ANTIOXIDANT AND CYTOTOXIC INVESTIGATIONS OF *BETA VULGARIS L.*

JAIME STELLA A/P MOSES RICHARDSON

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ABSTRACT

Beta vulgaris has been commonly consumed and traditionally used for various medicinal purposes. The present study scientifically evaluates the antioxidant and cytotoxic potential of the plant using various bioassays. The root part of *Beta vulgaris* was collected and extracted using methanol and then fractionated with hexane, ethyl acetate and water. The fresh root of *Beta vulgaris* was juiced and then subjected to evaporation under reduced pressure to form an extract. Chemical investigations were then directed to the ethyl acetate fraction and juice extract; fraction ET1, ET2 and ET3 were obtained from ethyl acetate fraction by isolation using high performance liquid chromatography (HPLC) technique while fraction purple and yellow were isolated from the juice extract using the same technique.

Antioxidant activity of the juice extract, crude methanolic, fractionated extracts (hexane, ethyl acetate and water) and sub-fractions [(ET1, ET2, ET3 – from ethyl acetate fraction), (purple and yellow fractions – from juice extract)] of the plant were evaluated. The antioxidant assays that measured the first line of defense mechanism were TBARS assay and metal chelating assay. The second line antioxidant defense capacity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, reducing power assay, superoxide dismutase (SOD) activity assay and Folin-Ciocalteu Assay. Third line of defense was evaluated by β-carotene bleaching assay and tyrosinase inhibitory assay (monophenolase and diphenolase activity). Only the crude extracts were studied for their cytotoxic activity based on the Neutral Red assay.

For the DPPH radical scavenging assay, the ethyl acetate fraction exhibited the highest activity (IC_{50} = 0.31 mg/ml), followed by the methanol extract, juice extract, water and hexane fractions. As for the sub-fractions, the purple fraction exhibited the highest

activity (IC_{50} = 0.13 mg/ml), followed by ET2, ET1, yellow and ET3. For the reducing power assay, the ethyl acetate fraction again showed the highest antioxidant activity with the highest potential of reduction in converting ferricyanide complex to ferrous form. This was followed by butylated hydroxyanisole (BHA), water fraction, methanol extract, hexane fraction and juice extract. Among the fractions, the purple fraction had the highest activity followed by ET1, ET3, ET2 and the yellow fraction. In the β-carotene bleaching assay, the hexane fraction showed the highest antioxidant activity followed by BHA, ethyl acetate fraction, ascorbic acid, juice extract, methanol extract and water fraction. In the metal chelating assay, all the extracts and sub-fractions had lower percentage of inhibition as compared to the standard (EDTA). In the SOD activity assay, the highest antioxidant activity was exhibited by ethyl acetate fraction (IC_{50} = 0.71 mg/ml), methanol extract, water fraction, juice extract and hexane fraction. As for the sub-fractions, excellent activities were reported by ET1 (IC_{50} = 0.21 mg/ml) and ET2 (IC_{50} = 0.24 mg/ml). Moderate activity was shown by the purple fraction. The yellow fraction and ET3 had low SOD activity. In the lipid peroxidation assay (TBARS), the inhibition rate of lipid peroxidation was highest for methanol extract followed by hexane fraction, juice extract, water fraction and ethyl acetate fraction. As for the sub-fractions, only the yellow fraction had higher inhibition towards lipid oxidation as compared to standards. In the tyrosinase inhibitory assay, monophenolase activity using L-tyrosine as substrate was tested. Crude extracts and fractions did not show higher activity than kojic acid but sub-fractions such as ET1, the purple and yellow fractions showed better activity than kojic acid. Tyrosinase inhibitory assay which used L-Dopa as the substrate (diphenolase activity) showed that the water fractions and juice extract at 20.00 mg/ml had better activity than kojic acid but all the other extracts had lower percentage of inhibition compared to the standard. All the sub-

fractions showed better activity than kojic acid at all the tested concentrations. Folin-Ciocalteau Assay which measures the reducing capacity of the extracts and fractions showed that ethyl acetate fraction had the highest reducing capability. This may be due to high phenolic content in this particular fraction. The lowest activity was exhibited by the juice extract. Hence, *Beta vulgaris L.* exhibited stronger antioxidant activities in comparison to the standards used in the reducing power assay, β-carotene bleaching assay, SOD assay, TBARS assay and tyrosinase inhibitory assay.

The crude and fractionated extracts were investigated for their effect on the cancer cell lines namely, hormone-dependent human breast (MCF7), human lung (A549), human colon (HCT116), human cervical (CasKi) and the human colon (HT29) carcinoma cell lines, using the Neutral Red Cytotoxicity assay. All the extracts showed poor inhibition towards the cancer cell lines tested (IC_{50} > 100.00 µg/ml) and the ethyl acetate fraction exhibited the lowest IC_{50} value of 69.50 µg/ml.

The identification of components in the sub-fractions through liquid chromatography - mass spectrometry - mass spectrometry (LC-MS-MS) showed the presence of known compounds such as betavulgarin, betanin and isobetanin in ET1; 2,15,17-tridecarboxybetanin and betagarin in ET2; ET3, betagarin in ET3. The yellow fraction showed the presence of vulgaxanthine I whilst the purple fraction contains betanin, isobetanin, neobetanin and decarboxylated betanin.

Thus, it can be concluded that *Beta vulgaris L.* is excellent as an antioxidant in the first, second and third line of defense mechanisms but did not show good *in vitro* cytotoxic ability. With its strong antioxidant properties, *Beta vulgaris L.* can be recommended for chemoprevention and regularly consumed in our diet to maintain good health.

ABSTRAK

Beta vulgaris L. kerap diambil dalam diet dan digunakan secara tradisional untuk pelbagai keperluan kesihatan. Dalam kajian ini, aktiviti antioksidan dan keupayaan sitotoksik dikaji secara saintifik dengan menggunakan pelbagai esei. Bahagian akar *Beta vulgaris L.* dikutip dan diekstrak dengan menggunakan metanol dan difraksikan dengan menggunakan heksana, etil asetat dan air. Bahagian akar *Beta vulgaris L*. yang masih segar juga dijus dan dijadikan sebagai ekstrak. Proses pengasingan dengan menggunakan teknik Kromatografi Cecair Prestasi Tinggi (HPLC) juga dilakukan pada fraksi etil asetat dan ekstrak jus. Penyiasatan kimia dilakukan kepada fraksi etil asetat dan juga ekstrak jus; fraksi ET1, ET2 dan ET3 diperoleh daripada fraksi etil asitat daripada proses pengasingan HPLC manakala, fraksi ungu dan fraksi kuning pula diperolehi daripada ekstrak jus.

Aktiviti antioksidan esktrak jus, ekstrak metanol, fraksi-fraksi heksana, etil asetat dan air dan juga fraksi-fraksi yang telah diasingkan [(ET1,ET2,ET3 daripada fraksi etil asetat) dan (fraksi ungu dan fraksi kuning dari ekstrak jus)] seterusnya dikaji. Assai antioksidan yang mengkaji tentang benteng pertahanan pertama antioksidan adalah assai TBARS dan assai pengikatan ion besi. Benteng pertahanan kedua antioksidan pula dikaji berdasarkan assai DPPH, assai kuasa penurunan, assai aktiviti SOD dan assai Folin-Ciocalteu. Mekanisma benteng pertahanan ketiga pula dikaji oleh assai pelunturan beta karotena dan assai perencatan enzim tirosina (aktiviti monophenolase dan aktiviti diphenolase). Kajian sitotoksik dijalankan menggunakan assai 'Neutral Red' untuk ekstrak mentah.

Berdasarkan assai DPPH (1,1-difenil-2 pikrilhidrazil), fraksi etil asetat telah memaparkan aktiviti yang tertinggi (IC_{50} = 0.31 mg/ml), diikuti dengan ekstrak metanol,

