CHAPTER 1

INTRODUCTION

1.1 Introduction

There is a long history of medicinal use of plants in Southeast Asian countries, some of which have proven useful to human as pharmaceuticals. This region therefore is a promising site for discovery of novel biologically-active substances from its flora (Houghton *et al.,* 2005). The notion of cancer prevention through antioxidant intervention arises from the fact that fruits and vegetables contain antioxidants and are linked to low cancer rates in those who consume them. Protection against DNA damage by plant food products has been demonstrated *in vitro*. There is strong evidence from observational epidemiology that fruits and vegetables in the diet are associated with a lower incidence of various cancers as for example, a research by Buchner *et al.* (2010), confirmed that consumption of a variety of fruits and vegetables can reduce lung cancer risk. It has been proven that it is the antioxidants in these foods that are the effective preventive agents. It is known that free radicals released during respiration can damage DNA, and oxidation damage to DNA can result in mutation. Exposure to radiation and chemicals can also generate free radicals. Fruits and vegetables contain substantial amounts of various natural compounds with antioxidant properties (Collins, 2005). Most of the active compounds can be found in the wood, bark, stem, leaf, fruit, root, flower and seeds of many plants.

Beta vulgaris L. is one of the prominent genus of flowering plant of the family Chenopodiaceae. This genus consists of several species such as *Beta nana, Beta adanensis, Beta atriplicifolia, Beta lomatogona* and others. *Beta vulgaris* has been chosen for the present research because of its various medicinal properties. *Beta vulgaris* has many subspecies such as *maritima, cycla, maritima var rubra and vulgaris* (Stephen, 2004).

It has been reported that *Beta vulgaris L.*, the beet root, has anti-cancer activity. The cancer chemo-preventive potential of the beet root is thought to be due to the betalains which are composed of two main groups: the red betacyanins and the yellow betaxanthins. Both are used as natural additives for food and are powerful radical scavengers. The betacyanins have been shown to inhibit the proliferation of tumor cells *in vitro* and the whole extract of the red beet root has been shown to induce phase-II enzymes (Ninfali *et al.,* 2007). Specifically there are growing interests in betaxanthins because of their antiradical and antioxidant activity (Trejo-Tapia *et al.,* 2008). Also present are some flavonol glycosides, identified as derivatives of apigenin which is a well-known anti-mitotic and apoptotic agent (Gil *et al.,* 1998).

There have been several phytochemical and biological studies conducted previously on *Beta vulgaris*. An *in vitro* test of the extract for anti-tumor promoting effect on Epstein-Barr virus early antigen induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), showed a high order of activity. *In vivo* anti-tumor promoting activity against mice skin and lung assay indicated that orally administered beetroot extract to ICR mice inhibited TPA-induced promotion of mice skin tumors, suggesting that beetroot is a useful cancer preventative agent (Kapadia *et al.,* 1996).

Some reports based on *in vitro* tests rank beet among the ten most potent vegetables with respect to their antioxidant activity (Vinson *et al.,* 1998; Halvorsen *et al.,* 2002; Ou *et al.,* 2002). There were enormous reports on an important pigment, betalain, which was found to be responsible for the antioxidant activity of the plant. These reports include ABTS free radical scavenging activity of by Escribano *et al.* (1998), which concludes that betacyanins have higher antiradical activity compared to betaxanthine due to its chemical structure. A report on 1,2-diphenyl-2-picrylhydrzyl (DPPH) radical scavenging activity and oxygen radical absorbance capacity (ORAC) assay showed that the plant has both high

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single electron transfer (SET) and hydrogen atom transfer (HAT) ability through the former assay and high HAT ability through the latter assay (Georgiev, *et al.,* 2010). There was also another report which measures the antiradical and antioxidant property by ABTS radical scavenging assay (TEAC) and FRAP assay by Gandia-Herrero *et al.* (2009) which showed high activity that relates to the phenolic hydroxyl group of the pigment. In a study by Kanner *et al.* (2001), linoleate peroxidation by cytochrome c was inhibited by betanin, catechin and α-tocopherol. Glizczynska-Swiglo *et al.* (2006) reported that betalains were more potent antioxidant than catechin and other flavonoids.

However, there have been no reports on a detailed study of the antioxidant and cytotoxic activity of the crude methanolic, hexane fraction, ethyl acetate fraction, water fraction and juice extract of *Beta vulgaris L.* Study on the crude extracts is important as it gives us the idea of what the antioxidant property based on human consumption as a whole. There also have not been studies in which the antioxidant properties were tested using various assay to understand the type of antioxidant mechanism exerted by these extracts i.e. the first, second and third line of antioxidant defense. Besides that, some fractions which have active compounds (betalains) were also isolated and tested on the various antioxidant assays to understand the underlying mechanism.

Cytotoxicity studies was carried out based on published work that showed high order of *in vitro* inhibitory effect of the extract on Epstein-Barr virus early antigen using Raji cells and significant tumor inhibitory effect on *in vivo* test against mice skin and lung bioassay (Kapadia *et al.,* 1995). Based on a research by Reddy *et al.* (2005), betanin showed excellent growth inhibition of breast, colon, stomach, CNS and lung cancer cell lines on MTT assay. Maizatul (2008) reported that the methanolic extract and water fraction showed excellent inhibitory effect (IC_{50}= 4.90 µg/ml and 5.90 µg/ml respectively)

on CaSki cell line. Based on these findings, a hypothesis that it might have cancer inhibition properties was made.

1.2 Objectives of Study

The main objective of the present study was to study the antioxidant and the cytotoxic activity of *Beta vulgaris L.*

The specific objectives of this study were as follows:

a) to investigate the antioxidant activity and antioxidant mechanism of the crude and fractionated extracts of *Beta vulgaris L.* using various antioxidant tests such as diphenylpicrylhydrazyl (DPPH) free radical scavenging assay, reducing power assay and β-carotene bleaching assay, metal chelating assay, superoxide dismutase (SOD) activity assay, thiobarbituric acid reactive species (TBARS) assay, Folin-Ciocalteau assay and tyrosinase inhibitory assay,

b) to conduct cytotoxicity studies on the crude extracts and fractionated extracts of *Beta vulgaris L.* against selected human cancer cell lines such as human breast carcinonma cell line (MCF7), human colon carcinoma cell line (HT29 & HCT-116), human cervical carcinoma cell line (CaSki) and human lung carcinoma cell line (A549),

c) to identify bioactive components of the active fractions. A bioassay-guided investigation will be attempted to determine the active chemical compounds. The chemical isolation will involve chromatographic techniques using high performance liquid chromatography (HPLC). The fractions isolated through this technique will then be identified by spectroscopic and spectrometric methods (LC-MS); and

d) to determine the antioxidant activity exhibited by the isolated sub-fractions from ethyl acetate and juice extract on various assays conducted earlier for the crude extracts of *Beta vulgaris L.*

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CHAPTER 2

Literature Review

2.1 Natural Products

Natural products has been studied and used to treat diseases since early human history. In the early 1900, most of the medicines were obtained from roots, barks and leaves. Humanity placed its faith in the belief that every sickness has a remedy in the plants of field and forest. Currently, natural product continues to be an important source of drugs and medicines. This fact is evident with about 60% of anticancer compounds and 75% of drugs for infectious diseases are either from natural products or natural product derivatives (McChesney, *et al.,* 2007).

Commercial evidence supports the demand for natural products. Among the 20 best-selling non-protein drugs in 1999, nine were either derived or developed from natural products for example simvastatin, lovastatin, enalapril, pravastatin, atorvastatin, augmentin, ciprofloxacin and many more. Natural product has been the key to discover new medicines (Harvey, 2000).

Plants, especially those possessing ethnopharmacological uses, have been the main source of early drug discovery. Current research on drug discovery from terrestrial plants mainly focuses on bioactivity guided isolation method. This type of research has led to discoveries of important anti-cancer agents such as paclitaxel from *Taxus brevifolia* (Mansukhlal *et al.,* 1971) and camptothecin from *Campthotheca acuminata* (Wall *et al.,* 1966)

Less than 10% of the biodiversity of the world has been researched for biological activities. Many more natural compounds are to be discovered. The real challenge is to access this natural chemical diversity.

2.2 The Family Chenopodiaceae

The Chenopodiaceae family or goosefoot family is a large plant family with 120 genera and 1500 species. It is found in many parts of the world and consists of many species that has agricultural importance. The agricultural importance includes: as crop plants, potential forage crops, weed species or host for insect pest species.

2.2.1 Taxonomic Classification of *Beta vulgaris*

The Scientific Classification of *Beta vulgaris* is as follows:

Kingdom: Plantae

Sub-kingdom: Tracheobionta

Super-division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Order: Caryophyllales

Family: Chenopodiaceae

Genus: Beta

Species: vulgaris

Sub-species: vulgaris

The common names of *Beta vulgaris L.* is listed in Table 2.1

Table 2.1: Common names of *Beta vulgaris L.* in different countries around the world

Country	Common Names
England	Beetroot, red beet, red beetroot, red-fleshed beetroot.
China	tian cai, hong tou cai, hong gen tian cai.
Denmark	rødbede.
Holland	kroot, kroten, rode biet.
Finland	punajuurikas.
France	betterave potagère, betterave rouge potagère
Germany	rote bete, rote rübe, rote rüben, rote beete, rote bete.
Israel	selek adom
Italy	barbabietola rossa, barbabietola da insalata, barbabietole da orto, bietola a radice rossa.
Japan	aka kabu, biito, kaensai, shokuyou biito
Portugal	beterraba, beterraba-vermelha, beterraba de salada, patarrábia, Terraba.
Russia	Svëkla stolóvaia, Svëkla obyknovennaia
Slovakia	rdezhe pesa
Spain	remolacha, remolacha colorada, remolacha de mesa, remolacha roja
Sweden	rödbeta

Figure 2.1: Whole plant of *Beta vulgaris L.*

Figure 2.2: *Beta vulgaris L.* purchased from Pasar Tani, Section 17, Petaling Jaya

2.2.2 *Beta vulgaris L.* (beetroot)

Beetroot (beets) are part of the Chenopodiaceae family. The Chenopodiaceae or goosefoot family of plants also includes other edible species, including spinach (*Spinacia oleracea*), quinoa (*Chenopodium quinoa*), orache or orach (*Atriplex hortensis*) and Good King Henry (*Chenopodium bonus-henricus*). *Chenopodium album* which is a member of this family is the fifth most common plant on the earth (Dinan, *et al.,* 1998).

Cultivated beets (*Beta vulgaris*) are biennials, although they are usually grown as annuals. Beetroot produces green tops and a swollen taproot during its first growing season. The nutrients stored in the taproot are used to produce flowers and seeds in the second season. Beets grow well in soil with a pH of 6.5-7.5 and will do well with boron supplement added to the soil – pelleted borax with 10% elemental boron (Schick, 2008). Cultivated beets thrive under a wide range of conditions and are easy to cultivate. Beetroot is one of the most popular vegetables grown on allotments and in gardens. It grows quickly, is highly productive, and is usually free of pests and diseases.

Beetroot has long been used as a common vegetable and in our daily cuisine. They have a complex flavor that can be described as earthly and rich. The famous food that involves beetroot is the famous Russian soup called Borscht. It has been a staple food during winter since 14th century. Pickled beets are also a common dish made by this vegetable (Schick, 2008).

Beetroot juice concentrate, beetroot red, is universally permitted as a food ingredient (Kujala *et al.,* 2002) as it is of plant origin and naturally harmless (Kapadia *et al.,* 1996). Beetroot has been used successfully to color products such as hard candies, yogurts, ice creams, salad dressings, ready-made frostings, cake mixes, meat substitutes, powdered drink mixes, gravy mixes, marshmallow candies, soft drinks and gelatin deserts (Kapadia *et al.,* 2003).

Beetroot is known to be a very good source of dietary folate (Jastrebova *et al.,* 2003). In recent research, folate was found to be able to able to prevent neural tube defects, play important role in preventing cardiovascular and cancer disease and act as antioxidants (Jiratanan and Hai Liu, 2004). Beetroot is also regarded as a good source of antioxidant and

phenols and also found to express anticancer (Ninfali *et al.,* 2007) and radio-protective properties (Bavec *et al.,* 2010). It is said to have good antioxidant property due to the presence of nitrogen compounds called as betalains which is divided into betacyanin that confers to the purple-violet color and betaxanthine which is present in yellow-orange color but present in lower amount as compared to betacyanin (Pitalua *et al.,* 2010). In comparison to other vegetables, the antioxidant capacity of beets was very high (Czapski *et al.,* 2009). Regular consumption of red beet could have prevention effect against oxidative stress related diseases (Kanner *et al.,* 2001).

Medicinally, beetroot has been employed as popular folk remedy of liver and kidney diseases. It has also been used to as a special diet in treating cancer (Kapadia *et al.,* 2003). Beetroot also is known to help with yellow jaundice and when the juice is put in the nostrils, it is helpful for ringing in the ears and toothaches. It was used to treat illnesses relating to digestion and blood. The Romans has long used beets as an herb for treatment of fever and constipation and as an aphrodisiac due to high presence of boron (Schick, 2008).

2.3 Betalains

Betalains is a term that introduced by Mabry and Dreiding (1968). It refers to the yellow (betaxanthine) and red (betacyanine) pigment of beet (Von Elbe, 1975). Betalains are water-soluble nitrogenous vacuolar pigments present in flowers and fruits of many caryophyllales with potent antioxidant properties (Devalraju *et al.,* 2007). It has pigments that are yellow, orange, pink, red and purple in colour. Most red colouration in plants is due to carotenoids and flavonoids. The red colour of most fruit and vegetables, such as strawberries, grapes and red cabbage, is due to anthocyanins, which are in the flavonoid class of pigments. Betalains accumulate in the cell vacuoles of flowers, fruits and leaves of the plants that synthesize them, mainly in the epidermal and subepidermal tissues (Kujala *et*

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al., 2002). They absorb visible radiation over the range of 476–600 nm with a maximum at 537 nm at pH 5.0 (Azeredo, 2009). Betacyanins act as reactive oxygen species (ROS) scavengers, limiting damage caused by wounding and pathogen infiltration in plant tissues ((Sepu´lveda-Jime´nez *et al.,* 2004). Betalains show antioxidant and radical scavenging activities (Escribano *et al.,* 1998). Gliszczynska-Swiglo *et al.* (2006) reported that, the free radical scavenging activity of betanin in the Trolox equivalent antioxidant capacity (TEAC) assay is pH dependent. The results suggests that the exceptionally high antioxidant activity of betanin is associated with an increase in its H-donation and electron donation ability when going from cationic state to mono-, di- and tri-deprotonated states present at basic solutions. Betalains are considered to be cationic antioxidant (Kanner *et al.,* 2001). Reports show that there is bioavailability for the human organism as they permeate from the alimentary tract to the blood stream (Netzel *et al.*, 2005).

Betalains replace the pigment anthocyanins in 13 families in the plant order Caryophyllales. It has various functions to be found in the plants. The functions are such as to attract animals in pollen transfer and fruit-eating animals for dispersal of the indigestible seeds. This is essential for plant propagation. Betacyanins were found to protect tissues from UV radiation. For plants like beetroot where it is growing under the ground, betalains acts to protect the plant from pathogens and virus. This fact is supported by the study by Kujala *et al.* (2000) that there is high concentration of betacyanin in peel, cron and flesh of the root. For plants such as *Salicornia europeae L.,* formation of pigment occurs when there is salt stress, hence betalains perform as osmolytes to maintain the physiological processes (Stintzing *et al.,* 2004)

In addition to *Beta vulgaris* (family Chenopodiaceae), betalains have been described from Cactaceae fruits (prickly pear), Amaranth seeds (Amaranthaceae), *Bougainvillaea*

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bracts (Nyctaginaceae), and flowers or other plant parts within the Aizoaceae, Basellaceae, Didieraceae, Phytolaccaceae and Portulaceae. Nine of the eleven families within the order Caryophyllales have plants containing betalains. Red beetroot and prickly pear (*Opuntia ficus-indica*) are the only edible sources of betalains (Stephen, 2004).

Betalain pigments were first isolated from the red roots of *Beta vulgaris*; the betalain class of pigments is in fact named after the plant genus *Beta*. Incidentally, bett is the Celtic word for red, although the published suggestions that this is how *Beta vulgaris* got its name are pure speculation. There are currently over fifty known betalain pigment molecules, which occur in flowers, fruits, shoots and roots. The betalains are subdivided into two structural groups: the red-violet betacyanins and the yellow betaxanthins.

Beetroot contains a complex mixture of betalain pigments. However, the characteristic purple-red-violet colour of beetroot is mainly derived from a betacyanin pigment called betanin. Betanin was first discovered in around 1920, while a crystalline form of betanin dye was produced in the 1960s. Up to 200 mg of betanin is typically found in one beetroot. It normally occurs at much higher levels in the roots of red beetroot than other betacyanin pigments. Like all betacyanins, betanin is metabolically derived from a molecule called 3,4-dihydroxyphenylalanine (L-DOPA). Betanin is formed from two L-DOPA molecules. The first undergoes a change to form betalamic acid. The second L-DOPA molecule is changed to cyclo-DOPA glucoside (CDG), which condenses with betalamic acid to produce betanidin. A change in structure, involving the addition of glucose, converts betanidin to betanin. The condensation with the closed structure of cyclo-Dopa extends the electronic resonance to the diphenolic aromatic ring. This extra conjugation shifts the absorption maximum from 480 nm (yellow, betaxanthins) to about 540 nm (violet, betacyanins) (Azeredo, 2006). The most common betacyanin is betanidin-

5-Ob-glycoside (betanin), the major pigment in red beets. After betanin, the yellow betaxanthin pigments vulgaxanthin-I and vulgaxanthin-II are the next most significant in beetroot (Stephen, 2004).

People prefer to use natural pigments which are considered harmless and even healthy. Synthetic colorants are being avoided in these times (Azeredo, 2009). The main commercial sources of betalains are powders and concentrates of red beet (*Beta vulgaris*) or cactus pear (*Opuntia ficusindica*) extracts (Georgiev *et al.,* 2008). Generally the amount of pure pigment to get the desired color is relatively small. The food colourant that is extracted from beetroot which is known as 'beetroot red' is commercialized in European Union and USA as food color. Beet colorants are available commercially as either juice concentrates which is produced by vacuum-concentration of beet juice to 60-65% of total solid, or in powder form that is produced by freeze drying or spray drying process (Azeredo, 2009).

