CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a type of disease characterized by abnormal cells which divide without control. These abnormal cells have the ability to invade other tissues.

Cell is the basic unit of life. Under normal condition, cells in the body will follow an orderly path of growth, division and death in order to keep the body stay healthy. When the cells become old or damaged, the cells will undergo apoptosis process. These cells will then be replaced with new cells. Oxidative DNA damage may produce gene mutation and resulting in heritable mutation (Chaudhary, 1994). When the DNA of a cell become damaged or changed, the normal cell growth, division and death is affected. Unlike normal cells, cancer cells do not undergo programmatic death (apoptosis) when they should. These cells will continue to grow and divide and lead to a mass of cells known as tumor.

Cancer cells are able to spread to the whole body through the blood and lymph systems. Cancer is classified by the organ or type of cell that it initiated from.

However, not all tumors are cancerous. Tumor can be classified as benign tumors and malignant tumors. Benign tumors are not cancerous. Cells in benign tumor will not spread to other parts of body. These types of tumors can be removed through surgery. The malignant tumors are cancerous. The cells in malignant tumor are able to spread to other parts of body through invasion. The process of spreading, growing, invading and destroying other healthy tissue part is known as metastasis.

Incorrect diet, genetic predisposition and environment are the factors that may lead to cancer (Reddy *et al*., 2003). In the year 2003, The Second Report of the National

Cancer Registry of Malaysia suggested that a total of 23,746 cancer cases were diagnosed among Malaysians. This total amount comprises of 10,473 males and 13,273 females. The rough estimate for females was 127.6 per 100,000 populations and 97.4 per 100,000 populations for the males. The age standardized incidence rate (ASR) for all cancers was 154.2 per 100,000 females and 134.3 per 100,000 males (The Second Report of National Cancer Registry, 2003). Cancer is the third causes of death in Malaysia.

2.1.1 Carcinogenesis

Oxidative damage on DNA will lead to mutation. Carcinogenesis may occur if there is severe or prolonged exposure of oxidative stress (Guyton and Kensler, 1993).

Carcinogenesis proceeds through three distinct stages which are known as initiation, promotion and progression.

The initiation stage starts when the DNA of a single somatic cell reacts with cancer-producing substance (carcinogen) and finally led to heritable mutation. This initiated mutation may let the initiated cell to grow either through the induction on cell proliferation or inhibition of apoptosis. The initiated cells escaped from the cellular regulatory mechanism. The initiation stage may remain dormant and may only be at risk for developing cancer at a later stage.

During the promotion stage, initiated cells are exposed to a tumor promoter. These cells will undergo phenotypical clonal expension. There are two types of tumor promotor known as external and internal stimuli. The tumor promoter can be either one. Only the initiated cells are stimulated to grow. The cells expansions are controlled by the direct effect of the tumor promotor and indirect effect of the tumor promoter on the

adjacent cells. The benign tumor is produced through the initiation and promotion step. These cells will undergo progression step known as malignant conversion. This process occurs over a long time and the growth rates are affected by hormones, vitamin and minerals and growth factor. Thus, a change in lifestyle and diet may have beneficial effect so that development of cancer will not happen during their life.

Malignant conversion process needs genetic alteration. At this stage, the cellular growth is deregulated thus proceeds uncontrolled. This stage involves progression and spread of the cancer cells, and the cellular expansion becomes very rapid. However, some inhibitors such as antioxidants have the ability to inhibit the growth of abnormal cells. During the malignant conversion process, a change in lifestyle and diet may have less impact of the developing of cancer. However, all these conditions will stop when the tumor becomes progressively more autonomous and controllable only by drastic intervention (Guyton and Kensler, 1993).

2.1.2 Carcinogen

Exposure to environmental carcinogen may lead to cancers. Natural and handmade chemicals, radiation and viruses are known as environmental carcinogens. Carcinogens can be divided into genotoxic carcinogens, procarcinogens and epigenic carcinogens. Genotoxic carcinogens are primary carcinogens; they will react directly with nucleic acids and affect the cellular constituents. Procarcinogens, induce carcinogenesis through metabolic activation to induce carcinogenesis. Epigenetic carcinogens are not genotoxic. Promotors (e.g. phorbol esters, saccharin), solid waste (e.g. asbestos), hormones (e.g. estrogens), immunosuppressants (e.g. purine analogues) and cocarconogens (e.g. catechol) are examples of epigenetic carcinogens (Timbrell, 2000). They are cancer-initiating compounds.

Genetic preposition have also been documented as carcinogen. For example, patients with genetic xeroderma pigmentosum are more susceptible to skin cancer (Reddy *et al*., 2003).

Certain diets lead to development of cancers. Moulds and aflatoxins (for example, in peanuts and maize), nitrosamines (in smoked meats and other cured products), rancid fats and cooking oils, alcohol, and additives and preservatives are (Reddy *et al*. 2003). A combination of foods may have a cumulative effect, and when incorrect diet is added to a polluted environment, smoking, UV radiation, free radicals, lack of exercise, and stress, the stage is set for DNA damage and cancer progression (Reddy *et al.* 2003).

2.1.3 Natural products and defense against carcinogenesis

The National Cancer Institute (USA) began to screen plant extracts with antitumor activity during the 1960s (Monks *et al*., 2002). The understanding of mechanism involved in carcinogenesis has lead to development of chemoprevention (cancer prevention through chemical intervention). In the 1970s, National Cancer Institute (NCI) established Laboratory of chemoprevention and has found the application of retinoid in suppressing malignant transformation (Sporn and Newton, 1979). Studies in cell-culture systems and animals' models identified that some natural products from plants can markedly influence the growth and development of cancer (Ames, 1983; Wattenberg, 1983). Vitamin C, vitamin A, vitamin E, retinoid and beta-carotene has demonstrated notable cancer-preventive activity in animal systems (Bertram *et al*., 1987).

An analysis indicates that over 60% of approved drugs for chemotherapeutic are derived from natural compounds (Cragg *et al*., 1997). A lot of natural compounds from

vegetables and herbs were found to exert chemopreventive properties against carcinogenesis. For example, *Rhizoma zedoariae* produces a compound called lemene. This compound is able to inhibit antitumor activity in human and murine tumor cells *in vitro* and *in vivo* (Zheng *et al.,* 1997). Another compound named bis-benzylisoquinoline obtained from *Coriolus versicolor* was found to inhibit *in vitro* proliferation of HL-60 cells or induction of apoptosis in promyelocytic leukemia (Dong *et al*. 1997).

2.1.4 Difference between natural products therapeutic and with conventional therapeutic

The interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/ or incorrect use of synthetic drugs results in side effects and other problems. Furthermore, a large percentage of the world's population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that "natural" products are harmless (Rates, 2001). Natural products are ideal candidates for new therapeutics due to their chemical diversity, structural complexity, lack of substantial toxic effects and inherent biological activity. Besides disrupt aberrant signaling pathways leading to cancer (i.e., proliferation, deregulation of apoptosis, angiogenesis, invasion and metastasis) natural products also synergize with chemotherapy and radiotherapy.

Conventional therapies using synthetic drugs may cause a lot of serious side effects and other problems. Chemotherapy drugs kill cells by damaging the RNA or DNA of the cells. Chemotherapy drugs are not highly specific toward the cancer cells. The drugs will kill all rapidly dividing cells (including normal and cancer cell). The "normal" most commonly affected cells are the blood cells, the cells in the mouth,

stomach and bowel, and the hair follicles; resulting in low blood counts, mouth sores, nausea, diarrhea, and/or hair loss. Natural compounds will only act selectively on the cancer cells. Thus, it will not destroy the normal cell.

Cancer cells and normal cells proliferate in response to proliferation signals. Cancer cells have higher proliferation signals as compared to the normal cells. Natural compounds able to interrupt the flow of information that promote higher proliferation signals and thus are generally not so damaging to normal cells as the treatments are target DNA itself (Boik, 2001).

The induction of apoptosis is a primary goal of anticancer therapies. Natural compound is an attractive anticancer therapy because it is applicable to all types of cancer cells. Thus, apoptosis rate can be increased by natural compounds.

Natural compounds act as anticancer agent through increasing of cytokine production, stimulate immune activity and reduce the production or activity that allowed immune evasion.

2.2 Free radicals

Free radicals are atoms or atomic groups which contained one or more unpaired electron in the outermost shell. Reactive oxygen species (ROS) are known as free radicals. Free radicals are highly reactive due to their low chemical affinity specificity. Free radicals will indiscriminately pick up electron(s) from the nearest stable molecules.

A free radical is formed when a stable molecule losses it's electron(s), thus a chain reaction will start and finally resulting in the disruption of a living cells. Free radicals will react with molecules such as proteins, lipid, carbohydrate and DNA in its vicinity. Free radicals will harm the cells and may result in cell death (McCord, 2000).

2.2.1 Types of free radical

Free radicals and non-free radical mimics are known as “reactive oxygen species” (ROS) or oxidant. Superoxide, hydroxyl, hydroperoxyl, alkoxyl, peroxyl and nitric oxide radicals are the main free radicals. Whilst, singlet oxygen, hydrogen peroxide and hypochlorous acid (Jacob, 1995) acts like a free radical although they are technically not free radicals.

i. Singlet Oxygen

Singlet oxygen is a mutagenic form of oxygen. It is a non-radical reactive oxygen species because it does not have an unpaired electron. It shows strong oxidizing activity when photoexcitable compounds (sensitizers) such as tetrapyrroles, chlorophyll, hemaproteins and reduced pyridine nucleotides (NADH) are present with oxygen molecules. Singlet oxygen is generated through radiation or enzymatically action of lipogenases or peroxidases. Singlet oxygen is generated during thermo-decomposition of dioxetanes (Briviba *et al*. 1997).

ii. Superoxide ($O_2^{\cdot -}$)

Superoxide anion is produced when an oxygen molecule accepted an electron. Mitochondrion is the major source of superoxide production. The internal mitochondria membrane is the first production site of superoxide radical. Superoxide is produced by some macrophages to aid in phagocytation.

Superoxide radical involves in the initiation and termination steps of lipid peroxidation. Superoxide is the precursors of the formation of lethal hydroxyl radical (Cheeseman and Slater, 1993).

iii. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is generated when a superoxide accepted two protons. Hydrogen peroxide is categorized as reactive oxygen species although it is not a free radical. The enzymatic reactions occurred in the microsomes, peroxysomes and mitochondria will also produce hydrogen peroxide. Hydrogen peroxide is an oxidizing agent. In the presence of transition metal ions, hydrogen peroxide is the main source to produce hydroxyl radicals (Cheeseman and Slater, 1993).

iv. Hydroxyl Radical ($^{\cdot}OH$)

Hydroxyl radical is a very reactive oxidizing radical. Hydroxyl radical is known as 'the radical's radical' (Reiter, 1995). It reacts with most biomolecules such as DNA, membrane lipid or carbohydrate (Dawson and Dawson, 1996; Reiter *et al*, 1995).

