CHAPTER I

INTRODUCTION

1.1 Statement of the problems

Oxidative stress has long been known to be involved in the pathogenesis of age-related and chronic diseases including cancer. Oxidative stress has been defined as an imbalance between oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular levels of reactive oxygen species (ROS) (Klaunig and Kamendulis, 2004). Oxidative DNA damage can trigger tumor initiation (Evans *et al.*, 2004). ROS can affect a number of cellular processes critical in tumor development such as cell proliferation, senescence, inflammation, metastasis, etc (Chandra *et al.*, 2000; Vaquero *et al.*, 2004; Urbano *et al.*, 2005; Ito *et al.*, 2006).

Epidemiological studies have shown an inverse association between consumption of vegetables and fruits and the risk of human cancers at many sites (Steinmetz and Potter, 1991a; Block *et al.*, 1992). It is believed that antioxidant properties of these vegetables and fruits protect cells from ROS-mediated DNA damage that can result in mutation and subsequent carcinogenesis (Lopaczynski and Zeisel, 2001).

Anthocyanins occur ubiquitously in the plant kingdom and confer the bright red, blue and purple colors to fruits and vegetables such as berries, grapes, apples, purple cabbage and purple rice. Epidemiologic studies suggest that the consumption of anthocyanins lowers the risk of cardiovascular disease, diabetes, arthritis and cancer due, at least in part, to their anti-oxidant and anti-inflammatory activities (Prior and Wu, 2006). Anthocyanins possess antioxidant activity, which is considered to be an important physiological function. Additively, anthocyanins are reported to have anticancer activity, anti-inflammatory activity, apoptotic induction effect, α-glucosidase inhibition activity, vision benefits and effects on collagen, blood platelet aggregation and capillary permeability and fragility (Hou, 2003).

Anthocyanins have been shown to exhibit anticarcinogenic activity against multiple cancer cell types *in vitro* and tumor types *in vivo*. Potential cancer chemopreventive activities of anthocyanins revealed from *in vitro* studies include radical scavenging activity, stimulation of phase II detoxifying enzymes, reduced cell proliferation, inflammation, angiogenesis, invasiveness and induction of apoptosis and differentiation. *In vivo* studies have shown that dietary anthocyanins inhibit cancers of the gastrointestinal tract and topically applied anthocyanins inhibit skin cancer (Wang and Stoner, 2008).

Cleistocalyx nervosum var. *paniala*, Ma-kiang, is a perennial tree belonging to the Myrtaceae family. It is found growing in scatter locations in some villages of the northern provinces of Thailand such as Chiang Mai, Chiang Rai, Lumphun, Lampang and Mae Hong Son. The rich purplish red color of the fruit *C. nervosum* is characterized by an anthocyanin profile and a major compound was cyanidin-3-glucoside (Jansom *et al.*, 2008). Surprisingly, there is no report on biological activity of *C. nervosum* fruit especially *in vivo* study, though *Syzygium cumini*, Luk-waa, belongs to the same family, was known to possess anti-diabetes, antibacterial, analgesic, anti-inflammatory and antioxidant effects (Banerjee *et al.*, 2005; Sharma *et al.*, 2006). Previous study demonstrated that the active antioxidant compounds in *S. cumini* were three major anthocyanin-glucosides of delphinidin, petunidin and malvidin (Kong *et al.*, 2003). From the above information, it can be expected that *C. nervosum* may have biological activities similarly to *S. cumini*.

About 60% of known chemical carcinogens were found to exert carcinogenic potential to the liver of rodents. It is well-known that the rat liver medium-term bioassay for carcinogens has much advantage among different bioassays. Based on the two-step initiation and promotion concept of liver carcinogenesis, the screening assays have the important advantage of easy detection of the preneoplastic enzyme-altered lesions which are widely accepted as early indicators of neoplastic development (Fukushima *et al.*, 2005). Diethylnitrosamine (DEN) is a powerful hepatocarcinogen that has been used as an initiating agent in some two-stages carcinogenesis protocols for hepatocarcinogenic studies. Phenobarbital (PB) is an antiepileptic drug that promotes hepatocarcinogenesis in rodents when administered

subsequent to an initiating carcinogen like DEN (Sreepriya and Bali, 2005; Scolastici et al., 2008). Chronic administration of PB elicits many effects on the liver, including development of hyperplasia and hypertrophy without increasing cell death and also stimulates cell proliferation in focal relative to non-focal areas of carcinogen-challenged tissues. The DEN-PB treated rat model of experimental hepato-carcinogenesis is so well defined that it makes itself a potential tool for studying mechanisms of cell growth, differentiation and cell death (Chakraborty *et al.*, 2007).

Glutathione-*S*-transferase placental form (GST-P) expression is a useful marker for preneoplastic and neoplastic rat liver lesions (Fukushima *et al.*, 2005). The detection of GST-P positive single cells and mini foci or large GST-P positive FAH is an important tool for analyzing relevant carcinogenic or anti-carcinogenic responses during the initiation and promotion stages of rat liver carcinogenesis (Dias *et al.*, 2008).

In this study, we hypothesized that *C. nervosum* extract containing antioxidant activities might reduce oxidative stress inducing hepatocarcinogenesis in rats using GST-P positive foci as the endpoint marker.

1.2 Literature reviews

1.2.1 Oxidative stress and reactive oxygen species

Oxidative stress refers to a cell's state characterized by excessive production of reactive oxygen species (ROS) and/or a reduction in antioxidant defenses responsible for their metabolism. This generates an imbalance between ROS production and removal in favor of the former. ROS can be produced by both endogenous and exogenous sources. Endogenous sources include oxidative phosphorylation, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Table 1-1). During mitochondrial oxidative metabolism, the majority of the oxygen consumed is reduced to water; however, an estimated 4% to 5% of molecular oxygen is converted to reactive oxygen species, primarily superoxide anion, formed by an initial one-electron reduction of molecular oxygen (Table 1-2). Superoxide can be dismutated by superoxide dismutase to yield hydrogen peroxide. In the presence of

partially reduced metal ions, in particular iron, hydrogen peroxide is subsequently converted through Fenton and Haber-Weiss reactions to a hydroxyl radical. The hydroxyl radical is highly reactive and can interact with nucleic acids, lipids, and proteins. Neutrophils, eosinophils, and macrophages are an additional endogenous source and are major contributors to the cellular ROS. Activated macrophages, through "respiratory burst" elicit a rapid but transient increase in oxygen uptake that gives rise to a variety of ROS, including superoxide anion, hydrogen peroxide, and nitric oxide (Table 1-1).

Table 1-1 Reactive oxygen and nitrogen species generation and removal in the cell (Klaunig and Kamendulis, 2004)

Cellular oxidants	**Source**	**Oxidative species**
Endogenous	Mitochondria	$O_2^{-\bullet}$, H_2O_2, $^{\bullet}OH$
	Cytochrome P450	$O_2^{-\bullet}$, H_2O_2
	Macrophage/inflammatory cells	$O_2^{-\bullet}$, $^{\bullet}NO$, H_2O_2, OCl^-
	Peroxisomes	H_2O_2
Exogenous	Redox cycling compounds	$O_2^{-\bullet}$
	Metals (Fenton reaction)	$^{\bullet}OH$
	Radiation	$^{\bullet}OH$
Cellular antioxidants		
Enzymatic	Nonenzymatic	
Superoxide dismutase	Vitamin E	
Catalase	Glutathione	
Glutathione peroxidase	Vitamin C	
Glutaredoxin	Catechins	
Thioredoxin		

In addition, peroxynitrite is formed from the coupling of nitric oxide and superoxide (Table 1-2). The release of the biologically active molecules, such as cytokines and reactive oxygen intermediates, from activated Kupffer cells, the resident macrophage of the liver, has been implicated in hepatotoxicological and hepatocarcinogenic events. More recently it has been demonstrated that the Kupffer cell may participate at the tumor promotion stage of carcinogenesis. These results provide evidence linking the products released from the activated Kupffer cell to the tumor promotion stage of the carcinogenesis process (Klaunig and Kamendulis, 2004).