Betalain pigments are easily degradable. Degradation could happen due to different factors. These factors includes structure and composition in which betaxanthine was found to be more stable compared to betacyanins, pH where betalains were found to be stable at a certain pH range (3-7) and at its maximum stability at pH 5-6, water activity in which the pigments are found to be stable in dry condition without presence of water, oxygen, where pigments are more stable in anaerobic conditions. Other factor includes light (UV or visible light), where betalain stability was found to be impaired in presence of light. Next factor would be presence of metals. Metal cations such as iron, copper and aluminium were found to accelerate the degradation of betalains. Temperature also affects the stability of betalains where at increasing temperature, increasing betalains degradation was found. On the other hand, antioxidants such as asorbic acid and isoascorbic acid were found to have the reverse

effect where it enhances the stability of betalains. The figure below shows some degradation pathways of betanin. (Azeredo, 2009)
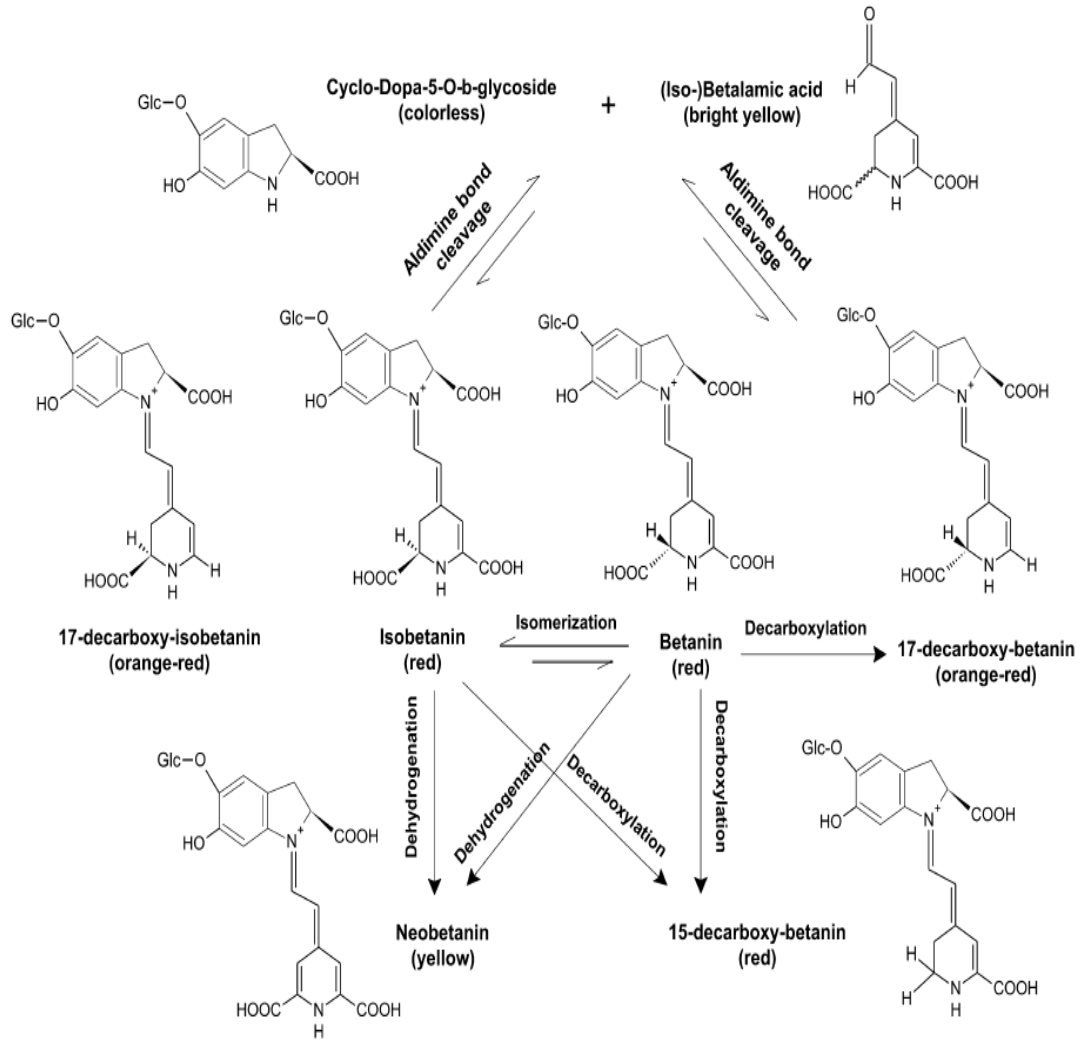
Figure 2.3: Some degradation pathways of betanin (Excerpted from Azeredo, 2009)
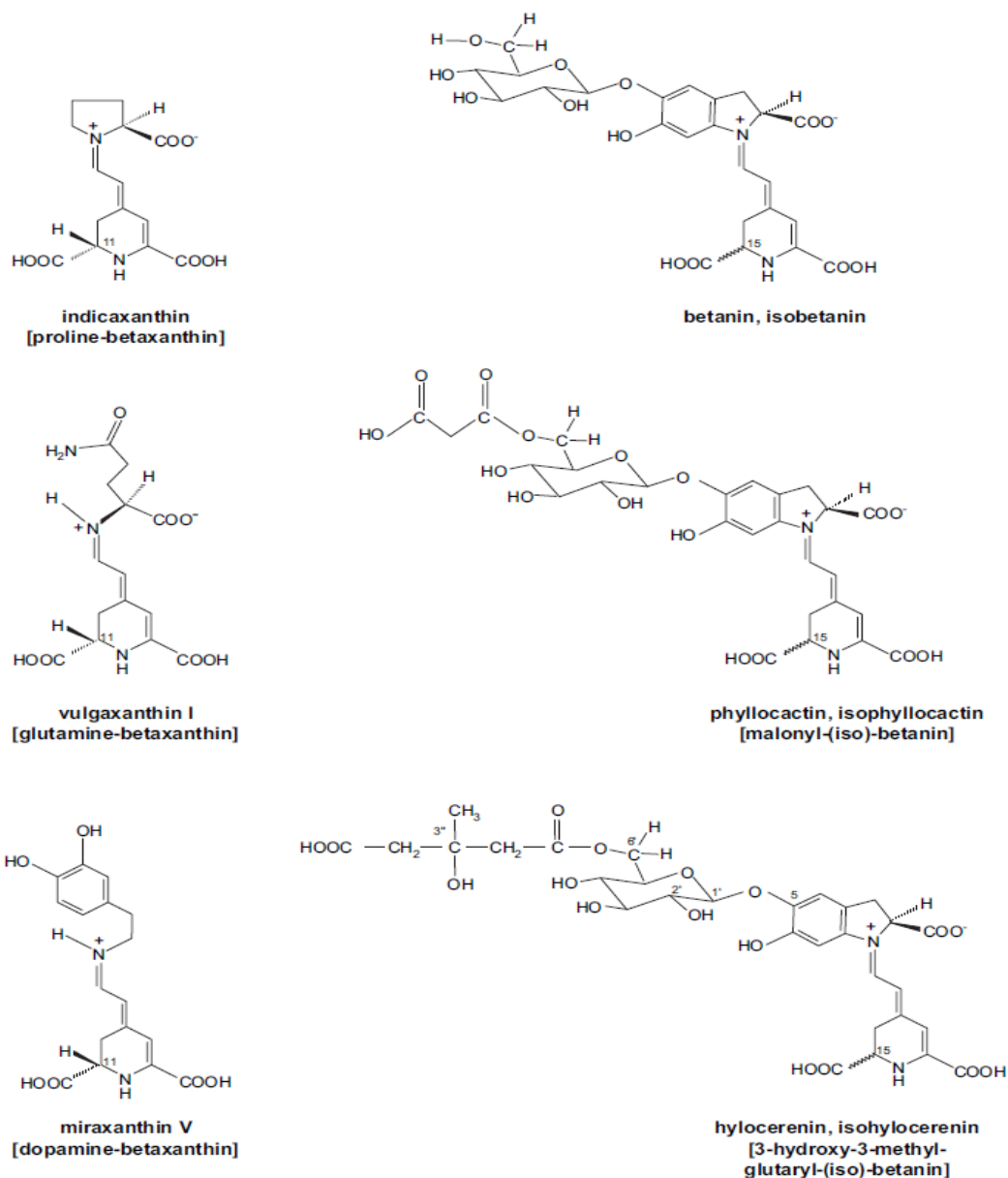
Figure 2.4: Predominant betaxanthine (left) and betacyanine (right) in fruits and vegetable from family Chenopodiaceae and Cactaceae (Excerpted from Stintzing and Carle, 2007)

Betalains were reported to have various desirable biological properties that cause so much of interest on its study. The biological properties include anti-inflammatory, hepatoprotective, cancer chemo-preventive and ability to lessen the oxidative stress and to protect LDLs from oxidation (Georgiev *et al.,* 2008).

2.4 Traditional Medicinal Uses of Beetroot

Beetroot is employed as a popular folk remedy to stimulate the immune system and for the treatment of liver and kidney diseases. It is also employed as a special diet in the treatment of cancer (Govinda *et al.,* 2003). The ancient Greeks and Romans used beetroot to help relieve high fever. During the dark ages in Europe, the juice of beetroot was always recommended when the person was unable to consume hard food. Beetroot is well known for its blood purifying properties as well. Its juice stimulates the liver, kidneys, gall bladder, spleen and bowels. Naturopaths believe that beetroot stimulates the lymphatic system and strengthens the immune system, especially when it comes to fighting colds. Chinese medicine suggests that consumption of beetroot strengthens the heart, sedates the body and purifies the blood. They especially recommend beetroot juice

2.5 Antioxidants

Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu *et al.,* 1998). Antioxidants also protect the human body from free radicals and reactive oxygen species (ROS) effects. They inhibit the progress of many chronic diseases as well as lipid peroxidation (Gulcin *et al.,* 2009). They also activate a battery of detoxifying proteins in the body (Chen *et al.,* 2009). It is an organic compound generally. Antioxidants are vitamins, minerals and enzymes (Muzaffar, 2007). Oxidation is a chemical reaction in which electron is transferred from a substance to an oxidizing agent. Oxidation process could produce free radicals that are harmful for the cells (Cayuela, 1994). Antioxidants could fight and destroy excess free radicals and repair oxidative damage in biomolecules. They basically delay or inhibit the start or propagation of oxidizing chain

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reactions (Vimala *et al*., 2003). Antioxidants are often reducing agents and this means they are often thiols and polyphenols. Antioxidants generally prolong the life span of a cell by protecting the membrane of the cell against free radical damages and this would stop the aging process. It is also found that antioxidants do help to extend the quality and length of life and reduce occurrence of diseases (Vimala *et al*., 2003).

Major action that is exhibited by antioxidants in cells is to prevent damage due to action of reactive oxygen species. The reactive oxygen species could be divided into two types. These include hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). Free radicals on the other hand could be substances like hydroxyl radical ($\cdot OH$). These molecules are extremely unstable and are highly reactive (Hani, 2007). Naturally generated reactive oxygen species (ROS) are molecules that can attack cell components and create several types of biological damage. They play important roles in the pathogenesis of various diseases ranging from carcinogenesis to aging (Towatana *et al*., 2006). Generally and naturally, all cells contain antioxidants to prevent this effect (Hani, 2007).

Antioxidant compounds in food play an important role as health protecting factor. Scientific evidences have shown that antioxidants reduce the risk of various chronic diseases and this includes cancer and heart disease. The major sources of antioxidant in foods are whole grains, fruits and vegetables. Food antioxidant that comes from plant source which contains vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized to possess characteristics that could reduce disease risks. Most of the antioxidant source that is gained by us comes from food source. (Prakash *et al*., 2007

Many clinical studies have suggested that antioxidants in fruits, vegetables, tea and red wine has the efficiency in reducing the incidence of chronic diseases including heart disease drastically (Prakash *et al*., 2007). Scientific studies have exhibited precisely on how antioxidants help to improve immune system, protect and treat degenerative diseases. It has been proven to defend body against diseases such as cardiovascular disease (CVD), cancer, lung diseases, rheumatoid arthritis, diabetes and Alzheimer's disease (Amez *et al*., 1993).

Basically, consuming a wide variety of antioxidant enzymes, vitamins, minerals and herbs may be the best method to provide our body with the most sufficient protection against the free radical damage (Hani, 2007).

Basic characteristics of a good antioxidant

A good antioxidant should possess the following characteristics:

(i) specifically quench free radicals;

(ii) chelate redox metals;

(iii) interact with (regenerate) other antioxidants within the "antioxidant network";

(iv) have a positive effect on gene expression;

(v) be readily absorbed;

(vi) have a concentration in tissues and bio fluids at a physiologically relevant level; and

(vii) work in both the aqueous and/or membrane domains.

(Valko *et al*., 2006)

2.5.1 Natural and Synthetic Antioxidants

There are two categories of antioxidant which is synthetic and natural. Synthetic antioxidants are compounds which has phenolic structures at various degrees of alkyl substitution. Natural antioxidants on the other hand could be phenolic compounds such as tocopherols, phenolic acids and flavonoids. Besides that, it could also be nitrogenous compounds such as alkaloids, amino acids and amines or could also be carotenoids (Velioglu *et al.,* 1998).

Recently there has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones. Natural antioxidant substances are presumed to be safe since they occur in plant foods and are seen to be more desirable than their synthetic counterparts (Chanwitheesuk *et al*., 2004). Possibility has been raised that some synthetic antioxidants may be toxic (Towatana *et al*., 2006). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are widely used in the food industry but restrictions are being imposed on these substances as it could damage liver and has carcinogenetic effect. The carcinogenic effect was exhibited in animal models. (Bin Li *et al*., 2007).

Plants generally contain natural antioxidants which help the plant to protect the fats, oils, protein and nucleic acid of the plant. The plant generally must protect itself from destruction caused by ultraviolet (UV) light from sunlight, pollutants, chemicals and cosmic radiations and besides that free radicals which are generated internally in the plants. Phenolic acid, alkaloids, amino acids, lignans, flavonoids are the natural antioxidant phytochemicals which are present in high amount in plants (Vimala *et al*., 2003). Plants that contain most antioxidants included members of several families, such as Rosaceae (dog

rose, sour cherry, blackberry, strawberry, raspberry), Empetraceae (crowberry), Ericaceae (blueberry), Grossulariaceae (black currant), Juglandaceae (walnut), Asteraceae (sunflower seed), Punicaceae (pomegranate) and Zingiberaceae (ginger) (Halvorsen *et.al.,* 2002). Natural antioxidants especially flavonoids has many biological effects. The biological effects that is exerted by this component are such as antibacterial, antiviral, anti-allergic, vasodilatory actions and anti-thrombotic. These are proven to promote many health benefits (Cook and Samman, 1996).

Tocopherol are widely used as safe natural antioxidants but they are not as effective as the synthetic antioxidants and the manufacturing cost is high (Osawa *et al*., 1980).

2.5.2 Plant Antioxidants

Natural products containing antioxidants from plants often called nutraceuticals are believed to modulate oxidative stress and prevent or delay degenerative disorders (Srivastava *et al.,* 2006). Plants are generally defined to have high source of antioxidants. This is why many times plants are encouraged to be consumed in our diet. Data from both scientific reports and laboratory studies show that plants contain a large variety of substances called "plant chemicals" or "phytochemicals" possesses antioxidant activity (Chanwitheesuk *et al*., 2005). Epidemiological studies have demonstrated an inverse association between intake of fruits and vegetables and mortality from age-related disease such as coronary heart disease and cancer. This could be strongly attributed to the antioxidant activities of these foods (Bin Li *et al*., 2007). The consumption of natural antioxidant phytochemicals was reported to have health benefits and recently there has been increasing interest in finding natural antioxidants from plant materials (Tachakittirungrod *et al*., 2006). The antioxidant phytochemicals from plants, particularly flavonoids and other

polyphenols have been reported to inhibit the propagation of free radical reactions and to protect the human body from disease. They are also found to have effects to retard the lipid oxidative rancidity (Tachakittirungrod *et al*., 2006). Besides that, plants also are well known to syntesize antioxidants like tocopherol, ascorbic acid and carotenoids (Chanwitheesuk *et al*., 2004). For people in developing countries, medicinal plants are popular because their products are safe and widely availabe at low cost. (Sawangjaroen *et al*., 2002)

All antioxidant substances have basic molecular similarities, but hundreds of them produce a rich variety of effects essential to cope with the multitude of free radical species in different surroundings. These substances basically have a phenolic chemical structure that is common. This phenolic structure can neutralize a reactive free radical species by readily giving up a hydrogen atom (Lu and Foo, 1995).

The major classes of important phenolics include the flavonoids, gallate esters (which also comprise a range of hydrolyzable tannins), lignans, coumarins, stilbenes and flavans. All these compounds would form a broad range of antioxidant activity that helps in the defence system against free radical attack in the plants (Lu and Foo, 1995). Clinical studies provides evidence that phenolic compounds that is present in cereals, fruits and vegetables are the major contributing factor in reducing incidence of chronic and degenerative diseases. People who consume a lot of these products are known to have lower risk towards those ailments.

Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases that has their etiology and pathophysiology in reactive oxygen species. The medicinal

properties of folk plants are mainly attributed to the presence of flavonoids, but may also be influenced by other organic and inorganic compounds such as coumarins, phenolic acids and antioxidant micronutrients, e.g., Cu, Mn, Zn (Atawodi, 200).

Natural antioxidants occur in all higher plants and in all parts of the plants (wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds). Hence, there have been recommendations to increase the daily intake of fruit and vegetables (Chanwitheesuk *et al*., 2005).

2.5.3 Types of Antioxidants

Antioxidants could also be categorized as preventive and chain-breaking antioxidants based on their mechanism of action. Both have the basic mechanism of scavenging and decreasing the effects that is exhibited by free radicals. They need to be constantly replenished.

The mechanism of action of antioxidants is divided into two categories i.e. preventative and chain-breaking.

i) Preventative Mechanism (Primary Antioxidant Defense)

Primary or preventive defenses diminish the initiation rate of radical reactions by decreasing free radical concentration or destroying it altogether (Cayuela, 1994). The radicals are destroyed even before it starts a chain reaction of oxidation. Oxidation could also be prevented by stabilizing transition metal radicals such as copper and iron. Generally, all aerobic organisms have elaborate defense mechanism to prevent the formation of toxic form of oxygen and also to remove peroxides. Many endogenous non-protein small antioxidant molecules are important in quenching free radicals. They act as

the first line of defense against reactive oxygen species (Ling, 2006). Examples of antioxidants that comes under this classification includes vitamin C, vitamin E, carotenoids such as β−carotene, lycopene, thiols such as glutathione and lipoic acids, ubiquinols, flavonoids, polyphenols and enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). These primary antioxidant enzymes work to prevent oxygen radical from performing its reaction and to reduce lipid peroxidation. In an overview, SOD would destroy $O_2 \cdot^-$ radicals, hydroperoxides would decompose H_2O_2 and peroxides would destroy LOOHs/ROOHs. Other enzymatic proteins such as DT-diaphorase or epoxide hydrolase are also considered to be primary antioxidant defenses (Cayuela, 1994).

ii) Chain Breaking Mechanism (Secondary Antioxidant Defense)

Chain propagation mechanism is a mechanism in which free radical either releases or steals an electron and this reaction would subsequently form a second radical. This radical which is newly formed would be unstable and then attacks another molecule. This chain reaction would create more unstable products or free radicals. This process would continue to occur until termination process occurs. The termination occurs when the radical is stabilized by an antioxidant. The antioxidants that are included in this class are a variety of enzymes and vitamins like vitamin C and E. Secondary defenses are primarily involved in repair of already damaged proteins and lipids that was not dealt with in the primary antioxidant defenses. The repair of membrane phospholipids, DNA and proteins is the responsibility of secondary antioxidant defense (Ling, 2006). Chain breaking antioxidants are also known as sacrificial antioxidants because of its strong property of donating electron to free radical or oxidizing the free radical before another target molecule is damaged (Elizabeth, 2007).

Antioxidants are also classified as enzymatic antioxidants and non-enzymatic antioxidants.

iii) Enzymatic Antioxidants

There are several types of endogenous enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR) and catalase (CAT) (Sen *et al.,* 2010). They serve as the primary line of defense in destroying free radicals. They play an important role in oxidative stress. One of the most effective intracellular enzymatic antioxidant is superoxide dismutases (SOD) (Valko *et al*., 2006).

iv) Non-Enzymatic Antioxidants

Non-enzymatic antioxidants are generally taken through our daily dietary intake. They are also known as metabolic and nutrient antioxidants. Non-enzymatic antioxidants are represented by vitamin C, vitamin E, thiol antioxidants such as gluthatione, the thioredoxin system and lipoic acid, carotenoids, flavonoids and selenium (Valko *et al*., 2006). Non-enzymatic antioxidants also can be further divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants are a sub-class of endogenous antioxidants that is produced by metabolism in the body like lipoid acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin and metal-chelating proteins. Nutrient antioxidants belong to exogenous antioxidant and cannot be produced in the body but must be provided through diet or supplements. Some examples are trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids and others. Vitamin E and C are the non-enzymatic antioxidants exist within normal cells as well as they can be supplied through diet (Sen *et al.,* 2010).