Hydroxyl radicals are generated from superoxide anion and hydrogen peroxide via the Fenton reaction and the Haber-Weiss (1934) reaction.

In the Haber-Weiss reaction, when hydrogen peroxide interacts with superoxide, a hydroxyl radical, a water molecule and a hydroxyl group are produced.

Whilst, in the Fenton reaction (Fenton, 1894; 1899), ferrous ions are produced when superoxide anions react with ferric ions. Ferrous ions will then react with hydrogen peroxide to produce hydroxyl radicals.

v. Hydroperoxyl Radical

Hydroxyl radical is a conjugate form of superoxide radical. It is generated through the protonation of superoxide radical. Hydroperoxyl radical is more reactive as compared to superoxide radical. The hydroperoxyl radical can easily diffuse through the lipophilic membranes. It attacks the lipid molecule. Hydroperoxyl radical have the ability to convert linolenic, linoleic and arachidonic acids to peroxides.

vi. Nitric Oxide ($NO^{\cdot}$)

Nitric oxide is a gaseous form of free radical. It is a poorly oxidizing agent. Nitrogen dioxide is produced when nitric oxide reacts with oxygen. Nitrogen dioxide may react with nitric oxide to produce nitrogen trioxide (N_2O_3).

When superoxide reacts rapidly with nitric oxide, peroxynitrite is produced. Peroxynitrite will mediate oxidation, nitrosation and nitration reactions. Production of nitric oxide is stimulated by cytokines, tumor necrosis factor or interleukins. The microbial and tumoricidal activities of macrophages can be reduced through inhibition of nitric oxide production.

vii. Peroxynitrite ($ONOO^-$)

When superoxide reacts with nitric oxide, peroxynitrite is produced. This is a radical-radical reaction.

Peroxynitrite radical is highly reactive. This radical will damage the cell through oxidation of lipids, protein and DNA.

viii. Hypochlorous acid (HOCl)

In the presence of enzyme myeloperoxides, activated polymorphonuclear cells will produce hypochlorous acid.

$$H_2O_2 + Cl^- \longrightarrow HOCl + OH^-$$

In the presence of transitional metal ions, HOCl will produce hydroxyl radical (Aruoma, 1994). Hydroxyl radical is produced when HOCl/ OCl^- reacts with a reductant. Superoxide radicals and ferrous ions are examples of reductant.

Hypochlorous acid will initiate lipid peroxidation (Panasenko *et al*., 1995) and causing damages to DNA.

2.2.2 Sources of free radicals

Free radicals are produced endogenously and exogenously (Finkel and Holbrook, 2000).

i. Endogenous sources of free radicals

Endogenous sources of free radicals include those that are generated and act intracellularly as well as those that are formed within the cell and are released into the surrounding area (Machlin and Bendich, 1987).

Reactive oxygen species are produced through autoxidation of catecholamines, haemoglobin, myoglobin, reduced cytochrome C and thiol. Superoxide is the primary radical which form during the autoxidation process.

Enzymatic activity of certain oxidases, peroxidases, lipoxygenases, aldehyde oxidase, cyclooxygenases, dehydrogenases, amino acid oxidase, xanthine oxidase and prostaglandin synthase will generate free radicals.

Superoxide is generated in the mitochondria, microsomes, peroxisomes and nuclei. The electron leakage from the electron transfer chain of mitochondria is the major sources of superoxide production. Microsomes and peroxisomes also produced hydrogen peroxide. During prolonged starvation, peroxisomal oxidation of fatty acid will generate hydrogen peroxide.

Iron and cooper facilitate the process of lipid peroxidation. In the Haber-Weiss reaction, transition metal ion such as Fe^{2+} will react with hydrogen peroxide to generate OH, OH^- and Fe^{3+}.

ii. Exogenous sources of free radicals

Exogenous sources of free radicals including drugs, tobacco smoke, pollutants, organic solvents, radiation, pesticides, hyperoxic environments, anesthetics and etc.

Some drugs produce free radicals. Some antibiotics showed pro-oxidant activities. Whilst, some drugs may deplete ascorbic acid and accelerate lipid peroxidation.

Radiotherapy may cause tissue injury in an organism. The electromagnetic radiation (X-rays) and particulate radiation (electrons, photons, neutrons, alpha and beta particles) will generate primary radicals.

Smoke from cigarette contains aldehyde, epoxide, peroxide and other free radicals that will damage the alveoli. Oxidants present in the tobacco may injure

the respiratory tract. Inhalation of inorganic particles such as asbestos, quartz, silica will mediate the production of free radical.

Ozone with two unpaired electrons act as a very powerful oxidizing agent although it is not a free radical. It will generate lipid peroxidation.

2.2.3 Oxidative stress

Oxidative stress occurs when there is an imbalance between productions of reactive oxygen species (exogenous sources or endogenous sources) against the antioxidant protection mechanism (enzymatic and non-enzymatic) in an organism. The excess free radicals will attack biological molecules such as DNA, proteins and lipids. Oxidative stress will lead to cell or tissue injury and causing chronic and degenerative diseases like atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others (Diaz *et al.*, 1997; Lang and Lozano, 1998; Halliwell, 2000; Metodiewa and Koska, 2000; Young and Woodside, 2001; Heinecke, 2003; Leibovitz and Siegel, 1980). Generally, free radicals will lead to DNA damage, protein damage and lipid damage.

i. DNA damage

DNA is the genetic material of a cell. Genetic material plays an important role in regulate the normal cell function, growth and repair the damage tissues. The cell will stay and grow healthily only if the DNA remains intact. The forms of DNA damage caused by free radicals are modification of all bases, production of base free sites, deletions, frame shifts, strand breaks, DNA protein cross- links and chromosomal rearrangements. DNA damage will lead

to the changes in the encoded protein and may lead to malfunctions of the protein. Free radicals will induce numerous lesions in DNA that cause deletions, mutation and other lethal genetic effects. Carcinogenesis may occur if there is severe or prolonged exposure of oxidative stress (Guyton and Kensler, 1993).

The growth of abnormal cells initiated when the chromosoin and nucleic acids are damaged by free radicals. The growth of abnormal cells is the first step in cancer development.

ii. Protein damage

Proteins are less susceptible to free radicals as compared to polyunsaturated fatty acids. Free radicals will only attack protein in the cell when the radicals are accumulated in large amount within the cell or when lipid peroxidation occurred. Protein damages may result in the further modification of enzyme activity (stimulation or inhibition) (White and Crawford , 1976).

Protein damages may lead to modifications in structure, enzyme activity and signaling pathways. Free radicals will damage the membrane transport protein and receptor protein. The transferring signal within a cell is modified when there are any changes in the receptor protein or gap junction proteins.

iii. Lipid damage

Cell membranes are rich in polyunsaturated fatty acid (PUFA). PUFAs are easily attacked by free radicals. Lipid peroxidation is a radical-chain reaction. Lipid peroxidation is known as oxidative degradation of polyunsaturated fatty

acids (PUFAs) which are found in cell membranes. Lipid peroxidation involves 3 sequences known as initiation, propagation and termination.

Lipid peroxidation starts when free radical abstract a hydrogen atom from a methylene groups in lipids. Hydroxyl radicals and iron-oxygen complexes are the initiator moieties of lipid peroxidation.

The presence of carbon double bond in the fatty acid helps the abstraction of hydrogen atom. Abstraction of hydrogen atoms results in an unpaired electron and formation of carbon radical in the initiation step. The carbon radical is very unstable, thus it tends to undergo molecular rearrangement in order to form a more stable conjugated diene. Under aerobic condition, the conjugated diene will bind to an oxygen molecule to form a peroxyl (or peroxy) radical, ROO·: During the propagation step, peroxyl radical will abstract hydrogen atom from another lipid molecule (adjacent fatty acid) to produce hydroperoxide (or peroxide). The chain reaction is continuing when another carbon radical is formed and then reacts to form another peroxyl radical. During the termination steps, these molecules will easily provide hydrogen and are easily abstracted by peroxyl radicals in favor of lipid molecules (Halliwell and Gutteridge, 1990). Lipid free radicals can react with a lipid peroxide to give a non –initiating and non-propagating species. Lipid peroxyl radicals can propagate into malodialdehyde (MDA). The structure and function of the membrane can be modified by MDA. Lipid peroxides may interact with cellular DNA and cause the formation of DNA-MDA adducts (Chaudhary *et al*., 1994).

The diagram below shows the mechanism of lipid peroxidation:

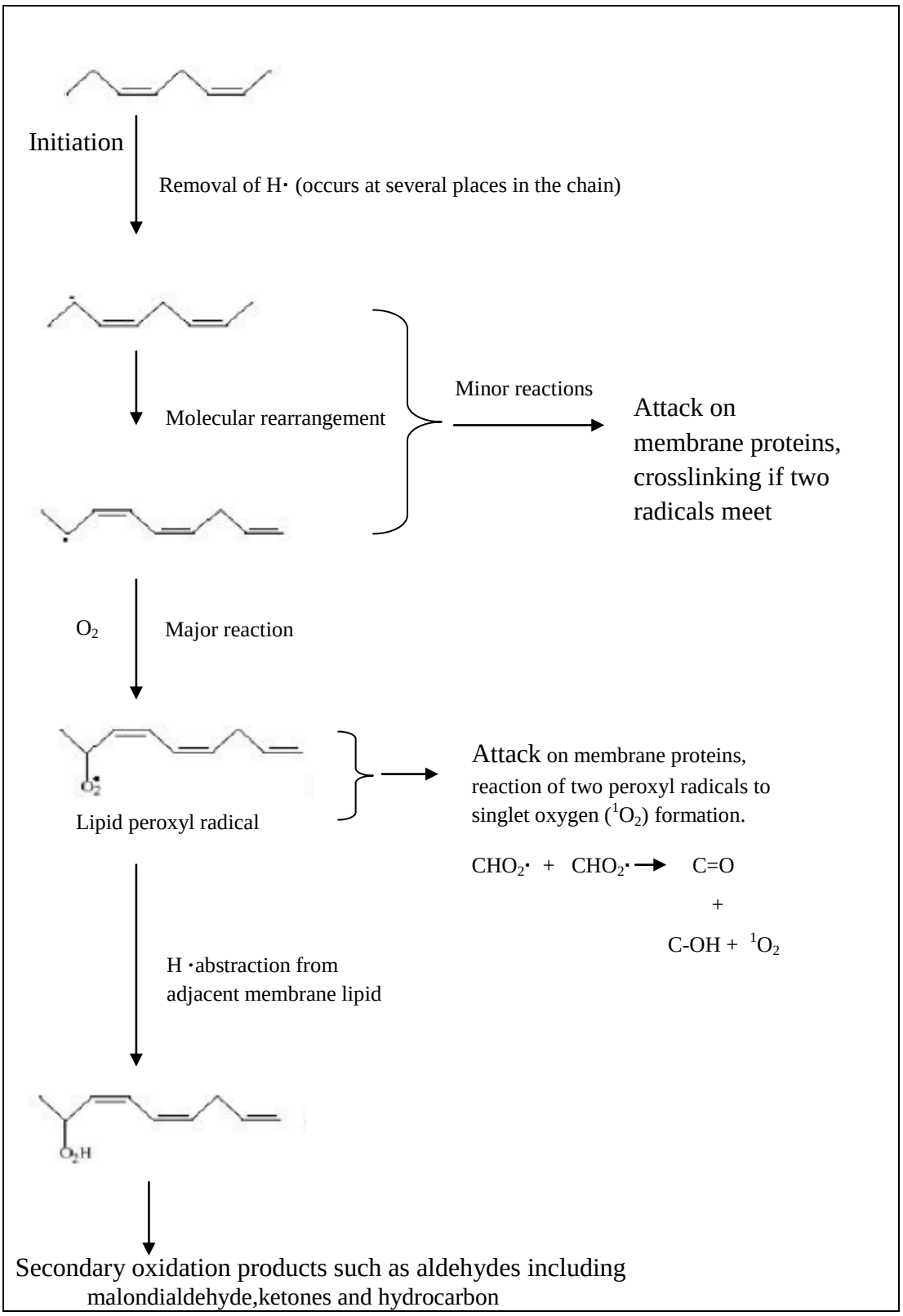


Figure 2.1: The mechanism of lipid peroxidation (Gutteridge and Halliwell, 1990)

2.2.4 Antioxidant

Antioxidants have the ability to donate their electron(s) to the free radicals. The chain reaction of oxidation is stopped when an antioxidant donates electron(s) to the free radicals. In order to eliminate the free radicals present in the body, our body has developed several endogenous antioxidant systems. There are two groups of antioxidant systems known as enzymatic system and non-enzymatic system.

The enzymatic systems include superoxide dismutase (SOD), glutathione peroxidase and catalase. While the non-enzymatic system include vitamin A, Vitamin E, Vitamin C, BHA (synthetic antioxidant), BHT (synthetic antioxidant) and etc.

Epidemiological studies showed that dietary antioxidant has protective role to the population. The population who consumed adequate amounts of fresh fruits and vegetables are at a lower risk for cancer, heart disease, and other degenerative diseases. According to Schafer and Buettner (2001), antioxidants and anti-inflammatory compounds have the ability to modify the redox environment of cancer cells and thus their behavior. According to Reddy *et al*. (2003) antioxidants have the potential to reduce the genetic instability of cancer cells and thus may be useful in treatment. The efficacy of chemotherapy can be increased by using antioxidants (Reddy *et al*., 2001).

2.2.5 Biological antioxidant defense mechanisms

i. Superoxide dismutase (SOD)

Superoxide dismutase plays a major role in inhibiting lipid peroxidation. Superoxide dismutase catalyses the breakdown of superoxide into oxygen molecule and hydrogen peroxide. Superoxide dismutase defenses the cells against

the toxic effect of oxygen radicals. It stops the production of peroxynitrite because it competes with nitric oxide (NO) for superoxide anion.

ii. Catalase

Catalase catalyses the breakdown of hydrogen peroxide into water and oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase works with superoxide dismutase to prevent free radical damages. Superoxide dismutase will convert the superoxide radical into hydrogen peroxide. Then, catalase will breakdown the hydrogen peroxide into water and oxygen.

iii. Glutathione peroxidase (GSH)

Glutathione peroxidase system consists of enzyme glutathione peroxidase, glutathione reductase, cofactor glutathione (GSH) and reduced nicotinamide adenosine dinucleotide phosphate (NADPH). Glutathione peroxidase catalyses the removal of hydrogen peroxide and lipid peroxides such as superoxide anion and hydroxyl radical (Michiels *et al*, 1994). Glutathione peroxidase reduces hydrogen peroxide to water molecule by oxidizing glutathione (GSH) (Equation 1). The rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase (Equation 2). The glutathione redox cycle is a central mechanism for the reduction of hydroperoxides in the cell.

$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O \quad \text{(Equation 1)}$$

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+ \quad \text{(Equation 2)}$$

2.2.6 Synthetic antioxidants and natural antioxidants

Antioxidants have the ability to terminate or retard oxidation process. Antioxidants act as electron donors, scavengers of free radicals and chelators of free catalytic metal.

There are two types of antioxidants namely synthetic antioxidants and natural antioxidants. The commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), propylgallate (PG), *tert*-butylhydroquinone (TBHQ) and butylatedhydroxytoluene (BHT) (Sherwin, 1990). BHA and BHT were found to cause liver damage and carcinogenesis in experimental animals (Grice, 1986; Wichi, 1988). BHA appeared to have tumor-initiating as well as tumor promoting action (Cheung *et al*., 2003). Recent studies showed tumor formation appears to involve only tumor promotion caused by BHA and BHT (Botterweck *et al*., 2000).

Fruits, vegetables and medicinal plants have been suggested as natural sources of antioxidants (Auddy *et al*., 2002; Choi *et al*., 2002; Mantle *et al*., 2000). According to Pokorny (1991), when compared to synthetic antioxidants natural antioxidants have many of advantages. For example; (i) they are readily acceptable by the consumers, (ii) they are considered to be safe, (iii) no safety tests are required by legislation, (iv) this natural antioxidant (not as a synthetic chemical antioxidant) is identical to the food which people have taken over a hundred years or have been mixed with food.

2.3 Natural products

Natural products are the basis of the earliest medicines. Natural products play an important role in drug discovery (Newman *et al*., 2000; Sneader, 1996; Buss *et al*., 2003; Weatherall 1990; Grabley and Thiericke, 2000; Mann and Murder, 2000). Natural products are chemical compounds or substances derived from plants, insects, marine organisms, microbes or animals. Phytosterols, acyl lipids, nucleotides, amino acids, and organic acid are known as primary metabolites which found in all organisms. They are needed to perform metabolic roles. Secondary metabolites are organic compounds which also present in living organism. The function(s) of secondary metabolites are remaining unknown.

Secondary metabolites can be characterized into various family based on their chemical structures. For example polyketides and fatty acids, terpenoids and steroids, phenylpropanoids, alkaloid, phenolic compounds, specialized amino acids and peptides and specialized carbohydrate. Studies have shown that secondary metabolites possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities (Halliwell, 1994; Mitscher *et al*., 1996; Owen *et al*., 2000; Sala *et al*., 2002).

Recently, the demand for alternative therapies and the therapeutic use of natural products is increasing. Many pharmaceutical agents have been discovered through screening natural products from plants, animals, microbes and marine organisms. This is due to the folk medicines suggesting that “natural” products are harmless (Rates, 2001). Besides this, natural products can be obtained easily. The conventional medicines are very expensive as compared to therapeutic use of natural products. Natural products can be an option for low income people. Obviously, natural products can be lead compounds allowing the design of new drugs.

2.3.1 The Portulacaceae family

Portulacaceae is a family of flowering plant. It is also known as the Purslane family. This family comprises of 20 – 30 genera with about 500 species. Portulacaceae family has a cosmopolitan distribution with the highest diversity in semi-arid region of the southern hemisphere in South America, Africa, Australia and a few species in Asia, Europe and North America. There are also a few species extending from north into the Artic region.

The Portulacaceae are sub-shrubs or herbs. They are annual, biennial or perennial plant. The plants are often succulent or fleshy. The leaves are alternate and sometimes secund or opposite, sub-opposite, sometimes rosulate or sub-rosulate exstipulate (except Portulaca and Talinopsis, with nodal or axillary hairs regarded stipular). True stipules are absent in the leaves and the nodes sometimes with axillaries scales and or hairs. Petiole is usually poorly defined or absent in the leaves. The leaf blade margins are mostly entire, occasionally dentate to crisp.

The inflorescences are usually terminal and less often axillaries. The inflorescences are cymes, racemose, paniculate or umbrellate, glomerate or spikelike forming heads of sessile flowers surrounded by involucres of leaves or reduced to solitary flowers. The bracts are inconspicuous.

The flowers are bisexual and have what appears to be a differentiated perianth consisting of 3 distinct or basally connate sepals and mostly 4 – 6 distinct or basally connate petals. Sometimes the flowers showed slightly irregular in Genus Montia. The petals are imbricate and often have brightly coloured and usually have short lived. Disk is usually absent. The stamens and petals are isomerous, opposite and sometimes adnate at the stamens may number 2 - 4 times as many as the petals. The stamens are fascicled

or adnate to petals. The filaments are linear, anthers 2- loculed, introrse and dehiscence longtitudianal.

The gynoecium consists of a single compound pistil of 2 – 3 carpels with 2 – 5 branched style and a superior or half interior ovary that has a single locule containing 1 to many basal ovules. The placentation is basal or free centrals. The stigma consists of 2 – 9 lobed.

The fruit look like a thin-walled capsuled. It is usually a circumscissile or loculicidal capsule. The seeds are usually produced in large amount. The seeds are in reniform or globose. Caruncle may be present in the seed. The endosperms are mostly copious and surrounded by embryo.

Some examples of the Genera in Portulacaceae family are Calandrinia, Calyptridium, Cistanthe, Claytonia, Lewisia, Montia, Phemeranthus, Portulaca, Portulacaria, Spraguea, Talinoppsis and Talinum.

2.3.2 The *Portulaca* genus

The *Portulaca* is an annual or parennial herbs. The stems grow erect to prostrate, branched, fleshy, suffrutescent or decumbet. Stem nodes may be absent or present. The nodes or leaf axils are with scales, britsles and or hairses. Sometimes are short and inconspicuoses. The roots are tuberous, fleshy, fibrous, or small taproots.