Metabolic activation and production of reactive oxygen species by cytochrome P450 has been proposed by Parke, *et al.* Reactive oxygen species can be produced from several sources during metabolism, including (*a*) through redox cycling in the presence of molecular oxygen, (*b*) through peroxidase-catalyzed single-electron drug oxidations, and (*c*) through "futile cycling" of cytochrome P450. Through the induction of cytochrome P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, arises following the breakdown or uncoupling of the P450 catalytic cycle. Cytochrome P450 2E1 is involved in the oxygenation of substrates such as ethanol, and is capable of generating a prolonged burst of reactive oxygen species near the site of substrate oxidation. Similarly, metabolism of Phenobarbital by P450 2B resulting in the uncoupling of the catalytic and subsequent release of superoxide anion. On the other hand, exogenous sources of ROS generation include those of various xenobiotics, chlorinated compounds, various transition metals and radiation, all of which have been documented to cause ROS induced damage to cellular macromolecules such as DNA, RNA, lipids and proteins both *in vitro* and *in vivo*. Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including nongenotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. The induction of oxidative stress and damage has been observed following exposure to xenobiotics of varied structures and activities. Chlorinated compounds, radiation, metal ions, barbiturates, phorbol esters, and some peroxisome proliferating compounds are among the classes of compounds that have been shown

to induce oxidative stress and damage *in vitro* and *in vivo* (Klaunig and Kamendulis, 2004; Franco *et al.*, 2008).

Oxidative stress has been implicated in various pathological conditions involving cancer, cardiovascular disease, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing. These diseases fall into two groups: (i) the first group involves diseases characterized by pro-oxidants shifting the thiol/disulfide redox state and impairing glucose tolerance so called "mitochondrial oxidative stress" conditions such as cancer and diabetes mellitus; (ii) the second group involves disease characterized by "inflammatory oxidative conditions" and enhanced activity of either NAD(P)H oxidase, leading to atherosclerosis and chronic inflammation, or xanthine oxidase-induced formation of ROS, implicated in ischemia and reperfusion injury (Valko *et al.*, 2007).

Table 1-2 Pathways for intercellular oxidant generation (Klaunig and Kamendulis, 2004)

1. Generation of reactive oxygen species via reduction of molecular oxygen
$O_2 + e^- \longrightarrow O_2^{-\bullet}$ (superoxide anion)
$O_2^{-\bullet} + H_2O \longrightarrow HO_2^{\bullet}$ (hydroperoxyl radical)
$HO_2^{\bullet} + e^- + H \longrightarrow H_2O_2$ (hydrogen peroxide)
$H_2O_2 + e^- \longrightarrow OH^- + {}^{\bullet}OH$ (hydroxyl radical)
2. Production of reactive nitrogen species
L-ARGININE + $O_2 \longrightarrow {}^{\bullet}NO$ (nitric oxide radical) + L-CITRULLINE
$O_2^{-\bullet} + {}^{\bullet}NO \longrightarrow ONOO^-$ (peroxynitrite)
$ONOO^- + CO_2 \longrightarrow ONOOCO_2^-$ (nitrosoperoxy carbonate)
$ONOOCO_2^- \longrightarrow {}^{\bullet}NO_2$ (nitrogen dioxide radical) + $CO_3^{-\bullet}$(carbonate anion radical)
3. Fenton reaction
$H_2O_2 + Fe^{2+} \longrightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$

1.2.2 Oxidative stress in the cancer process

Chemically induced cancer is a multistage process definable by at least three steps or stages; initiation, promotion and progression. The three stages model of carcinogenesis is shown in Figure 1-1. ROS can act in all these stages of carcinogenesis. Initiation stage involves a nonlethal and inheritable mutation in cells by interaction of a chemical with DNA. This mutation confers a growth advantage to that cell. For the mutation to be set a round of DNA synthesis must occur to lock in the mutation. The activation of the carcinogen to an electrophilic DNA-damaging moiety is a necessary step for this stage. ROS are believed to mediate the activation of such carcinogens through hydroperoxide-dependent oxidation that can be mediated by peroxyl radicals. This occurs with aflatoxin B_1, aromatic amines, and polycyclic aromatic hydrocarbon dihydrodiols. ROS or their byproduct of lipid peroxidation, such as malondialdehyde (MDA), can also directly react with DNA to form oxidative DNA adducts. The presence of carcinogen-DNA adducts and oxidative DNA adducts generated by chemical carcinogens suggest an interactive role of ROS in initiation. Therefore, ROS can have multiple effects in the initiation stage of carcinogenesis by mediating carcinogen activation, causing DNA damage, and interfering with the repair of the DNA damage. Promotion stage involves the selective clonal expansion of the initiated cell population through either increased cellular proliferation and/or inhibition of cell death. ROS are specifically generated in initiated cell populations such as preneoplastic foci in liver. Because ROS generation is related to P450 enzyme activity, oxidative stress may have an important role in the clonal expansion of these initiated cells. In fact, higher levels of ROS have been found in neoplastic nodules of rat liver than in the surrounding normal tissue. PB treatment enhanced this formation by increasing the mono-oxygenase system in the nodules. Another suggested source of ROS is from the oxidation of glutathione by γ-glutymyltranspeptidase in preneoplastic foci. These multiple sources of ROS may contribute to a persistent oxidative stress environment that results in pathophysiologic changes and allows for the selective growth of preneoplastic initiated cells. Many tumor promoters have a strong inhibiting effect on cellular antioxidant defense systems such as superoxide dismutase, catalase and glutathione. While a high level of

oxidative stress is cytotoxic to the cell and halts proliferation by inducing apoptosis or even necrosis, a low level of oxidative stress can in fact stimulate the cell division in the promotion stage and thus stimulate the promotion of tumor growth (Figure 1-2). This implies that production of ROS during this stage of carcinogenesis is the main line of ROS-related tumor promotion (Valko *et al.*, 2006). Tumor progression results in the development of malignant growth from benign lesions. In this stage oxidative stress may play a direct role in the development of cancer characteristics such as uncontrolled growth, genomic instability, chemotherapy resistance and invasion and metastasis. This persistent oxidative stress does not appear large enough to induce cell death because tumor cells have decreased cell sensitivity to oxidative stress. Cancer cells emerging from the multistep carcinogenic process with inactivated or deleted tumor-suppressor genes and/or activated oncogenes are much less dependent than normal cells on external growth factors because they can manufacture their own factors (Klaunig *et al.*, 1998).