2.5.4 Antioxidant scavenging enzymes

i) Superoxide Dismutase (SOD)

SOD (EC 1.15.1.1) has been described as one of the most effective intracellular enzymatic antioxidants (Valko *et al*., 2006). These are a family of metalloenzymes with different prosthetic groups, variable intracellular location and great tissue heterogeneity (Cayuela, 1994). SOD enzymes are present in almost all aerobic cells and in extracellular fluids. This enzyme scavenges superoxide radicals ($O_2 \cdot^-$) by catalyzing the conversion of two moles of these radicals into hydrogen peroxide and molecular oxygen as shown below. Eukaryotes contain two forms of this enzyme. A manganese containing version is located at the mitochondria and a copper-zinc-dependent cytosolic form. Both these enzymes perform dismutation reaction by quite similar mechanism. The oxidized form of the enzyme is reduced by superoxide to form oxygen. The reduced form of the enzyme, formed in this reaction, then reacts with a second superoxide ion to form peroxide, which takes up two protons along the reaction path to yield hydrogen peroxide (Stryer *et.al.,* 2003). The existence of a third form of SOD in the extracellular fluids was also found. They contain copper and zinc in their active sites. These proteins catalyze the $O_2 \cdot^-$ dismutation to produce $H_2O^-\cdot$ and O_2 at a rate 104 times higher than spontaneous dismutation at physiological pH (Cayuela, 1994). The dismutation of $O_2 \cdot^-$ to H_2O_2 and O_2 can be shown as follows:

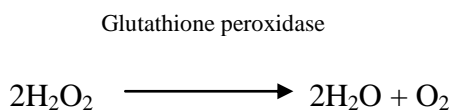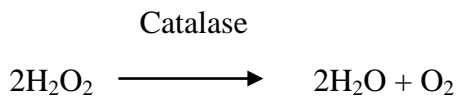$2O_2 \cdot^- + 2H+ \longrightarrow O_2 + H_2O_2$ (Stryer *et al.,* 2003)
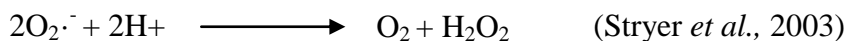
The importance of cell's defense against ROS is demonstrated by the presence of superoxide dismutase in all aerobic organisms. Escherichia coli mutants lacking this

enzyme are highly vulnerable to oxidative damage. Moreover, oxidative damage is believed to cause a growing number of diseases (Stryer *et al.,* 2003).

ii) Catalase (CAT)

Catalase is the enzyme that removes H_2O_2 from the cell when the latter is at high concentration. Catalase uses either an iron or manganese cofactor. This haemoprotein is localized within the peroxisomes. Like SOD, catalase is widely distributed in the tissues (Cayuela, 1994). The hydrogen peroxide formed by superoxide dismutase and by other processes is scavenged by catalase. It basically catalyzes dismutation of hydrogen peroxide to water and molecular oxygen (Stryer *et al*., 2003). The function of this enzyme is shared together with glutathione peroxidase. Both enzymes helps in detoxification of the oxygen-reactive free radicals by catalyzing the formation hydrogen peroxide derived from superoxide. It was found that liver, kidney and red blood cells possess high level of catalase (Lee, 2006).

$$2H_2O_2 \quad \xrightarrow{\text{Catalase}} \quad 2H_2O + O_2$$

$$2H_2O_2 \quad \xrightarrow{\text{Glutathione peroxidase}} \quad 2H_2O + O_2$$

iii) Glutathione Peroxidase (GPX)

The selenium-containing peroxidase glutathione peroxidase (EC 1.11.1.19) contains a single selenocysteine residue in each of the four identical subunits, which is essential for enzyme activity. GPX (80 kDa) catalyses the reduction of hydroperoxides using glutathione (GSH) and thereby protecting mammalian cells against oxidative damage. In fact,

glutathione metabolism is one of the most essential antioxidative defense mechanisms (Mates *et al*., 1999). It is an enzyme that catalyzes the reduction of H_2O_2 and organic free hydroperoxides requiring glutathione as co-substrate. Unlike catalase, it has a high substrate affinity. This peroxidase, containing four selenium atoms responsible for its catalytic activity, is located in the cytoplasm of eukaryotic cells although it can also be found within the mitochondrias. Many kinds of tissue exhibit glutathione peroxidase activity (Cayuela, 1994). There are five GPX isoenzymes found in mammals. Although their expression is ubiquitous, the level of each isoform varies depending on the tissue type (Mates *et al*., 1999). The reaction exhibited by this enzyme is as shown below:

$$H_2O_2 + GSH \longrightarrow GSSG + 2H_2O$$

$$ROOH + 2GSH \longrightarrow GSSG + ROH + H_2O \qquad \text{(Cayuela, 1994)}$$
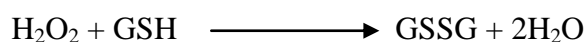
GSH = reduced glutathione

GSSG = glutathione reductase

iv) Glutathione Reductase (GR)

In every aerobic organism, the redox status of a certain cell depends on the maintenance of glutathione in its reduced state, GSH and prevention of its oxidation to the oxidized state, GSSG. During oxidation stress, there would be a decrease in GSH level and the level of GSSG would rise. Oxidation process of the free thiols such as GSH is deleterious to the cells. The cytosolic enzyme glutathione reductase (GR) is an important in the process of regeneration of GSH from GSSG (Ling, 2006).

2.5.5 Non-Protein Antioxidants (e.g. the vitamins)

i) Vitamin A

Vitamin A could be classified into vitamin A1 which is retinol and vitamin A2 which is also known as dehydroretinol. Vitamin A (retinol) is a precursor of retinal, the light sensitive group in rhodopsin and other visual pigments. Vitamin A may be found in various forms such as retinol, the form of vitamin A absorbed when eating animal food sources, is a yellow, fat-soluble, vitamin with importance in vision and bone growth. Since the alcohol form is unstable, the vitamin is usually produced and administered in a form of retinyl acetate or palmitate another form would be retinoids, a class of chemical compounds that are related chemically to vitamin A, which is used in medicine. Vitamin A supplementation was found to have positive effect on immune system as it could be a good immune stimulant. Vitamin A was found to aid the formation of antibodies in tears, saliva and other body fluids for the first line of defense against foreign bodies. Vitamin A has the ability to stimulate immune system; to react to tumor cells has implications in cancer prevention and treatment (Vimala *et al*., 2003).

ii) Vitamin E

Vitamin E consists of natural occuring tocopherols or tocotrienols. Vitamin E is an example of a phenolic antioxidant. It is a major lipid soluble vitamin (Ling, 2006). It has been claimed that α-tocopherol is the most important lipid-soluble antioxidant, and that it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This would remove the free radical intermediates and prevent the oxidation reaction from continuing. The oxidised α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other

antioxidants, such as ascorbate, retinol or ubiquinol. The significance of vitamin E has been subsequently proven as a radical chain breaking antioxidant that can protect the integrity of tissues and play an important role in life processes. Because it is lipophilic, vitamin E is especially useful in protecting membranes from lipid peroxidation (Stryer *et al*., 2003)

iii) Vitamin C (Ascorbic Acid)

Vitamin C may be the best known for its effects as an antioxidant and also for its role in maintaining proper immune function. Many of the beneficial effects of vitamin C are attributed mainly to antioxidant and free radical scavenging effects (Bruno, 2008). Ascorbic acid is an essential vitamin which is generally found in fruits and vegetables. It has few major roles such preventing free radical induced damage to the DNA and also acts as an antioxidant (Vimala *et al*., 2003). It is a water soluble vitamin and it basically functions better in an aqueous environment. The biochemical importance of vitamin C is primarily related to its reducing potential as an efficient free radical scavenger and in the regeneration of lipid soluble antioxidants by reducing their respective radicals (Chee, 2003).

The current recommended dietary allowance (RDA) for vitamin C for adult non-smoking men and women is 60 mg/d, which is based on a mean requirement of 46 mg/d to prevent the deficiency disease scurvy. However, recent scientific evidence indicates that an increased intake of vitamin C is associated with a reduced risk of chronic diseases such as cancer, cardiovascular disease, and cataract, probably through antioxidant mechanisms (Carr *et al*., 1999).

As compared to other water soluble antioxidants, vitamin C has been found to provide the most effective protection against plasma lipid peroxidation. Ascorbic acid has

been found to act both as an antioxidant and also pro-oxidant. In this perspective as an antioxidant, vitamin C has a sparing effect on the antioxidant actions of vitamin E and selenium. Excessive amounts of vitamin C can lead to it behaving as a pro-oxidant in the presence of transition metals iron (Fe^{3+}) and copper (Cu^{2+}). This is by generating cofactor of activated oxygen radicals during lipid peroxidation (Chee, 2003).

2.5.6 Phenolic Antioxidants

The antioxidant activity of phenolics was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen *et al*., 1999).

Flavonoids

Flavonoids are a class of secondary plant phenolics which has significant antioxidant and chelating properties (Sharififar *et al.,* 2008). It is present in fruits, vegetables, nuts, seeds and beverages of plant origin, which have been reported to exhibit anti-carcinogenic, anti-inflammatory and antimicrobial activities (Rey *et al.,* 2005). Flavonoids are a group of polyphenolic compound and have a variety of chemical structure and characteristics. Over 4,000 different flavonoids have been identified within the major flavonoid classes which include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones. Flavonoids are absorbed from the gastrointestinal tracts of humans and animals and are excreted either unchanged or as flavonoid metabolites in the urine and feces. Flavonoids are potent antioxidants, free radical scavengers, and metal chelators and inhibit lipid peroxidation. The structural requirements for the antioxidant and free radical scavenging functions of flavonoids include a hydroxyl group in carbon position three, a double bond between carbon positions two and three, a carbonyl group in carbon

position four, and polyhydroxylation of the A and B aromatic rings. Figure 2.5 shows some examples of flavonoid structures. Epidemiological studies show an inverse correlation between dietary flavonoid intake and mortality from coronary heart disease (CHD) which is explained in part by the inhibition of low density lipoprotein oxidation and reduced platelet aggregability (Cook and Samman, 1996).
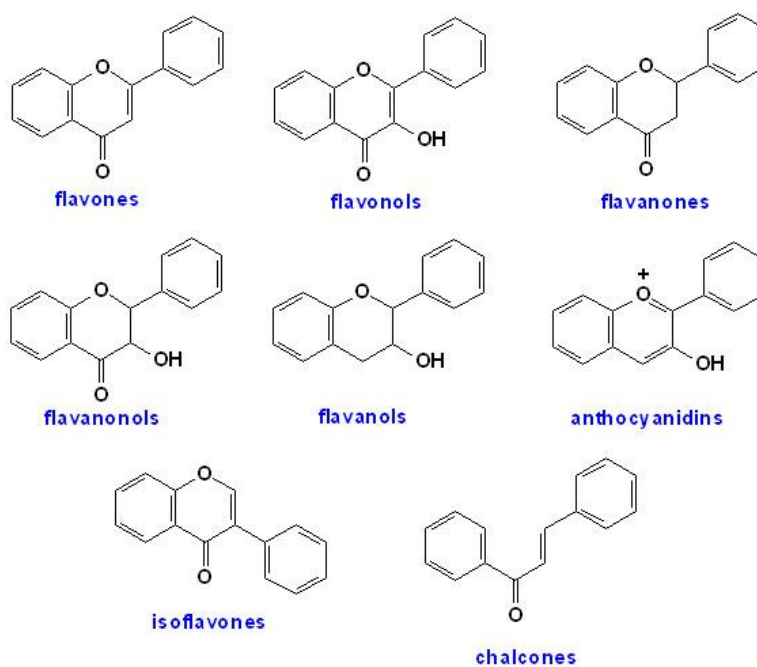
Figure 2.5: Some examples of flavonoids (excerpted from http://supplementscience.org/antioxidants.html)

2.5.7 Other Antioxidant Components including Uric acid, Selenium, and Carotenoids

Uric acid is a by-product of purine metabolism (Stryer *et al*., 2003). It is a powerful antioxidant and is a scavenger of singlet oxygen and radicals. The concentration of urate reduces oxo-heme oxidant which is formed by peroxide reaction with hemoglobin and protects erythrocyte from peroxidative damage that could eventually lead to lysis (Tan, 2006).

Selenium is a dietary micronutrient which is essential to be taken in our diet. Selenium functions as a constituent of the antioxidant enzyme glutathione peroxidase, which detoxifies products of oxidized fats, and is also found in the red blood cells (Bruno, 2008). Selenium is commonly found in grains, nuts, garlic, and yeast. It is an important component in synthesizing natural antioxidant enzymes. Selenium and vitamin E was found to work together for antioxidation process. Studies have also shown that selenium helps to prevent certain diseases like cancer, asthma and heart disease (Vimala *et al*., 2003).

Carotenoids are reported as excellent antioxidants. It has the capacity to protect oxidative damage to DNA, protein and lipids. They are also thought to be primarily responsible for the inhibition of cell proliferation (Reddy *et al.,* 2005). The most important carotenoids observed in plasma or serum is β-carotene, alfa-carotene, licopene, criptoxantin and lutein with some zeaxanthin. Besides its function as a precursor for formation of vitamin A, it has been also attributed to cancer preventive action. Action as an antioxidant is mainly via deactivation of oxygen molecular singlet in the peroxide radical (Oliveira *et al.,* 2004).

2.6 Oxidation Stress

Oxidative stress is a harmful condition occurring when there is an excess of ROS and/or a decrease in antioxidant levels. This condition may cause tissue damage by physical, chemical, psychological factors that lead to tissue injury in human and causes different diseases (Sen *et al.,* 2010). Oxidative stress is caused by free radicals. It is believed to be the primary factor in various degenerative diseases, such as, atherosclerosis, inflammation, carcinogenesis, aging and Alzheimer's disease (Chen *et al.,* 2009). This is a situation where

cells are damaged due to oxidation process. It involves adverse effects of oxygen on living tissue.

2.7 Free Radicals

Free radicals can be divided into reactive oxygen species (ROS), for example, superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), and reactive nitrogen species (RNS), namely, nitric oxide ($NO\cdot$) and peroxynitrate ($\cdot ONOO^-$). They are produced after stimulation from exogenous agents such as ultraviolet rays, ionizing radiation, chemical reactions and also from normal metabolic processes (Chen *et al.,* 2009). A free radical is a chemical species that contains one or more unpaired electrons. Free radicals are extremely reactive, so they have a very short half-life and low steady-state concentration (Cayuela, 1994). This broad definition includes the hydrogen atom (one unpaired electron), most transition metals and the oxygen molecule itself. O_2 has two unpaired electrons, each located in a different,*π anti-bonding orbital. These two electrons have the same spin quantum number and so if O_2 attempts to oxidize another atom or molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin so as to fit into the vacant spaces in the *π orbitals (Halliwell *et al*., 1984). The reactivity of a free radical can be stopped when it reacts with a non-radical species in the process, produces another free radical. This reaction is known as chain reaction. Chain reaction could be classified into different stages such as initiation, propagation and termination (Elisabeth, 2007).

2.7.1 Reactive Oxygen Species (ROS)

This includes oxygen ions, free radicals and peroxides both organic and also inorganic. It is very small and highly reactive due to presence of an unpaired electron at the valence shell. ROS are natural by product in metabolism of oxygen and also is important in cell signaling mechanism (Hani, 2007). In environmental stress condition, ROS level can increase and this could cause damage to cell structures. This situation is known as the oxidation stress. Cells protect themselves from oxidation stress by having natural antioxidant enzymes such as catalase and superoxide dismutases and also other antioxidant components. This stress however mediates several pathological processes which include the leakage of cell membranes, dysfunction of mitochondria, and depletion of ATP. These processes affects cells and DNAs and subsequently this will promote aging, tumor production, cancer, inflammatory diseases, malaria, neurodegenerative diseases, diabetes and etc (Ling, 2006). Examples of ROS are superoxide radical ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH \cdot$), singlet oxygen ($O_2 \cdot$) and others.

2.7.2 Superoxide radical

The free radical superoxide anion (O_2^-) is formed by the addition of one electron to ground state di-oxygen. This radical is also formed in aerobic cells due to electron leakage from the electron transport chain. The superoxide radical is also formed during the activation of phagocytes on bacteria. This radical is removed by superoxide dismutase enzyme in living organisms (Wettasinghe and Shahidi, 2000). This very reactive chemical species is unstable in aqueous solutions due to it being able to react spontaneously with itself producing hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (dismutation reaction) as shown below :-

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H+ \longrightarrow H_2O_2 + O_2$$
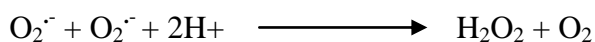
The superoxide radical can be in its protonated form as hydroxyl radical (HO$^{\cdot}$), which exhibits even higher reactivity. Nevertheless, at physiological pH the un-protonated form predominates (Cayuela, 1994).

2.7.3 Singlet Oxygen

Singlet oxygen ($^1O_2$) is not a free radical, but is a more reactive form of oxygen (Chee, 2003). A way of increasing the reactivity of oxygen is to turn the two parallel spinning electrons into antiparallel by means of an input of energy. This process produces singlet oxygen, which is highly reactive because spin restriction has been removed. There are two forms of singlet oxygen: delta singlet oxygen and sigma singlet oxygen. The former is the more important biologically due to its long life. However, it is not a free radical because it does not have unpaired electrons (the outer two electrons occupy the same orbital and have opposite directions). Sigma singlet oxygen, on the other hand, has electrons of antiparallel spins occupying different orbitals. This species has very high reactivity but a short half-life because it decays, immediately after being formed, to the, delta singlet oxygen state (Cayuela, 1994).

Irradiation on the skin is a major source of singlet oxygen and maybe the leading cause of lipid peroxidation in the epidermis. Singlet oxygen can diffuse to a considerable distance, and they could react with a variety of cellular components, including DNA (Chee, 2003).

Reaction by singlet oxygen is simplified as follows:

$$O_2 \xrightarrow{\text{hv}} {}^1O_2 \text{ (singlet oxygen)}$$
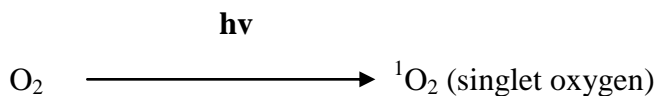
2.7.4 Hydrogen Peroxide

Addition of a second electron to superoxide anion (O_2^-) gives the peroxide ion, O_2^{2-}, which has no unpaired electrons and is not a radical. Any O_2^{2-} formed at physiological pH will immediately protonate to give hydrogen peroxide (H_2O_2) since the pKa of H_2O_2 is very high. In aqueous solution, O_2^{2-} undergoes the so-called dismutation reaction to form H_2O_2 and O_2. The overall reaction can be written as follows:

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$
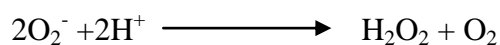
It is the sum of several stages. The rate of dismutation is faster at acidic pH values (Halliwell *et al*., 1984).

2.7.5 Hydroxyl Radical (·OH)

Hydroxyl radical is produced in living organisms. They are generally produced by two mechanisms. Reaction of transition metal ions with H_2O_2 and hemolytic fission of water upon exposure to ionizing radiation produces hydroxyl radicals. Hydroxyl radicals could attack all biological molecules and sets off free radical chain reactions as it is a powerful oxidant and can extract an electron from another molecule or hydroxylate another molecule. The major damage that could be caused by this radical is by causing changes in the DNA that would lead to mutation (Vimala *et al*., 2003).

Formation of hydroxyl radical is possible through Fenton's reaction as shown below:

$$O_2^- \cdot + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad \text{......................... (i)}$$

$$H_2O_2 + Fe^{2+} \longrightarrow \cdot OH + OH^- + Fe^{3+} \quad \text{.................... (ii)}$$

$$O_2^- \cdot + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2 \quad \text{...................... (iii)} \quad \text{(i) + (ii)}$$
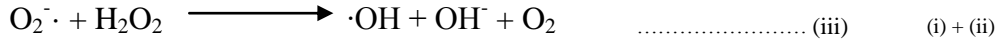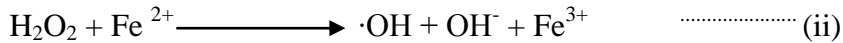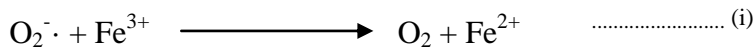
Superoxide radical will react with Fe (3+) which is the oxidized state, as the superoxide radical donates the electron to Fe (3+), the iron is reduced to Fe (2+) and now becomes available to react with hydrogen peroxide which accepts an electron from the Fe (2+) and then forms the hydroxyl radical and hydroxyl ion (Halliwell *et al*., 1984).

