BHP was introduced to the cells for another 5 hours. Later, cell viability was determined using neutral red uptake assay. The results showed that only the kernel extract displayed cytoprotective activity against oxidative damage caused by t-BHP in HepG2 cells, with a cytoprotective index value (concentration producing 50% protection) of 1.2 ± 0.1 µg/ml. Meanwhile, the positive control (quercetin) showed lower cytoprotective activity as shown by a higher cytoprotective index value of 5.3 ± 0.7 µg/ml. Previous studies have shown that polyphenols and polyphenol-rich-extracts display cytoprotective activity via direct scavenging of toxicants and/or induction of detoxification enzymes (i.e. induction of quinone reductase, glutathione reductase and superoxide dismutase) which leads to the elimination of toxicant-induced oxidative stress (Lee-Hilz et al., 2006; Martin et al., 1996; Muzolf-Panek et al., 2008).

Accordingly, in the present study, the protein expression of three detoxifying enzymes, namely quinone reductase (NQO1), glutathione reductase (GR) and methionine sulfoxide reductase A (MSRA) were determined after incubation of the HepG2 cells with the cytoprotective extract/compound for 24 hours. Protein expression was then determined using

western blotting assay. The results showed that NQO1 was induced by quercetin in a concentration dependent manner, but not by the *M. pajang* kernel extract. Quercetin has also been previously shown to induce the expression of NQO1 in mammalian cell lines (Uda et al., 1997, Yang and Liu, 2009). In addition, both quercetin and *M. pajang* kernel extract significantly induced the expression of glutathione reductase (GR), indicating that both materials (quercetin and kernel extract) may exert their cytoprotective action by a combination of direct (radical scavenging and reducing capacity) and indirect (cytoprotective protein up-regulation) antioxidant activities. t-BHP has been reported to exert its cytotoxic activity via glutathione (GSH) depletion (Lima et al., 2006); up-regulation of GR serves to maintain the GSH status and thereby provide protection against oxidative stress. Meanwhile, both quercetin and *M. pajang* kernel extract were shown to induce the expression of MSRA in HepG2 cells. To our knowledge, these are the first results to show the effect of plant extracts or compounds on the expression of MSRA in a mammalian cell line. As discussed in Chapter 3, the expression of MSRA has been associated with enhanced antioxidant defence mechanism via scavenging reactive oxygen species facilitating the cyclic interconversion of methionine and protein-methionine residues between oxidized and reduced forms.

As reviewed by Dinkova-Kostova and Talalay (2008) most cytoprotective proteins are activated by the Keap1/Nrf2/ARE system which leads to the activation of cytoprotective genes (Figure 5-1). Generally, when an inducer (cytoprotective compound) enters the cell, Nrf2 (nuclear factor

erythroid 2-related factor, a basic leucine zipper transcription factor responsible for both basal and inducible expression of cytoprotective genes) will be released from Keap1 (kelch-like ECH-associated protein 1, which acts as a sensor and chemical target for inducers). Nrf2, which undergoes nuclear translocation, and in heterodimeric combination with the small Maf transcription factors, binds to the ARE (antioxidant response elements; specific sequences that are present in the upstream regulatory of regions of phase 2 genes), and induces the basal transcriptional machinery to activate the transcription of cytoprotective genes (i.e. quinone reductase, glutathione reductase and methionine sulfoxide reductase A) (Figure 5-2).