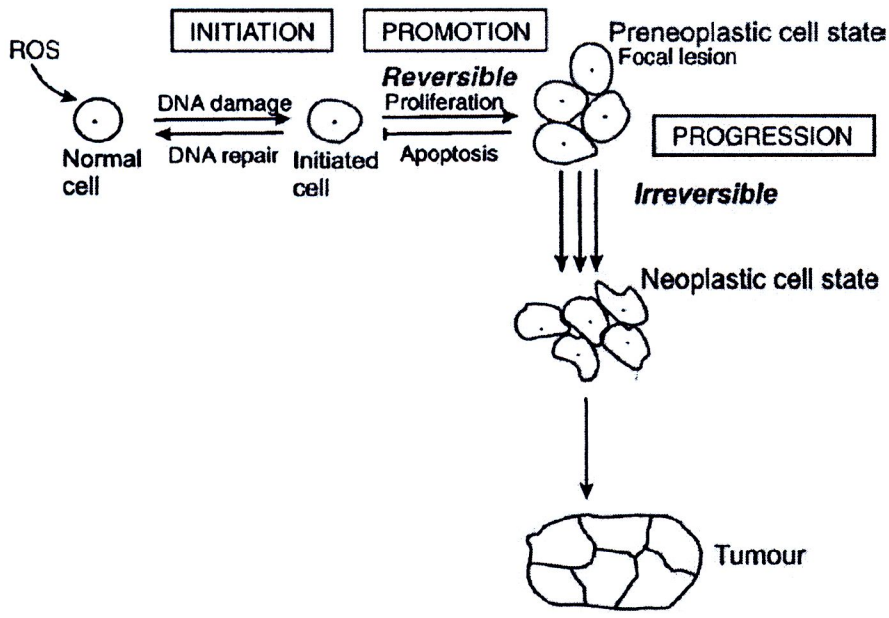

Figure 1-1 Three stages model of carcinogenesis (Valko *et al.*, 2006)

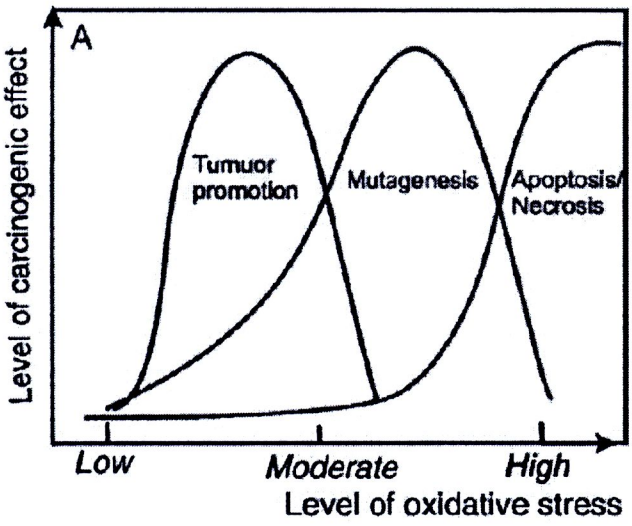

Figure 1-2 The level of carcinogenic effect and level of free radicals at various stages of carcinogenic process (Valko *et al.*, 2006)

1.2.3 Classification of carcinogens

Carcinogenic classification is by no means consensual. It is not easy to incorporate a carcinogenic compound into a certain group because the information obtained from different studies is increasingly complex. Some researchers classify them in function of their participation in each of the stages of carcinogenesis. In this way, incomplete carcinogens are mutagenic chemicals that initiate irreversible DNA damage. A complete carcinogen displays properties of both initiators and promoters simultaneously depending on the dosage and exposure time. Butterworth and his colleagues have classified chemical carcinogens in function of their mechanisms of action (Table 1-3).

A genotoxic carcinogen is one in which mutations are induced directly by covalent binding of the chemical or its metabolite to the DNA, or by directly altering chromosome structure or number. Not-directly genotoxic carcinogens are not DNA-reactive and are usually referred to by the term 'nongenotoxic'. Nongenotoxic carcinogens act as promoters and do not need metabolic activation. They do not react directly with DNA, do not raise adducts and show negative on mutagenicity tests

carried out *in vivo* and *in vitro*. Melnick *et al.* (1996) reviewed that exposure to these compounds favors the synthesis of other substances responsible for neoplasic development. These compounds promote effects on target cells which indirectly unchain neoplasic transformation or increase neoplasic development from genetically changed cells. Nongenotoxic carcinogens are classified as cytotoxic and mitogenic. Mitogenic compounds such as phorbol esters, dioxins, and phenobarbital induce cell proliferation in target tissue through interaction with a specific cellular receptor. Cytotoxic carcinogens cause cell death in susceptible tissues followed by compensatory hyperplasia, such as chloroform. If the carcinogen dose is high, some cells cannot survive. The more that nearby cells increase the number of cell divisions through regenerative procedures, the more likely it is that they will end up being prematurely recruited for the cell cycle and that the time available for reparation DNA will be inferior-this increases the probability of mutations occurring. On the other hand, necrosis cells are destroyed by the immune system and ROS, reactive nitrogen species (RNS) and proteolytic enzymes are produced. When production of ROS and RNS exceeds the cellular antioxidant capacity, it may cause oxidative damages to lipids, proteins, carbohydrates and nucleic acids, leading to carcinogenesis and cell death. Mitogenic compounds need to be present in certain concentrations to promote their activities. Contrastingly, the action of non-cytotoxic compounds is independent of their concentrations.

Diethylnitrosamine (DEN), known as *N*-Nitrosodiethylamine (NDEA), has been found in a variety of products that would result in human exposure, including mainstream and side stream tobacco smoke, meat and whiskey (Verna, *et al.*, 1996). Its chemical structure is shown in Figure 1-3. DEN has had extensive use as an experimental carcinogen, and although there is evidence of human exposure, no epidemiological studies have specifically investigated DEN-related human cancer. The International Agency for Research on Cancer review concluded that DEN was carcinogenic in all animal species tested, and that there was sufficient evidence of a carcinogenic effect to classify DEN as a probable human carcinogen (Group 2A), despite the lack of epidemiological data.

Table 1-3 Classification of carcinogens according to mode of actions (Butterworth *et al.*, 1995)

1. Genotoxic carcinogens
DNA reactive or DNA-reactive metabolites
Direct interaction to alter chromosome structure or number
May also be cytotoxic
Regenerative cell proliferation will enhance mutagenic/ carcinogenic activity
May also be mitogenic
Induced cell proliferation will enhance mutagenic/ carcinogenic activity
2. Nongenotoxic carcinogens
Cytotoxicants
Not DNA reactive
Cytolethal at carcinogenic doses
Induce regenerative growth
Mutations may occur secondary to regenerative cell proliferation
Accompanying critical effects may occur such as inflammation
Circulating growth factors may cause preferential growth of preneoplastic cells
Mitogens
Not DNA reactive
Not cytolethal at carcinogenic doses
Mitogenic stimulation of growth
May be acting through a specific receptor
Mutations may occur secondary to cell proliferation
May stimulate preferential growth of preneoplastic cells

DEN is a potent hepatocarcinogen in rats influencing the initiation stage of carcinogenesis during a period of enhanced cell proliferation accompanied by hepatocellular necrosis and induces DNA carcinogen adducts, DNA-strand breaks and in turn hepatocellular carcinomas (HCCs) without cirrhosis through the development of putative preneoplastic focal lesions (Verna, *et al.*, 1996). Chronic administration of a promoting agent, such as PB has many effects on the liver, including development of hyperplasia and hypertrophy without increasing cell death and has been shown to stimulate cell proliferation in focal relative to non-focal areas of carcinogen-challenged tissues (Chakraborty *et al.*, 2007). Nakae *et al.* showed that initiation with DEN-induced liver DNA-8-hydroxydeoxyguanosine adducts and suggested that oxidative stress participates in hepatocarcinogenesis. It appears that DNA-oxygen adducts or a concomitant alteration in the signaling pathway due to ROS may be important in hepatocarcinogenesis (Yadav and Bhatnagar, 2007).

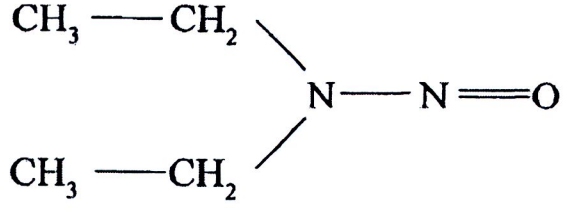

Figure 1-3 Chemical structure of diethylnitrosamine (Verna *et al.*, 1996)

Phenobarbital (PB) has been used widely as a sedative/hypnotic and for the treatment of epilepsy. PB is soluble in alcohol and slightly soluble in water and benzene. PB monosodium salt is more soluble in water and has been used in some of the studies. The structure of PB is shown in Figure 1-4.