ekstrak jus, fraksi air dan fraksi heksana. Bagi fraksi yang telah diasingkan, fraksi ungu telah menunjukkan aktiviti perencatan DPPH yang tertinggi (IC_{50} = 0.13 mg/ml) diikuti dengan fraksi ET2, ET1, kuning dan ET3. Dalam assai kuasa penurunan pula, fraksi etil asetat menunjukkan kuasa penurunan kompleks feriksianida kepada ion ferus yang tertinggi. Ini diikuti dengan fraksi air, ekstrak metanol, fraksi heksana, dan fraksi jus. Bagi fraksi yang diasingkan, penunjuk piawai menunjukkan aktiviti yang lebih baik. Antara fraksi, fraksi ungu memunjukkan aktiviti yang tertinggi. Dalam assai pelunturan beta karotena, fraksi heksana menunjukkan aktiviti tertinggi dan diikuti oleh fraksi etil asetat, ekstrak jus, ekstrak metanol dan fraksi air. Untuk fraksi yang diasingkan, aktiviti tidak begitu tinggi dan aktiviti piawai positif adalah lebih tinggi. Dalam assai pengikatan ion besi, semua ekstrak dan fraksi menunjukkan aktiviti lebih rendah berbanding piawai positif EDTA. Dalam assai aktiviti 'Superoxide Dismutase' SOD, aktiviti tertinggi ditunjukkan oleh fraksi etil asetat diikuti oleh ekstrak metanol, ekstrak jus dan fraksi heksana. Dalam fraksi yang diasingkan pula, fraksi-fraksi ET1 dan ET2 juga menunjukkan aktiviti yang amat baik. Aktiviti yang sederhana terhadap SOD ditunjukkan oleh fraksi ungu dan activiti yang lemah terhadap assai ini ditunjukkan oleh fraksi kuning. Dalam assai oksidasi lemak (TBARS) pula, perencatan terhadap oksidasi lemak yang tertinggi antara fraksi ekstrak dicatat oleh ekstrak metanol dan diikuti oleh fraksi heksana, ekstrak jus, fraksi air dan fraksi etil asetat. Bagi fraksi yang diasingkan pula, hanya fraksi kuning yang menunjukkan perencatan terhadap oksidasi lemak yang tinggi. Dalam assai perencatan enzim tirosinase, L-tyrosine digunakan sebagai substrat untuk menunjukkan aktiviti 'monophenolase' dan L-DOPA digunakan sebagai substrat untuk menunjukkan aktiviti 'diphenolase'. Ekstrak mentah tidak menunjukkan aktiviti yang lebih baik daripada penunjuk piawai, asid kojik, akan tetapi fraksi yang diasingkan; fraksi ET1, ungu dan kuning menunjukkan aktiviti yang

lebih baik daripada asid kojik. Bagi aktiviti diphenolase pula, fraksi air dan ekstrak jus menunjukkan aktiviti perencatan yang lebih tinggi daripada asid kojik pada kepekatan 20.00 mg/ml. Semua fraksi yang diasingkan mempunyai aktiviti yang lebih baik daripada penunjuk piawai bagi akitivi diphenolase. Dalam assai Folin-Ciocalteau yang memantau kuasa penurunan ekstrak, fraksi etil asetat menunjukkan kuasa penurunan yang tinggi. Ini mungkin mempunyai perkaitan yang bahawa fraksi ini mempunyai kandungan bahan kimia fenolik yang tinggi. Aktiviti terendah dalam assai ini dicatat oleh ekstrak jus. Oleh yang demikian, *Beta vulgaris L*. menunjukkkan aktiviti yang lebih baik daripada piawai positif dalam assai kuasa penurunan, assai pelunturan β-karotena, assai aktiviti SOD, assai TBARS dan assai perencatan enzim tirosina.

Assai sitotoksik Neutral Red digunakan untuk kajian sitotoksisiti. Semua ekstrak dan fraksi dikaji dengan sel kanser MCF7, HCT116, HT29, A549 dan CasKi. Hanya fraksi etil asetat menunjukkan aktiviti yang sederhana pada sel HT29 (sel karsinoma usus manusia) (IC_{50} = 69.50 μg/ml). Semua ekstrak dan fraksi lain menunjukkan perencatan yang rendah terhadap sel kanser yang dikaji.

Berdasarkan pengenalpastian menggunakan 'Liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS), sebatian-sebatian yang telah dikenali seperti betavulgarin, betanin dan isobetanin dapat dikenalpasti dalam ET1. ET2 pula menunjukkan kehadiran 2,15,17-tridekarboksibetanin dan betagarin. Di ET3, betagarin juga didapati hadir. Dalam fraksi kuning, vulgaxanthine I dikenalpasti hadir dan dalam fraksi ungu, kehadiran betanin, isobetanin, neobetanin dan dekarboksibetanin (betanin terdekarboksilat) dikenalpasti.

Daripada kajian ini, kita dapat membuat kesimpulan bahawa *Beta vulgaris L.* mempunyai mekanisma pertahanan primer, sekunder dan tertier. Akan tetapi ia tidak dapat

merencatkan sel kanser. Jadi, *Beta vulgaris L.* adalah penting utnuk menghindarkan kanser dan untuk mengekalkan kesihatan badan yang baik.

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%	Percentage
±	plus-minus
µg	microgram
µl	microlitre
µm	micrometer
CO_2	carbon dioxide
e.g.	for example
etc	et cetera
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric Ion
$FeCl_2$	ferrous chloride
$FeCl_3$	ferric chloride
g	gram
IC_{50}	concentration that causes 50% inhibition
m	meter
mg	milligram
min	minute
ml	mililitre
mm	milimetre
ºC	degree Celcius
U	units
v/v	volume by volume
w/v	weight by volume
α	alpha
β	beta
Ω	ohm
ABTS	2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid
BHA	butylated hyroxyanisole
BHT	butylated hyroxytoluene
SOD	superoxide dismutase
CAT	catalase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPPH	1,2-diphenyl-2-picrylhydrzyl
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FBS	foetal bovine serum
FCR	Folin Ciocalteu Reagent
FRAP	ferric reducing antioxidant power
GSH	glutathione
GSH.Px:	glutathione peroxidase
GSSG	glutathione disulfide
GST	glutathione S transferase

HAT	hydrogen atom transfer
HPLC	high performance liquid chromatography
LC-MS-MS	liquid chromatography-mass spectrometry-mass spectrometry
LOOH	lipid hydroperoxides
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium
NR	neutral red
ORAC	oxygen radical absorbance capacity
PBS	phosphate buffered saline
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
rpm	revolution per minute
SD	standard deviation
SET	single electron transfer
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEAC	trolox equivalent antiradical capacity
UV	ultraviolet
XO	xanthine oxidase

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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

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.*

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*

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*

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 „bœtroot 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)

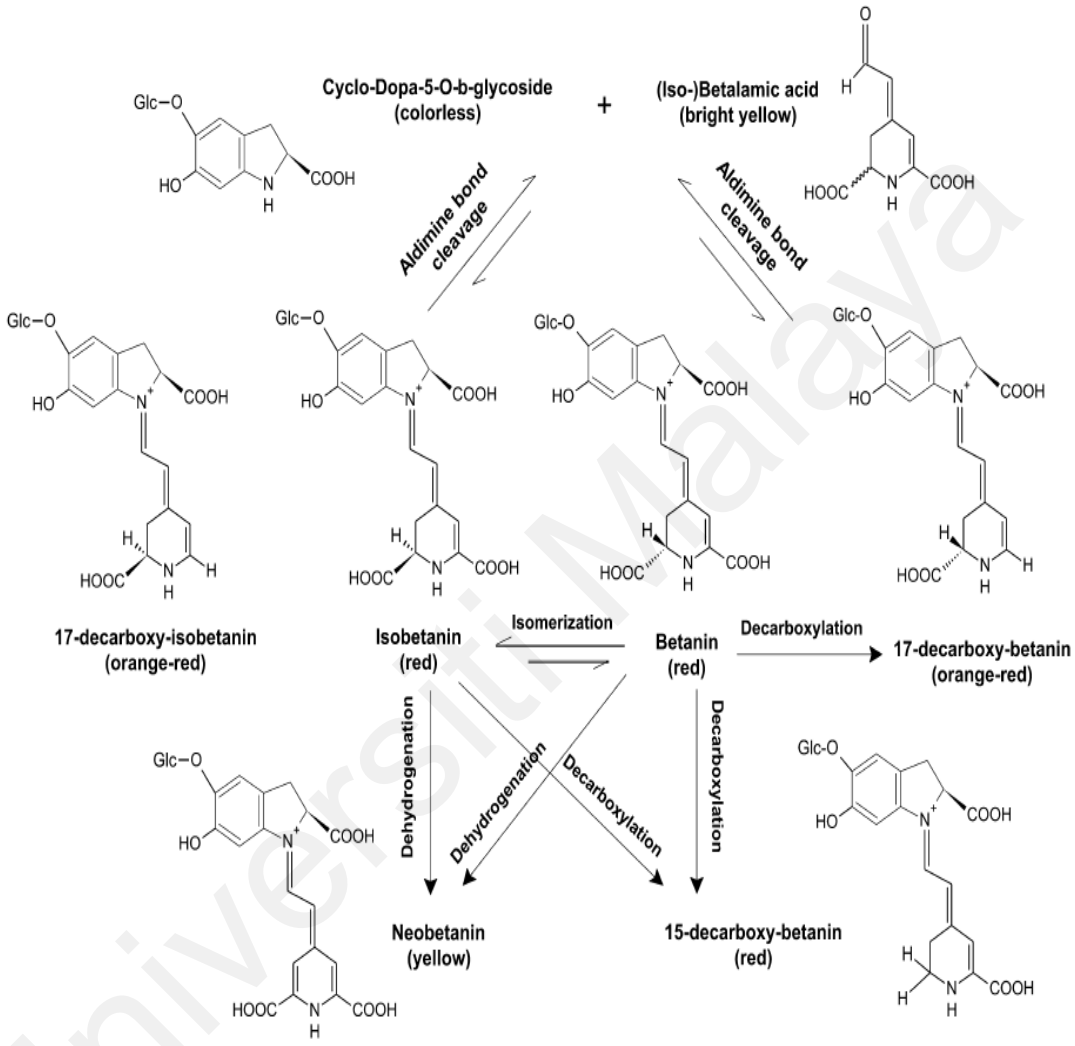


Figure 2.3: Some degradation pathways of betanin (Excerpted from Azeredo, 2009)