The hydroxyl radical is a very reactive species, more reactive than superoxide radical. It is the most toxic of all oxyradicals (Vimala, 2003). It is a strong oxidizing agent that can react with every type of molecule in the body at a very fast rate (Chee, 2003)

$$H_2O_2 + O_2 \cdot \longrightarrow H_2O + O_2 + \cdot OH$$

$$H_2O_2 \longrightarrow H_2O + \cdot OH$$
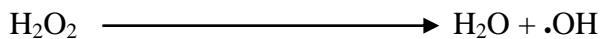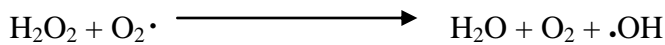
2.8 Lipid Peroxidation

Lipid peroxidation is defined as the oxidative breakdown of polyunsaturated fatty acids. It is also accepted as a general mechanism of cellular injury and cellular death (Sheu *et al.,* 2003). It has received great deal of attention in the area of food science (Lee *et al.,* 1999). Lipid oxidation has positive and negative effects. At low levels, the peroxidation products of lipids are responsible for the desirable aroma of fried foods and some of the characteristic flavor properties of cooked meats, roasted nuts, and etc. On the other hand, lipid peroxidation not only poses problems in development of rancidity in processed foods

and also causes serious damage to the human body (Gulcin *et al.,* 2009). Oxidation of muscle lipids involves the polyunsaturated fatty acids which are located in the membrane of muscle cells (Lee *et al.,* 1999). Formation of toxic compounds, off flavor and deterioration of nutritional value of food may be caused by oxidation of lipids. Lipid peroxidation contains a series of free radical-mediated chain reaction processes. Presence of transition metals such as iron can promote towards lipid oxidation (Lee *et al.,* 1999). Lipid peroxidation could produce secondary metabolites such as alkanes, alcohols, acids and carbonyls (Figure 2.6). These products are highly reactive and could react with biological components such as DNA, protein and others and lead to cancer and mutagenesis (Osawa, 1995). The ferrous ion speeds up the lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + \cdot OH$). Fe^{3+} ions also produce radicals from peroxides, although the rate is 10-fold less than that of Fe^{2+} ions (Koksal and Gulcin, 2008). Lipid peroxidation could also cause problems like membrane damage, heart disease, cancer and also aging (Gulcin *et al.,* 2009).
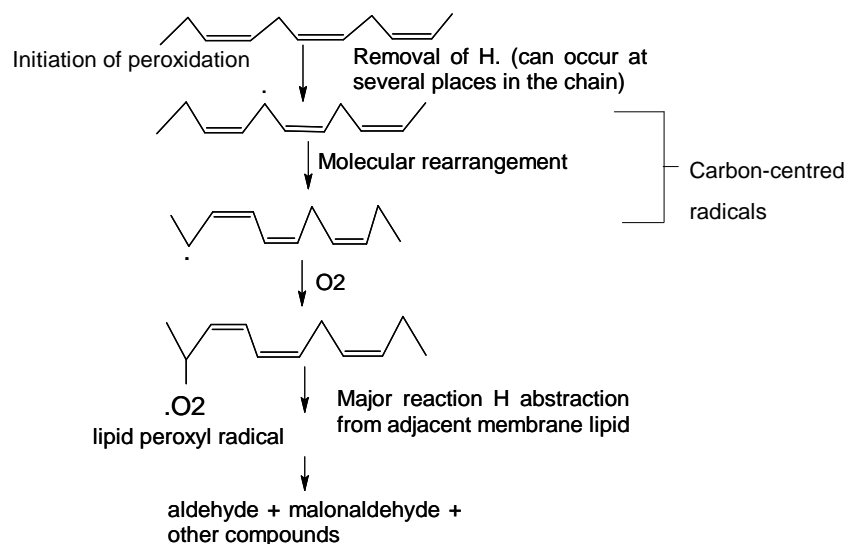
Figure 2.6: Steps of Lipid Oxidation

Free radicals that abstract hydrogen from fatty acids produce hydroperoxides (initial product of oxidation that decompose to produce secondary products e.g. malonylaldehyde that gives off flavor and color). Hydroperoxides are not stable molecules and they break down through a number of mechanisms. Figure 2.6 above shows how hydroperoxides acts in producing aldehydes, ketones, alcohol and hydrocarbons (secondary products).

2.9 Free Radical & Diseases

The balance between antioxidation and oxidation is believed to be a concept for maintaining a healthy biological system. Physiological burdens by free radicals causes imbalance in homeostatic phenomena between oxidant and antioxidant in the body. This imbalance causes a condition called oxidation stress. Oxidation stress leads to various diseases such as stroke, diabetes, cancer, atherosclerosis, neurodegenerative diseases such as Alzheimer disease, Parkinson's disease (Leterier *et al.,* 2008). Various diseases could be caused by ROS and RNS. These diseases include cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis, and ageing. Research has been focused on this link (Valko *et al*., 2006). ROS have been also implicated in many lung diseases, including acute respiratory syndrome associated with exposure to oxidants, *e.g.*, asbestos, nitrogen dioxide, ozone, paraquat, hyperoxia, carbon tetrachloride, and the anticancer drugs bleomycin and adriamycin. In addition, oxidative stress, superoxide production and an imbalance in antioxidant enzymes has been related with much other specific pathology as chronic granulomatous disease, Down's syndrome, diabetic complications, hepatitis, rheumatoid arthritis, Influenza virus, ulcer, pneumonia, HIV infection, cataract and glaucoma (Mates *et al*., 1999).

2.10 Antioxidant Bioassay Systems and Principles

Many methods have been employed generally to measure antioxidant activities and to compare the antioxidant activity of foods. Recently, oxygen radical absorbance capacity assays and enhanced chemiluminescence assays have been used to evaluate antioxidant activity of foods, serum and other biological fluids. Methods used needs special equipment and some technical analysis in order for the analysis to be conducted effectively. Normally, the analytical methods measure the free radicals like 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide anion radical ($O_2 \cdot^-$), the hydroxyl radical (OH·) or the peroxyl radical (ROO·). These various methods can give different results and this depends on the specific radical that is being used as the reactant. Besides this method, there are also many other methods in determining resistance of lipid or lipid emulsions to oxidation in the presence of a certain antioxidant that is being tested (Prakash *et al.,* 2001).

There are many types of antioxidant bioassay systems that are being used in recent days. Some of those bioassays includes autoxidation of linoleic acid in a water-alcohol system, xanthine/xanthine oxidase superoxide scavenging system, DPPH (1,2-diphenyl-2-picrylhydrazyl) free radical scavenging system, tyrosinase inhibitory assay system, ferric reducing/antioxidant power (FRAP) assay system, trolox equivalent antioxidant capacity (TEAC) assay system, thiobarbituric acid (TBA) assay system, beta-carotene-linoleate model system. All these methods differ in the way they work. Each method relates to different radicals being used, different mechanism, and the point of measurement. Basically there are two types of assay overall which is either the inhibition assay which includes the scavenging ability of antioxidants and the next one would be the presence of an antioxidant system in which it is present during the generation of the radical (Vimala *et al*., 2003).

The *in vitro* approach is simple and provides impressive comparative information between different plants. Antioxidant properties of mixtures of compounds, rather than micronutrients in isolation could be measured. Generally, mixtures are presented in the human body. However, there are limitations to *in vitro* methods. The real level of antioxidant analyzed depends on the variety of plant. The way the plant was cultivated, how it's harvested or stored and the condition it is grown (organic or synthetic), affects the antioxidant content. *In vitro* methods in antioxidant testing have several drawbacks such as it tells us nothing on the bioavailability and metabolism i.e., how efficiently the compounds are taken up from the gut, how they are transported to tissues and into cells and how quickly they are excreted (Collins, 2005). Hence, *in vitro* studies are preliminary studies in the process of a drug discovery.

2.10.1 DPPH (1,2-diphenyl-2-picrylhydrzyl) free radical scavenging system

Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay (Sharma and Bhat, 2009). DPPH, is a stable free radical, and has been used to evaluate free radical-scavenging capacities of natural antioxidants. Unlike the free radicals generated in the laboratory such as $O_2\cdot$ and $\cdot OH$, use of stable free radical has the advantage of being unaffected by side reactions such as metal chelation or enzyme inhibitions (Wettasinghe and Shahidi, 2000). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation gives rise to the deep violet color which is characterized by an absorption band in ethanol solution centered at about 520 nm.

If a solution of DPPH is mixed with a substance that can donate a hydrogen atom, then it will form a reduced form. The violet color would be lost and would be a pale yellow color formation due to the presence of the picryl group. If the DPPH radical is represented by Z· and the donor molecule is represented by AH, the reaction would proceed as follows,

$$Z· + AH \rightarrow ZH + A·$$
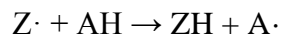
When ZH is in the reduced form and the A· is the free radical produced, the radical would then undergo further reaction to control the overall stoichiometry. The overall stoichiometry is the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant.

The reaction shown provide link to the reactions taking place in an oxidising system such as autoxidation of lipid or other unsaturated substance. The DPPH molecule is represented by the free radicals (Z·) that is formed in the system whose activity is to be suppressed by the substance AH (Molyneux, 2004).
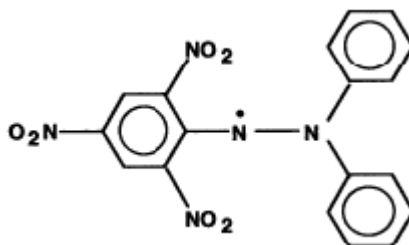
Figure 2.7: Diphenylpicrylhydrazyl (free radical)
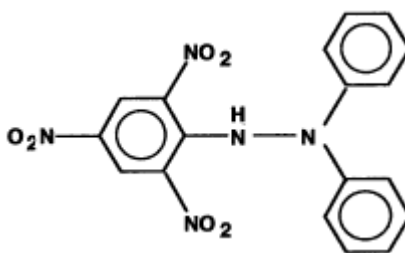
Figure 2.8: Diphenylpicrylhydrazine (non-radical)

The concentration needed to reduce the DPPH radical to 50% (IC_{50} value) is used as the parameter to measure the antioxidant activity in this assay. The lower the IC_{50} value, the higher the antioxidant power (Parejo *et al.,* 2000). This value is calculated by plotting inhibition percentage against extract concentration (Deng *et al.,* 2011).

In comparison to other methods, the DPPH assay has many advantages, such as good stability, credible sensitivity, simplicity and feasibility (Deng *et al.,* 2011).

2.10.2 Reducing Power assay system

In this assay, the yellow colour of the test solution changes to various shade of green and blue depending upon the reducing power of each extract. The presence of reductants (i.e. antioxidants) in the herbal extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. In other words, the $FeCl_3/K_3Fe(CN)_6$ system offers a sensitive method for the "semi-quantitative" determination of dilute concentrations of polyphenolics, which participates in the redox reaction (Amarowicz, 2004). The ferrous complex has an intense blue color. This could be monitored by measuring the change in the absorption. Formation of ferrous ions (Fe^{2+}) was measured spectrophotometrically at 700 nm, with higher absorbance values indicative of

greater reducing capacity of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions (Lue *et al.,* 2010). The change in absorbance refers to the total reducing power of the antioxidants which would donate electrons that is present in the reaction mixture.

The reaction that takes place could be represented as follows:

$$Fe^{3+} + antioxidant \longrightarrow Fe^{2+} + oxidized\ antioxidant$$

$$Fe^{2+} + Fe(CN)_6^{3-} \longrightarrow Fe[Fe(CN)_6]^- \ or$$

$$Fe(CN)_6^{3-} + antioxidant \longrightarrow Fe(CN)_6^{4-} + oxidized\ antioxidant$$

$$Fe(CN)_6^{4-} + Fe^{3+} \longrightarrow Fe[Fe(CN)_6]^- \qquad (Muhammad,\ 2007).$$
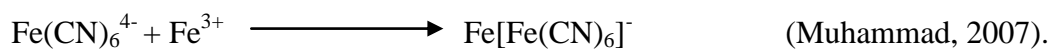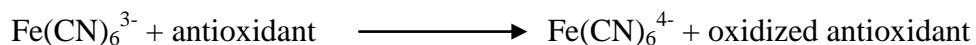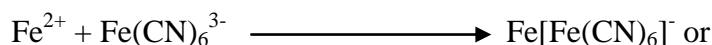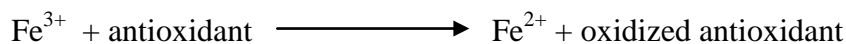
An assay like this gives an indication on how easily a given antioxidant donates electrons to reactive radical species and promotes the termination of free radical chain reactions. The ability of the antioxidant to reduce Fe^{3+} to its more active Fe^{2+} form might also be indicative of its ability to act as a pro-oxidant in the system (Lue *et al.,* 2010).

2.10.3 β-Carotene Bleaching Assay

In the β-carotene bleaching assay, oxidation of linoleic acid releases linoleic acid peroxide as free radicals that oxidize β-carotene resulting in discoloration, thus decreasing the absorbance value. A linear relationship was found between the ability of the sample extract to inhibit oxidation and antioxidant capacity (Ikram *et al.,* 2009).

Linoleic acid hydroperoxides attack the β-carotene molecule and, as a result, it undergoes rapid decolorization. The corresponding decrease in absorbance can be monitored spectrophotometrically. The presence of antioxidant extracts can hinder the

extent of β-carotene bleaching by acting on the free radicals formed in the system (Jayaprakasha *et al.,* 2001).
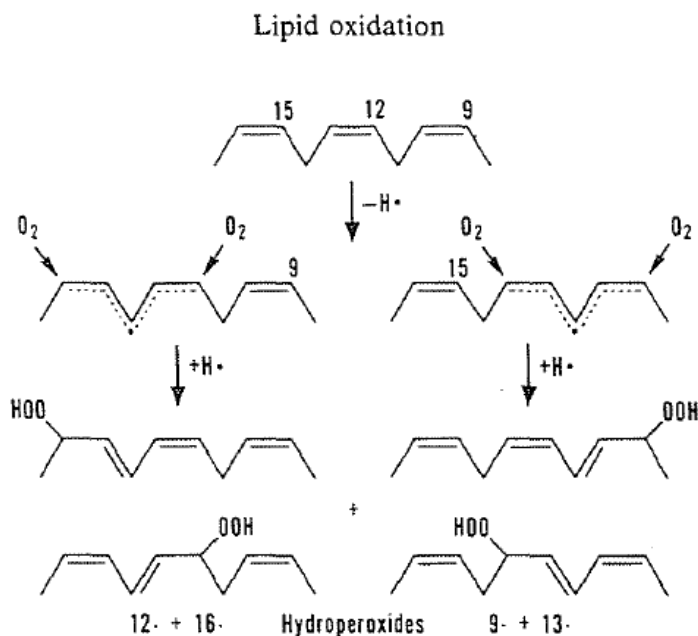
Lipid oxidation

Excerpted from Frankel, 1980

Figure 2.9: Mechanism of linoleate autoxidation

Lipid oxidation (Fig 2.9) is a complex phenomenon induced by oxygen in the presence of initiators such as heat, free radicals, light, photosensitizing pigments and metal ions. It occurs via three reaction pathways:

(i) Non-enzymatic chain autoxidation mediated by free radicals,

(ii) Non-enzymatic and non-radical photo-oxidation, and

(iii) Enzymatic oxidation

Antioxidants counteract oxidation in two different ways,

- by protecting target lipids from oxidation initiators or

- by stalling the propagation phase

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Tests used to measure antioxidant power require assessment of the extent of oxidation of the lipid substrate in the presence or absence of a potential antioxidant molecule or plant extract (Laguerre *et al.,* 2007). Four different measurement strategies can be used to directly assess the antioxidant capacity of a molecule toward a lipid substrate; these involve measuring oxygen depletion, substrate loss, secondary oxidation products and formation of primary products.

The β-carotene bleaching assay belongs to type (ii). β-Carotene exhibits antioxidant activity by suppressing singlet oxygen, scavenging peroxide radicals, and directly reacting with peroxy radicals thus stabilizing membrane lipids from free radical attack.
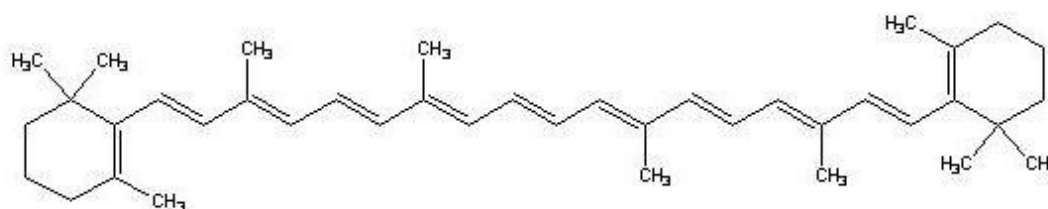
Figure 2.10: Chemical structure of β-carotene

This UV-spectrophotometric technique initially developed by Marco and modified by Miller involves measuring β-carotene bleaching at 470 nm resulting from β-carotene oxidation by linoleic acid degradation products. Tween 80 is used for dispersion of linoleic acid and β-carotene in the aqueous phase. Linoleic acid oxidation is non-specifically catalyzed with heat (50°C). The addition of an antioxidant-containing sample, individual antioxidants, or plant extracts, results in retarding β-carotene bleaching (Laguerre *et* al., 2007).

All chemical or biological structures consisting of unsaturated lipid compounds such as triacylglycerols, carotenoid pigments, cholesterol, lipoproteins and biological

membranes are prone to oxidation. The most important end products of free radical reactions are malondialdehyde (MDA) and conjugated dienes (CD). MDA and CD have various cytotoxic effects, including enzyme inactivation and inhibition of DNA, RNA and protein synthesis, which may result in many diseases (Cao *et al.,* 1995). Recognition of lipid peroxidation involvement in the pathogenesis of a disease is of importance, because the deleterious effects of this process might be prevented by administration of free radical scavenging systems or antioxidants (Ismail Dikici *et* al., 2005).

2.10.4 Metal Chelating Assay

The metal binding (chelating) capacity of a test sample is investigated by assessing the ability of the antioxidants to compete with the indicator ferrozine to complex with ferrous ion (Fe^{2+}) in solution. The ability to form iron complexes is an important characteristic in food. Foods need to have this characteristic because oxidative processes are strongly influenced by trace metal compounds. Transition metal like iron reacts with either lipid peroxides or hydrogen to produce alkoxyl radicals and hydroxyl radical compounds. This is also known as Fenton reaction. These radicals are very reactive and will speed up the oxidative degradation processes (Lue *et al.,* 2010). The metal ion chelating capacity plays a significant role in the antioxidant mechanism because it prevents oxyradical generation and the consequent oxidative damage (Srivastava *et al.,* 2006). The results were expressed as a percentage of inhibition of the ferrozine-Fe^{2+} complex formation (Koksal and Gulcin, 2008). Compounds containing structures of two or more of the following functional groups: –OH, –SH, -COOH, –PO_3H_2, C=O, –NR_2, –S– and –O– in a favourable structure–function configuration will have chelation activity. Molecules like organic acids, malic, tartaric, oxalic, succinic, and phytic acid and some flavonoids like quercetin and rutin are known for their ability to chelate ions (Yuan *et al.,* 2005).

2.10.5 Superoxide dismutase (SOD) Activity Assay

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes. It catalyzes the dismutation of superoxide anion ($O_2 \bullet^-$) into hydrogen peroxide and molecular oxygen. SOD activity could be determined by several direct and indirect methods that have been developed. The prominent indirect method is by using nitroblue tetrazolium (NBT). This method is used as it is convenient and easy to use. This method has several disadvantages because the formazan dye has poor water solubility and false positive results as it interacts with reduced form of xanthine oxidase. In our experiment we used a kit to enable fast and easy measurement of the SOD activity in the test sample. SOD Assay Kit –WST was used. It used Dojindo's highly water soluble tetrazolium salt, WST-1 [(2-(4-lodophenyl)-3-(4– nitrophenyl)-5-(2,4-disulfopheynyl)-2H-tetrazolium, monosodium salt)] that produces a water soluble formazan dye upon reduction with a superoxide anion. The enzyme working solution in SOD assay kit consists of xanthine oxidase which catalyzes the conversion of xanthine into uric acid and superoxide anion in the presence of oxygen while reducing WST-1 into WST-1 formazan. If an antioxidant, like SOD is present in the mixture, the superoxide anion radical formed will be converted into oxygen and hydrogen peroxide and WST-1 will not be reduced into WST-formazan and there is no colour change from pink into yellow. The yellow colour of WST-1 formazan has an absorption wavelength value of 450 nm. As the absorbance at 450 nm is proportional to the sum of superoxide radical in the reaction mixture, thus, it can be used to determine the SOD inhibition by measuring the decrease in the color development at 450 nm. SOD enzyme acts to inhibit the xanthine oxidase (XO) enzyme. If this enzyme (XO) is inhibited molecular oxygen production

would be reduced. The explanation above could be summarized by reaction pathway in Fig. 2.11.
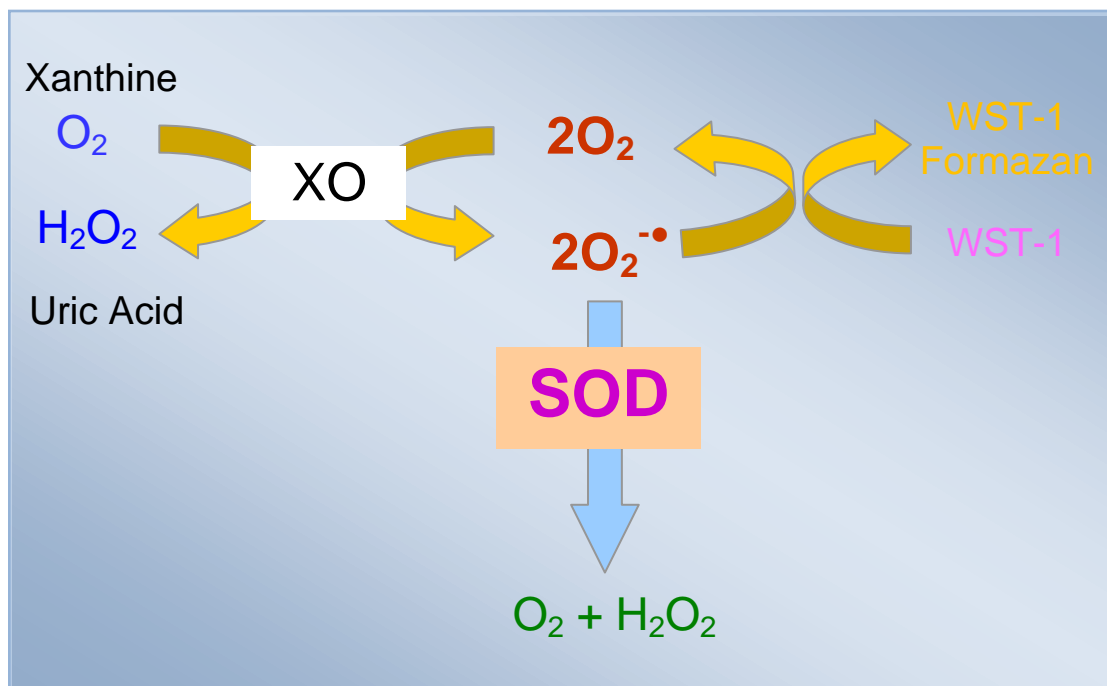
Figure 2.11: Reaction pathway of SOD activity assay

(Excerpted with slight modification from SOD Determination Kit Manual by Sigma)

2.10.6 TBARS (Thiobarbituric acid Reactive Species) Assay

The TBA test is a colorimetric technique in which the absorbance of a red chromogen formed between TBA and malondialdehyde is measured (Hanachi and Golkho, 2009). Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 1 sample of plant. During the oxidation process, peroxide is gradually decomposed to malondialdehyde which is of lower molecular weight, the amount of which is measured by the thiobarbituric acid (TBA) assay (Abas *et al.,* 2006). MDA is a product of lipid peroxidation by thiobarbituric acid reaction. The concentration of MDA was calculated from the absorbance at 532 nm (Bor *et al.,* 2003).The higher the concentration of

MDA in the test system, the higher the lipid oxidation that occurred. The binding of MDA-TBA gives a color that could be measured by spectrophotometer. Antioxidants stop the hydrogen atom abstraction from a fatty acid, which leads to less formation of hydroperoxides. It is well known that phenolic compounds act as hydrogen donors in that reaction mixture and therefore, the formation of hydroperoxides would be decreased (Hanachi and Golkho, 2009).