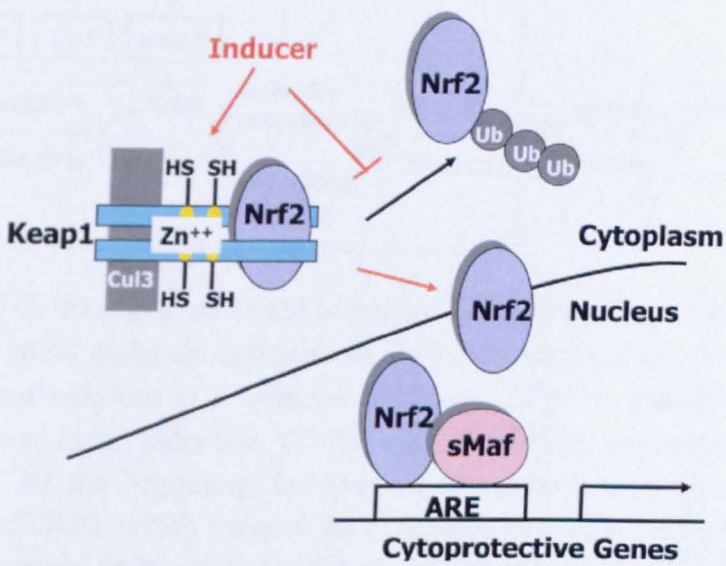

Figure 5-1. Mechanism of induction of cytoprotective genes. In the absence of an inducer the sensor protein Keap1 forms a complex with cullin 3 (Cul3) and represses transcription factor Nrf2 by presenting it for ubiquitination (Ub) and subsequent proteasomal degradation. When an inducer enters the cell, it modifies the highly reactive cysteine residues of Keap1 resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2, which then undergoes nuclear translocation, and in heterodimeric combination with small Maf transcription factors, binds to the ARE and recruits the basal transcriptional machinery to activate transcription of cytoprotective genes (Adapted from Dinkova-Kostova and Talalay, 2008).

From this consideration, induction of GR (Ernster and Forsmark-Andree, 1993) and probably MSRA by quercetin and *M. pajang* kernel extract might also regenerate lipid soluble direct antioxidant ubiquionols from their oxidized form that are very crucial to protect membrane phospholipids and lipoprotein from peroxidation and probably MSRA (Figure 5-2).