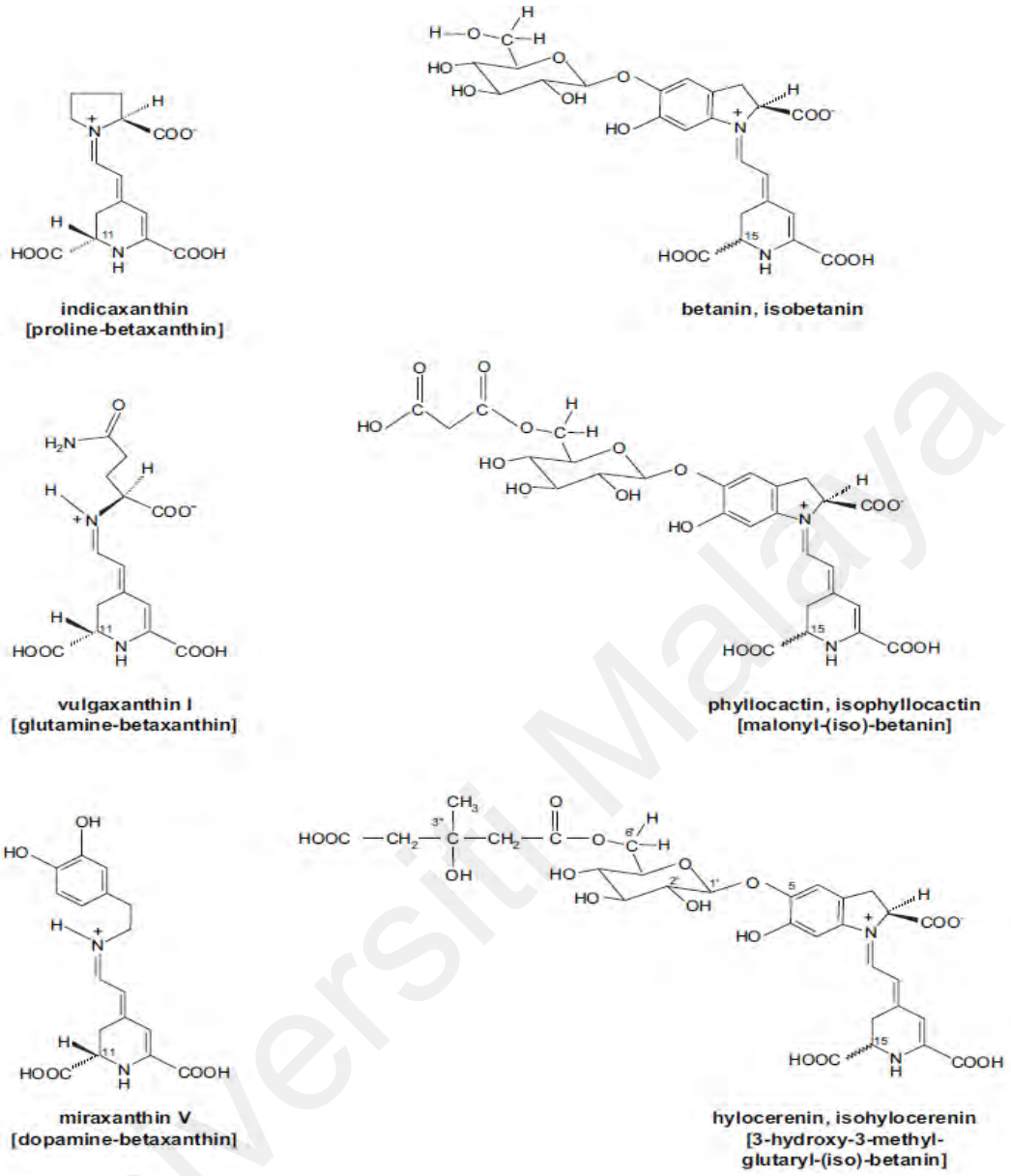


Figure 2.4: Predominant betaxanthine (left) and betacyanine (right) in fruits and vegetable from family Chenopodiaceae and Cactaceae (Excerpted from Stintzing and Carle, 2007)

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

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 (·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_2 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).

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

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

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:

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

$$ROOH + 2GSH \longrightarrow GSSG + ROH + H_2O$$ (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).

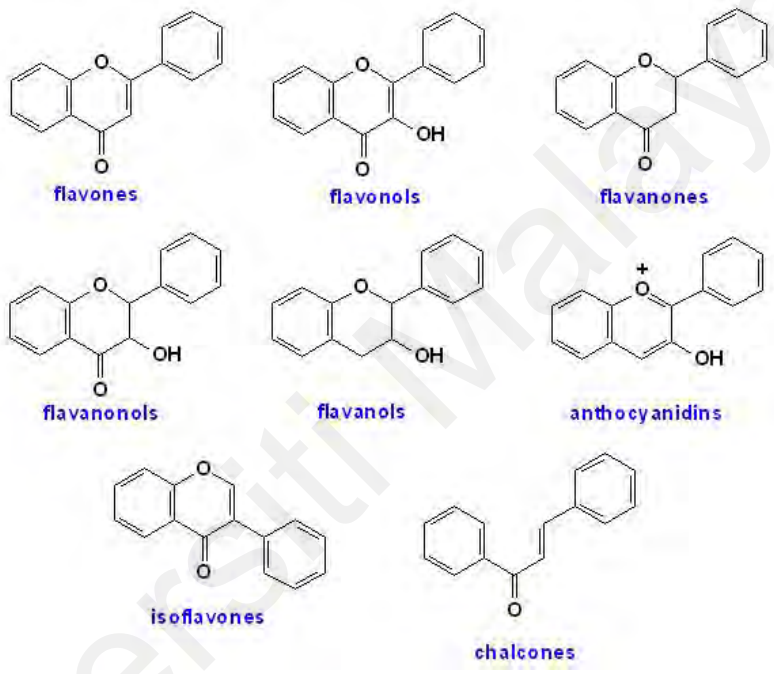


Figure 2.5: Some examples of flavonoids (excerpted from http://supplementscience.org/antioxidants.html)

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:

$$O_2 \xrightarrow{\mathbf{hv}} {}^1O_2 \text{ (singlet oxygen)}$$

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:

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

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

$O_2^{-}\cdot + H_2O_2 \longrightarrow \cdot OH + OH^{-} + O_2$ ..... (iii) (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)

$H_2O_2 + O_2^{\cdot} \longrightarrow H_2O + O_2 + \mathbf{.}OH$

$H_2O_2 \longrightarrow H_2O + \mathbf{.}OH$

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 ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} +$ **.**OH + **.**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).

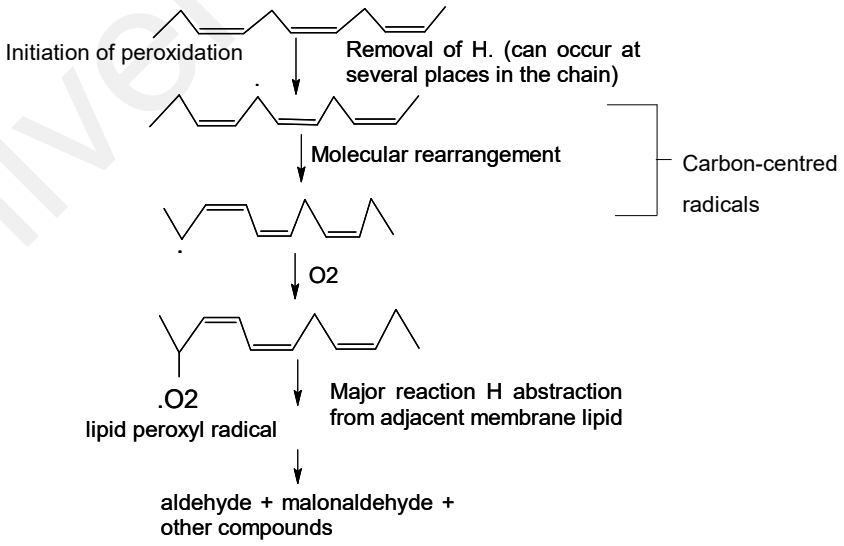


Figure 2.6: Steps of Lipid Oxidation

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 ($OH^{\cdot}$) or the peroxyl radical ($ROO^{\cdot}$). 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 ·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,

$$Z\cdot + AH \rightarrow ZH + A\cdot$$

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).

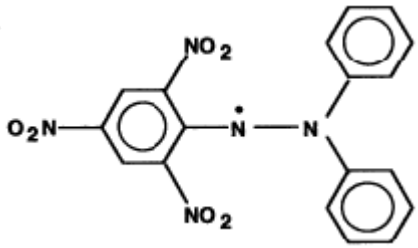


Figure 2.7: Diphenylpicrylhydrazyl (free radical)

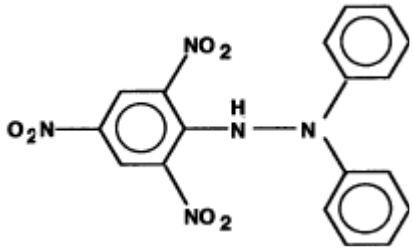


Figure 2.8: Diphenylpicrylhydrazine (non-radical)

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

$Fe(CN)_6^{4-} + Fe^{3+} \longrightarrow Fe[Fe(CN)_6]^-$ (Muhammad, 2007).