The International Agency for Research on Cancer has evaluated PB and found sufficient evidence for carcinogenicity in experimental animals, but insufficient evidence in humans. Chronic administration of PB produces hepatocellular adenomas in rats and hepatocellular adenomas and carcinomas in mice. PB is not considered to be DNA reactive but is a liver tumor promoter when administered after various

initiating carcinogens such as DEN. In previous studies, a dose-response effect of PB on the increase of hepatic cancer was observed, exhibiting a threshold at low doses (Kitagawa, *et al.*, 1984; Pitot, *et al.*, 1987; Maekawa, *et al.*, 1992). PB fed in mice (Lee, 2000) and rats (Kinoshita, *et al.*, 2003) in low doses has occasionally been reported to exhibit an inhibitory effect on hepatocarcinogenesis initiated with the potent carcinogen DEN. Some studies have shown that growth of preneoplastic lesions by PB may be attributed to inhibition of apoptosis, increased oxidative stress by reactive oxygen species and spontaneous aberrations in DNA replication and repair. In addition to cytochrome P450s, PB induces several other enzymes such as NADPH cytochrome P450 reductase and transferases (Tharappel *et al.*, 2008).

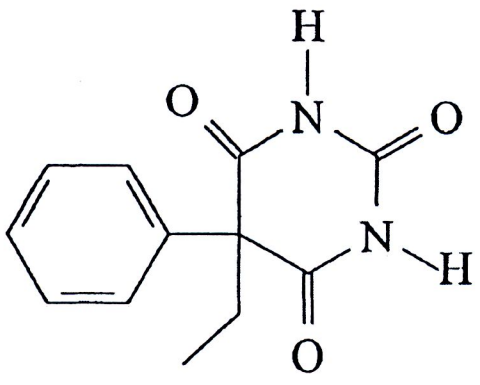

Figure 1-4 Chemical structure of phenobarbital (Whysner *et al.*, 1996)

1.2.4 Medium-term bioassay system

Long-term *in vivo* carcinogenic tests using rodents are generally considered as the most reliable method for the prediction of carcinogenic potential in man. However, they must satisfy costly regulatory guidelines for appropriate facilities, duration, maintenance of animals, sufficient numbers of rodents and histopathological examination. It is impossible to apply such comprehensive carcinogenicity *in vivo* testing to the large number of compounds introduced into our environment. Therefore, attention has concentrated on development of *in vitro* short-term tests with the aim of predicting the carcinogenicity of test compounds within a short period

This has allowed very large numbers of chemicals to be tested for mutagenicity in bacterial and other systems (Ishidate, *et al.*, 1988; Grant and Salamone, 1994), but comparison of different test results has indicated that mutagens do not necessarily possess carcinogenicity (Zeiger, E., 1987). While the *Salmonella* mutagenicity test or Ames test has gained widespread acceptance as an initial assay for the identification of potentially hazardous compounds, it cannot distinguish mutagenic carcinogens from mutagenic noncarcinogens. To bridge the gap between long-term carcinogenicity tests and short-term screening assays, a medium-term bioassay system based on induction of glutathione-*S*-transferase placental form (GST-P) positive liver cell foci (Figure 1-5) in rats has been developed (Ito, *et al.*, 1988). It contains two types of system, one for detection of initiation potential and the other for demonstration of promoting activities (Sakai *et al.*, 2002). The numbers and sizes of the GST-P positive foci which are analyzed and expressed as values per unit liver section (1 cm^2). When the yield of GST-P positive foci is significantly enhanced over the control value, a chemical is judged to possess carcinogenenic or promoting potential for the liver (Fukushima, *et al.*, 2005).

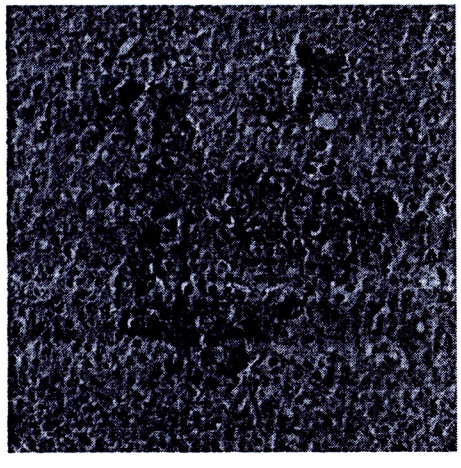

Figure 1-5 Glutathione-*S*-transferase placental form (GST-P) positive foci as an marker of liver medium-term bioassay system

1.2.5 Antioxidant defense system

Under normal physiological conditions, cells are capable of counterbalancing the production of reactive oxygen species with antioxidants (Table 1-1). Endogenous cellular antioxidant defenses are mainly enzymatic and include superoxide dismutase, glutathione peroxidase, and catalase. Nonenzymatic antioxidants such as vitamin E, vitamin C, β-carotene, glutathione and coenzyme Q function to quench reactive oxygen species. When the redox balance is shifted in favor of cellular oxidants, oxidative damage to nucleic acids, lipids, or proteins can result and produce modification to cell function and cell viability. Interestingly, many of the cellular antioxidants are regulated in part by the redox status of the cell (Klaunig and Kamendulis, 2004).

The cellular defense mechanisms can be divided into at least two levels according to their functions (Figure 1-6) (Lykkesfeldt and Svendsen, 2007). As a first level of defense against oxidants, the cell is equipped with a so-called antioxidant network. Antioxidants are capable of donating electrons to oxidants, thus quenching their reactivity under controlled conditions and making them harmless to cellular macromolecules. The antioxidants thereby become radicals themselves, but these are less stable and are not capable of inducing cellular damage. The oxidized antioxidants are subsequently recycled to their active reduced state by a number of efficient cellular processes fuelled by energy from NADPH. This recycling is the key to the power of the antioxidant network, which would otherwise deteriorate rapidly (Lykkesfeldt *et al.*, 2003).

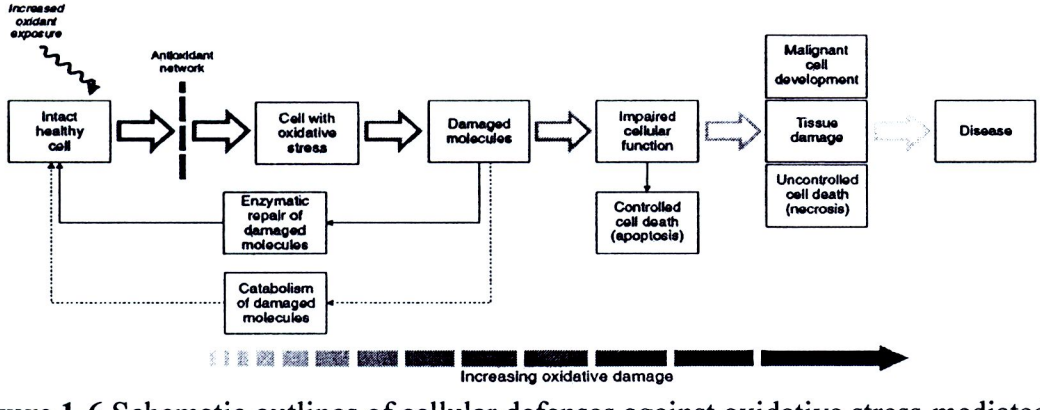

Figure 1-6 Schematic outlines of cellular defenses against oxidative stress-mediated cellular damage (Lykkesfeldt and Svendsen, 2007)

Furthermore, the defense mechanisms could be classified into 2 types which compose of enzymatic systems and chemical scavengers (Martfnez-Cayuela, 1995).

1. Primary defenses

Primary or preventive defenses diminish the initiation rate of radical reactions by decreasing free radical concentration.