The leaves grow alternate or sub-opposite, congested and involucre like immediately proximal to inflorescence; blade terete, subterete, or flattened. Usually the leaves are 4 – 3 mm long. The inflorescences are usually terminal in clusters, or axillary on short branches. The flowers are sessile or subsessile, usually open only in sunshine; the sepals are broadly clasping at base, herbaceous to scarious, falling from top of

capsule; the petals ephemeral, 5 - 7, usually distinct, margins usually entire. The stamens are 4 – 100 and adnate to the base of petals. The ovary is half-interior and the stigma consist of 2 – 9 lobed. The fruits are usually circumscissile, sessile, globose or loculicidale capsule. The seeds are many, mostly are glossy black, or iridescent gray. They are less often brown in colour. The seeds are reniform to cochleate; seed coat smooth or variously sculptured, granular to stellate-tuberculate or spiny.

The Genus *Portulaca* comprised of about 150 species. The species mainly found in arid tropical or subtropical region, particularly in Africa and South America. There are few species which extending into temperate region, 5 species are found in China (Lu and Michael, 2003).

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Caryophyllales
Family: Portulacaceae
Genus: *Portulaca*
Species: *oleracea*

2.3.3 *Portulaca oleracea (P. oleracea)*

i. Plant description

Portulaca oleracea is an annual, succulent herb in the family Portulacaceae. This plant is given the term 'Global Panacea' by Dweck (2001). *P. oleracea* are commonly known as Purslane, Pursley, Pusley, or Wild Portulaca. In Malaysia, this plant is known as '*Gelang Pasir'*. It is found in temperate and tropical regions.

The stems are growing in prostrate form and has radial grow pattern. The stems are from stout taproot, multiple from base, prostrate, glabrous, branching, succulent and herbaceous, not articulated and usually are flushed red or purple in colour. The stems able to grow up to 8^{th} degree branching. The stem is circular in with smooth an even surface. The stem consists of distinct epidermis, broad cortex and pith.

The leaves grow alternate, subalternate or opposite. The leaves are glabrous, thick, succulent, sessile or very short petiolate (+/-1 mm), obovate to spatulate (4 – 3 mm long to 13 mm wide), tapering slightly to base, entire and rounded to truncate at tip.

The flowers are bright yellow, distinct, grown in cluster of 3 – 5, 0.4 – 0.5 cm in diameter, surrounded by involucres 2 – 6 bracts. The flower comprise of 5 petals, each petal is 3 – 4 mm long by 2 – 3 mm broad and slightly connate at base. The flower has 2 sepals which is 3 – 4 mm long for each. The petals are fused to the base of ovary. The number of stamen is 6 – 10. The filaments are 1 mm long, translucent and glabrous. The anthers are yellow, 2 - 3 mm broad. The calyx tube is to 2 mm long, glabrous, green, 2-lobed, with transverse groove. The lobed are subequal to unequal, to 4 mm long, glabrous and acute. The stigma comprised of 4 – 6 lobed and the ovary is glaborous. The placentation is free central. The fruits are in capsule with the size of 4 – 9 mm. The fruits are circumsessile at or just below the middle. The seeds are glossy black when mature and the size is 0.5 – 0.8 mm in diameter. The seed is usually tuberculate with

white scar at one end. The roots are thick taproot, long and have many fibrous. Lateral roots also present in the plant.

P. oleracea is growing throughout the temperate and tropical areas of the world. *P. oleracea* is growing in an open, disturbed area. The plant requires hot season and high light intensity to grow well. The plant is drought resistant. This plant grows the best in pH range of 5.6 – 7.8.

The origin site of *P. oleracea* is uncertain; possibly it is from an arid climate such as North Africa (Chapman *et al*., 1974). The post- Columbian human helps to disperse *P. oleracea* to the new world.

P. oleracea have specialized water storage in the stem and leaves (Vengris *et al.*, 1972). During drought, the leaves will senesce but the stems will remain alive. During winter, *P. oleracea* will survive as dormant seeds.

P. oleracea is a C4 plant. The C4 metabolism allows *P. oleracea* to optimize photosynthesis in conditions of high heat and bright sunlight while enduring periods of limited water availability (Koch and Kenneddy, 1982).

The flowers are self-pollinating and nonpomitic. The flowers open according to temperature and light conditions. Usually the flowers open in the morning to noon hours on hot and bright day. The flowers will only last for one day.

Figure 2.2 : The appearance of *P. oleracea*

Figure 2.3 : The leaves of *P. oleracea*

Figure 2.4 : The flower of *P. oleracea*

Figure 2.5 : The seeds of *P. oleracea*

ii. Traditional Uses

It is eaten as a salad and vegetable all around the world. This plant is edible with a slightly acidic and salty taste similar to spinach. It is widely used as a pot herb in the Mediterranean, Central European and Asian countries. In traditional folk medicine it is use to treat sore nipples, gastric ulcers, tonsillitis, headache, stomach ache, painful urination, enteritis, mastitis, lack of milk flow in nursing mothers and in postpartum bleeding. Externally it is used to treat burns, earache, insect stings, inflammations, skin sores, ulcers, itching skin, eczema and abscesses.

In Africa, the whole plant is considered antiphlogistic (takes the heat out). It has been used in prescriptions as an antidiabetic. It is used as anthelmintic for children to expel roundworm. The leaf infusion has been used as a vegetable for its antiscorbutic properties (Dweck, 2001).

In China, *P. oleracea* is considered to have blood-cooling and haemostatic properties and so used internally in bleeding bacillary dysentery (Keys, 1976), haemotochezia (bloody stool), bleeding haemorrhoids (Keys, 1976) and metrorrhagia. It is also used as antiphlogistic (Reid, 1993), diarrhoea, haemorrhoids, enterorrhagia (Keys, 1976). It is also described as antidote; refrigerant; antidysenteric (Reid, 1993). The dried herb is boiled into tea/soups (Cai *et al*., 2004).

In Malaysia, *P. oleracea* possesses anti-scorbutic, emollient and sedative properties (Leyel, 1987). The aerial parts of *P. oleracea* are used medicinally to alleviate pain and swelling (Okwuasaba *et al*., 1987), and as an antiseptic (Chan *et al*., 2000).

iii. Biological activities

P. oleracea is reported to be used in traditional medicine preparation, but there is not much recorded data on the biological and limited work has been done on cytotoxic studies against various cancer cell lines.

The water extracts of *P. oleracea* have been certified safe for daily consumption as a vegetable (Yen *et al*., 1995). *P. oleracea* also exhibits a wide range of pharmacological effects including antibacterial (Zhang *et al*., 2002); analgesic, antiinflammatory (Chan *et al.,* 2000), skeletal muscle- relaxant (Parry *et al*., 1993), bronchodilatory effect (Malek *et al*., 2004) and wound healing (Rashed *et al.,* 2003) activities and anti-fatigue activities (Yue *et al*., 2005; Dong *et al.,* 2005; Ling, 2004). It is reported that extracts of *P. oleracea* has inhibitory effect on lipopolysaccharide (LPS) and interferon-γ (IFN- γ) induced NO production (Abas *et al*., 2006).

There are several reports on the antioxidant capacities of *P. oleracea*. Lim and Quah (2007) determined the antioxidant activity of six cultivars of *P. oleracea* using the Folin–Ciocalteu method, the total phenol content (TPC) of methanolic extracts of six cultivars of *P. oleracea* were analyzed. The antioxidant activity of the plant was measured using 1,1-diphenyl-2-picrylhydrazyl, ferric-reducing antioxidant power (FRAP) and β-carotene bleaching (BCB) assays. The total phenolic content of the cultivars of *P. oleracea* ranged from 127 ± 13 to 478 ± 45 mg GAE/100 g of fresh weight of plant. Lim and Quah showed that all the ornamental cultivars may provide a new source of antioxidants for use in food and medicinal purposes.

Ercisli *et al*. (2008) evaluated the *in vitro* antioxidant and antibacterial activities of methanol extracts from *P. oleracea* leaves from Turkey. The methanol extracts exhibited EC_{50} 54.33 ± 1.26 μg/ml in the DPPH assay. The total lipids in *P. oleracea* leaves were 5.83 %. The dominant fatty acid was linolenic acid (56.33 %) followed by

linoleic (14.01 %), palmitic (9.72 %), oleic (8.83 %), myristic (5.04 %) and stearic (4.36 %) acids. The methanolic extract showed antibacterial activities against *Bacillus subtilis, Pseudomonas syringae* pv. *tomato, Vibrio cholerae* and *Yersinia pseudotuberculosis.* The water extract of *P. oleracea* did not show any antibacterial activity against the studied bacteria.

Sanja *et al*. (2009) investigated the antioxidant activity of the methanolic extract of *P. oleracea.* The IC_{50} value of methanolic extract for DPPH radical assay was 12.67 ± 1.2 μg/ml, 667.12 ± 16.02 μg/ml for nitric oxide scavenging assay and 182.02 ± 9.64 μg/ml for superoxide scavenging assay.

Chen *et al.* (2009) showed the polysaccharides of *P. oleracea* could significantly scavenged superoxide anion, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide and hydroxyl radicals. *P. oleracea* polysaccharides able to inhibit the haemolysis of red blood cell (RBC). *P. oleracea* polysaccharides can be used to prevent some free radical associated health problems such as ovarian cancer and others diseases.

Wang and Yang (2010), demonstrated that betacyanins from *P. oleracea* are able to ameliorate cognition deficits and attenuate oxidative damage induced by D-galactose in the brains of senescent mice. At the dose of 50 or 100 mg/kg body weight, betacyanins can significantly reverse the cognitive impairments and increased the activities of antioxidant enzymes in the mouse brain. Betacyanins have potential in the treatment of neurodegenerative diseases or work as an antiaging therapy.

Yoon *et al*. (1999) showed the aqueous extract of *P. oleracea* has tumoricidal activity against KATO III (human gastric carcninoma cell line) and COLO 320 HSR cells (human colon adenoma cell line) in a dose dependent manner. However, the aqueous extract of *P. oleracea* did not showed tumoricidial activity against non-tumorous cell lines, L292 (murine lung connective tissue) and W138 (human ling diploid cell) cells.

Results from Gholamreze *et al.* (2009) suggested that *P. oleracea* extract had protective effect against cisplatin-induced renal toxicity. It might serves as a novel combination agent with cisplan to reduce the injury of renal.