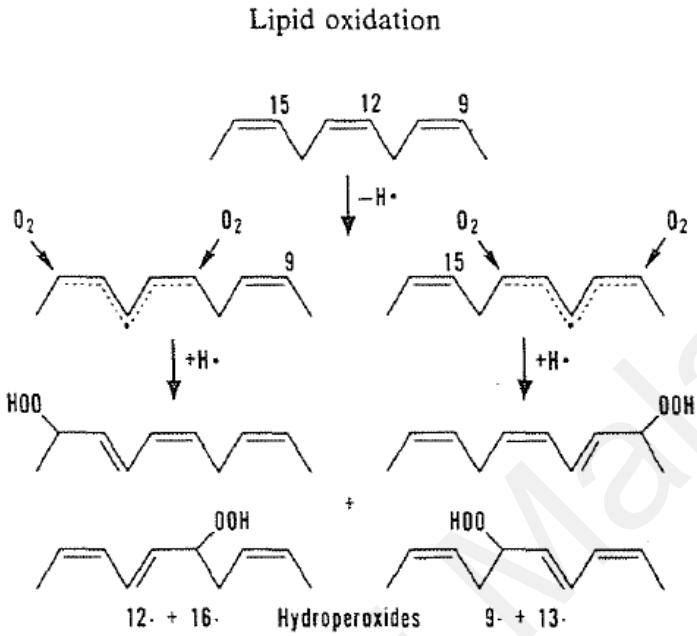
An assay like this gives an indication on how easily a given antioxidant donates electrons to reactive radical species and promotes the termination of free radical chain reactions. The ability of the antioxidant to reduce Fe^{3+} to its more active Fe^{2+} form might also be indicative of its ability 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).



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

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.

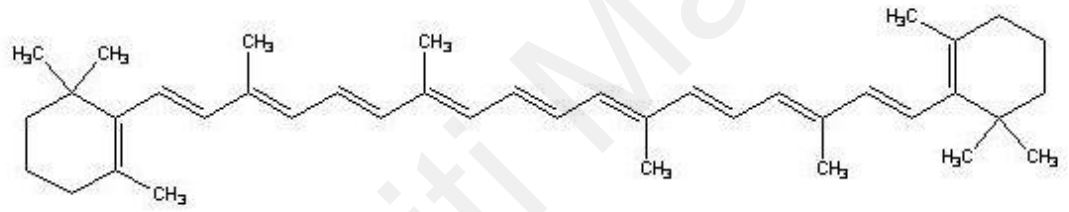


Figure 2.10: Chemical structure of β-carotene

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-Iodophenyl)-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.

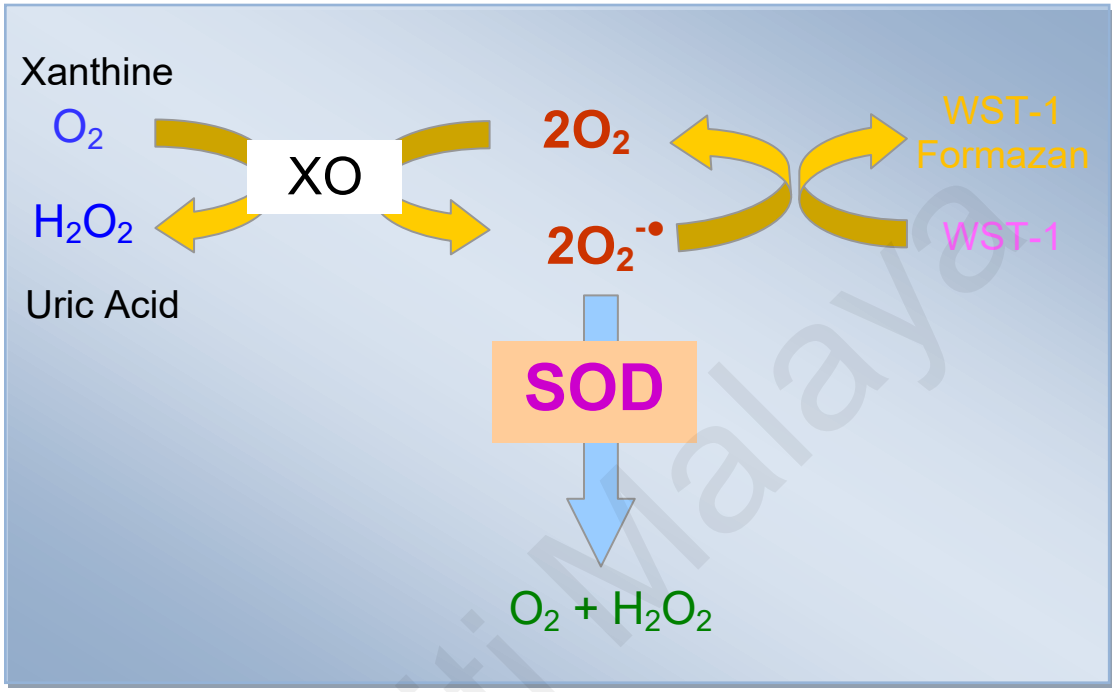


Figure 2.11: Reaction pathway of SOD activity assay

(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

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:

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

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.