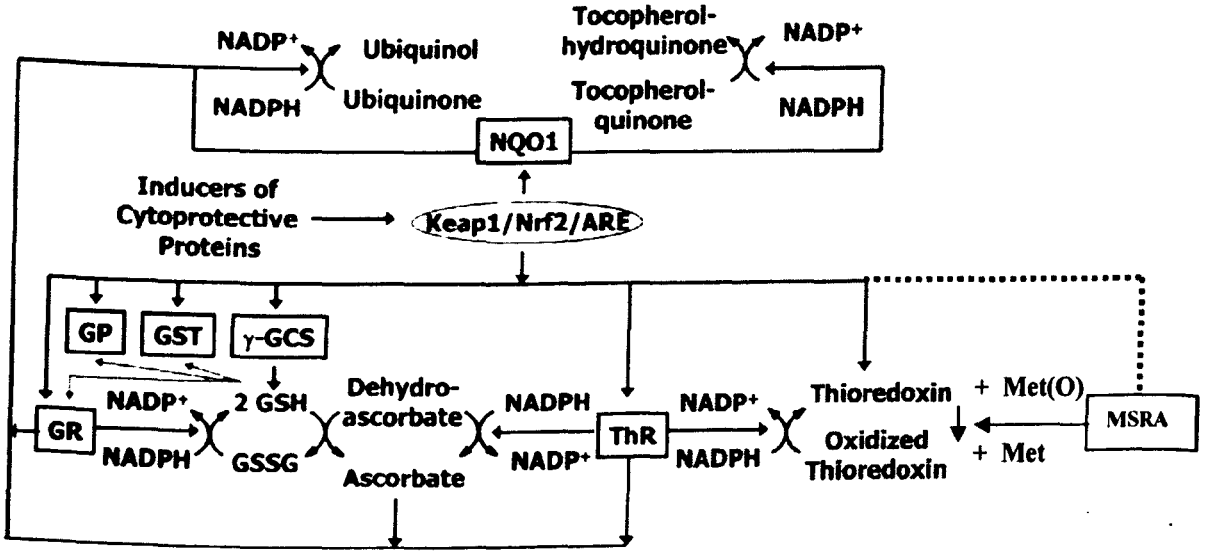

Figure 5-2. Example of possible antioxidant defence mechanism of action between small molecule antioxidants (i.e glutathione (GSH), thioredoxin) and antioxidant enzymes (i.e. quinone reductase (NQO1), glutathione reductase (GR) thioredoxin reductase (ThR) and methionine sulfoxide reductase A (MSRA). At the beginning, inducers of cytoprotective proteins activate the Keap1/Nrf2/ARE which induces the expression of cytoprotective proteins. GR which is crucial in generating GSH, is also up-regulated. In addition, GSH is also induced which is involved in the reduction of dehydroascorbate to maintain the level of ascorbate in its reduced state. MSRA plays an important role to maintain the level of methionine in its reduced state by converting methionine sulfoxide back to its reduced state via catalyzing the thioredoxin and at the same time ThR converts the oxidized thioredoxin back to its reduced state. At least 3 antioxidant enzymes involved in generating ubiquinol (a lipid soluble antioxidant which protects phospholipids and lipoproteins from peroxidation) which are NQO1, GR and ThR. NQO1 is also involved in maintaining the antioxidant tocopherol-hydroquinine in its reduced state. (Adapted with modification from Dinkova-Kostova and Talalay, 2008).

With regards to heat shock protein, the expression of both HSP 27 and HSP 70 were increased on exposure to quercetin, but not on exposure to the kernel extract of *M. pajang* in HepG2 cell line. Urani et al. (2005) reported that heat shock proteins could be induced by compounds at high non-cytotoxic concentration probably as consequences of proteotoxicity. As reviewed by Parcell and Lindquist (1994) studies on heat shock protein have revealed how the cell senses stress and the heat shock proteins play an important role in protecting against protein damage. While prolonged exposure to extreme conditions (i.e heat, xenobiotics) is harmful and results in cell death, less extreme condition led to activation of heat shock response and other stress responses which results in cytoprotection against stress-induced oxidative damage. In addition, exposure to diverse forms of stress (i.e. heat or chemical stress) has a cross-protective effect against normally lethal exposure to different forms of stress (Marimoto and Santoro, 1998). However, more studies are needed to investigate the involvement of heat shock proteins in cytoprotective mechanism.

5.4 Anticancer activity

While some plant extracts display potential antioxidant properties, they have also been shown to display cytotoxic effects in a wide range of cancer cell lines (Boivin et al. 2009; Ju et al., 2004) and therefore it was postulated that the extracts of *M. pajang* and *A. odoratissimus* might display cytotoxic properties in selected cancer cell lines. In addition, some researchers have suggested the potential use of antioxidant chemicals in interfering at the very early stage in

tumor progression by preventing tumor angiogenesis. Antioxidants have been generally known to prevent the occurrence of cancer via scavenging of reactive oxygen species. However, since angiogenesis may be regulated by free radicals, peroxides and redox sensitive transcription factors (e.g. NF-κB), reduction of redox signaling may also induce the expression of anti-angiogenic proteins which lead to the reduction of tumour invasion of solid tumor (Khan and Marajver, 2007).

In the present study, alcoholic extracts of *M. pajang* kernel, peel and flesh extracts as well as alcoholic extracts from *A. odoratissimus* seed and flesh were studied for their anti-cancer potential against proliferation *in vitro* of human liver cancer cells (HepG2), human colon cancer cells (HT-29), human ovarian cancer cells (Caov3) and human breast cancers (MCF-7 and MDA-MB-231) using MTT assay as a marker for proliferation. As expected, *M. pajang* kernel extract was shown to induce a broad cancer-inhibition spectrum in the panel of cancer cell lines. Prominent results were shown in breast cancer cell lines (IC_{50} for MCF-7: 23.0 μg/ml and MDA-MB-231: 30.5 μg/ml). Many polyphenol phytochemicals present in the kernel of *M. pajang* have been shown to inhibit the growth of breast cancer cells (Indap et al., 2008; Manthey and Guthrie, 2002; Noratto et al., 2009), and so it is likely that at least some of the growth inhibitory effect of the extract can be ascribed to these phenolic compounds. The possible anticancer mechanisms of *M. pajang* kernel extract were further elucidated in the subsequent studies.