Chen *et al.* (2010) isolated a water-soluble polysaccharide (POP1) from *P. oleracea* using chlorosulfonic acid method and N, N-Dicyclohexylcarbodiimide (DCC) as a dehydration-condensation agent. Sulfated modification on the (POP1) produced four sulfated derivatives namely POP1-s1, POP1-s2, POP1-s3 and POP1-s4. All sulfated derivatives significantly inhibited the *in vitro* growth of HepG2 cells and Hela cells. This indicated that sulfated modification could enhance cytotoxicity of POP1 on tumor cells.

iv. Chemical investigation

P. oleracea contains a lot of nutrients. Some of the reported biologically active compounds include organic acids, oxalic acid (Mathams and Sutherland, 1952), anthraquinone glycosides, alanine, alkaloids, saponins, tannins, coumarins, flavonoids, cardiac glycosides, N-trans-feruloyltyramine (Mizutani *et al*., 1998) and catechol. *P. oleracea* was reported to contain also urea, calcium, iron, phosphorous, manganese, copper and fatty acids, (Ezekwe *et al*., 1999; Garti *et al*., 1999; Hussein, 1985; Mohamed and Hussein, 1994 and Simopoulos *et al.*, 1992). Simopoulos *et al.* (1992) found the occurrence of glutathione; glutamic acid; and aspartic acid in the plant.

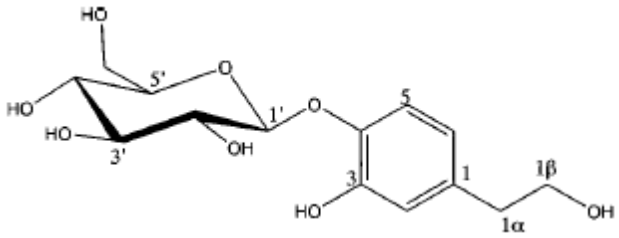
There is limited information on the chemical investigations of the *P. oleracea.* Monoterpene glycoside known as Portuloside A was isolated by Sakai *et al.* (1996). Portuloside A was found to be (3S)-3-(3, 7- dimethylocta-1, 7-dien-6-only)-β-D-glucopyranoside using spectroscopic method. To date, two acylated betacyanins, oleracins I and II, have been isolated from stems of *P. oleracea* (Imperato, 1975). Upon

treatment with aqueous citric acid, Olecacin I will convert to Oleracin II, which is diastereoisomer of Oleracin I, and both appear as a mixture.

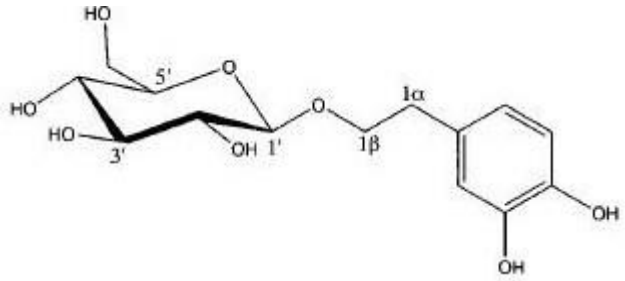
P. oleracea gum (POG) was extracted from the leaves of *P. oleracea* by Garti *et al*. (1999). POG is a water-soluble anionic, low molecular weight polysaccharide (gum) with surface, interfacial, and emulsification properties. POG forms small emulsion droplets and stable emulsions at low oil and low gum concentrations and seems to have higher potential as an emulsifier than other hydrocolloids. POG can be used as a food emulsifier.

P. oleracea has been reported to be rich in α-linolenic acid and β-carotene (Liu *et al*., 2000). The levels of α-linolenic acid are reported to be in the range of 97- 160 mg/100g. The total fatty acid content ranged from 0.6 to 0.9 mg/g in stems, 80 to 170 mg/g in seeds and 1.5 to 2.5 mg/g of fresh mass in leaves. The β-carotene content ranged from 22 to 30 mg/g fresh mass in leaves.

Youngwan *et al.* (2003) isolated two biphenolic glucosides from *P. oleracea* using column chromatography and reversed-phase HPLC. Compound namely 3-hydroxy-1-(2-hydroxyethyl) phenyl-4-O-β-D-glucopyranoside and 2-(3, 4-dihydroxyphenyl) ethyl-O-β-D-glucopyranoside were isolated using column chromatography and reversed-phase HPLC. 3-hydroxy-1-(2-hydroxyethyl) phenyl-4-O-β-D-glucopyranoside was obtained as a colorless gum and was determined to have composition $C_{14}H_{20}O_8$ by HRFABMS and ^{13}C NMR analyses. Whilst, 2-(3,4-dihydroxyphenyl)ethyl-O-β-D-glucopyranoside with molecular formula $C_{14}H_{20}O_8$ was isolated as a colorless gum.



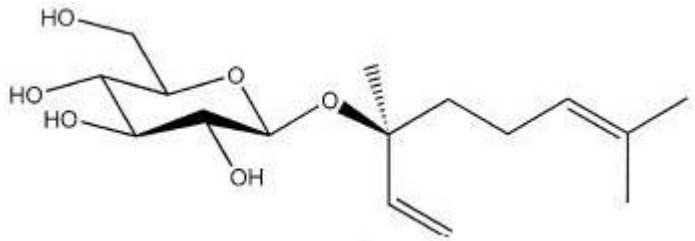
3-hydroxy-1-(2-hydroxyethyl) phenyl-4-O-β-D glucopyranoside



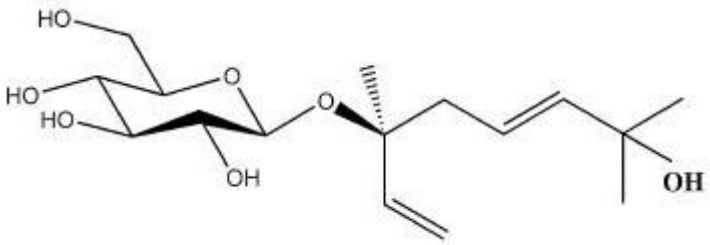
2-(3,4-dihydroxyphenyl)ethyl-O-β-D-glucopyranoside

Figure 2.6: The structures of phenolic glucosides (Youngwan *et al.*, 2003)

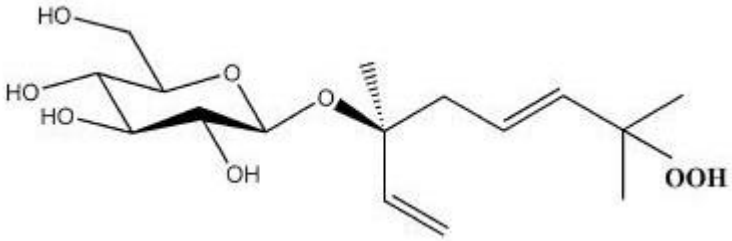
Youngwan *et al*. (2003) isolated three monoterpene glucosides from the methanol extract of *P. oleracea.* (3*S*)-3-O-(β-Dglucopyranosyl)- 3,7-dimethylocta-1,6-dien-3-ol, was isolated as a colorless gum which analyzed as $C_{16}H_{28}O_6$ by a combination of HRFABMS and ^{13}C NMR spectrometry. (3*S*)-3-O-(β-D-glucopyranosyl)-3, 7-dimethylocta-1,5-dien-3, 7-diol with molecular formula $C_{16}H_{28}O_7$ was isolated as a colourless gum. Another compound Portuloside B, (3*S*)-3-O-(β-D-glucopyranosyl)-3,7-dimethyl-7-hydroperoxyocta-1,5-dien-3-ol, was obtained as a colorless gum which analyzed as $C_{16}H_{28}O_8$.



(3*S*)-3-O-(β-Dglucopyranosyl)-3,7-dimethylocta-1,6-dien-3-ol



(3*S*)-3-O-(β-D-glucopyranosyl)-3, 7-dimethylocta-1,5- dien-3, 7-diol



(3*S*)-3-O-(β-D-glucopyranosyl)-3,7-dimethyl-7-hydroperoxyocta-1,5-dien-3-ol (Portuloside B)

Figure 2.7: **The structures of monoterpene glucosides (Youngwan *et al.*, 2003)**

Chen *et al.* (2003) determined the presence of noradrenaline and dopamine in different parts of *P. oleracea* using high-performance liquid chromatographic (HPLC) technique coupled with photodiode array (PDA). Five flavonoids namely kaempferol, apigenin, myricetin, quercetin and luteolin) were determined in different parts of *P.*

oleracea using capillary electrophoresis with electrochemical detection (CE–ED) (Xu *et al.,* 2006).

Asia *et al.* (2004) isolated β-sitosterol, β-sitosterol-glucoside, N,N'-dicyclohexylurea, and allantoin from the methanol extract of *P. oleracea*. Allantoin is a colorless crystals obtained from the methanol fraction of *P. oleracea*. It was identified based on the spectroscopical data. Whilst, N,N'- dicyclohexylurea was identified based on spectral data (D'agostino *et al.,* 1987; Tasi *et al.,* 1997). β-sitosterol glucoside was identified through comparison of IR, MS, ^{1}H-NMR and ^{13}C-NMR data with published data (Aquino *et al.,* 1998; Hamed, 1996; Kusano *et al.,* 1973; Misra and Tiwari, 1973). β-Sitosterol was identified through comparison of the spectral data with published data (Tasi *et al.,* 1997).

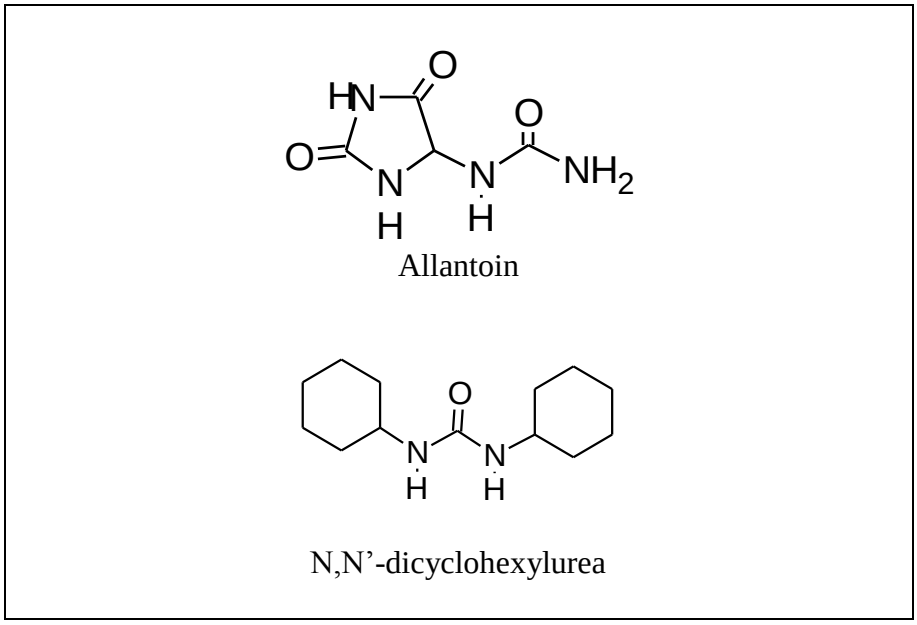


Figure 2.8: The structures of Allantoin and N,N'-dicyclohexylurea (Asia *et al.*, 2004)

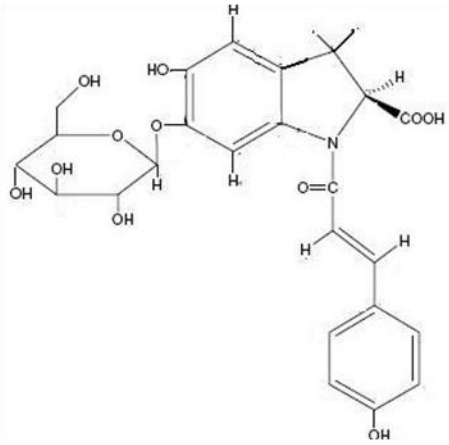
Xiang *et al.* (2005) isolated five alkaloids from *P. oleracea*, namely oleraceins A, B, C, D and E. Oleracein A, 5-hydroxy-1-p-coumaric acyl-2, 3-dihydro-1H-indole-2-carboxylic acid-6-O-β-D glucopyranoside was isolated as water-soluble yellow powder. The molecular formula of Oleracein A was determined to be $C_{24}H_{25}O_{11}N$. Oleracein A showed yellow-brown fluorescence under UV light at 365 nm and the colour was retained when exposed to ammonia vapor.