1.1 Antioxidant enzymes

Superoxide dismutases (SOD)

These are a family of metalloenzymes with different prosthetic groups, variable intracellular location. The prevalent isozymatic form CuZnSOD has been found in almost all eukaryotic cells. SODs catalyze the $O_2^{-\bullet}$ dismutation to produce H_2O_2 and O_2 at a rate 10^4 times higher than spontaneous dismutation at physiological pH (reaction 1).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Catalase (CAT)

CAT, located in peroxisomes, is the enzyme that removes H_2O_2 from the cell when the latter is at high concentration (reaction 2). CAT is responsible for the removal of high levels of hydrogen peroxide.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2 \qquad (2)$$

Glutathione peroxidase (GPx)

GPx localized in the cytosol and mitochondria. GPx is an enzyme that catalyzes the reduction of H_2O_2 and organic free hydroperoxides requiring glutathione as co-substrate (reactions 3 and 4). Unlike CAT, it has a high substrate affinity. GPx, containing four selenium atoms responsible for its catalytic activity, is located in the cytoplasm of eukaryotic cells although it can also be found within mitochondria. Many kinds of tissue exhibit GPx activity.

$$H_2O_2 + 2\ GSH \rightarrow GSSG + H_2O \qquad (3)$$

$$ROOH + 2\ GSH \rightarrow GSSG + ROH + H_2O \qquad (4)$$

Glutathione reductase (GR)

It is a cytosolic protein with a tissue distribution similar to that of GPx. The enzyme reduces GSSG utilizing NADPH generated by various systems (reaction 5).

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+ \qquad (5)$$

1.2 Small molecules

There are a certain number of small molecules, widely distributed in biological systems, which can scavenge oxygen free radicals non-enzymatically. GSH, vitamin C, uric acid, taurine and hypotaurine are some of these small molecules.

GSH can either act by reducing peroxides to H_2O and GSSG by means of GPx (reactions 3 and 4) or react directly with oxygen radicals first forming the thiyl radical and later GSSG. Like GSH, vitamin C may reduce oxygen free radicals. Dehydroascorbate formed in this reaction may be reduced by GSH. Uric acid is an antioxidant which traps free radicals very effectively. This compound is an important protector against oxidation in plasma. It has been demonstrated that taurine and hypotaurine also have a protective function preventing damage by free radicals. These compounds are present in many biologic fluids.

Glutathione is important to the overall cellular redox balance. Because cellular glutathione concentration is about 500 to 1000 fold higher than the other redox regulating proteins, changes in the ratio of reduced to oxidized glutathione are directly reflective of intracellular redox alterations (Schafer and Buettner, 2001). Glutathione, the most abundant low molecular weight thiol in mammalian cells, is present in GSH and GSSG forms (Meister and Anderson, 1983). The GSH is 10 to 100 fold higher than GSSG. An increase in intracellular GSSG can arise from the breakdown of H_2O_2 by GPx (Cotgreave *et al.*, 1988). Because of the relatively low concentration of GSSG in the cell compared with GSH, a minor elevation in the oxidation of GSH to GSSG can result in a significant elevation in intercellular GSSG levels. GSSG can be

reduced to GSH by the NADPH-dependent glutathione reductase as well as via the thioredoxin/glutaredoxin systems. GSH also modulates the activity of thiol-dependent enzymes that contain cysteine residues sensitive to redox changes (Klatt and Lamas, 2000). GSH also is used as a cofactor for antioxidant enzymes such as GPx, involved in the reduction of peroxides, including membrane lipids peroxides formed upon oxidative insults. The GSH/GSSG ratio is normally closely regulated. Disruption of this ratio is involved in several cellular reactions involved in signal transduction and cell cycle regulation under conditions of oxidative stress; the GSH/GSSG ratio tends to decrease either through an increase in the level of glutathione disulfide or a decrease in GSH (Cotgreave and Gerdes, 1998; Herlich and Bohner, 2000). The redox balance can be maintained, however, even in the face of an oxidative stress by increasing glutathione reductase activity or via elimination of GSSG from cells (Schafer and Buettner, 2001).

1.3 Other proteins

Other enzymatic proteins such as DT-diaphorase or epoxide hydrolase are also considered to be primary antioxidant defenses.

Transition metals are involved in hydroxyl radical generation through the Fenton-type reactions that they catalyze. Nevertheless, when these metals are linked to proteins, they are unable to carry out this catalysis. Free iron concentration is kept low by binding to transferrin or lactoferrin, glycoproteins that transport iron in the circulation, or to ferritin, which stores it. Albumin and caeruloplasmin transport copper in plasma, but the latter does not prevent interaction between this metal and $O_2^{\cdot\cdot}$ or H_2O_2 to generate $OH^{\cdot}$.

2. Secondary defenses

Secondary or chain-breaking defenses, on the other hand, trap propagator radicals, stopping their harmful effects in the early stages.

2.1 Enzymes

Many glutathione transferases that show peroxidase activity dependent on glutathione are defense systems against lipid peroxidation. These enzymes, which metabolize hydroperoxides of low molecular mass, but not H_2O_2, need the activity of the phospholipase A_2 in order to function. There are phospholipase A_2 isozymes in every cellular type, all of them playing a major role in the metabolism of membrane phospholipids. Ursini *et al.* have reported the existence of an enzyme with peroxidase activity, named phospholipid hydroperoxide glutathione peroxidase, which is capable of reducing lipid hydroperoxides without the action of phospholipase A_2. Different oxidoreductases that catalyze reduction reactions of thiol and other protein groups when these are oxidatively damaged are protective enzymes against oxygen free radicals. Oxidatively modified proteins can be involved in reactions harmful to cells. As a result, the degradation of irreparably damaged protein contributes to the host defense. Macroxyproteinase and other mammalian proteolytic enzymes have been demonstrated to be responsible for this process.

Nuclear enzymes for DNA repair may be considered to be defense systems against oxidative injury by oxygen free radicals. For example, when an apurinic or apyrimidinic site is formed by oxidative damage and the DNA replication is stopped, DNA polymerase I and DNA ligase act repairing the break. There are other enzymes that have a critical role in protecting the cellular DNA and the flux of genetic information, as in the cases of endonucleases and glycosylases. Activities of these enzymes have been described in human as well as in other eukaryotic organisms. However, the specific role of these proteins in repairing DNA is still unclear. As with proteins, when the DNA damage is too great to be repaired, cells have to be eliminated. Cytotoxicity can be initiated by the poly (ADP-ribose) synthetase when this lowers NAD^+ levels. This enzyme, which is stimulated by DNA strand breaks, transfers ADP ribose groups of NAD^+ to amino acids or to other ADP-ribose groups previously linked to the protein. In this way, the DNA unpacking is allowed.

2.2 Other molecules

Vitamin E, also known α-tocopherol, the major lipid-soluble antioxidant present in all cellular membranes, protects against lipid peroxidation. It reacts with oxygen free radicals donating a hydrogen ion and converting them to less reactive forms. The tocopheryl radical may also be directly reduced by the ascorbic acid-GSH redox couple. β-carotene, the most efficient scavenger of singlet oxygen known in nature, has a synergistic action with vitamin E, but, while β-carotene acts at low oxygen pressure, vitamin E acts at high pressures. Vitamin E protects conjugated double bonds of β-carotene from oxidation. Finally, bilirubin, a waste product, must be mentioned. It has recently been suggested that bilirubin breaks the chain of damage propagation by reacting with the oxygen free radicals which caused this damage.

A second and highly important level of defense is the ability to detect and repair or remove oxidized and damaged molecules. Included in this part of the defense is a series of DNA repairing enzymes capable of detecting oxidized bases or misincorporations, cutting them out and inserting the correct undamaged base in the DNA. The extent of this activity is enormous; it has been estimated that the DNA of each living cell is subjected to 10,000 to 100,000 oxidative modifications per day, more than 99.99% of which are enzymatically repaired. Other means of second level defense include catabolism of nonfunctional or modified proteins and lipids.