There are some setbacks in this assay. It is reported that some compounds apart from MDA that can react with thiobarbituric acid. A high level of specificity in the results could only be achieved if the MDA-TBARS compounds are analysed by HPLC (Collins, 2005).

2.10.7 Folin-Ciocalteu Assay (Total Phenolic Content Assay)

Unlike the name of the assay which says that it measures the total phenolic content of a certain test sample, recent publications (Huang *et al.,* 2005 and Prior *et al.,* 2005) suggest that it not only measures phenols but also reducing agents and possibly metal chelators. The basic mechanism is based on oxidation/reduction reactions. Folin-Ciocalteu reagent is made by first boiling for 10 h, the mixture of sodium tungstate, sodium molybdate, concentrated hydrochloric acid, 85% phosphoric acid, and water. After boiling, lithium sulfate is added to the mixture to give an intense yellow solution. Contamination of reductants leads to a green color, and the addition of oxidants such as bromine can restore the desired yellow color (Huang *et al.,* 2005). Although the chemical nature of the reagent is not certain, the assay is reproducible, simple and convenient.

2.10.8 Tyrosinase Inhibitory Assay

Tyrosinase (EC 1.14.18.1) enzyme is also known as polyphenol oxidase (PPO) (Ha *et al.,* 2005). It is involved in the initial step of melanin synthesis (Chiari *et al.,* 2010). This

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protein, which catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanin (L-DOPA) (monophenoloase activity) and the consequent oxidation of L-DOPA to dopaquinone (diphenolase activity), is widespread in many organisms such as vertebrates, invertebrates, plants and microorganisms (Chen & Kubo, 2002). The enzyme has three domains, and the central domain contains two copper binding sites and the copper binding sites are the active site in the tyrosinase catalytic reaction (Matsuura *et al.,* 2006). Tyrosinase catalyzes two different reactions by using molecular oxygen, the hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase or catecholase activity) (Matsuura *et al.,* 2006). Tyrosinase causes enzymatic browning in plants, and it may cause undesirable changes in colour, flavour and nutritive value of plant-derived foods and beverages. Tyrosinase catalyses melanin biosynthesis in human skin and epidermal hyperpigmentation (Maisuthisakul and Gordon, 2009). Melanogenesis is a major defense mechanism in human skin against the ultraviolet light of the sun. Abnormal melanin pigmentation such as melasma, freckles, ephelide, and senile lentigines is a serious aesthetic problem (Matsuura *et al.,* 2006). Tyrosinase is responsible for browning in plants and is considered to be deleterious to the color quality of plant-derived foods and beverages. This unfavorable darkening from enzymatic oxidation results in a loss of nutritional and market values and has been of great concern (Kubo and Kinst-Hori, 1999,b). The importance of tyrosinase inhibitors has also become increasingly high in medicinal and cosmetic products in relation to hyperpigmentation and also in skin whitening products (Maisuthisakul and Gordon, 2009). Tyrosinase is one of the most important enzymes in the insect molting process, and its investigation on the inhibitors may be important in finding alternative insect control agents (Miyazawa *et al.,* 2003). Tyrosinase inhibitors are much in demand as they have broad applications. There is an increasing interest in using plants as a source of natural

tyrosinase inhibitors as they are mostly free of harmful side effects and are rich in bioactive chemicals (Maisuthisakul and Gordon, 2009). A large number of tyrosinase inhibitors have already been reported. For example, kojic acid, known as one of the most popular tyrosinase inhibitors, has been widely used as a skin whitening and anti-browning agent (Matsuura *et al.,* 2006). Although there is a large number of tyrosinase inhibitor, only a few of them are used today, because many of them show side effects or low effectiveness. Previously, sulphites were widely used as tyrosinase inhibitors for their anti-browning effect but, their use has been regulated in order to ensure consumer safety since these preservatives affect the nutritional quality of foods and can cause allergic reactions and gastrointestinal distress. Various anti-tyrosinase compounds derived from plants are considered free of harmful side effects and it is readily available at low cost (Chiari *et al.,* 2010).

In this experiment, the extent of inhibition by the testing samples was expressed as the concentrations of sample needed to inhibit 50% of the enzymatic activity (IC_{50}) (Fu *et al.,* 2005). Kojic acid was used as the positive reference standard (Masamoto *et al.,* 2003).

2.11 Cancer

Cancer has been given various definitions. It is a set of diseases characterized by unregulated cell growth leading to invasion of surrounding tissues and spread (metastasis) to other parts of the body (King, 2000).

Cancer is a leading cause of mortality worldwide and the failure of conventional chemotherapy to effect major reductions in the mortality indicate that new approaches are critically needed. The new science of chemoprevention has appeared as an attractive alternative to control malignancy. This is a pharmacological approach to intervention in

order to arrest or reverse the process of carcinogenesis. In experimental chemoprevention studies, attempts are made to identify agents which could exhibit any or a combination of the following characteristics: (i) prevent the initiation of tumors, (ii) delay, or arrest the development of overt tumors, (iii) extend the cancer latency period, (iv) reduction in cancer mortality, metastasis, and (v) in some cases the prevention of recurrence of secondary tumors. At present, a major focus of research in chemoprevention of cancer includes the identification, characterization, and development of a new and safe cancer chemopreventive agent (Govinda *et al.,* 2003).

2.11.1 Carcinogens

The majority of human cancers result from exposure to environmental carcinogens; these include both natural and manmade chemicals, radiation, and viruses. Carcinogens may be divided into several classes: (1) Genotoxic carcinogens, if they react with nucleic acids. These can be directly acting or primary carcinogens, if they are of such reactivity so as to directly affect cellular constituents. (2) Alternatively, they may be procarcinogens that require metabolic activation to induce carcinogenesis. (3) Epigenetic carcinogens are those that are not genotoxic. Molecular diversity of the cancer-initiating compounds ranges from metals to complex organic chemicals, and there is large variation in potency. The variation in structure and potency suggests that more than one mechanism is involved in carcinogenesis. It is also clear that apart from exposure to carcinogens other factors such as the genetic predisposition have been documented. Thus, patients with the genetic xeroderma pigmentosum are more susceptible to skin cancer. Furthermore, incidence of bladder cancer is significantly higher in those individuals who have the slow acetylator phenotype, especially if they are exposed to aromatic amines. Carcinogens in the diet that trigger the initial stage include 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. 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. On the protective side, we know that a diet rich in fruit, vegetables, and fibre is associated with a reduced risk of cancer at most sites (Reddy *et al.,* 2003).

Table 2.2: Types of carcinogens

Type	Example
1. Genotoxic carcinogen Primary, direct-acting alkylating agents	Dimethylsulfate, ethylene imine, b-propiolactonel
2. Procarcinogens Polycyclic aromatic hydrocarbons Nitrosamines Hydrazine Inorganic	Benzo[a]pyrene Dimethylnitrosamine 1,2-Dimethylhydrazine Cadmium, plutonium
3. Epigenetic carcinogens Promoters Solid state Cocarcinogens Immunosuppressants Hormones	Phorbol esters, saccharin, bile acids Solid state Asbestos, plastic Estrogens Purine analogues Catechol
4. Unclassified Peroxisome proliferators	Chofilbrate, phytalate esters

2.11.2 Carcinogenesis

Carcinogenesis is the malignant transformation of a cell or group of cells (Cayuela, 1994). The transformation of a normal cell into a cancerous cell is believed to proceed through many stages over a number of years or even decades. The stages of carcinogenesis include initiation, promotion, and progression. The first stage involves a reaction between the

cancer-producing substance (carcinogen) and the DNA of tissue cells. There may be a genetic susceptibility. This stage may remain dormant, and the subject may only be at risk for developing cancer at a later stage. The second stage occurs very slowly over a period ranging from several months to years. During this stage, a change in diet and lifestyle can have a beneficial effect so that the person may not develop cancer during his or her lifetime. The third and final stage involves progression and spread of the cancer, at which point diet may have less of an impact. Preventing initiation is an important anti-cancer strategy, as are the opportunities to inhibit cancer throughout the latter stages of malignancy. One of the most important mechanisms contributing to cancer is considered to be oxidative damage to the DNA. If a cell containing damaged DNA divides before the DNA can be repaired, the result is likely to be a permanent genetic alteration constituting a first step in carcinogenesis. Body cells that divide rapidly are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division. Mutagenic changes in the components of signaling pathways lead to cellular transformation (cancer).

2.11.3 Multistage Carcinogenesis

The development of fully malignant tumor involves complex interactions between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, immunological, etc). Carcinogenesis can proceed through few stages includes initiation, promotion (appearance of benign tumor) and progression (conversion of benign to malignant tumor).

The transition between stages can be enhance or inhibited by different types of agent. These aspects show that formation of malignant tumor involves various factors, various cellular genes and various types of changes in gene structure and function.

Agents that initiate the carcinogenic process often do so by damaging cellular DNA. The genetic alterations brought about by the initiating agent require cell proliferation, triggered by the promoting agent, to transform a single potential cancer cell into a multicellular tumor. But, additional cellular changes are required over and above simply expanding a pool of initiated cells. The promoting agent on its own can generate proliferative changes but not cancers.

Promotion occurs by different pathways in different cells, but two common features are altered cell proliferation and the formation of new blood vessels. Tumors will grow to about 1mm diameter in the absence of new capillaries (angiogenesis) but further expansion requires the production of angiogenic growth factors by the cancer cells. Progression reflects multiple changes in growth regulatory mechanisms. These include altered sensitivity to adjacent cells, local growth factor production, changes in receptors that initiate signal transduction and alterations to the downstream transduction pathways. The culmination of these events in autonomous cell growth is an ability to grow outside their normal environment and to metastasize to other parts of the body. (Roger, 2000).

2.11.4 Cancer Chemoprevention

Cancer chemoprevention is the inhibition of development of invasive cancer by using pharmacological or natural agents that would prevent the metabolic activation of procarcinogens (Hong and Sporn, 1997, Sreekanth *et al.,* 2007). The mechanism of action of the antiproliferative effects are as follows:

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a) Alterations in the cell differentiation pattern are induced. This plays an important role in metastatic progression and invasiveness of tumors.

b) Induction of apoptosis or pre neoplastic cell expansion block.

c) Metabolic activation of carcinogens is stopped by scavenging ROS. (Sreekanth *et al.,* 2007)

d) Inhibit carcinogen uptake, formation or activation of carcinogen

e) Inhibit oncogene activity

f) Restore immune response

g) Restore tumore suppressor function

(Kelloff, 2000)

Understanding mechanisms of cancer helps us better devise strategies to block two or more key pathways in the cancer process, and thus limit or reverse many types of cancer (Steele and Kelloff, 2005).

2.12 Neutral Red Assay

In Vitro Neutral Red Cytotoxicity Assay (NR) is a technique invented to determine the cytotoxicity effects of a series of extracts. The neutral red cytotoxicity assay is introduced by Borenfreund and Puerner (1984) initially. The assay has been used to determine the relative acute cytotoxicities of a broad spectrum of chemical test agents, to establish structure-toxicity relationships for series of related chemicals, to study metabolism-mediated cytotoxicity to evaluate interactions between combinations of test agents, to evaluate differential and selective toxicities of cancer chemotherapeutics and other pharmaceuticals and to study temperature toxicity interactions.

NR assay quantitates cell viability and can be used to measure cell replication, cytostatic effects, or cell death depending on the seeding density. This assay does not measure the total number of cells, but it does show a reduction in the absorbance related to loss of viable cells and is readily automated. The test is very sensitive, specific, and readily quantifiable.

The assay based on the binding of neutral red, a weakly cationic, supravital dye, to the lysosomal matrix of viable cells by non-ionic diffusion. The dye will be accumulated intra-cellulary in lysosome. After incubation for 3 hours to allow for the dye uptake, the cells are rapidly washed with a solution of 0.5% formaldehyde:1% calcium chloride. Damaged or dead cells lose their ability to retain NR, which is then removed during this wash procedure. The dye is then extracted from the intact, viable cells with a solution of 1% acetic acid : 50% ethanol. The plate is later left to agitate on a microplate shaker for 30 minutes. Spectrophotometric quantitation of the extending dye with a microtiter well reader equipped with 540 nm filter was shown to be linear with the number of surviving, undamaged, viable cells (Harvey *et al.,* 1993). An increase or decrease in the number of cells or their physiological state results in an associated change in the amount of dye incorporated by the cells in the culture. Neutral red tends to precipitate, so the medium with stain is usually centrifuged before use (Freshney, 2000).

2.13 Isolation and Identification

2.13.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a highly sensitive method of detection and quantification of any chemicals in a particular sample using ultraviolet and visible absorbance (Hanachi and Golkho, 2009). HPLC normally uses small particle size for the stationary phase. This results in a fairly large backpressure when the mobile phase is passed through this bed. The result of this, the

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only way to achieve flow of the mobile phase is to use pump systems. Pressure of HPLC can reach up to 200 bars. Nowadays, modern instrumentation can handle up to 400 bars.

Typically, a HPLC system consist of solvent reservoir, injection system, column, HPLC pump, detector, sample collector (optional) and a computer serving as a data station for the detector information as well as a way to control and automate the HPLC pump.

A more advanced instrument consists of two or three pump as well as a number of detectors to analyse the sample. The importance of having more than a pump is that solvent gradients could be programmed, i.e., the solvent composition can be changed continuously throughout the chromatography. In this case, separation of a much larger range of compounds is possible because the mobile phase can be adjusted to the changing polarity of the mixture. As an additional benefit, chromatographic peaks are sharper and hence they elute in a smaller volume from the column and the separation can be done in a much shorter time period.

Almost every HPLC system would be equipped with an UV detector to detect compounds of interest. In older days, these UV detectors were single wavelength detectors but with cheaper hardware, photodiode-array detectors permits the scanning of the full UV-visible range (210nm-650nm) are more popular now. In separation of natural products, it is a great advantage because based on the compounds involved; there can be a very large difference in their UV maxima.

Natural products, such as sugars, that absorb in the range of the solvent systems are detected using refractive-index (RI) detectors or evaporative light-scattering detectors (ELSD). MS and NMR can also be used to detect compounds and these techniques are always used in combination with UV detection.

Generally, HPLC is used for analytical purpose. With either reversed-phase or normal phase materials, column sizes are typically 25 cm in length and 4.6 mm in diameter.

The normal load for an analytical column is below 1 mg for the overall mixture. But, up to 5 mg of sample are possible for certain cases. Semi-preparative separations can be achieved using larger columns; column diameter is increased to 10 mm or more. For a 10 mm column, a maximum load of about 50 mg is possible. A preparative column up to 50 mm in diameter allows separation of a few grams of material.

For normal-phase HPLC, there is a variety of different surface chemistry commercially available. The most commonly used are silica columns. Others are such as alumina packing or modified silica columns such as diol, cyano or amino phases. The benefit of the modified silica phases is their ability to equilibrate quickly unlike silica or alumina phases. If it is classified according to retention power, the normal phase materials can be grouped as follows: alumina>silica = amino>diol>cyano (Leland *et al.,* 2006).

2.13.2 Liquid Chromatography – Mass Spectrometry (LC-MS)

This is a powerful analysis technique for sensitive and selective mass detection in characterizing complex samples. The development of LC-MS system is a difficult process as there is a necessity to remove the solvent. Some of the analytes are non- volatile and could also be thermally labile and must be presented in gaseous form. It took a long time to invent a viable machine to be used and to make LC-MS as a routine technique (Christian, 1994). Although LC-MS still needs further improvements, the technique has clearly reached a point where it now makes very valuable contributions to organic trace analysis. It is important technique for non-volatile, polar and low thermally stable compounds (Linscheid and Westmoreland, 1994).

The normally used interfaces include electrospray ionization (ESI) source, thermospray ionization (TSI), atmospheric pressure chemical ionization (APCI), and particle beam ionization. The choice of the interface used in the sample analysis is mainly

on the polarity and thermal stability of the tested material. For polar, ionic and very large molecules, ESI method is employed. APCI method is suitable for large molecules and non-polar compounds. Thermospray is used for polar and non-polar compounds, but is been replaced by atmospheric techniques. For samples which are volatile, small polar and non-polar molecules, particle beam ionization is useful. (Christian, 1994)

ESI is a soft technique, which in most cases, produces stable ions and low spontaneous fragmentation, remaining the respective parent ion in a high proportion (Almela, *et al.,* 2006). It is a promising technique in bioscience to measure molecular weights of proteins, nucleic acid and etc. Much attention has been given to this particular technique. The difference to thermospray is that the spray is formed by charging the aerosol to such an extent that the droplets explode by coulomb repulsion into smaller droplet, which eventually can be dried to yield highly charged molecules (Linscheid and Westmoreland, 1994). This technique can produce multiply charged ions, number of charges increases with increasing molecular weight. For example multiple charged sites are produced on proteins and peptides. This technique enables determination of large molecular weights. Electrospray is an excellent technique for charged, polar, or basic compounds (Christian, 1994).

Quadrupole mass filter is the most popular mass analyzer for LC-MS because of its low cost and compactness (Christian, 1994). The development of the time of flight spectrometers has opened up a new area in terms of sensitivity, but interfacing to chromatography is not straight forward (Linscheid and Westmoreland, 1994).

LC-MS technique has been extensively used in wide areas for example in environmental applications (pesticides analysis, organometallic compounds analysis and polyaromatic hydrocarbon (PAHs), technical products, biomedical research (amino acids,

saccharides, peptides and proteins) and natural products (lipid, secondary metabolites and alkaloids) (Linscheid and Westmoreland, 1994).

CHAPTER 3

MATERIALS & METHODS

3.1 Materials

The fresh root of the plant *Beta vulgaris L.* was obtained from the Pasar Tani, Seksyen 17, Petaling Jaya, Kuala Lumpur, Malaysia. These samples were harvested from Cameron Highlands, Pahang, Malaysia. The roots were washed, weighed and cut with the skin on. The flesh of the root was cut into thin slices to ensure easy and fast drying.