Cell cycle analysis was conducted using flow cytometry. The DNA content of the cells (after incubation of the cells with *M. pajang* kernel extracts) as assessed by flow cytometry could be categorised into 4 different stages, Sub-G_1 (apoptosis), G_0/G_1 (living cells), G_2-M (transition of the cells to mitosis phase) and S phase (synthesis phase). In MCF-7 cells, results showed that *M. pajang* kernel extracts induced an accumulation of cells in the G_0/G_1 phase and thus inhibited the transition of cells into S phase, followed by increases in the proportion of cells in sub-G_1 phase. In MDA-MB-231 cells, the extract initially (by 24 hours) caused cell-cycle arrest at G_2/M, and later (by 72 hours) caused a substantial increase in the proportion of cells in sub-G_1 phase. The increase of cells in sub-G_1 reflected the occurrence of cells in apoptosis phase. It seems that the *M. pajang* extract displayed 2 distinct mechanisms regarding the cell cycle events. This might be due to the oestrogen-related-mechanisms since one cell is hormone dependent (MCF-7) another one is non-hormone dependent (MDA-MB-231). Similar trend of results was observed by Choi and Kim (2008) in that diadzein (which belongs to the isoflavone subclass of flavonoids and is found in fruits and nuts) was shown to induce strong G_2-M cell cycle arrest in MDA-MB-435 (which lack of oestrogen receptors) as compared to MCF-7 (which has oestrogen receptor). The down-regulation of CDK1 (cdc2) has been associated with the arrest of cell cycle events at G_2/M. CDK1 has been shown to play an important role (by forming a complex with cyclin B) to trigger the G_2/M transition. Many flavonoids which possess similar molecular structure have been shown to display different mechanisms of anti-cancer action in breast cancer cell lines (Balabhadrapathruni et al., 2000).

Annexin V-FITC and Propidium iodide staining revealed that the early apoptosis occurred as early as 36 hours post-incubation with values of 14.01% for MCF-7 cells and 24 hours for MDA-MB-231 cells with the value of 16.51%. This apoptosis appears to be caspase-2 and caspase-3 dependent in MCF-7; and caspase-2, caspase-3 and caspase-9 dependent in MDA-MB-231. These findings suggest *M. pajang* kernel extract has potential as a potent cytotoxic agent in both hormone and non-hormone dependent breast cancer cell lines. The mechanisms for the cytotoxic effects might be associated with activation of caspases and G_2-M cell cycle arrest leading to the induction of apoptosis.

5.5 Safety issues

In concern with the human safety, both fruits have been consumed by local people regularly during the fruit season. No adverse effects or toxicity have been reported for both *M. pajang* and *A. odoratissimus* in humans or animals. The kernel seed of *M. pajang* can be regarded as safe since it is eaten with the flesh as a pickle by the local community although it is not known if the process of making 'pickle' might modulate the changes the phytochemicals in the kernel. The same goes to the seed of *A. odoratissimus* where the roasted seeds are eaten by local people. In addition, the observation that the kernel of *M. pajang* is eaten with the flesh as a pickle by the indigenous community is a re-assuring sign of low systemic toxicity, as is the report by Rukmini and Vijayaraghavan (1984) of a multi generation study in rats in which a diet

containing 10% mango kernel oil produced no adverse effects. The findings that addition of 25% mango kernel to the diet of growing lambs was without effect on body weight gain (Anigbogu, 2003; Saiyed et al., 2003) and that addition of 20% mango kernel to the diet of chickens was also without effect on weight gain or mortality (Odunsi and Farinu, 1997) are similarly suggestive of a lack of significant systemic toxicity of extracts of seed kernel from *Mangifera* species. However, possible toxicity effects of bambangan kernel extracts at high dose and/or long term exposure should be considered.