Finally, if the extent of the oxidative damage exceeds the capacity of repair and removal, the organism is equipped with one final weapon, controlled cell suicide or apoptosis (Payne et al., 1995). The ability to induce programmed cell death is of major importance in a variety of bodily functions, including control of tissue growth, and is apparently under control by several signaling pathways. However, one of these appears to be that apoptosis is induced by increased oxidative stress and thus constitutes a final resort to encapsulate and isolate the damaged cells (Payne et al., 1995).

1.2.6 Mechanisms of chemopreventive agents

Because carcinogenesis comprises three different stages, initiation, promotion and progression, many potential chemopreventive agents can be categorized broadly as blocking agents, which prevent the initiation stage, or suppressing agents, which arrest or reverse the promotion and progression of cancer, probable by affecting or disturbing crucial factors that control cell proliferation, differentiation, senescence or apoptosis. Many natural products from our daily consumption of fruits, vegetables and tea beverages, such as resveratrol from grapes, sulforaphane from broccoli, genistein from soy, curcumin from turmeric powdered food preparations, and epigallocatechin-3-gallate (EGCG) from green tea, can be considered blocking or suppressing agents (Manson, 2003). The mechanisms that are likely to underlie the effectiveness of these dietary chemopreventive compounds are summarized in Table 1-4. There appear to be several different cellular and molecular mechanisms that underlie the blocking and suppressing effects of chemopreventive compounds, and many of these compounds appear to possess both blocking effects and suppressing effects. Thus, the anti-carcinogenic function of these compounds might be attributed to a combination of their cytoprotective effect on normal cells and their cytotoxic effect on preneoplastic and/or neoplastic cells (Chen and Kong, 2005).

Early epidemiological studies have suggested that the reduction of cancer risk related to the consumption of fruits and vegetables. Moreover, diets high in fiber-containing foods are associated with a reduced incidence of cancer, especially cancer of the colon. Thus, increasing evidence exists that plant-based food possesses cancer-preventive properties. The chemopreventive potential of health-promoting phytochemicals is expected to combine antioxidant, anti-inflammatory, immune enhancing, and anti-hormone effects. Additionally, modifications of drug-metabolizing enzymes, influences on the cell cycle and cell differentiation, induction of apoptosis and suppression of proliferation and angiogenesis are often playing roles in the initiation and secondary modification stages of neoplastic development (Tsuda *et al.*, 2004; Bhat and Singh, 2008; Gopalakrishnan and Tony Kong, 2008). Plant chemicals thus interfere with tumor initiation, promotion and progression by acting directly on carcinogen activation, tumor cell proliferation and physiological

conditions affecting the tumor growth, respectively. As multiple mechanisms are involved in the protective effects, it is difficult to identify the relative contributions of various components of a plant-based diet to overall cancer risk reduction (Steinmetz and Potter, 1996). Moreover, the synergism among these compounds may account for the final beneficial effect (Tsuda *et* al., 2004; Thangapazham *et al.*, 2006; Hodek *et al.*, 2009).

For a rational implementation of chemoprevention strategies it is essential not only to assess safety and efficacy of candidate chemopreventive agents in preclinical models and in humans but also to understand their mechanisms of action.

Three levels of prevention in connection with the possible mechanisms of cancer chemopreventive agents (De Flora and Ferguson, 2005).

1. Primary prevention

Primary prevention, having the goal of preventing the occurrence of the malignant disease, includes inhibition of mutation and cancer initiation, either in the extracellular environment or inside cells, followed by inhibition of tumor promotion.

1. Inhibition of mutation and cancer initiation in extracellular environment or nontarget cells
    - 1.1. Inhibition of uptake of mutagens/carcinogens
    - 1.2. Inhibition of the endogenous formation of mutagens and carcinogens: Inhibition of the nitrosation reaction and modification of the intestinal microbial flora.
    - 1.3. Complexation, dilution and/or deactivation of mutagens/carcinogens outside cells: By physical or mechanical means, by chemical reaction and by enzyme-catalyzed reaction.
    - 1.4. Favoring absorption of protective agents
    - 1.5. Stimulation of trapping and detoxification in nontarget cells
2. Inhibition of mutation and cancer initiation in target cells
    - 2.1. Modification of transmembrane transport: Inhibition of cellular uptake and stimulation of extrusion outside cells.

2.2. Modulation of metabolism: Inhibition of activation of promutagens/ procarcinogens by Phase I enzymes, induction of Phase I detoxification and Phase II conjugation pathways, or acceleration of decomposition of reactive metabolites and stimulation of activation, coordinated with detoxification and blocking of reactive metabolites.

2.3. Blocking or competition: Trapping of electrophiles by either chemical reaction or enzyme-catalyzed conjugation, antioxidant activity and scavenging of reactive oxygen species and protection of DNA nucleophilic sites.

2.4. Inhibition of cell replication

2.5. Maintenance of DNA structure and modulation of DNA metabolism and repair: Increase of fidelity of DNA replication and repair, stimulation of repair and/or reversion of DNA damage, inhibition of error-prone repair pathway, correction of hypomethylation, inhibition of histone deacetylation and blocking of telomerases or inhibition of their activity.

2.6. Control of gene expression: Targeted inactivation of oncogenes, inhibition of oncogene expression, inhibition of oncogenes sequences or activity, neutralization or post-translation modification of oncogene products, replacement of deleted tumor suppressor genes by antidiotypic antibodies, mimicking the DNA binding of tumor suppressor genes by antidiotypic antibodies and killing of cells lacking tumor suppressor genes

3. Inhibition of tumor promotion

3.1. Inhibition of genotoxic effects

3.2. Antioxidant activity and scavenging of free radicals

3.3. Anti-inflammatory activity: Cyclooxygenase inhibition, lipooxygenase inhibition and inhibition of inducible nitric oxide synthase.

3.4. Inhibition of proteases

3.5. Inhibition of cell proliferation: Inhibition of ornithine decarboxylase, promoting proteasomal degradation of cyclins and interference with multiple signaling pathways.

3.6. Induction of cell differentiation

3.7. Modulation of cell apoptosis
3.8. Signal transduction modulation
3.9. Protection of intercellular communications

Table 1-4 Potential mechanisms of dietary chemopreventive compounds

Function	Examples	Source
Blocking agents		
Enhance detoxification of carcinogens	Curcumin	Turmeric
	Sulforaphane	Cruciferous vegetables
	Indole-3-carbinol	Cruciferous vegetables
Inhibit cytochrome P450-mediated activation of carcinogens	Isothiocyanates	Cruciferous vegetables
Antioxidant activity (scavenge free radicals)	Selenium	Nuts and meat
	Vitamin E	Vegetable oils
'Trap' carcinogens and prevent their interaction with DNA	Flavonoids	Fruits and vegetables
Suppressing agents		
Disrupt the cell cycle and/or induce apoptosis	EGCG	Green tea
	Quercetin	Onions and tomatoes
	Resveratrol	Grapes
	Curcumin	Turmeric
	Sulforaphane	Cruciferous vegetables
Modulate hormone activity	Genistein	Soy beans
Modulate nuclear receptors	Vitamin D	Fish
	Retinoids	Eggs and milk
Suppress gene expression by DNA methylation	Folic acid	Fruits and vegetables

2. **Secondary prevention**

Secondary prevention exploits a variety of mechanisms aimed at inhibiting progression of a timely diagnosed benign tumor towards malignancy.

1. Inhibition of tumor promotion
    1.1. Inhibition of tumor progression
    1.2. Antioxidant activity and scavenging of free radicals
    1.3. Inhibition of proteases
    1.4. Signal transduction modulation
    1.5. Effect on the hormonal status: Selective estrogen receptor modulation, aromatase inhibition, selective blocking of prostaglandin E_2 receptors, decrease in ovarian hormones by dietary isoflavones, inhibiting the pituitary secretion of luteinizing hormone, preventing conversion of testosterone into dehydrotestosterone by 5α-reductase and selective androgen receptor antagonism.
    1.6. Effect on the immune system
    1.7. Inhibition of angiogenesis
    1.8. Antineoplastic activity by mechanical, physical, chemical, or biological means

3. Tertiary prevention

Tertiary prevention has the goal of preventing local relapses of the disease and of inhibiting invasion and metastasis.