Oleracein B, 5-hydroxy-1-ferulic acyl-2, 3-dihydro-1H-indole-2-carboxylic acid-6-O-β-D-glucopyranoside, with molecular formula $C_{25}H_{27}O_{12}N$ was isolated as a water-soluble yellow colour compound. Oleracein B showed yellow-brown fluorescence under UV light at 365nm and turned bright yellow when treated with ammonia vapor.

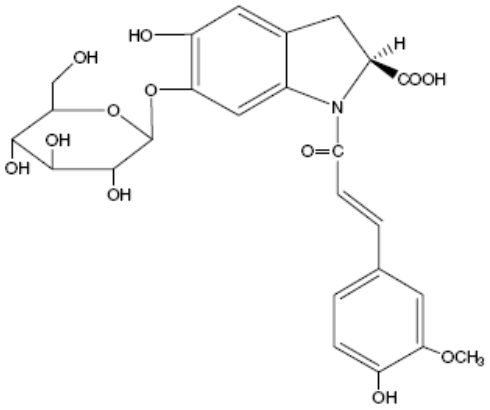
Oleracein C, 5-hydroxy-1-(p-coumaric acyl-7'-O-β-D-glucopyranose)- 2,3-dihydro-1H-indole-2-carboxylic acid-6-O-β-D-glucopyranoside, was isolated as a water-soluble yellow colour compound with molecular formula $C_{30}H_{35}O_{16}N$.

Oleracein D, 5-hydroxy-1-(ferulic acyl-70-O-b-D-glucopyranose)-2, 3-dihydro-1H-indole-2-carboxylic acid-6-O-b-D-glucopyranoside is a water-soluble yellow colour compound with molecular formula $C_{31}H_{37}O_{17}N$.

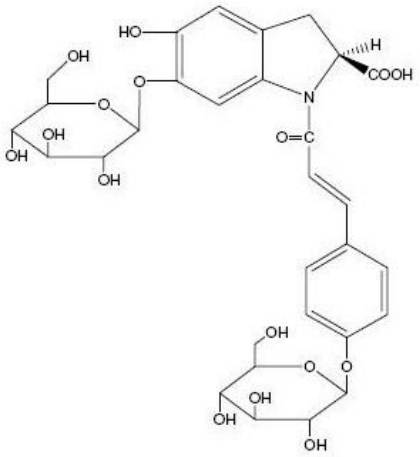
Oleracein E, 8, 9-dihydroxy-1, 5, 6, 10 b-tetrahydro-2H-pyrrolo [2, 1-a]isoquinolin-3-one, was isolated as pale- white powder. Oleracein E was colourless under natural light and turned pink when exposed to iodine. The molecular formula of Oleracein E was $C_{12}H_{13}O_3N$.



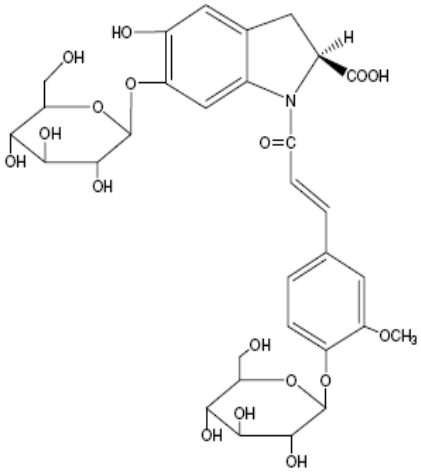
Oleracein A



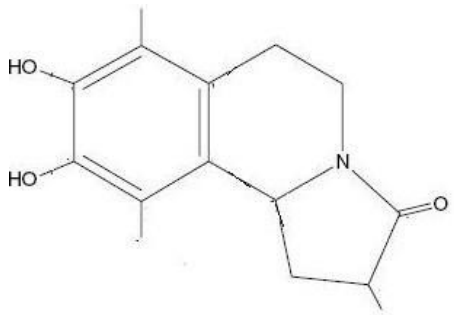
Oleracein B



Oleracein C



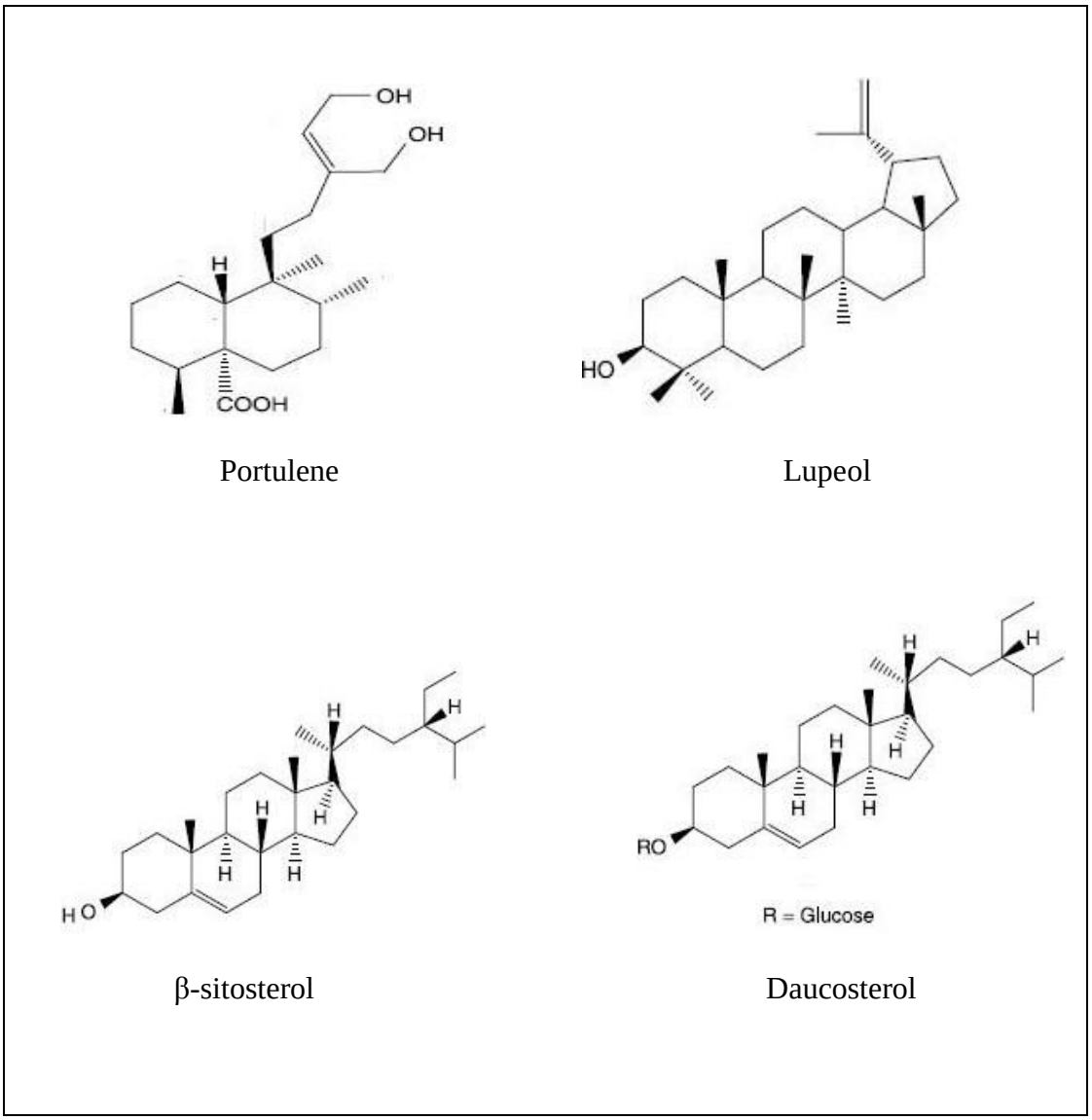
Oleracein D



Oleracein E

Figure 2.9: The structures of oleracein A-E (Xiang *et al.*, 2005)

Ehab *et al.* (2008) isolated lupeol, β-sitosterol, daucosterol and a new diterpene namely portulene from the chloroform extract of *P. oleracea*. *P. oleracea* extract showed antibacterial and antifungal activities. Portulene was isolated as white amorphous powder with molecular formula $C_{20}H_{34}O_4$. Lupeol, β-sitosterol, and daucosterol were identified by comparison of their spectral data with those reported in the literature.



Portulene Lupeol

β-sitosterol Daucosterol

Figure 2.10: The structures of compounds isolated by Ehab *et al.* (2008)

A compound named as portulacerebroside A, (2*S*, 3*S*, 4*R*)-2-[(2'*R*, 4*E*)-2'-hydroxy-hexacosenoylamino]-3, 4-dihydroxy-hexadecane-1-*O*-*β*-*D*-glucopyranoside was isolated by Xin *et al*. (2008). Portulacerebroside A is a white waxy solid compound with melting point 146 - 148 ℃ has molecular formula $C_{48}H_{93}NO_{10}$.