5.6 Limitations of study

i. Different extraction methods were used for antioxidant and phytochemicals analysis (See Chapter 2) and cytoprotection (Chapter 3) as well as cytotoxic (Chapter 4) activities. Methanol extracts were used for the antioxidant and phytochemicals assessment. However, ethanol extracts were used to examine the cytoprotection and cytotoxic activities. The reason for this matter is that methanol is highly toxic to human where else ethanol is regarded as safer to human. Thus, different solvent was used in the cell-related experiments to eliminate the toxicity effects of the solvent.

ii. Due to the difference of the extraction solvents, different compounds might be extracted using different solvent. However, compounds of interest (polyphenols) could also be isolated by both methanol and ethanol. Thus, the use of both solvents should not be a problem for a matter of comparison and correlation.

iii. t-BHP induced cells were not studied for the expression of cytoprotection proteins. Thus, the expression of cytoprotective proteins might not play a direct role in the cytoprotection mechanism against toxicant. However, high expression of cytoprotective proteins (i.e. NQO1 and GR) has been shown to display protective effects against oxidative damage which the correlation between the induction of cytoprotective proteins and their role in the mechanisms of the cytoprotection action have been discussed in Chapter 3 and 5.

iv. Passage number of all cells used was in the ranges of 10 to 40. Different passage number might influence the response of the cells to the extracts or bioactive compounds.

v. Samples collection (places, variety and weather) might influence the phytochemicals and bioactivity of the fruit extracts. Thus, different activity might be displayed if different batches of plant extracts were used.

5.7 Conclusion

In conclusion, the results of this study have indicated that *M. pajang* kernel displayed highest antioxidant activity as compared to other part of the fruits as assessed in non-cellular antioxidant assay (DPPH free radical scavenging activity and FRAP assay). *M. pajang* also contained high total phenolic content (approximately 10% of its total weight) and HPLC study on the kernel revealed the occurrence of at least 10 polyphenol phytochemicals such as phenolic acids (gallic, coumaric, sinapic, caffeic, ferulic, chlorogenic)

and flavonoids (naringin, hesperidin, rutin, diosmin) which are recognized as potent antioxidants with demonstrable health benefits properties. *M. pajang* kernel extract displayed excellent cytoprotective against t-BHP induced oxidative damage in HepG2 cell line. *M. pajang* kernel extract has been shown to exert it cytoprotective effects via combination of direct antioxidant activity (radical scavenging and reducing activity) and indirect (induction of MSRA and GR protein expression) antioxidant activities. *M. pajang* has also displayed cytotoxic effects in all cancer cell lines tested, particularly in both cancer cell lines (MCF-7 and MDA-MB-231). Cell cycle arrest, induction of apoptosis and activation of caspases has been affiliated with the possible anticancer mechanism of action. These findings point to the potentially beneficial properties of the kernel extract of *M. pajang* in various areas of human health. This possibility needs to be explored further.

5.8 Recommendations for future research

i. Bioactive compounds should be isolated to study the phytochemical(s) which might be responsible for the potent antioxidant, cytoprotection and cytotoxic activities.

ii. Proteins which involve in the apoptosis (i.e bax and bcl-2), cell cycle event (i.e. Cdc2 and cyclin B1) and antioxidant mechanism (Nrf2 and Keap1) should be further examined.

iii. *In vivo* animal study should be conducted in cancer induced rats to determine the efficacy and possible toxicity of the extracts or pure compounds.

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APPENDIX

1. Buffers for Western Blotting

1.1. Electrophoresis running buffer (10x)

30.3 g Tris, 144 g Glycine, 10g SDS dissolved in 2 litre distilled H_20

1.2. Transfer buffer for western blotting

30.3 g Tris, 144g Glycine dissolved in distilled water. Methanol was then added (2 litres) and the solution was made up to 10 litres with distilled water.

1.3. TBST buffer 1 litre

25 mM Tris, 125 mM NaCl dissolved in 1 litre of distilled water and adjust to pH 7.6 and then add 0.1% (v/v) Tween 20

Table A: Correlation (Pearson correlation test; r value) between the antioxidant activity and the phytochemicals content.

	DPPH free radical scavenging activity	FRAP
Total phenolic content	0.558	0.985
Total flavonoid content	0.815	0.663
Total anthocyanin content	0.000	0.000