1. Inhibition of invasion and metastasis
    1.1. Antioxidant activity and scavenging of free radicals
    1.2. Signal transduction modulation
    1.3. Inhibition of cell proliferation
    1.4. Modulation of cell apoptosis
    1.5. Induction of cell differentiation
    1.6. Inhibition of angiogenesis
    1.7. Effect on cell-adhesion molecules

1.8. Inhibition of proteases involved in basement membrane degradation and modulation of the interaction with the extracellular matrix

1.9. Activation of antimetastasis genes

1.2.7 Anthocyanins

Chemistry of anthocyanins

Anthocyanins belong to the widespread class of phenolic compounds collectively named flavonoids. They are water-soluble glycosides and acylglycosides of anthocyanidins, which are polyhydroxy and polymethoxy derivatives of the 2-phenylbenzopyrylium (flavylium) cation (Figure 1-7) (Wu *et al.*, 2004; Prior and Wu, 2006). The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule. There are 17 known naturally occurring anthocyanidins or aglycones which are listed in Figure 1-8. Only six anthocyanidins are common in higher plants such as pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp). The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3, 5-diglycosides and 3, 7-diglycosides. 3-Glycosides occur about two and half times more frequently than 3, 5-diglycosides. So, the most widespread anthocyanin is cyanidin 3-glucoside (Kong *et al.*, 2003). Proanthocyanidins (PAs) are oligomeric and polymeric flavan-3-ols. The size of proanthocyanidin molecules can be described by their degree of polymerization. PAs containing (epi) catechin or (epi) gallocatechin as subunits are named procyanidins or prodelphinidins, respectively.

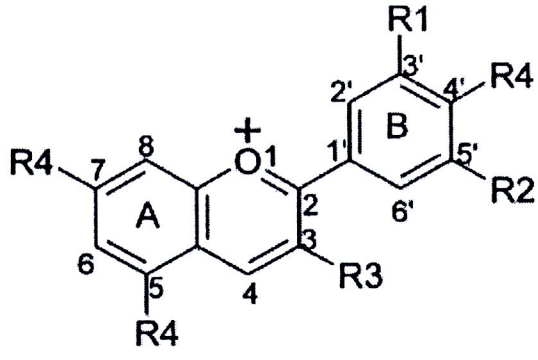

Figure 1-7 General anthocyanins structure

Pharmacokinetics and metabolism of anthocyanins

In spite of the biologic activities of anthocyanins demonstrated *in vitro*, the efficiency *in vivo* would depend on absorption, metabolism, tissue distribution, and excretion (Scalbert and Williamson, 2000). There is still considerable uncertainty about the exact mechanism of the absorption of dietary phenolic compounds from the gastrointestinal tract. In general, two mechanisms are involved in flavonoids absorption in the intestine. Flavonoids are present as glycoside conjugates in vegetables, and they can be deglycosylated by endogenous β-glycosidase (Gee, *et al.*, 2000) or by gut microflora enzymes, and further absorbed by passive diffusion of the aglycone form (Hollman and Katan, 1999). The second mechanism would be the absorption of the glycosidic form by direct interaction with the hexose transport pathway (Matsumoto, *et al.*, 2001). Several studies have shown that anthocyanins are quickly absorbed in the stomach (Talavéra, *et al.*, 2003) and small intestine (Talavéra, *et al.*, 2004). Although the mechanism of absorption of anthocyanins in the stomach is unclear, Passamonti, *et al.* (2003) suggested the involvement of an anion translocator such as bilitranslocase, expressed in the gastric epithelium. The anthocyanins are present in plasma mainly in the intact form and as a minor level methylated and/or conjugated with glucuronic acid (Ichiyanagi, *et al.*, 2005; Talavéra, *et al.*, 2005). Matsumoto *et al.* (2001) demonstrated that cyanidin-3-glucoside (cy-3-glu) and cy-3, 5-diglycoside were excreted in their unchanged forms in human urine, in agreement

Name	Substitution pattern							Color
	3	5	6	7	3'	4'	5'	
Apigeninidin	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	OH	OH	OH	OH	H	OH	H	Orange
Capensinidin	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanidin	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	H	OH	H	OH	OH	OH	H	Orange
Malvidin	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	OH	OH	H	OH	H	OH	H	Orange
Peonidin	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidin	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	H	OH	H	OH	OH	OH	OH	Red

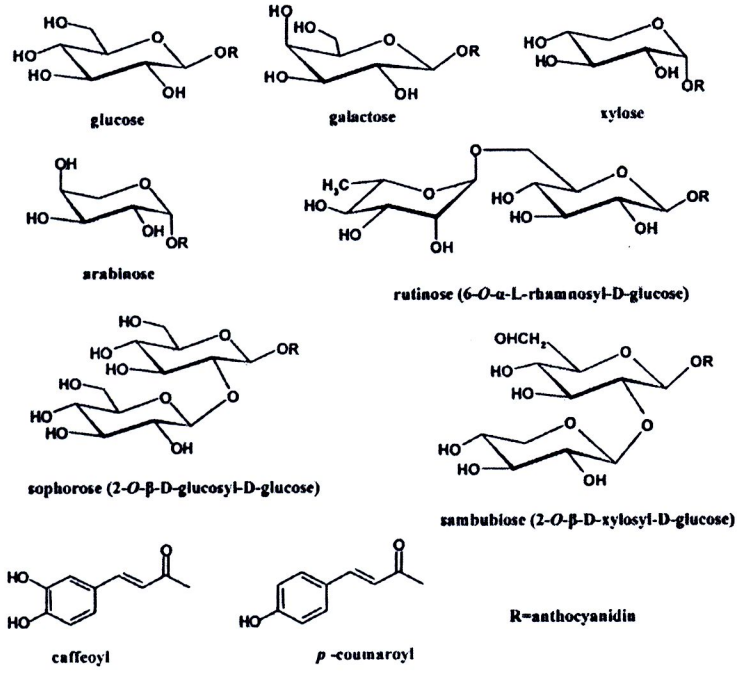

Figure 1-8 Structures of known naturally occurring anthocyanidins, sugar moieties, and acylated substitutes (Kong *et al.*, 2003; Wu *et al.*, 2004; Ovando *et al.*, 2009)

with the finding of Cao *et al.* (2001). Some of the metabolites found after administration of cy-3-glu were produced during absorption by the enterocyte, and other metabolites were produced in the liver and kidney.

The bioavailability, pharmacokinetics of distribution, and metabolism of anthocyanins in animals and in humans have been studied. In general, in both animals and humans, the anthocyanins are absorbed as intact glycosides, and their absorption and elimination are rapid. However, the efficiency of their absorption is relatively poor. The researchers investigated the absorption and metabolism of black raspberry anthocyanins in humans when administered orally at high doses (2.69 ± 0.085 g/day). Peak plasma levels of the four anthocyanins in black raspberries were observed within 2 h of oral berry treatment and their elimination from plasma followed first-order kinetics. They were excreted both as intact anthocyanins and as methylated derivatives in the urine within 4-8 h of berry ingestion. Overall, less than 1% of the administered dose of the berry anthocyanins was absorbed and excreted in urine. Similar results have been obtained in studies of the absorption and metabolism of anthocyanins in rodents. Anthocyanins have been shown to inhibit malignant cell growth, stimulate apoptosis and modulate oncogenic signaling events *in vitro* in the 10^{-6} to 10^{-4} M concentration range.