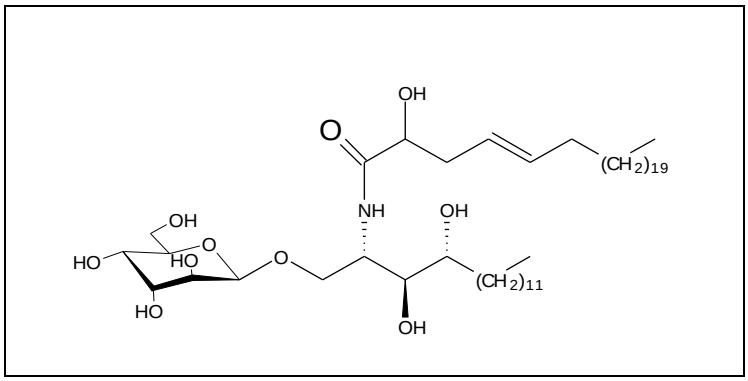


Figure 2.11: The structure of portulacerebroside A (Xin *et al*., 2008)

Oliveira *et al*. (2009) found that water was the major constituent of *P. oleracea*. The average water content in the stems was 90.5 % while the leaves were 97.8 %. The fat values varied from 0.11 % to 0.57 %. There were 27 fatty acids found in the leaf samples. Linolenic acid was the most abundant fatty acid, ranging from 27.7 to 39.1 %, followed by palmitic (19.3 – 24.3 %) and oleic acids (11.6 – 19.5 %). Fumaric, aconitic, citric, malic and oxalic acids were also in the plants. Oxalic and citric acids were the most abundant organic acid, whilst aconitic acid was present in the lowest content. 3-Caffeoylquinic and 5-caffeoylquinic acids were also present in the plant. 3-Caffeoylquinic acid was found in abundance in the leaves while 5-caffeoylquinic acid was found in abundance in the stems.

Table 2.1 : Compounds isolated from *P.oleracea*

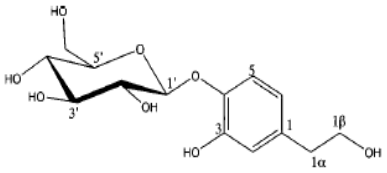
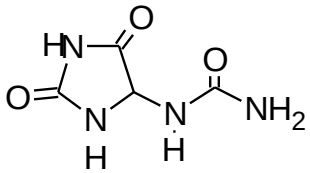
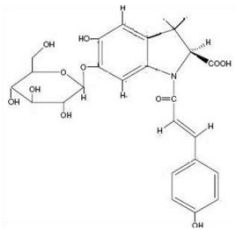
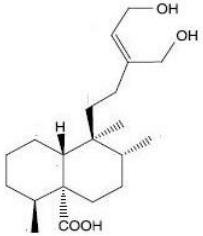
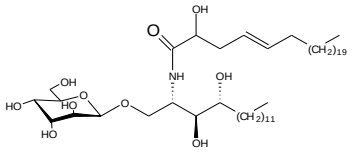
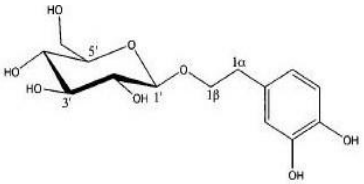
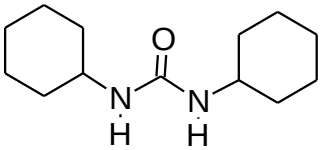
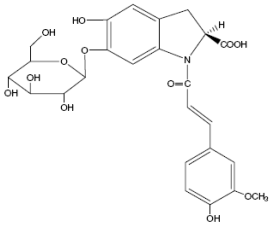
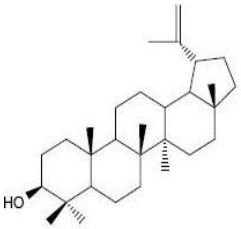
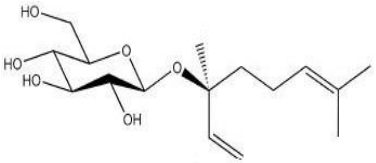
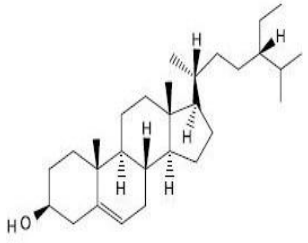
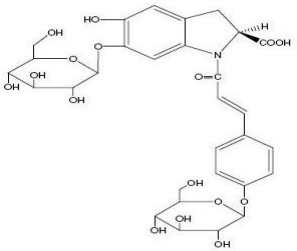
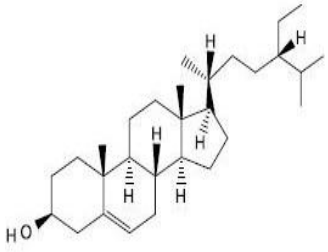
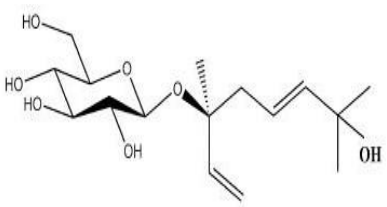
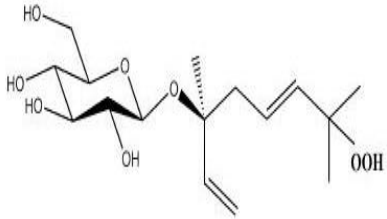
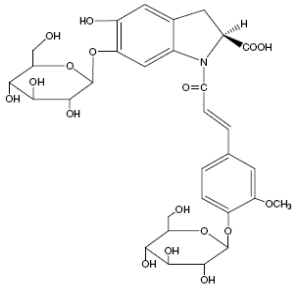
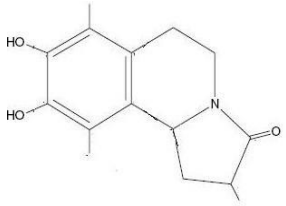
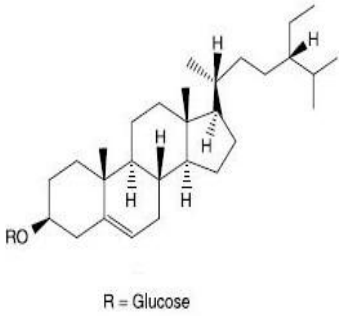
Youngwan *et al.* (2003)	Asia *et al.*, (2004)	Xiang *et al.* (2005)	Ehab *et al.* (2008)	Xin *et al.* (2008)
 3-hydroxy-1-(2-hydroxyethyl) phenyl-4-O-β-D glucopyranoside	 Allantoin	 Oleracein A	 Portulene	 Portulacerebroside A
 2-(3,4-dihydroxyphenyl)ethyl-O-β-D-glucopyranoside	 N,N'-dicyclohexylurea	 Oleracein B	 Lupeol	
 (3*S*)-3-O-(β-Dglucopyranosyl)-3,7-dimethylocta-1,6-dien-3-ol	 β-sitosterol	 Oleracein C	 β-sitosterol	

Table 2.1, Continued

 (3*S*)-3-O-(β-D-glucopyranosyl)-3, 7-dimethylocta-1,5- dien-3, 7-diol  (3*S*)-3-O-(β-D-glucopyranosyl)-3,7-dimethyl-7-hydroperoxyocta-1,5-dien-3-ol (Portuloside B)		 Oleracein D  Oleracein E	 Daucosterol	

2.4 Bioactivity assays

Biological activity describes the beneficial or adverse effects of a test sample on living matter. A material / test sample is considered bioactive if it has interaction with or effect on any cell tissue in the human body. *In vitro* condition antioxidant activities of a material can be assessed using various antioxidant assays namely DPPH radical scavenging assay, lipid peroxidation assay, ferric reducing ability of plasma (FRAP) assay, superoxide radical scavenging assay, and reducing power assay. The antioxidant activities of *P.oleracea* extracts were investigated using three assays: DPPH, reducing power and β-carotene bleaching assays. Whilst, the cytotoxic activity of *P.oleracea* extracts were investigated using the neutral red cytotoxicity assay.

2.4.1 Methods to determine antioxidant activity

Commonly, there are two major groups of assay that used to determine the total antioxidant capacity. The first group of assay is based on a single electron transfer reaction. The antioxidant capacity is monitored through a change in colour as the oxidant is reduced. The degree of colour change is correlated with the sample's antioxidant concentration. The second group of assays is based on a hydrogen atom transfer reaction. In these assays, the antioxidant and substrate (probe) are competing for free radicals (Huang *et al*., 2005).

Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay, the copper reduction (CUPRAC) assay and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay are known as electron transfer reaction assays (Tabart *et al*., 2009). Meanwhile, the oxygen radical absorbance capacity (ORAC) assay, the total peroxyl radical trapping antioxidant parameter (TRAP) assay and the crocin bleaching assay are known as hydrogen atom transfer reaction assays (Tabart *et al*., 2009). The total antioxidant scavenging capacity

(TOSC) assay , the electrochemiluminescence assay and the chemiluminescence assay are not included in these two major groups of assay (Huang et al., 2005; Prior *et al.*, 2005).

The determination of antioxidant capacity in food should take into account the overall concentrations and compositions of diverse antioxidants because the total antioxidant capacity is due to the combined activities of diverse antioxidants, including phenolic (Tabart *et al*., 2009).

2.4.2 Principle of 1,1-diphenyl-2-picrylhydrazyl (DPPH) Assay

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging capacity assay is based on a single electron transfer reaction principle .The molecule of 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical (Figure 2.1). The free radical DPPH is deep violet in color and posses a characteristic absorption at 520 nm.

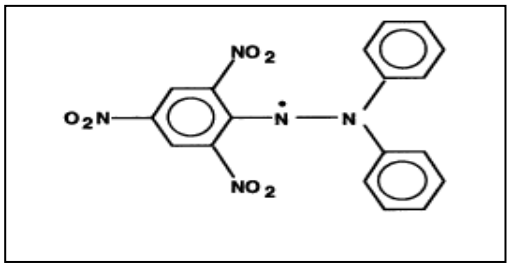


Figure 2.12 : Diagram of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH)

When a solution of DPPH is mixed with a substance that can donate an electron, the DPPH free radical will lose its deep violet colour and change into pale yellow (reduced form).

Foti *et al.,* (2004) showed the kinetic analysis for rates of the reaction of DPPH · and phenols in methanol and ethanol, leading to the conclusion that the rate determining step for the reaction is the slow process of formation of phenoxide anions.