3.2 Methods

3.2.1 Extraction

i) Preparation of crude methanolic extract of *Beta vulgaris* and its fractions (Houghton and Raman, 1998)

Fresh *Beta vulgaris L.* were cut into small pieces and dried in oven at 60ºC for 3 days. The temperature is chosen because the root is very succulent and damp. The temperature should be a bit higher in order to ensure optimal drying as if it is left in a damp condition for a long time without proper drying condition; there are fungal growths on the plant sample. Concerns about the stability of the pigment in this high temperature of drying is addressed by comparing the activity of the extract from the dried samples and also from the juice extract where the extract is prepared by direct juicing of the fresh sample without drying the sample first. The dried samples were ground into fine powder using a blender. The finely ground samples (465.5 g) were weighed in a conical flask. Methanol was added into the conical flask and the sample was soaked for 3 days. The volume of methanol used was just enough to cover all the samples. The extracting solvent was decanted, treated with

anhydrous sodium sulphate to remove water and finally evaporated using a rotary evaporator to give a gummy reddish crude methanolic extract (64.7 g). The crude methanolic extract was further partitioned by extraction with hexane until the solvent became almost colorless (decrease in color gradually). The decrease in color gradually means that the compounds that are hexane soluble are in less quantity in the crude extract and eventually when hexane is added into the methanol extract, the color of hexane doesn't change. This serves as an indicator to stop partitioning with the hexane solvent and proceed partitioning with the other polar solvents. The hexane layer was then treated with anhydrous sodium sulphate and evaporated to give a yellowish hexane fraction (0.1 g). The remaining residue was then further partitioned with ethyl acetate and water at a ratio of 1:1. The ethyl acetate and water layer was separated using a separating funnel. The separated fractions were evaporated using rotary evaporator after treating with anhydrous sodium sulphate. Water layer formed at the bottom of the funnel whereas ethyl acetate layer at the top. Water layer was eluted first into a conical flask followed by ethyl acetate in another flask. The water layer was repeatedly extracted with ethyl acetate until the ethyl acetate layer became colorless. The excess ethyl acetate was evaporated using rotary evaporator until a yellow color extract (1.6 g) was obtained. The water fraction was freeze dried or evaporated with a rotary evaporator in small batches with temperature ranging 45-50ºC. Water layer was not treated with anhydrous sodium sulphate and was freeze dried or evaporated. The process was summarized in Figure 3.1. Evaporation using a rotary evaporator needed a rotary evaporator with strong vacuum pump. Since this process took a longer period of evaporation time, samples were prepared in small batches to avoid long evaporation time that might alter the components present in the fraction. The extracts were weighed and kept in glass vials and stored in refrigerator at a temperature 4ºC prior to use. The fractionation of methanol extract was summarized in Figure 3.2.

ii) Preparation of Juice Extract of *Beta vulgaris* (Kujala *et al.,* 2002)

The roots of *Beta vulgaris L.*, together with its peel, was washed and cut into small pieces. They are then juiced using an electronic juicer. No water was added before juicing as the root was very succulent and was easy to be juiced. Purplish red juice was collected from the juicer and was kept in -20ºC prior to freeze drying or evaporation. This is to avoid the juice being spoilt. Evaporation was done using a rotary evaporator at a temperature of 40ºC. Since the rotary evaporation process took quite some time, the evaporation was done in small batches of sample to avoid long time sample exposure to a high temperature that might affect the compounds in the sample. A purplish red sample was obtained and kept in the -20ºC to avoid changes in the morphology and for stabilization purposes. Freeze drying is certainly a better technique in preparing the juice extract. In our research the technique was not used due to unavailability of the equipment.

```
  ┌──────────────────────┐
  │  Dried ground roots of │
  │    Beta vulgaris L.    │
  └──────────┬───────────┘
             │       1. Soaked in methanol; 3 days at
             │          room temperature (28ºC)
             │       2.  Filtration
             │       3.  Removal of excess methanol
             │           in filtrate (rotary evaporator)
             ▼
  ┌──────────────────────┐
  │  Dark, Gummy, Reddish  │
  │   Methanolic Extract   │
  └──────────────────────┘
```
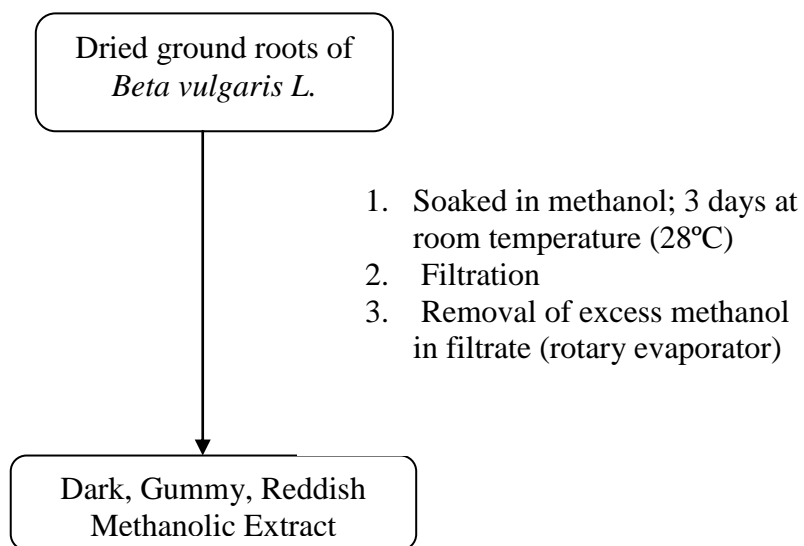
Figure 3.1: Flow chart of extraction of Crude Methanolic Extract

```
┌─────────────────────────┐
│    Methanol Extract     │
└─────────────────────────┘
         │
    ┌────┴─────────────────────────────┐
    ▼                                   ▼
┌──────────────┐              ┌──────────────────┐
│ Hexane Layer │              │ Hexane Insoluble │
│              │              │      Layer       │
└──────────────┘              └──────────────────┘
    │                              │
    ▼                         ┌────┴──────────┐
┌──────────────────┐          ▼               ▼
│ Hexane Fraction  │    ┌─────────────┐  ┌──────────────┐
└──────────────────┘    │ Water Layer │  │ Ethyl Acetate│
         \              │             │  │    Layer     │
          \             └─────────────┘  └──────────────┘
           \                  │               │
            \                 ▼               ▼
             \         ┌──────────────┐ ┌──────────────┐
              \        │Water Fraction│ │Ethyl Acetate │
               \       └──────────────┘ │  Fraction    │
                \            │          └──────────────┘
                 ▼           ▼              │
              ┌──────────────────────────────▼──┐
              │      Bioassay Screening         │
              └─────────────────────────────────┘
```
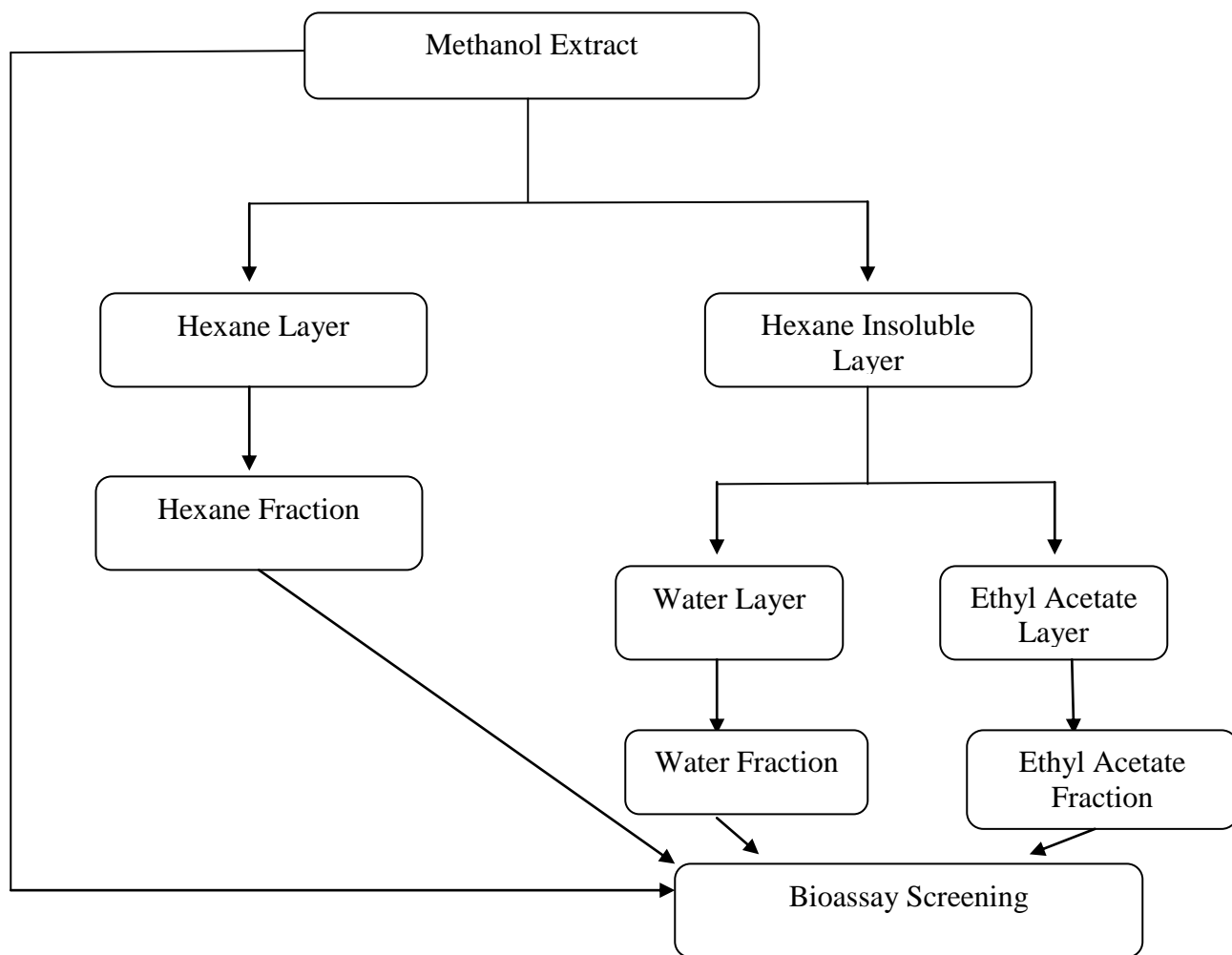
Figure 3.2: Flow chart of fractionation of Methanol Extract

3.2.2 Antioxidant Assays

i) DPPH (1,2-dipheyl-2-picrylhydrzyl) Radical Scavenging Assay

a) Concept of DPPH (1,2-diphenyl-2-picrylhydrzyl) free radical scavenging system

DPPH is a nitrogen centered free radical which is stable. It has a violet color which would
change to yellow when reduction occurs by hydrogen or electron donation. Compound or
substances that can perform this reduction reaction is classified as antioxidants and acts as

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radical scavengers. In order to demonstrate the potential antioxidant activity of the extracts of *Beta vulgaris L.* (methanol extract, hexane fraction, ethyl acetate fraction, water fraction and juice extract), sub-fractions (ET1, ET2, ET3, yellow and purple), and the positive standard (BHA and ascorbic acid), DPPH free radical scavenging activity were tested against the stable DPPH free radical. The stable DPPH free radical is widely used to evaluate antioxidant activity of plant samples or substances in a relatively short time as compared to other available methods. The positive DPPH test result suggests that the samples possess scavenging capability towards free radicals.

b) Preparation of Stock Plant Sample

A stock solution of 20 mg/ml (0.02 gm extract + 1ml methanol/distilled water) of each extract was prepared in a micro centrifuge tube and was wrapped in aluminium foil. The crude extracts and fractions (methanol, hexane, ethyl acetate, juice and water) and other fractions (ET1, ET2, ET3, purple, and yellow) were dissolved in methanol or distilled water. Methanol or distilled water was used as the diluting solvent. If the extracts were to be stored, it is stored in a refrigerator. Normally the dilution with methanol for the extracts is done on the day a particular test is to be conducted. This is to ensure that the correct volume and concentration of the extract is obtained as methanol is easily evaporated.

Preparation of other reagents are described in Appendix 2 part Preparation of Reagents.

c) Procedure

The method described was of Brand-Williams *et al.,* 1995 with modifications.

Ascorbic acid and BHA as positive reference standard

Ascorbic acid and butylated hydroxyanisole (BHA) were used as the positive reference standards in the DPPH assay. The radical scavenging activity of various concentration of ascorbic acid was determined by taking the spectrophotometric absorbance at 520 nm.

Reaction mixtures were prepared according to the Table 3.1, which shows the various volumes for ascorbic acid/BHA and methanol to produce a certain required concentration of ascorbic acid.

Table 3.1: Reaction mixture of ascorbic acid/BHA, DPPH and methanol for DPPH assay

Concentration of Ascorbic Acid/BHA (µg/ml)	Volume of Methanol (µl)	Volume of Ascorbic Acid/BHA (400 µg/ml)(µl)	Volume of DPPH solution (8 µg/ml) (µl)
200.00	475.00	500.00	25.00
100.00	725.00	250.00	25.00
50.00	850.00	125.00	25.00
25.00	912.50	62.50	25.00
12.50	943.75	31.25	25.00
6.25	959.38	15.63	25.00
3.12	967.19	7.81	25.00
1.56	971.09	3.91	25.00
Control	975.00	-	25.00

DPPH (25.00 µl) was added to total up the volume of each of the reaction mixture to 1000 µl. The reaction mixtures were incubated for 30 minutes in a dark room (light sensitive reaction) at room temperature. This is to allow them to react. The absorbance at 520 nm was taken and recorded after the incubation period. Methanol was used as blank and DPPH radical without any antioxidant is used as a control.

Pre-screening of DPPH Radical Scavenging Effect of *Beta vulgaris L. subsp. vulgaris* extracts

Reaction mixtures containing extracts (20 mg/ml), DPPH (8 mg/ml) and methanol was prepared according to the Table 3.2.

Table 3.2: Reaction mixture for pre-screening containing crude extracts, DPPH and methanol

Concentration of extract/fraction (mg/ml)	Volume of Methanol (µl)	Volume of fraction/extract (µl)	Volume of DPPH solution (µl)
5	725.00	250.00	25.00
Control	975.00	-	25.00

The assay procedures were repeated as similar to the previous testing using ascorbic acid or BHA as described above.

d) Determination of Percentage of Inhibition

The percentage of inhibition of the test samples were calculated by using the following formula:

$$\% \text{ of inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100\%$$

OD control was the absorbance of the control and OD sample was the absorbance of the extract/standard.

Screening of DPPH radical scavenging activity on extracts of *Beta vulgaris L.*

For extracts which had percentage inhibition of 50% or more, they are classified as the positive extracts at 5.00 mg/ml. They were re-tested for their antioxidant activity at lower concentrations. The concentrations were 5.00 mg/ml, 4.00 mg/ml, 3.00 mg/ml, 2.00 mg/ml and 1.00 mg/ml. The IC_{50} values for these concentrations were determined. Reaction mixtures containing positive extracts (20.00 mg/ml), DPPH (8.00 mg/ml) and methanol were prepared according to Table 3.3 below.

Table 3.3: Reaction mixture containing methanol, DPPH and extracts/fractions at concentration 5.00mg/ml -1.00mg/ml

Concentration of Extracts/Fractions (mg/ml)	Volume of Methanol (µl)	Volume of Crude Extracts (µl)	Volume of DPPH solution (µl)
5.00	725.00	250.00	25.00
4.00	775.00	200.00	25.00
3.00	825.00	150.00	25.00
2.00	875.00	100.00	25.00
1.00	925.00	50.00	25.00
Control	975.00	-	25.00

All tests were run in triplicates and readings obtained were averaged. IC_{50} for each extract was extrapolated from the graph of % of inhibition against concentration of samples. IC_{50} value (mg/ml) is the effective concentration at which DPPH were scavenged by 50% and was obtained by interpolation from linear regression analysis. This method is used to determine IC_{50} for all the test samples.

If the obtained results with the above lowest concentration which was 1mg/ml still showed a percentage of inhibition which is 50% or more (positive extracts), then the test was run again with lower concentrations. The concentrations that were used are 1.00 mg/ml, 0.80 mg/ml, 0.60 mg/ml, 0.40 mg/ml, 0.20 mg/ml, 0.10 mg/ml and 0.05 mg/ml. The IC_{50} value for these concentrations were then determined. Reaction mixtures containing positive extracts (20.00 mg/ml), DPPH (8.00 mg/ml) and methanol was prepared according to Table 3.4 below.

Table 3.4: Reaction mixture containing methanol, DPPH solution and crude extracts at lower concentration i.e. 1.0 mg/ml – 0.05 mg/ml

Concentration of Crude Extracts (mg/ml)	Volume of Methanol (µl)	Volume of Crude Extracts (µl)	Volume of DPPH solution (µl)
1.00	925.00	50.00	25.00
0.80	935.00	40.00	25.00
0.60	945.00	30.00	25.00
0.40	955.00	20.00	25.00
0.20	965.00	10.00	25.00
0.10	970.00	5.00	25.00
0.05	972.50	2.50	25.00
Control	975.00	0	25.00

All tests were run in triplicates and the results obtained were averaged. IC_{50} for each extract was extrapolated from the graphs that were plotted with % of inhibition against concentration of samples in Table 3.4.

Fractions that were isolated from ethyl acetate fraction and juice extract using HPLC technique was subjected to antioxidant screenings. The sub-fractions were prepared at following concentrations using serial dilution method from a stock solution (20.00 mg/ml). The reaction mixture containing methanol, sub-fractions and DPPH solution were then prepared based on the Table 3.5 below.

Negative control does not have the tested sample (extracts/sub-fractions) but has all other reagents.

The sub-fractions are tested in lower concentrations as compared to the crude extracts because they are in purified form as compared to the crude extracts, so the purified fractions which contain less chemical components would be a good antioxidant if it's able to exert its activity in lower concentrations.

Table 3.5: Reaction mixture containing methanol, DPPH and sub-fractions at concentration 1.00 mg/ml - 0.05 mg/ml

Concentration of Sub-fractions (mg/ml)	Volume of Methanol (µl)	Volume of Sub-fractions (µl)	Volume of DPPH solution (µl)
1.00	925.00	50.00	25.00
0.80	935.00	40.00	25.00
0.60	945.00	30.00	25.00
0.40	955.00	20.00	25.00
0.20	965.00	10.00	25.00
0.10	970.00	5.00	25.00
0.05	972.50	2.50	25.00
Control	975.00	0.00	25.00

ii) Reducing Power Assay

a) Concept of Reducing Power Assay

Reducing power assay monitors the reduction of ferricyanide complex (Fe^{3+}) to ferrous (Fe^{2+}) form. Fe^{2+} can be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. In this assay, the yellow colour of the test solutions change to various shades of

green and blue depending upon the reducing power of each extract. The higher the antioxidant capacity of the extract, the higher the absorbance would be.

b) Preparation of Plant Stock Solutions

Stock solutions of each type of extracts was prepared and wrapped in aluminium foil. The extracts were dissolved in methanol (Systerm) or distilled water.

Preparation of other reagents are described in Appendix 2 part Preparation of Reagents

c) Procedure

The reducing power assay of the extracts and fractions was determined and carried out according to the method that was described by Oyaizu (1986). Briefly, each extract and fraction in different amounts i.e. 5.00 mg, 10.00 mg, 15.00 mg and 20.00 mg was dissolved in 1.00 ml of methanol/distilled water. It was then added with 2.50 ml of 0.2M phosphate buffer (pH 6.6) and 2.50 ml of 1% (w/v) solution of potassium ferricyanide (Sigma). The mixture was then incubated in a water bath at 50ºC for 20 minutes. After that, 2.50 ml of 10% (w/v) trichloroacetic acid solution (Sigma) was added and the mixture was then centrifuged at 650 x g for 10 minutes. A 2.50 ml aliquot of the upper layer was combined with 2.50ml of distilled water and 0.50 ml of a 0.1% (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power. Mean values from three independent samples were calculated for the extracts. For isolated compounds/fractions, investigations were done at lower concentrations i.e. 5.000, 1.000, 0.500, 0.100, 0.050, 0.025 and 0.010 mg/ml. Graph of absorbance (nm) versus concentration (mg/ml) was plotted and comparison of result was done between extracts, fractions or sub-fractions with standard.

The table below summarizes the preparation of reaction mixture for reducing power assay.

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Table 3.6: The summary of preparation of reaction mixture for reducing power assay

Reagent	Volume (ml)
BHA/Extracts/Sub-fractions	1.00
0.2M Phosphate buffer (pH 6.6)	2.50
Potassium Ferricyanide solution (1.0%)	2.50
Trichloroacetic Acid (10.0%)	2.50
Distilled water	2.50
Ferric Chloride solution (0.1%)	0.50

iii) β-carotene bleaching assay

a) Concept of β-carotene bleaching assay

This assay monitors the degree of bleaching of β-carotene from orange to a lighter colour. The fading is due to loss of double bond in the compound which is caused by oxidation of hydroperoxides (free radical) from linoleic acid. Degree of bleaching to β-carotene can be monitored by taking absorbance at 470 nm (Tenpe *et al.,* 2008). Antioxidants hinders β-carotene bleaching extend by neutralizing linoleate free radical & other free radical in system. The higher the antioxidant content in a reaction mixture, the lower will be the color fading.

b) Preparation of Plant Stock Solution

Stock solutions of 4.00 mg/ml, 8.00 mg/ml, 12.00 mg/ml 16.00 mg/ml, and 20.00 mg/ml of each crude extracts and fractions were prepared and wrapped in aluminium foil. The crude methanol extract, hexane, and ethyl acetate fractions were dissolved in methanol. The water fraction was dissolved in distilled water. The juice extract was also dissolved in distilled or deionized water. The sub-fractions (ET1, ET2, ET3, Purple and Yellow) were prepared in the concentrations of 4.000, 2.000, 1.000, 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml. They were prepared by serial dilution from a stock concentration. They were prepared by dilution with distilled water or deionized water.