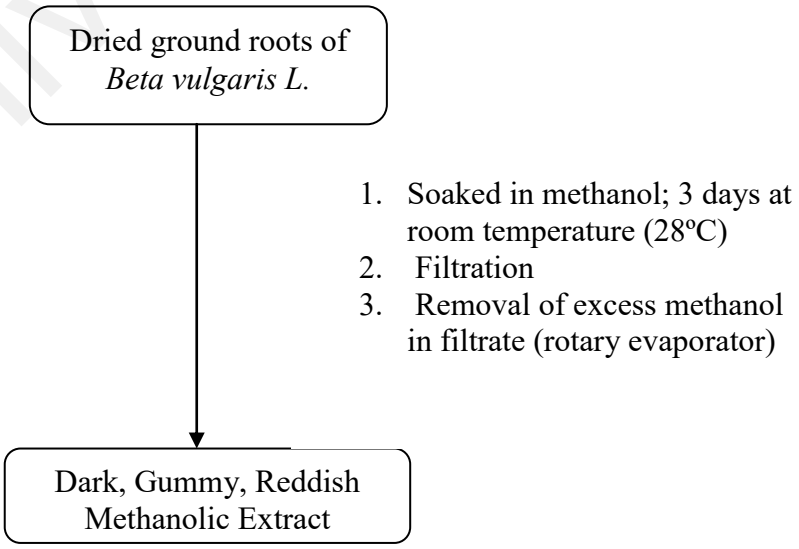


Figure 3.1: Flow chart of extraction of Crude Methanolic Extract

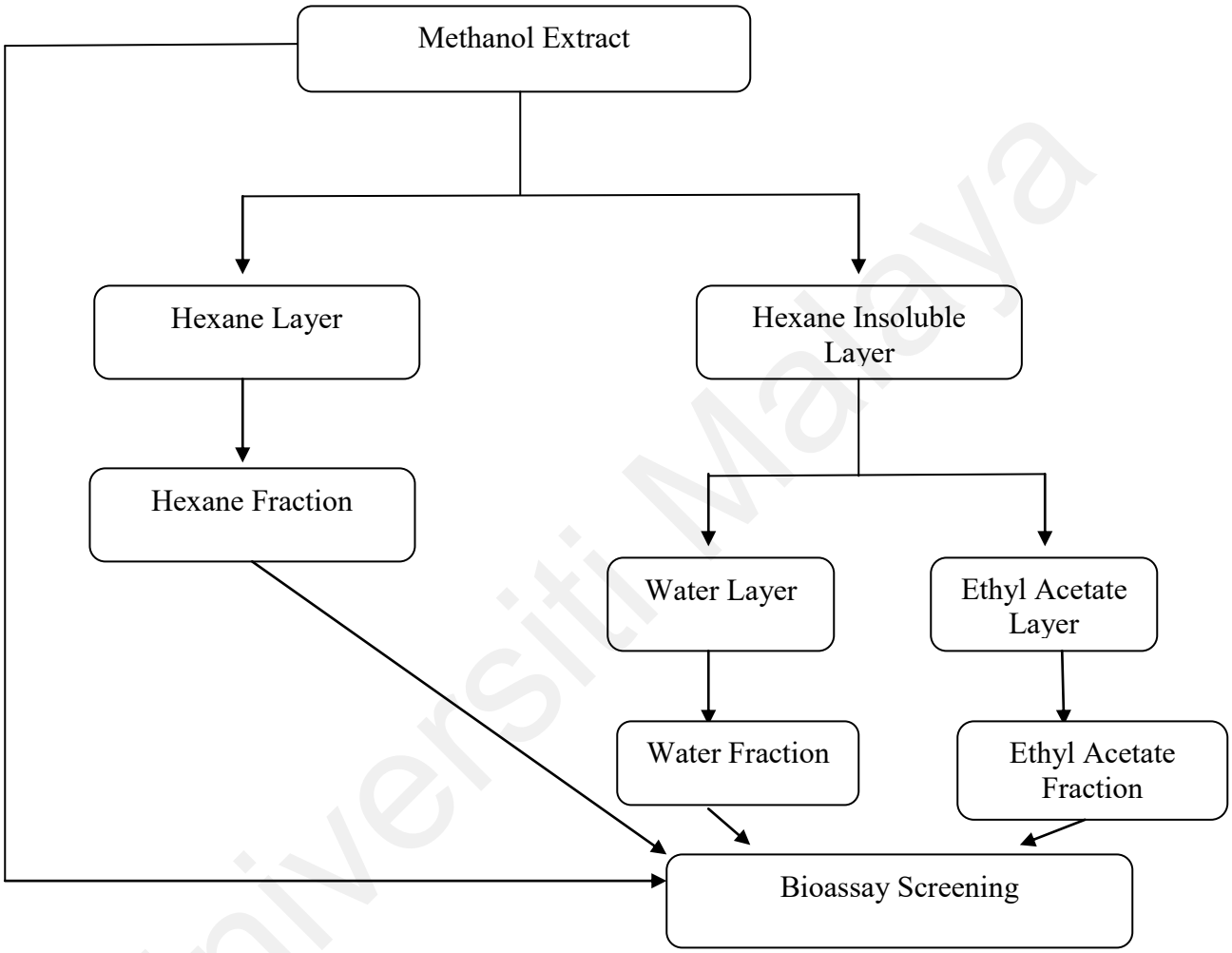


Figure 3.2: Flow chart of fractionation of Methanol Extract

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

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.

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	1.00
Emulsion of Linoleic Acid and Tween 80 in distilled oxygenated water	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

T: 120 min

$$\textbf{Antioxidant activity} = \frac{\mathbf{R}_{\text{control}} - \mathbf{R}_{\text{sample}}}{\mathbf{R}_{\text{control}}} \times \mathbf{100\ \%}$$

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:

$$\mathbf{R_{OR}} = \frac{\mathbf{R}_{\text{sample}}}{\mathbf{R}_{\text{control}}}$$

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, $FeCl_2$, and ferrozine.

Concentration of extract (mg/ml)	Volume of deionised H_2O (ml)	Volume of crude extract (µl)	Volume of $FeCl_2$ 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, $FeCl_2$, and ferrozine.

Concentration of sub-fractions (mg/ml)	Volume of deionised H_2O (ml)	Volume of crude extract (ml)	Volume of $FeCl_2$ 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-Iodophenyl)-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_2O	-	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) =

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

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:

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

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 37^{o}C 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 37^{o}C 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 37^{o}C 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^{o}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^{o}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^{o}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.

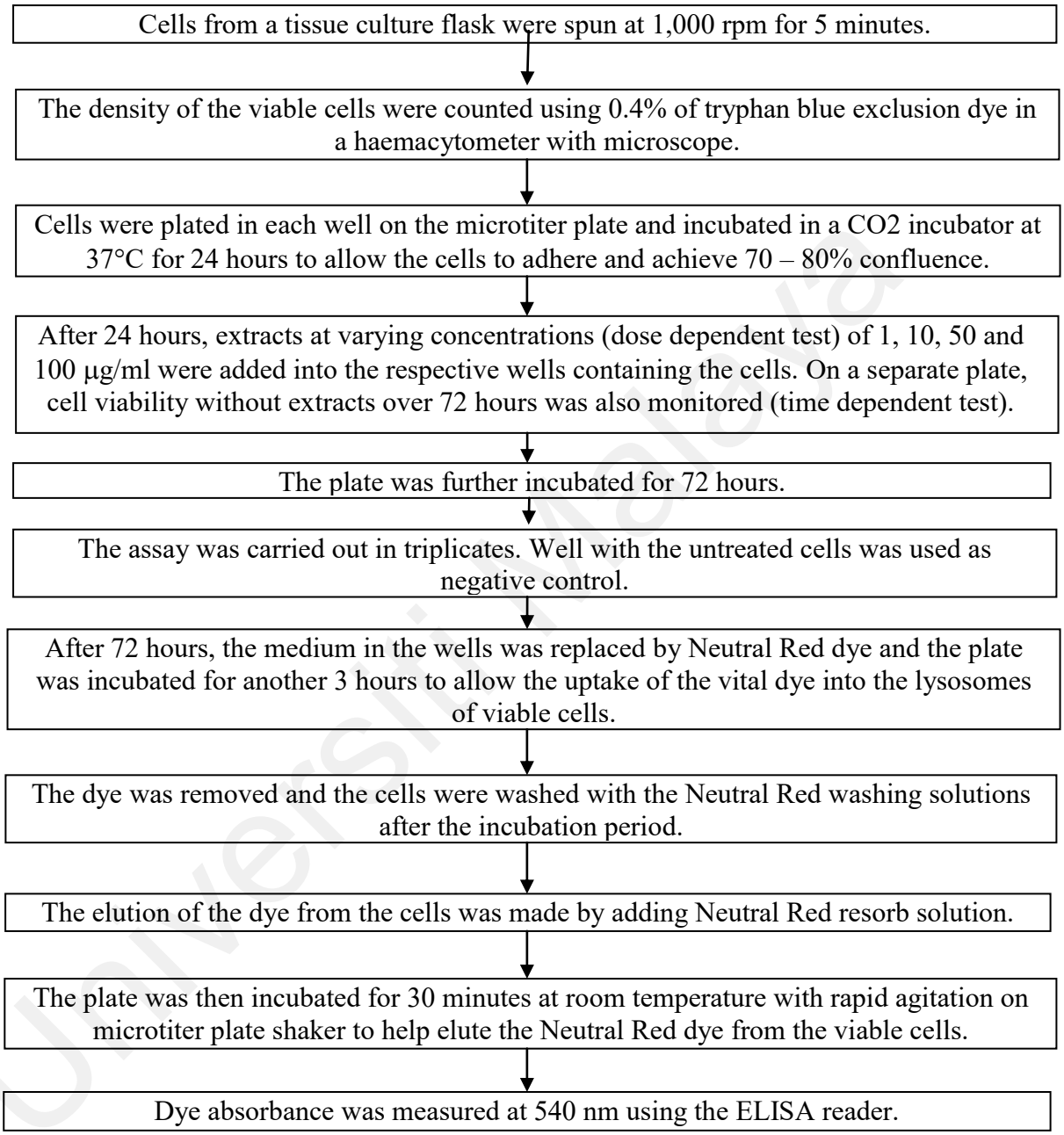


Figure 3.3: Neutral Red Cytotoxic Assay

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.

CHAPTER 4

RESULTS

4.1 Extraction

4.1.1 Extraction of *Beta vulgaris L.*

The yield of the dried sample from fresh sample is shown in Table 4.1. The yield of extracts in extraction process is tabulated in Table 4.2.

The yield of dried sample was only 9.52%. This is due to the reason that the plant is very succulent and in the drying process, all the water was lost.

Table 4.1: Weight of fresh and dried sample of *Beta vulgaris L.*

Description	Weight (g)
Fresh sample	4888.80
Dried sample	465.50
% of yield of dried sample	9.52%

From 100.00 g of dried sample, 64.70 g of methanol extract was obtained and from this, 90.00% was water fraction, 2.47 % was ethyl acetate fraction and only 0.15% was hexane fraction, Table 4.2.

Table 4.2: Yield of extracts of *Beta vulgaris L.*

Solvent	Weight (g)	Percentage (%)
Methanol	64.70 (100 g dried sample)	100.00%
Hexane	0.10	0.15
Ethyl acetate	1.60	2.47
Water	60.00	92.74

4.1.2 Extraction of *Beta vulgaris L.* juice extract

50.00 ml of *Beta vulgaris L*. juiced yielded 5.00 g of crude total juice extract after rotary evaporation.

4.2 Antioxidant Screening of Extracts and Sub-fractions of *Beta vulgaris L.*

The extracts of *Beta vulgaris L*. and its sub-fractions from HPLC were evaluated for antioxidant activities using DPPH free radical scavenging assay, reducing power assay, β-carotene bleaching assay, metal chelating assay, SOD activity assay, TBARS assay (lipid peroxidation assay), tyrosinase inhibitory assay and Folin-Ciocalteau assay (total phenolic content determination assay). Different assays were employed to study the antioxidant activity of the extracts and sub-fractions to determine the difference of the antioxidant activity in each assay. Each assay works and tests on different mechanism and acts differently on the extracts and sub-fractions. By conducting many assays that has different mechanism, we can understand in greater depth the antioxidant activity possessed by the plant. *Beta vulgaris L*. have been generally noted for its high antioxidant property but based on our knowledge, no antioxidant testing have been done on different assays to deduce the pattern of the activity exhibited by the plant on these assays.