This is then followed by the fast electron transfer of the phenoxide anions to the DPPH：The cascade of the reaction is as follows where ArOH refers to aromatic phenyl group, ArO^- as phenoxide ion, H^+ as hydrogen ion, ArO·as phenoxide radical.

$$ArOH \rightleftharpoons ArO^- + H^+ \quad (1)$$

$$ArO^- + DPPH\cdot \longrightarrow ArO\cdot + DPPH^- \quad (2)$$

$$DPPH^- + H^+ \longrightarrow H\text{-}DPPH \quad (3)$$

$$ArO\cdot + DPPH\cdot \longrightarrow Products \quad (4)$$

2.4.3 Principle of reducing power assay

The principle of reducing power assay is based on a single electron transfer reaction.

$$Fe^{3+} + e^- \longrightarrow Fe^{2+}$$

The reducing power assay measures the ability of antioxidants to reduce the ferric 2, 4, 6-tripyridyl-s-triazine complex [Fe (III)-(TPTZ)2]$^{3+}$ to the intensely blue colored ferrous complex [Fe (II)-(TPTZ)2]$^{2+}$ (Benzie and Strain, 1996). The reductones will change the yellow colour of the test solution into various shades of green and blue. The reductones (antioxidants) causes the conversion of Fe^{3+}/ ferricyanide complex to ferrous form. The concentration of Fe^{2+} can be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. The reducing power is indicated by the increase in absorbance value.

2.4.4 Principle of β-carotene bleaching assay

The β-carotene bleaching assay is based on the ability of the plant extracts to decrease the oxidative losses of β-carotene/ linoleic acid emulsion. When linoleic acid is incubated at 50˚C, hydroperoxides (free radicals) is produced. These free radicals will attack β-carotene. During the oxidation process, β-carotene loses its double bond. Thus the compound loses its chromophore and orange colour. The presence of antioxidants in the plant extracts can hinder the extent of β-carotene bleaching. The antioxidant compounds will neutralize the linoleate free radicals and any other free radicals formed within the system. The degradation rate of β-carotene depends on the antioxidant compounds that present. In the presence of antioxidant compounds, β-carotene will retain their colour and absorbance for a longer time, but in the absence of antioxidant in the samples, the β-carotene will lose its colour and the absorbance will decrease rapidly. Thus, there was a correlation between bleaching of β-carotene and degradation rate. Extract with lowest β-carotene degradation rate exhibited the highest antioxidant activity.

2.5 *In vitro* cytotoxic activity tests

The use of cell culture system offers many of advantages as compared to whole animal models (*In vivo* system). A number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). *In vitro* testing systems are inexpensive, sensitive, with reproducibility of test conditions and rapid.

2.5.1 Methods to determine cytotoxic activity

In vitro cytotoxicity assay can be used for general screening of chemicals and predict human toxicity (Clemedson and Ekwall, 1999; Scheers *et al*., 2001). The viability of the treated cells depends on the test agent and the cytotoxicity assay used (Weyermann *et al*., 2005).

The microtiterplates (96-well format) is the most convenient and modern assay because it allows many samples to be analyzed rapidly and simultaneously (Weyermann et *al*., 2005). Colorimetric and luminescence based assays allow samples to be measured directly in the plate by using a microtiterplate reader or ELISA plate reader (Weyermann *et al.*, 2005). The Lactate Dehydorgenase (LDH) leakage assay, protein assay, the neutral red assay, MTT assay and ATP content of treated cells are examples of cytotoxicity assays.

The LDH leakage assay is based on the measurement of lactate dehydrogenase (LDH) activity in the extracellular medium after the cell membrane damage. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that present in cells. When the plasma membrane is damaged, lactate dehydrogenase is rapidly released into the cell culture medium (Korzeniewski and Callewaert, 1983). The first step is the reduction of NAD^+ to $NADH/H^+$ by the LDH catalyzed conversion of lactate to pyruvate. After that, the catalyst (diaphorase) will transfer H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red formazan (Decker and Lohmann-Matthes, 1988; Lappalainen *et al*., 1994; Nachlas *et al.*, 1960). LDH leakage assay is reliable, fast and simple evaluation (Decker and Lohmann-Matthes, 1988).

The MTT assay is based on the measurement of metabolic activity of viable cells. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt. According to Smith (1951), tetrazolium salts are reduced only

by metabolically active cells. The tetrazolium salts are converted to an insoluble purple formazon by the cleavage of tetrazolium ring by succinate hydrogenase within the mitochondria (Fotakis and Timbrell, 2006). This formazon product accumulates in healthy cell because it is impermeable to healthy cells (Fotakis and Timbrell, 2006). Recent studies showed that NADH or NADPH within the cell and out of the mitochondria also catalyzed the reduction of MTT (Berridge and Tan, 1992). Further modification of the initial protocol described by Mossmann (1983) was proposed (Denizot and Lang, 1986; Hansen *et al*., 1989) in order to improve the repeatability and sensitivity of this assay.

The neutral red assay has been used for identification of cell viability in cultures (DeRenzis and Schechtman, 1973). After exposure to toxicants, the number of viable, uninjured cell is quantified. The living cells will take up neutral red (2-methyl-3-amino-7-dimethylamino-phenazine), which is concentrated within the lysosomes of the cell. From the quantification, the cell numbers has been shown to be linear to the dye extracted from the cells, both direct cell counts and by protein determinations of cell populations (Borenfreund and Puerner, 1985, 1986).

The protein assay is an indirect measurement of cell viability since it measures the protein content in viable cells that are left after washing of the treated plates (Fotakis and Timbrell, 2006).

Adenosine triphosphate (ATP) that is present in all metabolically active cells can be determined in a bioluminescent measurement (Weyermann *et al*., 2005). The bioluminescent method utilizes an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin (Weyermann *et al*., 2005). The emitted light intensity is linearly related to the ATP concentration (Crouch, 2000; Crouch and Slater, 2001; Crouch *et al.,* 1993; Slater, 2001).

2.5.2 *In vitro* cytotoxicity assay

The terminology cytotoxic, antitumor and anticancer has different meaning. Thus it is important to distinguish between these terms.

Cytotoxic compounds are compounds that are toxic to cells in the culture. These compounds may not show any selective toxicity to cancer cells as against normal cells. Cytotoxic compounds may be cytocidal (kill cells) or cytostatic (stop the cell growth, reversible or irreversibly).

Antitumor compounds are compound that show positive activity in an *in vivo* tumor system. Thus, antitumor compound show selectivity against tumor cells.

Anticancer compounds are compounds that are effective against cancers in human. Thus, human clinical trials are needed to determine if any antitumor compound has anticancer activity. The proliferation of cancer cells can be inhibited by anticancer compounds. These compounds are able to bind to the DNA in the cancer cells and inhibit certain enzymes that are required for continual growth of cancer cells and finally alter the morphology of cancer cells (immunomodulators) or cause total death (cytotoxic drugs) (Derelanko, 1995).

A cytotoxic agent is toxic to tumor cells *in vitro* (laboratory culture). This agent is said to have anti-tumor activity if it is toxic to tumor cell *in vivo*. The terminology anticancer is for the materials that toxic to tumor cells in clinical trials. The routine testing of extract fraction for antitumor activity is frequently done via an *in vitro* cytotoxicity assay. Cytotoxicity assay is popular for initial test because it is an *in vitro* bioassay, rapid and only small amount of extract is required. Fractions showing activity in this system are leading to purification of the active principles.

Many experiments are carried out *in vitro* in order to determine the potential cytotoxicity of the compounds being studied, either because the compounds must be shown to be non toxic or because they are designed as anticancer agent and cytotoxicity may be crucial to their action.

2.5.3 Principle of *in vitro* neutral red cytotoxicity assay

Neutral Red (NR) cytotoxicity assay is a cell survival/ viability chemosensitivity assay. In this assay, the viable cells will incorporate and bind to the neutral red, which is a supravital dye. Initially this assay is used to evaluate the acute toxicities of chemicals in mammalian cells. The cytotoxicity is expressed as a concentration dependent reduction of the uptake of the neutral red after chemical exposure. It provides a sensitive, integrated signal of both cell integrity and growth inhibition.

Neutral red (2-methyl-3-amino-7-dimethylamino-phenazine) is a weak cationic supravital dye. It can penetrate through cell membranes by non-ionic diffusion. It will accumulate intracellulary in the lysosomes of living cells. After the incubation with toxic agent, neutral red will bind with ionic sites in the lysosomal matrix. The cells will uptake the neutral red through passive diffusion across the plasma membrane. The alternation of the cell surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually becomes irreversible. Such changes brought about by the action of xenobiotic result in a decreased uptake and binding of neutral red. Thus, the viable, damaged or dead cells can be distinguished. This is the basis of the assay.

The xenobiotic will alter the surface of the lysosomal membrane and lead to lysosomal fragility and other changes. Thus it decreases the uptake and subsequent retention of the dye. The dyes are not able to retain in the dead or damaged cells after

the washing and fixation steps. Neutral red is quantified spectrophotometrically at 540 nm using an ELISA reader (Weyermann *et al*., 2005). The amount of neutral red is compared to the amount of dye extracted from control cell cultures.

Quantification of the extracted dye by spectrophotometry has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Borenfreund and Puerner, 1985). Neutral red assay provide information on the general, overall, toxic potential of a chemical.

Different cytotoxicity assays might produce different result. Both MTT assay (methyl tetrazolium) and neutral red assay are the most sensitive cytotoxicity assay that show statistically significant difference between the treated cells and the controls (Fotakis *et al*., 2006). Although the NR assay and MTT assay yielded comparable ranking of cytotoxicity data, the optical density absorbance with the neutral red assay was twice that obtain with the MTT assay and thus fewer cells are required for analysis.

2.6 Identification techniques

2.6.1 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is used for identification and quantitation of volatile and semivolatile organic compounds in complex mixtures or determination of molecular weights and (sometimes) elemental compositions of unknown organic compounds in complex mixtures.

In order to use GC-MS, the samples must be in solution for injection into the gas chromatograph. The solvent must be volatile and organic (for example, hexane or dichloromethane). As an identification method, when a given molecular species is

impacted with an electron beam, a family of positive particles is produced. The mass distributions of the particles are characteristic of the parent species.

2.6.2 Liquid chromatography tandem-mass spectrometry (LC-MS/MS).

Liquid chromatography-mass spectrometry (LC-MS) is a hyphenated technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS/MS is used for the specific detection and potential identification of chemicals in a complex mixture. (http://www.wikipedia.org/wiki/Liquid_chromatography-mass_spectrometry)

2.6.3 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) is used to study the chemical structure of the compounds by determining the carbon and hydrogen atoms' position by using the ^{13}C and ^{1}H method.

The principle of NMR usually involves two sequential steps. The first step is the alignment (polarization) of the magnetic nuclear spins in an applied, constant magnetic field $\mathbf{H}_0$. While the second step is the perturbation of this alignment of the nuclear spins by employing an electro-magnetic, usually radio frequency (RF) pulse. The required perturbing frequency is dependent upon the static magnetic field ($\mathbf{H}_0$) and the nuclei of observation.