Method of preparation of other reagents are described in Appendix 2

c) Procedure

Antioxidant activity was determined by measuring the coupled oxidation of β-carotene and linoleic acid, as described by Hammerschmidt and Pratt (1978). 1.00 ml of β-carotene solution in chloroform (0.20 mg/ml) was pipetted into a flask, which contained 0.02 ml linoleic acid and 0.20 ml Tween 80. After removal of the chloroform by evaporation, 50.00 ml of distilled oxygenated water (which was bubbled by an air pump overnight) were added to the flask followed by vigorous swirling using a magnetic stirrer. Five ml aliquots of this emulsion were placed in test tubes which contained 0.20 ml of extracts at varying concentrations. Samples were read against a blank containing the emulsion minus the carotene. A reading at 470 nm was taken immediately (t=0) and then at 20-min intervals for 120 min. The test systems are placed in oven at 50˚C during the 2 hours the test was conducted.

The table below summarizes the preparation of reaction mixture for β-carotene bleaching assay.

Table 3.7: The summary of preparation of reaction mixture for β-carotene bleaching assay

Reagent	Volume (ml)
BHA/Extracts/Fractions/Sub-fractions Emulsion of Linoleic Acid and Tween 80 in distilled oxygenated water	1.00 5.00

d) Determination of Antioxidant activity

The antioxidant activity of each of the test samples was calculated according to the following formulae:

$$R = \frac{\ln (a/b)}{T_{120}}$$

a: Initial absorbance reading at time = 0 min

b: final absorbance reading at time = 120 min

Antioxidant activity = $\dfrac{\textbf{R}\text{ control} - \textbf{R}\text{ sample}}{\textbf{R}\text{ control}}$ **x 100 %**

where **R** is degradation rate

Control in this bleaching test was the system with no added plant extract. The tests were run in triplicates and the reading obtained was averaged. Graphs of concentration of extracts versus antioxidant activity were plotted.

e) Determination of the Oxidation Rate Ratio

The oxidation rate ratio, R_{OR} is calculated based on the formula below:

$$\textbf{R}_{OR} = \dfrac{\textbf{R}\text{ sample}}{\textbf{R}\text{ control}}$$

R sample and R control are the same as used for the calculation of the antioxidant activity above (Method based on Suja, *et al.,* 2005)

iv) Metal Chelating Assay

a) Concept of Metal Chelating Assay

Metal chelating assay was performed based on the method described by Ng *et al.,* 2009 with slight modifications. This assay evaluates the ability of extracts/fractions to chelate ferrous ion and prevent the formation of ferrozine-Fe^{2+} complex. Ferrozine-Fe^{2+} complex have a red colour and it is non-soluble complex. In presence of antioxidants, which chelates the ferrous ions instead of the ferrozine, the formation of the red colour would be reduced

and a soluble complex would be produced and the ferrous ions would be retained in the solution. The colour reduction could be monitored by taking absorbance at 562nm.

b) Procedure

EDTA as the positive standard

EDTA (Sigma) was used as the positive standard for this assay. Reaction mixture of EDTA, deionised distilled water, ferrous chloride ($FeCl_2$) (Systerm) and ferrozine (Sigma) were prepared according to Table 3.8.

Table 3.8: Reaction mixture of EDTA, deionised distilled water, $FeCl_2$ and ferrozine

Concentration of EDTA (mg/ml)	Volume of deionised H_2O (ml)	Volume of EDTA (µl)	Volume of $FeCl_2$ 2 mM (µl)	Volume of ferrozine 5 mM (µl)
1.00	3.70	40.00	100.00	200.00
2.00	3.70	80.00	100.00	200.00
3.00	3.70	120.00	100.00	200.00
4.00	3.70	160.00	100.00	200.00
5.00	3.70	200.00	100.00	200.00
Control	3.70	-	100.00	200.00

The reaction mixture was shaken vigorously and left standing at room temperature for 10 minutes. It was then transferred to cuvettes. The absorbance reading was measured at 562 nm using a double beam spectrophotometer. All tests were carried out in triplicates and the readings were averaged.

Metal chelating activity of crude extract

Crude extracts were tested at concentration of 1.00 mg/ml, 2.00 mg/ml, 3.00 mg/ml, 4.00 mg/ml and 5.00 mg/ml hence they are prepared at the respective concentrations. Reaction mixture of crude extract, deionised distilled water, ferrous chloride ($FeCl_2$) and ferrozine were prepared according to Table 3.9.

Table 3.9: Reaction mixture of crude extract, deionised distilled water, FeCl₂, and ferrozine.

Concentration of extract (mg/ml)	Volume of deionised H_2O (ml)	Volume of crude extract (µl)	Volume of FeCl₂ 2 mM (µl)	Volume of ferrozine 5 mM (µl)
1.00	3.70	1000.00	100.00	200.00
2.00	3.70	1000.00	100.00	200.00
3.00	3.70	1000.00	100.00	200.00
4.00	3.70	1000.00	100.00	200.00
5.00	3.70	1000.00	100.00	200.00
Control	3.70	-	100.00	200.00

Sub-fractions (ET1, ET2, ET3, Purple and Yellow) were tested at concentration of 1.000, 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml. They were prepared by serial dilution from a particular stock concentration. Reaction mixture of sub-fractions, deionized distilled water, ferrous chloride ($FeCl_2$) and ferrozine were prepared according to Table 3.10.

Table 3.10: Reaction mixture of sub-fractions, deionised distilled water, FeCl₂, and ferrozine.

Concentration of sub-fractions (mg/ml)	Volume of deionised H_2O (ml)	Volume of crude extract (ml)	Volume of FeCl₂ 2 mM (ml)	Volume of ferrozine 5 mM (ml)
1.000	3.700	1.000	0.100	0.200
0.500	3.700	1.000	0.100	0.200
0.100	3.700	1.000	0.100	0.200
0.050	3.700	1.000	0.100	0.200
0.025	3.700	1.000	0.100	0.200
0.010	3.700	1.000	0.100	0.200
Control	3.700	-	0.100	0.200

The reaction mixture was shaken vigorously and left standing at room temperature for 10 minutes. It was then transferred to cuvettes. The absorbance reading was measured at 562 nm. All tests were carried out in triplicates and the readings were averaged.

Table 3.11 summarizes the preparation of reaction mixture for Metal Chelating assay.

Table 3.11: The summary of preparation of reaction mixture for metal chelating assay

Reagent	Volume (ml)
BHA/Extracts/Sub-fractions	1.00
Deionized water	0.50
24mM $FeSO_4$	0.50
20% Trichloroacetic acid	0.50
0.8% 2-Thiobarbituric acid	1.00

c) Determination of percentage of inhibition

The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below.

% of inhibition = [(Abs Control − Abs Sample) / Abs Control] x 100

Abs Control = absorbance value of control

Abs Sample = absorbance value of sample

v) Superoxide dismutase (SOD) activity assay

a) Concept of SOD Activity Assay

Superoxide dismutase (SOD) scavenging system was conducted using SOD assay kit-WST. This kit had WST Solution (5.00 ml), Enzyme Solution (100.00 µl), Buffer Solution (100.00 ml) and Dilution Buffer (50.00 ml). SOD assay kit − WST allows convenient SOD assaying by utilizing Dojindo's highly water soluble tetrazolium salt, WST 1-(2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt) that produces a water-soluble yellow colored formazan dye upon reduction by a superoxide anion. Hence, if there is high content of superoxide anion in the system, more yellow

colored formazan dye would be formed. In presence of SOD like materials (antioxidants), XO will be inhibited and formation of superoxide anions would be inhibited.

b) Preparation of Sample/Standard

Sample was prepared at various concentrations. Preparation was done using serial dilution method from a particular concentration of stock sample to the desired concentrations in the assay. For crude extracts, extracts were prepared at the following concentrations: 5.00, 4.00, 3.00, 2.00 and 1.00 mg/ml. If the extract had percentage inhibition more than 50% at the lowest concentration of 1.00 mg/ml, then the extract is subjected to test at lower concentrations i.e. 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml. For sub-fractions, the fractions were prepared at 1.000, 0.500, 0.100, 0.050, 0.025, 0.010 mg/ml. Standard, BHA, was prepared at 5.000, 4.000, 3.000, 2.000, 1.000, 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml. The wide range of concentration was prepared for the standard, was for comparison purpose.

Method of preparation of other reagents are described in Appendix 2.

c) Procedure

The experiment was carried out in a 96-well plate according to the method described in the kit's manual. The well in the plate were divided into 4 categories - sample, blank 1, blank 2 and blank 3. Each sample should have their respective blank 1, blank 2 and blank 3. Sample solution (20.00 µl) at various concentrations were placed in the sample well and also blank 2 well. Blank 2 was set separately if the sample was colored. Next, 20.00 µl of double distilled water were placed in blank 1 and blank 3 well. WST Working Solution (200.00 µl) was then pipeted into all the wells (sample, blank 1, blank 2 and blank 3) using a multi channeled pipetor. Next, 20.00 µl of Dilution Buffer was added to blank 2 and blank wells. Finally, Enzyme Working Solution was added into well that is designated for sample and blank 1. Enzyme Working Solution was placed in the last order because once this was

added, the reaction starts. Then, the plate was incubated in an incubator at 37ºC for 20 minutes. Absorbance was read at 450 nm using an ELISA reader. Calculation was carried out based on the formula below based on (d)

Table 3.12: Amount of each solution for sample, blank 1, blank 2 and blank 3.

Material	Sample	Blank 1	Blank 2	Blank 3
Sample Solution	20 µl	-	20 µl	-
ddH$_2$O	-	20 µl	-	20 µl
WST Working Solution	200 µl	200 µl	200 µl	200 µl
Enzyme Working Solution	20 µL	20 µl	-	-
Dilution Buffer	-	-	20 µl	20 µl

d) Determination of percentage of inhibition

SOD activity (% inhibition rate) =

$$\{[(\,A_{blank\ 1} - A_{blank\ 3}) - A_{sample} - A_{blank\ 2})] / (A_{blank\ 1} - A_{blank\ 3})\} \times 100$$

A_{blank1} = Absorbance of Blank 1

A_{blank2} = Absorbance of Blank 2

A_{blank3} = Absorbance of Blank 3

A_{sample} = Absorbance of Sample

IC_{50} value was calculated from the correlation of the dose response linear regression. The lower the IC_{50} value, the higher the inhibition rate.

vi) Determination of Total Phenolic Content (Folin-Ciocalteau Assay)

Method based on Singleton and Rossi, 1965 with modifications

a) Concept of Folin-Ciocalteau Assay

Although there were many claims that this assay measures the total phenolic content of a particular test sample, but this is not exactly true. According to Huang *et al.,* 2005, the assay actually measures the reducing capacity of a test sample. When Folin-Ciocalteu

reagent is added to the tested sample at the final stage, a blue color would be formed. One-or two-electron reduction reactions lead to blue species, which possibly is $(PMoW_{11}O_{40})^{4-}$ .Hence the higher the presence of reducing components (antioxidants), the higher would be the intensity of the hue of the blue color that is formed. The absorbance reading is taken at 765 nm and then calculations were done in the form of gallic acid equivalence per gram of sample.

b) Procedure

Gallic Acid Calibration Curve Preparation

Gallic Acid Calibration Curve is prepared based on the reagent mixtures described in the following table:

Table 3.13: The volume of gallic acid and distilled water for gallic acid curve preparation

Phenol Concentration (mg/l gallic acid)	Volume of phenol (gallic acid) stock (ml)	Volume of distilled water (ml)
0	0.000	1.000
25.000	0.005	0.995
50.000	0.010	0.990
75.000	0.015	0.985
100.000	0.020	0.980
150.000	0.030	0.970
200.000	0.040	0.960
250.000	0.050	0.950
500.000	0.100	0.900
1000.000	0.200	0.800

A calibration plot, using gallic acid concentrations ranging from 25.000 to 1000.000 mg/l was prepared. Gallic acid stock solutions (Sigma-Aldrich) in volumes ranging from 0.005 to 0.200 ml were pipetted out into test tubes. The final volume was made to 1.000 ml with distilled water in each test tube according to table above. Different concentrations of the resultant gallic acid solution and negative control (distilled water instead of gallic acid)

were mixed with 1.580 ml of distilled water. Folin-Ciocalteu's phenol reagent (0.100 ml) was added to each test tube.

After 3 min, 0.300 ml of saturated sodium carbonate (Na_2CO_3) solution (~35 %) was added to the mixture. The reaction mixtures were incubated at 40ºC for 30 min. The blank contained only methanol. The absorbance was determined at 765 nm with a spectrophotometer. The gallic acid calibration plot was obtained by plotting the absorbance against concentration of gallic acid (mg/l). The table below summarizes the preparation of reaction mixture for phenolic content determination.

Table 3.14: The summary of preparation of reaction mixture for Folin-Ciocalteau assay

Reagent	Volume (ml)
Gallic acid solution/ Extracts	0.02
Distilled water	1.58
Folin-Ciocalteau's phenol reagent	0.10
Saturated sodium carbonate (Na_2CO_3) solution	0.30
Total volume	2.00

Procedure to determine the total phenolic content in test extracts and positive reference standard.

BHA was used as positive reference standard in the study. All the test extracts and BHA standard were prepared at concentration of 20.00 mg/ml as stock extracts. The extracts (or BHA standard) (0.02 ml) at different concentrations (4.00, 8.00, 12.00, 16.00 and 20.00 mg/ml) and control (methanol or distilled water were used instead of extract) were mixed with 1.58ml of distilled water. Folin-Ciocalteau's phenol reagent (0.10 ml) was then added to each test tube. After 3 min, 0.30 ml of saturated sodium carbonate (Na_2CO_3) solution (~35%) was added to the mixture. The reaction mixtures were incubated at 40ºC for 30 min. The blank contained only methanol. The absorbance was determined at 765 nm with a spectrophotometer. The blank contained methanol. All extracts were assayed in

triplicate. The concentrations of phenolic compounds in the extracts of *Beta vulgaris*, expressed as gallic acid equivalents (GAEs), were measured according to the method by Taga *et al.* (1984) with some modifications. The results were mean values and were expressed as mg GAE (gallic acid equivalents)/L.

vii) Thiobarbituric Acid Reactive Species (TBARS) Assay

a) Concept of TBARS Assay (Lipid Peroxidation Assay)

The TBARS assay was performed based on the method described by Ukrisdawithid *et al.* (2008). The experiment works on the principal where TBARS i.e. TBA-MDA, a product of lipid peroxidation of egg yolk suspension, was measured using an ELISA micro plate reader at wavelength 532nm when lipid peroxidation occurs to the egg yolk. Sample which has the effect to inhibit the lipid peroxidation will have lower MDA formation and hence lower TBA-MDA (pink colored complex). Hence the higher the inhibition activity of a certain sample towards lipid peroxidation, the higher would be the antioxidant activity that it possesses.

b) Sample/Standard Preparation

Crude extracts were prepared at varying concentrations of 20.000, 16.000, 12.000, 8.000, and 4.000 mg/ml. Isolated fraction from ethyl acetate fraction and juice extract were prepared at a lower concentrations i.e. 4.000, 2.000, 1.000, 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml. Standard, BHA and ascorbic acid, were prepared at concentrations of 20.000, 16.000, 12.000, 8.000, 4.000, 2.000, 1.000, 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml to enable comparison between other extracts. Preparation of different concentration was done through serial dilution method. Method of preparation of other reagents was described in Appendix 2.

c) Procedure

BHA and ascorbic acid were used as standard. Egg yolk suspension (0.50 ml) was mixed with 1 ml of extract/standard/sub-fractions and 0.50 ml of 24mM $FeSO_4$ in a centrifuge tube. The mixture was then incubated at 37ºC for 15 minutes. After incubation, the water bath was set to 100ºC and once the water bath had attained the required temperature, 0.50 ml of trichloroacetic acid (20%) and 1.00 ml of 2-thiobarbituric (0.8%) acid was added to the reaction mixture. The reaction mixture was mixed and incubated at 100ºC for 15 minutes. After that, the reaction mixture was centrifuged at 3500 rpm for 20 minutes. The supernatant was pipetted out and placed in a 96 well plate and the absorbance of the color of the supernatant was measured at 532 nm. The inhibition of lipid peroxidation was calculated based on the formula below:

$$\text{Inhibition of Lipid Peroxidation (\%)} = \frac{(A - B)}{A} \times 100$$

A = Absorbance of reaction mixture without extract

B = Absorbance of reaction mixture with extract

The table below summarizes the preparation of reaction mixture for TBARS assay

Table 3.15: The summary of preparation of reaction mixture for TBARS assay

Reagent	Volume (ml)
BHA/Extracts/Sub-fractions	1.00
Egg yolk suspension	0.50
24mM $FeSO_4$	0.50
20% Trichloroacetic acid	0.50
0.8% 2-Thiobarbituric acid	1.00

viii) Tyrosinase Inhibitory Assay

a) Concept of Tyrosinase Inhibitory Assay

This assay was performed based on method of Huey-Ko *et al.,* (2008), with some minor modifications. Tyrosinase is the enzyme that causes oxidation and melanogenesis. The inhibitors of this enzyme have many potential benefits. The enzyme has monophenolase (L-Tyrosine as substrate) activity which converts tyrosine to 3-(3,4-dihydroxyphenyl)alanine (dopa) and diphenolase (L-DOPA as substrate) activity which converts dopa to dopaquinone. When there is an inhibition in the activity of the enzyme, the black color chromogen that is formed through oxidation would be reduced and there would be a fall in the absorbance values at 532 nm.

b) Preparation of Sample

Sample

Sample of crude extracts were prepared at varying concentrations of 20.000, 15.000, 10.000, 5.000, and 1.000 mg/ml. Sample of sub-fractions were prepared at varying concentrations of 1.000, 0.500, 0.100, 0.050, 0.025 and 0.010 mg/ml. Preparation was done by serial dilution from stock solution.

Standard (Kojic Acid)

Kojic acid was used as the standard, was prepared at concentrations 20.000, 15.000, 10.000, 5.000, 1.000, 0.500, 0.100, 0.050, 0.025, and 0.010 mg/ml. Preparation was done by serial dilution from stock solution.

Method of preparation of other reagents is described in Appendix 2.

c) Procedure

The test was conducted in 96 well plates to enable readings to be taken at a particular time frame with ease. The samples (20.00 µl) or standard (kojic acid) (20.00 µl) at various concentrations was pipetted into the wells. Next, 80 µl of 0.1M phosphate buffer (pH 6.8)

was pipetted into the entire well. Mushroom tyrosinase with concentration 1000 Units/ml was added pipetted into all the wells at a volume of 20.00 μl and the reaction mixture was then pre-incubated at 37ºC for 5-10 mins with gentle agitation. After the incubation period, 80.00 μl of L-tyrosine (2.0 mM) (monophenolase activity) or 80.00 μl of L-Dopa (12.0 mM) (diphenolase activity) was pipetted into the entire well. Reaction started upon addition of substrates. Absorbance must be taken immediately at time 0 minute and subsequently at 1 minute intervals for 15 minutes at 490 nm for both the monophenolase and diphenolase activity. Reaction mixture for control has all the components except the substrate, i.e. L-tyrosine for monophenolase activity and L-Dopa for diphenolase activity. The substrate was substituted with the diluting solvent of the sample instead. Differences in absorbance between each time measured and time zero were calculated and the percentages of inhibition were determined with respect to control.

The table below summarizes the preparation of reaction mixture for tyrosinase inhibitory assay.

Table 3.16: The summary of preparation of reaction mixture for tyrosinase inhibitory assay

Reagent	Volume (μl)
Kojic Acid/Extracts/Sub-fractions	20.00
Mushroom Tyrosinase (1000 Units/ml)	20.00
0.1M Phosphate Buffer (pH 6.8)	80.00
L-Dopa/L-Tyrosine	80.00

Percentage of inhibition for monophenolase activity is calculated based on the formula below:

$$\text{Percentage of Inhibition (\%)} = \frac{A - B}{B}$$

A = Absorbance of control at time 10 minutes

B = Absorbance of sample at time 10 minutes

Percentage of inhibition for diphenolase activity is calculated based on the formula below:

Percentage of Inhibition (%) = $\dfrac{A - B}{B}$

A = Absorbance of control at time 2 minutes

B = Absorbance of sample at time 2 minutes

Graph percentage of inhibition (%) versus concentration (mg/ml) is plot. IC_{50} value is determined from the graph.