4.2.1 DPPH (1,2-dipheyl -2-picrylhydrzyl) Free Radical Scavenging Activity

The extracts of *Beta vulgaris L*. were first pre-screened at 5.00 mg/ml and if it has positive scavenging results, it is then screened at lower concentrations. Positive scavenging results are when the extracts have 50 or more percentage of inhibition of the DPPH free radical. On the other hand, sub-fractions are not tested such, they are straight tested at low concentrations (<1.0 mg/ml), this is due to the reason that active components should possess high activity at low concentrations when is present in the isolated form. The

percentage of inhibition is plotted into a dose-response curve and the IC_{50} value is extrapolated from the graph. Free-radical scavenging activities of ascorbic acid, BHA, extracts and sub-fractions of *Beta vulgaris L.* were expressed as the percentage inhibition and results were shown below. The absorbance reading at 520 nm of the reaction mixtures of antioxidant assay for ascorbic acid, BHA, extracts, sub-fractions were taken in triplicates.

Table 4.3 Percentage inhibition of DPPH by ascorbic acid

Concentration of ascorbic acid (µg/ml)	Percentage inhibition (%)*
200.00	90.46 ± 0.17
100.00	90.11 ± 0.09
50.00	89.35 ± 0.37
25.00	77.70 ± 1.33
12.50	63.35 ± 3.86
6.25	35.00 ± 3.48
3.12	10.89 ± 0.97
1.56	8.73 ± 0.57

* Percentage inhibition as DPPH free radical scavenging activities as a mean of triplicate experiments ± standard deviation (SD)

Table 4.4 Percentage inhibition of DPPH by BHA

Concentration of BHA (µg/ml)	Percentage inhibition (%)*
200.00	88.49 ± 0.23
100.00	85.20 ± 1.09
50.00	82.93 ± 0.36
25.00	78.62 ± 0.68
12.50	45.10 ± 2.87
6.25	22.31 ± 3.01
3.12	8.69 ± 0.25
1.56	6.90 ± 2.12

* Percentage inhibition as DPPH free radical scavenging activities as a mean of triplicate experiments ± standard deviation (SD)

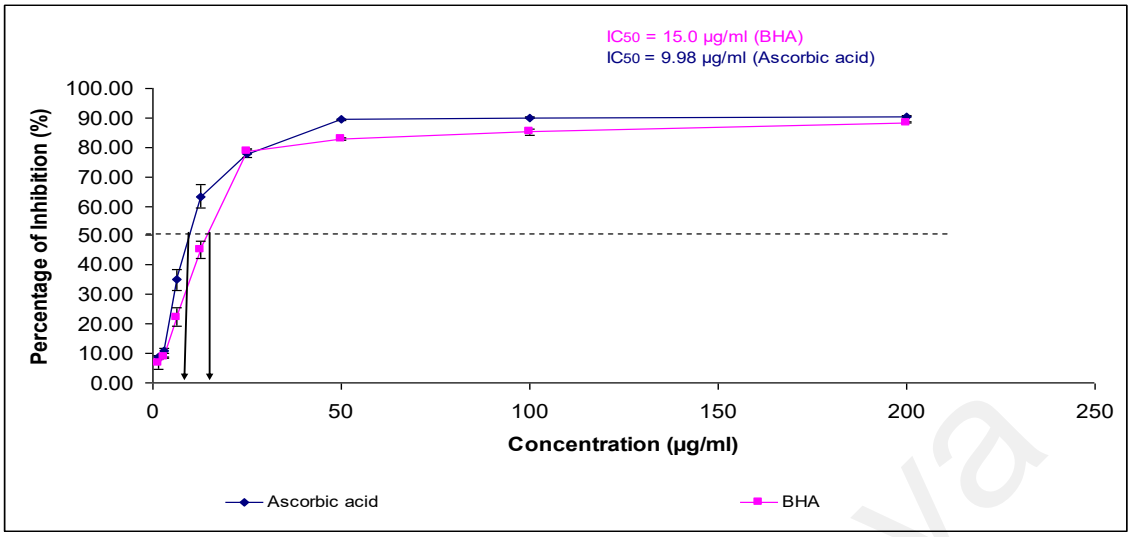


Figure 4.1: The determination of IC_{50} values of ascorbic acid and BHA as positive standard references in DPPH free radical scavenging assay

ii) Extracts of *Beta vulgaris L.* (Methanol extract, Hexane fraction, Ethyl acetate fraction, Water fraction and Juice Extract and Fractions)

Pre-screening of Extracts and Fractions of *Beta vulgaris L.*

The pre-screening results of crude methanol extract and its fractions were tabulated in Table 4.5. Pre-screening results showed that all the five extracts of *Beta vulgaris L.* had a percentage of inhibition higher than 50% at the highest concentration i.e., 5.00 mg/ml. It was further screened at lower concentrations.

Table 4.5: Pre-screening of *Beta vulgaris L.* extracts on DPPH Free Radical Scavenging Assay

Concentration of Extract (mg/ml)	Percentage inhibition (%)*				
	Methanol Extract	Hexane Fraction	Ethyl acetate Fraction	Water Fraction	Juice Extract
5.00	96.54 ± 0.08	86.31 ± 1.22	85.06 ± 1.12	81.69 ± 0.07	97.72 ± 1.91
Control	-	-	-	-	-

* Percentage inhibition as DPPH scavenging activities as a mean of triplicate experiments ± standard deviation (SD)

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

The extracts of *Beta vulgaris L.* were screened at concentrations of 5.00, 4.00, 3.00, 2.00 and 1.00 mg/ml. The percentage inhibition obtained were averaged, tabulated and shown in Table 4.6 and in Figure 4.3.

Table 4.6: Screening of *Beta vulgaris L.* extracts on DPPH Free Radical Scavenging Assay

Concentration of Extract (mg/ml)	Percentage Inhibition (%)*				
	Methanol Extract	Hexane fraction	Ethyl acetate fraction	Water fraction	Juice Extract
5.00	96.54 ± 0.08	86.31 ± 1.22	85.06 ± 1.12	81.69 ± 0.07	97.72 ± 1.91
4.00	94.45 ± 0.65	84.57 ± 0.02	84.17 ± 0.49	81.01 ± 0.16	95.09 ± 4.99
3.00	91.30 ± 0.19	83.00 ± 0.11	84.16 ± 0.94	80.27 ± 0.24	95.51 ± 0.52
2.00	88.78 ± 0.10	76.99 ± 2.24	83.99 ± 0.05	78.22 ± 1.93	92.12 ± 1.39
1.00	85.41 ± 0.04	16.47 ± 1.07	83.57 ± 0.90	11.19 ± 2.49	60.24 ± 2.02
Control	-	-	-	-	-

* Percentage inhibition as DPPH scavenging activities as a mean of triplicate experiments ± standard deviation (SD).

iii) Testing of active extracts (methanol extract, ethyl acetate fraction and juice extracts) at lower concentrations

Testing at lower concentrations (from 1.00 mg/ml to 0.05 mg/ml) was done to extracts which had percentage inhibition higher than 50% at 1.00 mg/ml concentration. This was done to determine the IC_{50} value. In this case, methanol, ethyl acetate and juice extract was tested and was tabulated at Table 4.7. The IC_{50} values were determined from Figure 4.2 for each extracts.

Table 4.7: Screening of active extracts of *Beta vulgaris L.* by the DPPH Free Radical Scavenging Assay

Concentration of Extract/Fraction (mg/ml)	Percentage Inhibition (%)*		
	Methanol Extract	Ethyl acetate Fraction	Juice Extract
1.00	85.41 ± 0.04	83.57 ± 0.90	60.24 ± 0.69
0.80	84.71 ± 0.61	80.63 ± 1.16	58.68 ± 1.60
0.60	54.61 ± 1.77	78.70 ± 1.99	50.46 ±2.86
0.40	32.41 ± 1.11	74.84 ± 2.86	27.10 ± 3.17
0.20	10.55 ± 2.40	17.44 ± 1.84	19.89 ± 3.40
0.10	7.96 ± 0.58	12.67 ± 3.33	12.70 ± 1.83
0.05	9.15 ± 3.52	6.07 ± 3.55	7.99 ± 2.72
Control	-	-	-

* Percentage inhibition as DPPH scavenging activities as a mean of triplicate experiments ± standard deviation (SD)