Studies of the uptake of anthocyanins in humans after their consumption as mixtures suggest that they reach levels of 10^{-8} to 10^{-7} M in human blood, or far below the levels required to exhibit anti-carcinogenic effects *in vitro*. Thus, it is unclear whether the concentrations *in vivo* are sufficient to elicit anti-carcinogenic effects in humans (Cooke, *et al.*, 2005; Wang and Stoner, 2008).

Biological activities of anthocyanidins

Anthocyanidins have been shown to inhibit malignant cell survival and oncogenic signalling with reasonable potency. In terms of inhibition of neoplastic cell survival, anthocyanidins have shown greater potency than their glycosylated counterparts. Anthocyanidins have been shown to inhibit the growth of embryonic fibroblasts and of cancer cells derived from malignant human tissues from a variety of origins including lung, breast, uterus, vulva and colon (Meiers *et al.*, 2001; Hou *et al.*,

2005; Hyun and Chung, 2004). Among anthocyanidins, delphinidin possessed the highest growth-inhibitory activity. At a structural determinant in anti-proliferative activity suggesting dependence of potency on the presence of hydroxyl groups on ring B of the anthocyanidin molecule. Inconsistent with this inference is the finding that in some cell types the potency of malvidin was equivalent to, or greater than, that of delphinidin (Hou *et al.*, 2005; Hyun and Chung, 2004).

The aglycones generated from the most abundant anthocyanins, cyanidin, delphinidin, malvidin, pelargonidin and petunidin, were growth inhibitors of human stomach, colon, lung, breast and CNS cancer cells (Zhang *et al.*, 2005). Delphinidin suppressed COX-2 by blocking MAPK-mediated pathways with the attendant activation of nuclear factor-kB (NF-kB), activator protein-1 (AP-1) and C/EBPd. These findings provide the first molecular basis that anthocyanidins with ortho-dihydroxyphenyl structure may have anti-inflammatory properties through the inhibition of MAPK-mediated COX-2 expression (Hou *et al.*, 2005).

Chemopreventive effects of anthocyanidins based on the molecular mechanisms of anticarcinogenesis, anti-inflammation, and apoptosis induction of malignant cells (Hou *et al.*, 2004) are presented in Figure 1-9.

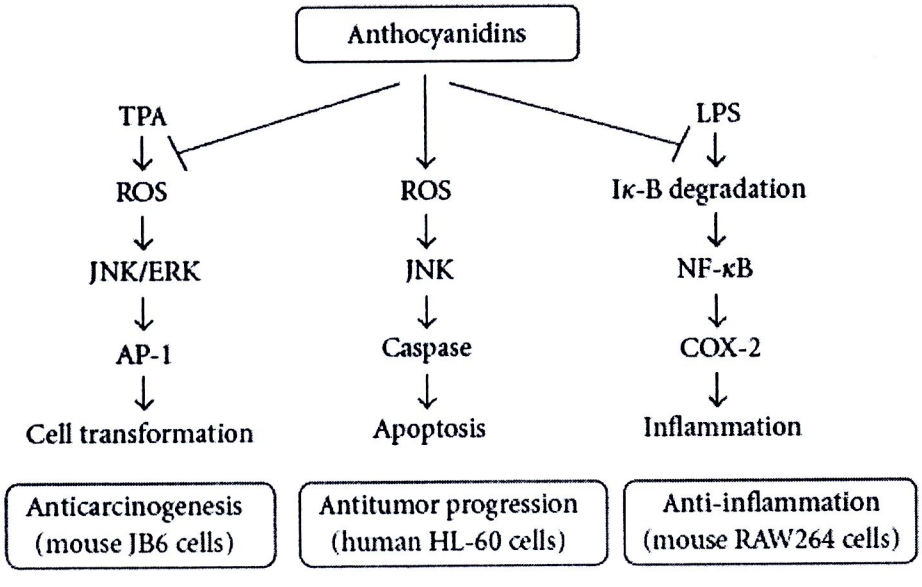

Figure 1-9 A schematic molecular view of cancer chemoprevention by anthocyanidins. (Hou *et al.*, 2004).

Biological activity of anthocyanins

Anthocyanins have been shown to be strong antioxidants, and may exert a wide range of health benefits through antioxidant or other mechanisms (Wang, *et al.*, 1999; Zheng and Wang, 2003; Feng *et al.*, 2007). Several studies have reported beneficial effects of anthocyanins in the treatment of various microcirculation diseases resulting from capillary fragility. Anthocyanins may also prevent cholesterol-induced atherosclerosis and inhibit platelet aggregation. Other biological properties such as anti-inflammatory and anticarcinogenic activities have also been described for anthocyanins. The positive effects of anthocyanins could be related to their potent antioxidant activity demonstrated in various *in vitro* studies (Felgines *et al.*, 2002; Hassimotto *et al.*, 2005) that are related to their ability to inhibit lipid peroxidation by radical scavenging activity and metal chelating properties. Studies on the relationship between anthocyanin structure and antioxidant capacity have shown that different patterns of hydroxylation and glycosylation in anthocyanidins appear to modulate their antioxidant properties (Rice-Evans *et al.*, 1996). Anthocyanins can react with reactive oxygen species (ROS) and thus interrupt the propagation of new free radical species. The double bonds present in the phenolic ring, the hydroxyl side chains, and even the glycosylation contribute to the scavenging activity. Anthocyanins, particularly cyanidin glycosides, have been found to possess a broad spectrum of biological activities, including scavenging effects on activated carcinogens and mutagens and effects on cell cycle regulation (Zheng, *et al.*, 2003; Lazzé, *et al.*, 2004). Previous studies suggest that the antitumor activities of cyanidin glycosides could be related to their ability to induce apoptosis and that these products may have potential as agents for cancer treatment as well (Wenzel, *et al.*, 2000; Fimognari, *et al.*, 2004; Lazzé, *et al.*, 2004; Ding, *et al.*, 2006). All of these activities are thought to be related to the antioxidant properties of the cyanidins (Kahkonen, *et* al., 1999; Zheng, *et* al., 2003; Wang, *et* al., 2005).

1.2.8 *Cleistocalyx nervosum* var. *paniala*

Cleistocalyx nervosum var. *paniala*, also known as Ma-kiang; Figure 1-10, is a perennial tree belonging to the Myrtaceae family. It is found growing in scatter locations in some villages of the northern provinces of Thailand such as Chiang Mai, Chiang Rai, Lamphun, Lampang and Mae Hong Son. Ma-kiang fruit is sour and slightly astringent with scant smell. The rich purplish red color of *C. nervosum* is characterized by an anthocyanin profile and a major compound was cyanidin 3-glucoside (Jansom, *et al.*, 2008). The researchers reported that total phenolic compounds of C. *nervosum*, which storage at 0, 4 and 10 °C, was stable. However, anthocyanin content and antioxidant activities were varying by temperature of storage (Patthamakanokporn, *et al.*, 2008). The previous report showed *C. nervosum* extract exhibited low cytotoxic activities against HeLa, COR L23 and MRC-5 (IC_{50} > 50 μg/ml) (Jansom, 2007). The previous study has been reported that *C. nervosum* was significantly enhanced NK cells activity and suggested that *C. nervosum* has stimulating activity on human lymphocytes and could be clinically useful for modulating immune functions of the body (Sriwanthana *et al.*, 2007). Recently, there is no any report about *in vivo* study on the biological activities of *C. nervosum*.

Figure 1-10 *Cleistocalyx nervosum* var. *paniala* (Ma-kiang)

1.3 Objectives of the study

1. To determine chemical constituents of aqueous extract of *C. nervosum*.
2. To evaluate the acute and subacute toxicities of aqueous extract of *C. nervosum*.
3. To investigate *in vitro* and *in vivo* antioxidant activity of aqueous extract of *C. nervosum*.
4. To investigate effect of aqueous extract of *C. nervosum* on oxidative stress induced early stages of hepatocarcinogenesis in rat.