3.2.3 Cytotoxicity Studies

i) Neutral Red Cytotoxicity Assay

Concept of Neutral Red Cytotoxicity Assay

The neutral red cytotoxicity assay was introduced by Borenfreund and Puerner (1984) initially. It is also known as neutral red (NR) *in vitro* cell viability assay. The assay quantitates cell viability and can be used to measure cell replication, cytostatic effects, or cell death depending on the seeding density. This assay does not measure the total number of cells, but it does show a reduction in the absorbance related to loss of viable cells and is readily automated. The assay based on the binding of neutral red, a weakly cationic, supravital dye, to the lysosomal matrix of viable cells by non-ionic diffusion.

ii) Cell Lines

The Human Colon Carcinoma cells (HCT 116), MCF-7, (an estrogen positive (ER^+) human mammary adenocarcinoma cells), CaSKi, (a human epidermal carcinoma of cervix cell line), HT29 (a human colon cancer cell line), A549 (a human lung cancer cell line) and HCT-116 (a human colon cancer cell line) were purchased from the American Tissue

Culture Collection (ATCC, USA). The viability of the cells was checked before and after treatment using tryphan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (-196ºC) prior to use.

iii) Cryopreservation of Cells

When the cells are not needed, they were frozen for later use. 50% of FBS, 20% of DMSO and 30% of Basic Medium were added to exponentially growing cells in sterile centrifuge tube where it was spun down at 1000 rpm for 5 minutes using a bench centrifuge. The cells were re-suspended in 3.00 ml of fresh cryopreservation solution and aliquotted into sterile provials (Falcon, USA), in 1.00 ml volumes. The provials were left to stand in ice and placed in a polystyrene cup and kept in -70ºC vapour phase of liquid nitrogen tank for about 4-24 hours. Provials were then transferred into liquid nitrogen (-196ºC).

vi) Revival of Cells

The provial of cells was removed from liquid nitrogen and plunged into a beaker of ice. It was then, transferred to a 37ºC water bath for quick thawing. The cells were transferred into 1 ml of 20% supplemented basic media in a polypropylene tube (Falcon, USA) and spun at 100% for 5 minutes. The supernatant was discarded and the pellet re-suspended in 1 ml of 20% supplemented basic medium and incubated in a 25 ml tissue culture flask (Falcon, USA) at 37ºC in a 5% CO_2 incubator (Shel Lab water-jacketed).

vii) Maintenance of Cells

HT-29, MCF-7, CaSKi and A549 cell was maintained in 10% supplemented RPMI Basic Media. HCT-116 was maintained in 10% supplemented McCOY'S 5A Medium containing 10% fetal bovine serum. The culture was incubated in a 5% CO_2 incubator (Shel Lab water-jacketed) kept at 37ºC in a humidified atmosphere. The culture was sub-   cultured every 2 or 3 days and routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for any contamination. Sub-culturing was done when the cell growth was heavy.

vii) Subcultivation of Cells

Adherent cells were attached and formed a single layer in the culture flask. Confluent cells were washed twice using phosphate buffer saline (PBS, PAA Lab, Austria). The cells were detached from the flask by incubating in 1.00 ml of 0.25% trypsin-EDTA solution and 3 ml of PBS solution for 5-10 minutes at 37°C and then sharply tapped to release the cells from attachment. The floating cells were transferred into a centrifuge tube (Falcon, USA), which contained 1.00 ml 10% supplemented medium and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded carefully and 2.00 ml of 10% supplemented medium was added to the pellet. The cells were split and transferred into different flasks containing 7.00 ml of culture media each. The flask was then further incubated.

ix) Cell Plating and Incubation of Cells with Plant Extract

Medium in tissue culture flask was initially discarded. PBS (5.00 ml) was then added into the flask. The solution was used to rinse the internal surfaces of flask gently. PBS was drained away carefully. Another batch of 5.00 ml of PBS was added again into the tissue culture flask. The flask was rinsed gently again. Later, PBS was drained away carefully. Three (3.00) ml PBS and 1 ml EDTA were added into the tissue flask and mixed well. The flask was later incubated in a 5% CO_2 incubator at 37°C for 10 minutes. The cells were observed under inverted microscope.

The concentration of a cell suspension may be determined by placing the cells in an optically flat chamber under a microscope. The cell number within a defined area of known depth was counted and the cell concentration was derived from the count. A monolayer culture is tryptinized or a sample from suspension culture is taken. The viable cells were counted by 0.4% tryphan blue exclusion in a haemocytometer chamber. The cells were counted on the microscope and calculate cell concentration. Then, the cells were plated in 96-well microtiter plate (Nunc) in a volume of 190µl. The plate was incubated in a CO_2

incubator at 37°C for 24 hours to allow the cells to adhere and achieve 60-70% confluence at the time of the addition of the test agents. It was incubated for 24 hours. The extracts of *Beta vulgaris L.* was then added to the wells at six different concentrations of 1.00, 10.00, 25.00,50.00,75.00,100.00 µg/ml of each extracts of *Beta vulgaris L.*. The plates were incubated with the cells for 72 hours. Negative control was the well with the untreated cells but must contain DMSO at the highest concentration used in the test samples. The test is conducted in triplicates for each concentration.

x) NR Dye Addition and Absorbance Reading

The media was replaced with medium containing 50.00 µg/ml Neutral Red at the end on the previous incubation period of cell plating. The plates were incubated for another 3 hours to allow the uptake of the vital dye into the lysosomes of viable and injured cells.

The media were removed and the cells were washed with the Neutral Red washing solution after the incubation period. Damaged or dead cells lose their ability to retain NR, which was then removed during this washing procedure. The dye is then extracted from the intact, viable cells with a solution of 1% acetic acid: 50% ethanol (resorb solution). The plate was later left to agitate on a microplate shaker for 30 minutes. Spectrophotometric quantitation of the extending dye with a microtiter well reader equipped with 540 nm filter was shown to be linear with the number of surviving, undamaged, viable cells (Harvey, *et al.,* 1993). Neutral red tends to precipitate, so the medium with stain is usually centrifuged before use. (Freshney, R.I., 2000).

Three replicate plates were used to determine the cytotoxicity activity of each extract. The average data from triplicates were expressed in terms of killing the percentage relative to negative control.

xi) Calculation of Percentage of Inhibition

The percentage of inhibition of each of the test samples was calculated according to the formula:

$$\% \text{ of Inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

where OD = optical density

The concentration of extract which causes 50% inhibition or cell death is determined by calculating the IC_{50}. IC_{50} value for each extract was extrapolated from the graphs plotted using the OD values obtained. The extracts that gave IC_{50} of 20µg/ml or less were considered active (Geran *et al.,* 1972).

xii) Cell Enumeration

Haemocytometer was used to determine the number of cells per unit volume of a suspension. Haemocytometer is a type of counting chamber. Preparation of the haemocytometer begins with cleaning it with a lens paper. The coverslip is also cleaned. Coverslips for haemacytometer are made especially a bit thicker than the conventional coverslips for microscopy. This is because they must be durable enough to overcome the surface tension of a drop of liquid. The coverslip would be placed over the counting surface prior to putting on the cell suspension. The suspension is placed into one of the V-shaped wells with a pipet. The area under the coverslip fills by capillary action. Enough liquid should be placed so that it covers properly. Haemocytometer is then placed on the microscope and counting grid is brought into focus at low power. Cells in the centre grid (with the smallest boxes) are counted for calculation.

A drop of diluted cell suspension (1:10) was placed on a haemocytometer. The dead cells were stained blue (tryphan blue) while the living cells were not stained. The

haemocytometer was placed under a microscope and then unstained living cells were counted under the 20X objective.

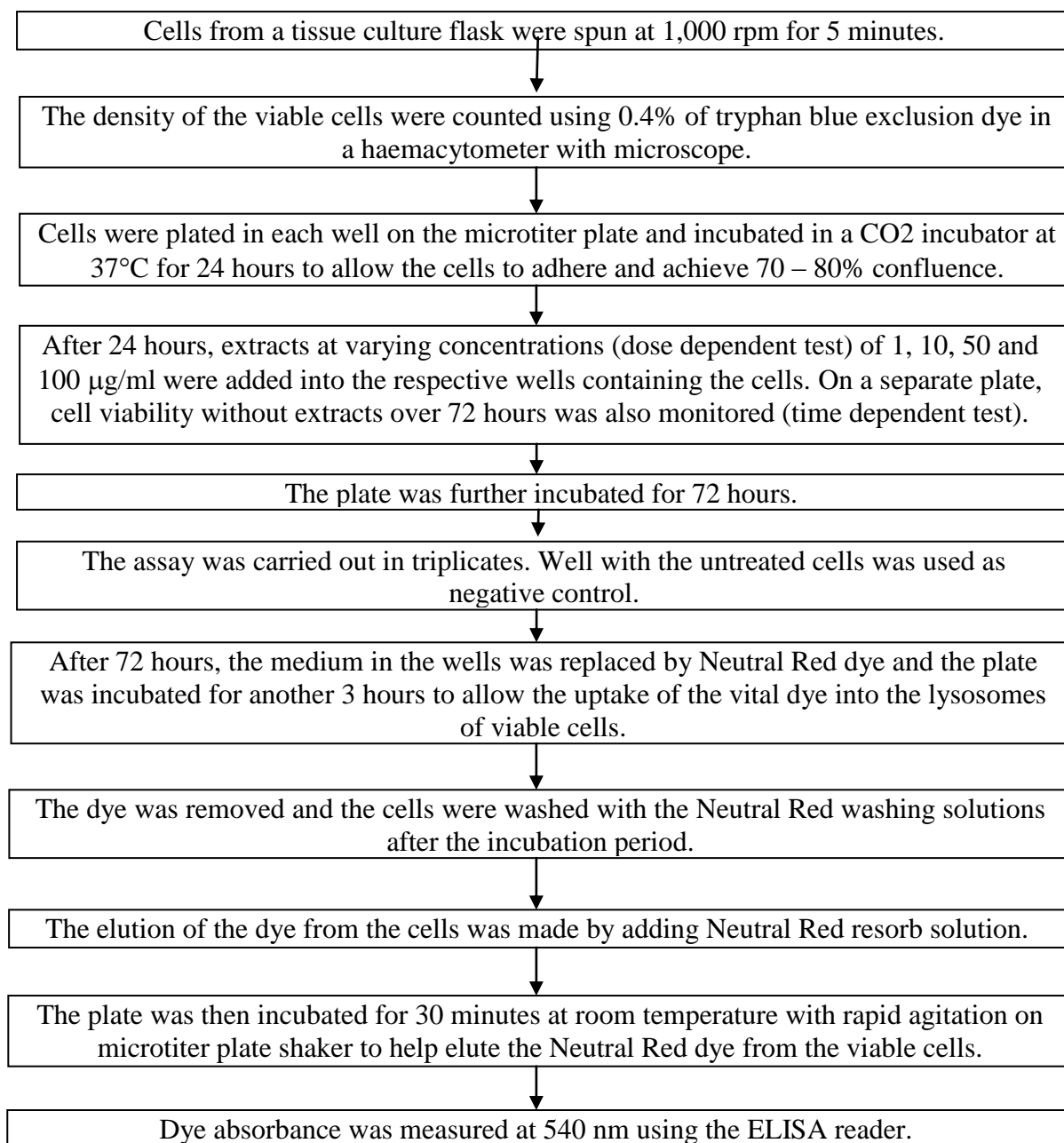The number of living cells in 1ml of the culture media was calculated using the following formula:

N (Number of cells in Tissue Culture Flask) = n x D x 10^4

n = Number of cells in box Grid 3 (centre grid in haemacytometer) ($1mm^2$ box)

D = Dilution Factor of cell and stain = 100µl: 900µl

xii) Summary of the Neutral Red Cytotoxicity Assay

The cytotoxic assay protocol is summarized in Figure 3.3.

Cells from a tissue culture flask were spun at 1,000 rpm for 5 minutes.

The density of the viable cells were counted using 0.4% of tryphan blue exclusion dye in a haemacytometer with microscope.

Cells were plated in each well on the microtiter plate and incubated in a CO2 incubator at 37°C for 24 hours to allow the cells to adhere and achieve 70 – 80% confluence.

After 24 hours, extracts at varying concentrations (dose dependent test) of 1, 10, 50 and 100 µg/ml were added into the respective wells containing the cells. On a separate plate, cell viability without extracts over 72 hours was also monitored (time dependent test).

The plate was further incubated for 72 hours.

The assay was carried out in triplicates. Well with the untreated cells was used as negative control.

After 72 hours, the medium in the wells was replaced by Neutral Red dye and the plate was incubated for another 3 hours to allow the uptake of the vital dye into the lysosomes of viable cells.

The dye was removed and the cells were washed with the Neutral Red washing solutions after the incubation period.

The elution of the dye from the cells was made by adding Neutral Red resorb solution.

The plate was then incubated for 30 minutes at room temperature with rapid agitation on microtiter plate shaker to help elute the Neutral Red dye from the viable cells.

Dye absorbance was measured at 540 nm using the ELISA reader.

Figure 3.3: Neutral Red Cytotoxic Assay

3.2.4 Chemical Isolation and Identification of Compounds

High Performance Liquid Chromatography (HPLC) based on the method by Kujala *et al.,* 2002 was used.

i) Profiling of the extracts using analytical HPLC system

Profiling of extracts and fraction (water fraction from fraction of 100% methanol extract), 100% water fraction from dried plant sample, juice extract and ethyl acetate fraction was done using HPLC (Waters Delta Prep) system consisting of a Waters Prep LC controller, Waters 2487 Dual λ Absorbance Detector (UV Detector) and Waters 2414 Refractive Index Detector (RI Detector). Chromatographic analysis was performed on a 250mm x 4.0mm I.D. Purospher, 5µm, LiChrocart RP-18 column. Two solvents, Acetonitrile (D) and formic acid/water (0.4:99.6, v/v) (C) were used. The injection volume was 20 µl and the flow rate 1.0 ml min^{-1}

ii) Preparation of eluting solvent

All solvents for HPLC were degassed prior to use. A mobile phase comprising of acetonitrile and formic acid/water (0.4:99.6, v/v) was prepared. This solvent system is the suitable solvent system for a good separation (Kujala *et al., 2002*). 996.00 ml of ultra-pure water and 0.40 ml of formic acid were mixed in a 1000.00 ml volumetric flask. The resultant solutions were then degassed using a vacuum pump and filter apparatus with a filtering membrane (PTFE filter membrane or nylon filter for water/buffer). The degassed solvent was poured into the reservoir system.

iii) Sample Preparation

Analytical HPLC

0.005 g (5.000 mg) of extract was diluted with the diluting solvent (ultra-pure deionized water or methanol) and was filtered using a syringe filter (0.45 µm).

Preparative HPLC

0.05 g (50.00 mg) of extract was diluted with the diluting solvent (ultra-pure deionized water or methanol) and was filtered using a syringe filter (0.45 µm). Sample could be prepared at higher concentrations based on the intensity of the peaks shown in the chromatogram.

iv) HPLC Condition

The flow rate was set at 1.00 ml/min and the flow of elution was allowed using the initial solvent system through the column for 5-10 minutes for conditioning purposes. Then, the flow rate was set at 1.00 ml/min for sample running. 20.00 µl of sample was prepared in a syringe. The sample syringe was put into the injection port hole and was injected.

Detail of the chromatographic system used was shown as below:

Chromatographic system : High Performance Liquid Chromatography system (Waters Delta Prep) equipped with Waters Prep LC controller, Waters 2487 Dual λ Absorbance Detector (UV Detector) and Waters 2414 Refractive Index Detector (RI Detector).

Mobile phase : Acetonitrile: Formic acid/water (0.4/99.6 v/v)

Detector : Waters 2487 Dual λ Absorbance Detector (UV Detector)

Column flow rate : 1.00 ml/min

Run time   : Depends on the sample

Wavelength   : Depends on the sample

Injection volume   : 20.00 µL

v) Method for Juice Extract and 100% Water fraction from Dried Plant Sample

Column:

Analytical : Lichrocart RP-18, Purospher STAR (100 x 4.6 mm, 5 µm, Merck)

Semi-Preparative: Lichrocart RP-18, Purospher STAR (250 x 4.6 mm, 5 µm, Merck)

Mobile Phase:

A = Water/formic acid (99.6:0.4) v/v

B = Acetonitrile

Method:

From time 0-5 minutes, there would be a isocratic flow of 100% water/formic acid, at time 5-50 minutes, gradient flow of 0-20% of acetonitrile. At time 50-70 min, gradient flow of acetonitrile increases from 20-70% subsequently. At time 70-90 min, the flow was set into isocratic mode of 70% acetonitrile. At the final stage of the run, from time 90-95 mins, 70% acetonitrile decreased to 0% gradually through gradient flow.

Flow rate: 1.00 ml/min (analytical), 4.70 ml/min (semi-prep)

Detection wavelength: 477 and 538 nm

vi) Method for Ethyl acetate fraction

Column:

Analytical: Lichrocart RP-18, Purospher STAR (100 x 4.6mm, 5 µm, Merck)

Semi-Preparative: Lichrocart RP-18, Purospher STAR (250 x 4.6 mm, 5 µm, Merck)

Mobile Phase:

A = Water/formic acid (99.6:0.4) v/v

B = Acetonitrile

Method:

From time 0-10 minutes, there was an isocratic flow of 20% acetonitrile, at time 10-70 minutes, gradient flow of 20-85% of acetonitrile. At time 70-90 min, isocratic flow of acetonitrile was set at 85% acetonitrile. At the final stage of the run, from time 90-95 mins, 85% acetonitrile decreased to 0% gradually through gradient flow.

Flow rate: 1.00 ml/min (analytical), 4.70 ml/min (semi-prep)

Detection wavelength: 280 nm

vii) Preparative HPLC

Preparative HPLC or in short prep-HPLC was done in order to collect the fractions or compounds based on the chromatogram that was observed previously in the analytical HPLC. The major peaks that were observed in the analytical HPLC were targeted for collection. In preparative HPLC, a different column, which has larger diameter, was used. This column was used in order to produce mass separation and collection of the fractions/compounds. The same method was used, the injection volume was raised to 100.00 µl and the column flow rate is adjusted to 4.70 ml/min. The column flow rate was calculated based on the formula shown below. The eluent was collected based on the chromatogram; fractions/compounds were collected at the initial formation of the peak, throughout the peak and a few seconds after the end of the peak. Eluent were collected in 250.00 ml Duran bottles and were kept in freezer until evaporation process using rotary evaporator.

viii) Evaporation and analysis of the collected fractions/compounds

The evaporation of the collected fractions/compounds was done using a rotary evaporator. The fractions/compounds were then transferred into a glass vial and then subjected to further identification procedure such as LC-MS-MS. The fractions/compounds collected were also subjected to bioactivity screenings.

ix) Chemical Stabilization of the Purple Fraction

The purple fraction which contains pigments loses its color (disintegrates) if it was left in the freezer without any change in its chemical stability. Stabilization of pigment was done based on Reynoso *et al.,* 1997. The pigment was stable at pH 5.5. Thus HCl was added to lower the pH (acidic) and NaOH was added to increase the pH (alkaline). After pH 5.5 was achieved, 0.1% ascorbic acid crystal was added to the purple fraction. The fractioned was stored in -20ºC and the vial that the fraction was kept was wrapped with aluminium foil to avoid any contact of the sample with light. This method seemed to be an effective method of keeping the sample stable until further use. Betalains were known to be sensitive to oxidation, which has an impact on their color stability. Therefore, compounds such as ascorbic and citric acids have been used to counteract this phenomenon due the fact that ascorbic acid is a good stabilizer for its scavenger oxygen capacity in a closed system and citric acid can chelate metal ions such as iron which promote oxidation (Reynoso *et al.,* 1997). In this experiment, ascorbic acid was used because it was found that the addition of ascorbic acid to the pigment extract protected their color stability.

x) Liquid Chromatography – Mass Spectrometry – Mass Spectrometry (LC-MS-MS)

The LCMS/MS method, full scan with MS/MS data collection was used. Positive ionization mode was set. The column that was used is Phenomenex Aqua C-18 with

dimension 50.0 mm x 2.0 mm x 5.0 µM. The mobile phase was water and methanol with 0.2 % formic acid and 2 mM ammonium formate. Rapid screening was performed with 10 min run time. LCMS machine model information is, Applied Biosystems 3200Q Trap LCMS/MS with Shimadzu ultra-pure liquid chromatography (UPLC) system. The method or gradient of the run program is as below:

A = water

B = methanol with 0.2% formic acid and 2 mM ammonium formate

10% A to 90% B from 0.01 min to 5.0 min, hold for 2 min and back to 10% A in 0.1 min and re- equilibrated for 3 min.