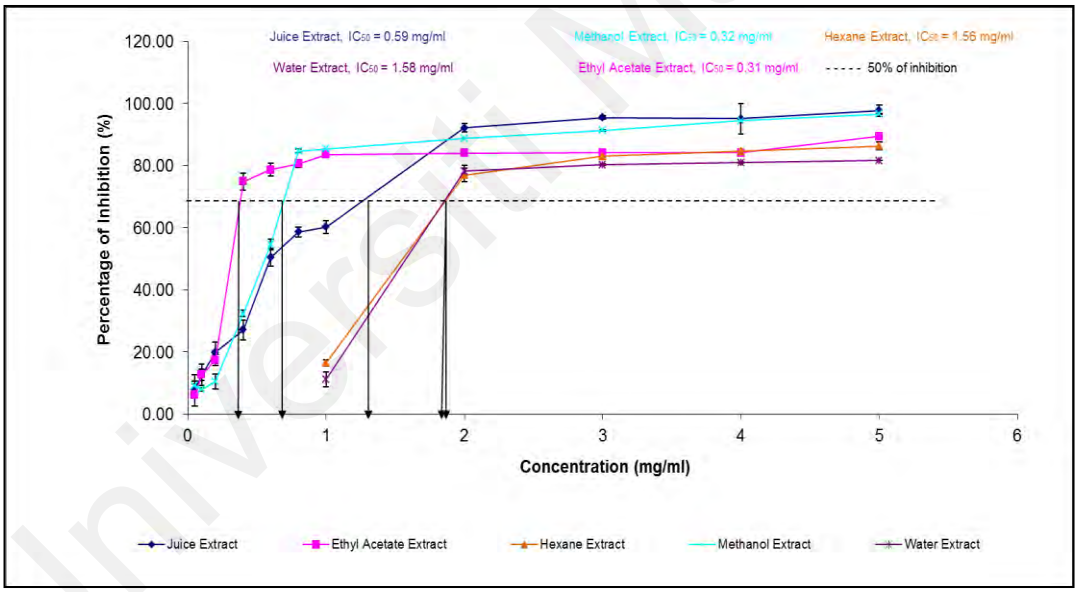


Figure 4.2: The determination of IC_{50} values of the active crude extracts of *Beta vulgaris L.* in the DPPH free radical scavenging assay

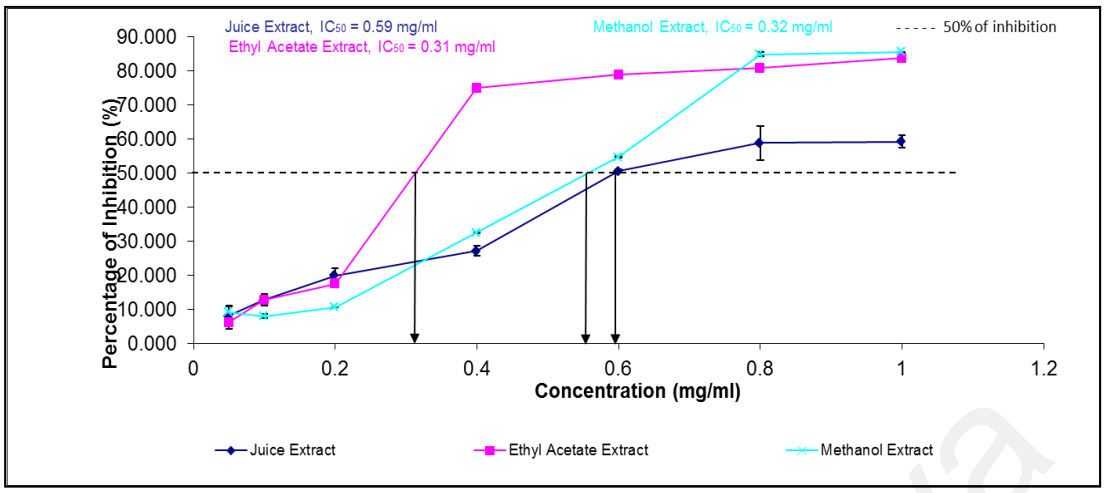


Figure 4.3: The determination of IC_{50} values of active crude extracts of *Beta vulgaris L.* which was tested at lower concentrations in the DPPH free radical scavenging activity assay

iv) Comparison IC_{50} Value Between Methanol extract, Hexane fraction, Ethyl Acetate fraction, Water fraction and Juice Extract of *Beta vulgaris L* with standards on DPPH Free Radical Scavenging Activity

The comparison of IC_{50} value within the extracts showed that the ethyl acetate fraction had the lowest IC_{50} value (0.310 mg/ml) and hence the highest antioxidant activity. This is followed by the methanol, juice and hexane fraction with IC_{50} values of 0.315, 0.590 and 1.560 mg/ml respectively. The water fraction gave a value 1.575 mg/ml and hence, had the lowest antioxidant activity. However, standards BHA and ascorbic acid exhibited excellent IC_{50} values at 0.015 and 0.010 mg/ml respectively. Comparison between the extracts and fractions showed that the fraction ethyl acetate has lower IC_{50} value as compared to crude extracts but all the other fractions has higher IC_{50} value as compared to the crude extracts.

Table 4.8 Summary of IC_{50} values of the active crude extracts and positive standard, ascorbic acid and BHA in the DPPH free radical scavenging activity assay

Extracts of *Beta vulgaris L.*	IC_{50} (mg/ml)
Methanol extract	0.320
Ethyl Acetate fraction	0.310
Hexane fraction	1.560
Water fraction	1.580
Juice extract	0.590
BHA (standard)	0.015
Ascorbic acid (standard)	0.010

v) Screening of sub-fractions from ethyl acetate fraction and juice extracts of *Beta vulgaris L.*

The sub-fractions were obtained through separation using HPLC technique. The sub-fractions from ethyl acetate fraction were represented as ET1, ET2 and ET3 whereas the fractions from juice extract were named as purple and yellow fractions. The sub-fractions were tested in concentrations ranging from 1.00 to 0.05 mg/ml. The results of the DPPH free radical scavenging activity of the sub-fractions were tabulated at Table 4.9.

ET1 had the highest percentage inhibition at 1.00 mg/ml (80.44% ± 0.35) and lowest percentage inhibition at concentration 0.05 mg/ml (0%). The percentage of inhibition decreased gradually with decreasing concentrations. ET2 also had the highest percentage of inhibition at 1.00 mg/ml (77.67% ± 0.51) and lowest percentage at 0.05 mg/ml (0%). ET3 also had the same effect with 77.67% ± 0.04 to be the highest percentage of inhibition at 1.00 mg/ml and lowest percentage of inhibition at 0% at 0.05 mg/ml. For purple fraction, highest percentage of inhibition was 128.78% ± 0.82 at 1.00 mg/ml and lowest percentage of inhibition at 0.05 mg/ml (0%). As for yellow fraction, the highest inhibition percentage was at 1.00 mg/ml (81.78% ± 0.35), and lowest at 0.10 mg/ml with 19.95% ± 0.07 inhibition.

All fractions exhibited dose-dependent response. Comparison within the fraction shows that, purple fraction had the highest percentage of inhibition (128.78% ± 0.82) at concentration 1.00 mg/ml. As for the lowest percentage of inhibition at concentration 0.05 mg/ml was exhibited by fraction ET2. IC_{50} values were determined based on Figure 4.4.

Table 4.9: The screening of sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* on DPPH free radical scavenging assay

Concentration of Sub-fractions (mg/ml)	Percentage Inhibition (%)*				
	ET1	ET2	ET3	Purple	Yellow
1.00	80.44 ± 0.35	77.02 ± 0.51	77.67 ± 0.04	128.78 ± 0.82	81.78 ± 0.35
0.80	77.49 ± 0.76	72.18 ± 0.08	29.48 ± 2.33	139.29 ± 0.30	65.06 ± 0.17
0.60	74.15 ± 0.94	72.06 ± 0.06	17.92 ± 3.82	131.85 ± 2.26	53.28 ± 0.06
0.40	25.62 ± 2.26	39.76 ± 0.05	2.19 ± 3.33	90.24 ± 0.71	31.36 ± 0.21
0.20	0	7.66 ± 0.07	8.31 ± 5.19	61.26 ± 0.19	32.21 ± 0.52
0.10	0	2.61 ± 0.09	1.55 ± 9.53	44.75 ± 0.32	19.95 ± 0.07
0.05	0	0	0	0	22.60 ± 0.14
Control	-	-	-	-	-

* Percentage inhibition as DPPH scavenging activities as a mean of triplicate experiments ± standard deviation (SD)

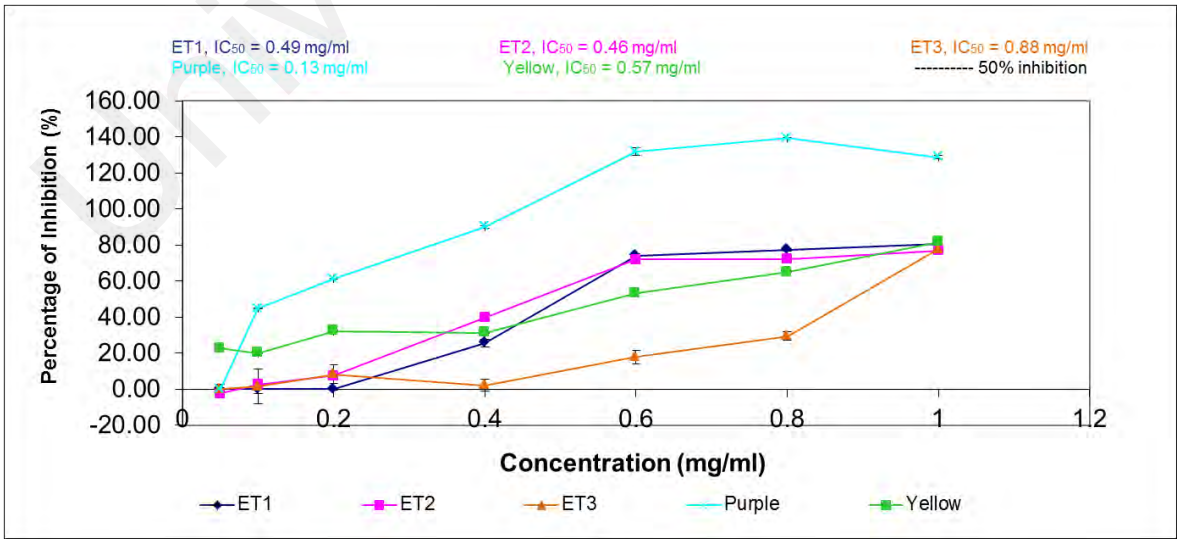


Figure 4.4: The determination of IC_{50} values of sub-fractions of ethyl acetate fraction and juice extracts of *Beta vulgaris L.* in DPPH free radical scavenging assay

vi) Comparison of IC_{50} value of fractions isolated from ethyl acetate fraction (ET1, ET2 and ET3) and juice extract (Purple and Yellow) with standards on DPPH free radical scavenging activity.

The comparison between the fractions shows that the Purple fraction has the lowest IC_{50} value (0.13 mg/ml) and hence the highest radical scavenging activity compared to the other fractions. This is followed by ET2 fraction which has an IC_{50} value of (0.46 mg/ml), ET1 fraction (0.49 mg/ml) and Yellow fraction (0.57). The highest IC_{50} value belongs to ET3 fraction with the value 0.88 mg/ml. As compared to the positive standards, BHA and ascorbic acid, the IC_{50} values obtained by the fractions were quite high. The results are summarized in Table 4.10.

Table 4.10: Summary of IC_{50} values of the sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* and positive standard, ascorbic acid and BHA in the DPPH free radical scavenging activity assay

Sub-fractions	IC_{50} (mg/ml)
ET1	0.490
ET2	0.460
ET3	0.880
Yellow	0.570
Purple	0.130
BHA	0.015
Ascorbic acid	0.010

4.2.2 Reducing Power Assay

The reducing power in this study was determined according to the method of Oyaizu (1986). Ascorbic acid and butylated hydroxyanisole (BHA) were used as positive references standard antioxidant compounds for comparison with the samples extracts.

i) BHA and Ascorbic acid as positive standard

Reductive capabilities of the plant extracts were evaluated with reference to BHA and ascorbic acid. Compounds at higher concentration had greater reductive capabilities and gave higher absorbance of the reaction mixture. Table 4.11, 4.12 and Figure 4.5 below shows the reducing power of BHA and ascorbic acid at varying concentrations. The reducing power of both standard compounds showed a small increment with increasing concentrations. Both standards exhibited very high absorbance values at 700 nm. The data showed that the reducing power of BHA is slightly higher than standard ascorbic acid. The highest reducing power of BHA and ascorbic acid was at concentration 20.000 mg/ml. The lowest reducing power was at 5.000 mg/ml.

The reducing power of BHA was tested from highest (20.000 mg/ml) to lowest (0.010 mg/ml) concentration for comparison with the crude extracts, fractions, and sub-fractions. The highest reductive capabilities were observed at concentration 20.000 mg/ml (3.465 ± 0.010) and the lowest reductive capabilities at 0.010 mg/ml with lowest absorbance value (0.131 ± 0.001).

The reducing power of ascorbic acid was tested from highest (20.000 mg/ml) to lowest (0.010 mg/ml) concentration for comparison with the crude methanol extract, fractions, and sub-fractions. The highest reductive capabilities were observed at concentration 20.000 mg/ml with an average absorbance value of 3.14 and the lowest reductive capabilities at 0.010 mg/ml with lowest absorbance value (0.131). The absorbance value increases with increasing concentrations, and hence the reducing capability.

Table 4.11: The reducing power of BHA at absorbance of 700 nm

Concentration of BHA (mg/ml)	Absorbance at 700nm*
20.000	3.465 ± 0.008
15.000	3.285 ± 0.063
10.000	3.175 ± 0.023
5.000	3.145 ± 0.010
1.000	2.897 ± 0.030
0.500	2.597 ± 0.024
0.100	0.818 ± 0.002
0.050	0.468 ± 0.000
0.025	0.219 ± 0.006
0.010	0.131 ± 0.001

* Absorbance value at 700 nm of reducing power assay as a mean of triplicate experiments ± standard deviation (SD)

Table 4.12: The reducing power of ascorbic acid at absorbance of 700 nm

Concentration of ascorbic acid (mg/ml)	Absorbance at 700nm*
20.000	3.142 ± 0.017
15.000	2.933 ± 0.001
10.000	2.853 ± 0.001
5.000	2.791 ± 0.039
1.000	2.853 ± 0.006
0.500	2.708 ± 0.001
0.100	0.692 ± 0.000
0.050	0.335 ± 0.001
0.025	0.194 ± 0.002
0.010	0.092 ± 0.001

* Absorbance value at 700 nm of reducing power assay as a mean of triplicate experiments ± standard deviation (SD)

ii) Comparison of reducing power activity between the positive standards

The comparison of reducing power activity between the positive standards, BHA and ascorbic acid is shown in Figure 4.5. At lower concentrations, 0.010 mg/ml to 1.000 mg/ml, the reducing power activity exhibited was almost similar to each other. But, towards higher concentrations (5.000-20.000 mg/ml), BHA exhibited slightly higher reductive capabilities. As a whole, both of the standards had high reductive capabilities and serve their purpose as positive reference. The reducing power of BHA and ascorbic acid was in dose dependent manner.

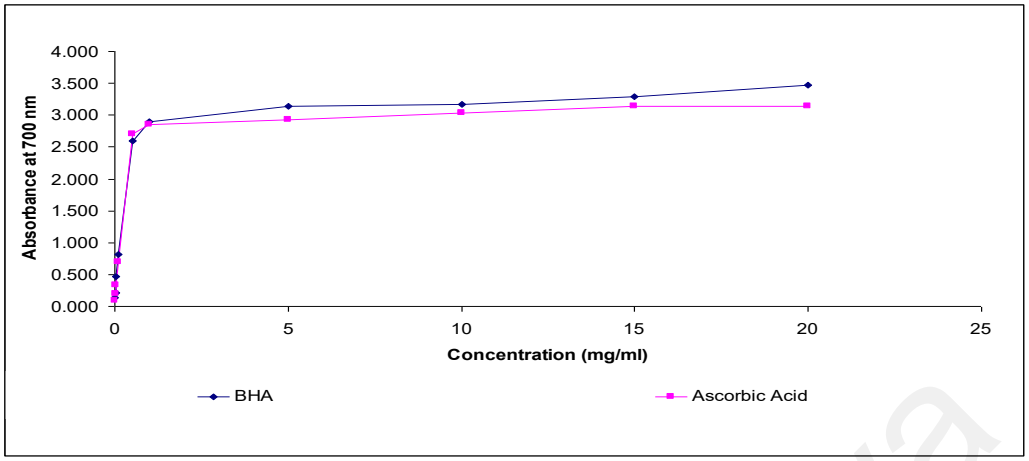


Figure 4.5: Comparison between the reductive ability of BHA and ascorbic acid

iii) Reducing power of extracts of *Beta vulgaris L.*

As illustrated in Figure 4.6, all the extracts of *Beta vulgaris L*. exhibited dose-dependent manner reductive capabilities. Ethyl acetate fraction showed even better reductive capability than the standards ascorbic acid and BHA at higher concentrations (10.000-20.000 mg/ml) with absorbance value 3.292A ± 0.000 to 3.767A ± 0.002. In fact, this was the highest reducing power noted among the extracts. At concentration 5 mg/ml, BHA had the highest absorbance value (2.791A ± 0.039), followed by ascorbic acid and then ethyl acetate fraction. Methanol extract with absorbance value ranging 2.249A ± 0.070 to 3.275A ± 0.002 and water fraction with absorbance value ranging from 1.949A ± 0.021 to 3.287A ± 0.041 had almost similar activities. Methanol and water fractions exhibited higher reductive capabilities than ascorbic acid at concentrations 10-20 mg/ml. Hexane fraction on the other hand had moderate reducing capabilities among the extracts with absorbance value 1.408A ± 0.172 to 3.290A ± 0.000. The lowest reducing capabilities among the extracts were exhibited by juice extract with absorbance value ranging from 1.438A ± 0.013 to 0.264A ± 0.001.

Table 4.13: The reducing power of extracts of *Beta vulgaris L.* at absorbance of 700 nm

Concentration of Extract (mg/ml)	Absorbance at 700 nm*				
	Methanol Extract	Hexane Fraction	Ethyl acetate Fraction	Water Fraction	Juice Extract
20.000	3.275 ± 0.002	3.290 ± 0.000	3.767 ± 0.002	3.287 ± 0.041	1.438 ± 0.013
15.000	3.138 ± 0.017	2.454 ± 0.054	3.464 ± 0.007	3.197 ± 0.072	1.009 ± 0.004
10.000	3.049 ± 0.003	1.853 ± 0.340	3.292 ± 0.000	3.091 ± 0.055	0.697 ± 0.004
5.000	2.249 ± 0.070	1.408 ± 0.172	2.816 ±0.049	1.949 ± 0.021	0.264 ± 0.001
Control	-	-	-	-	-

* Absorbance value at 700 nm of reducing power assay as a mean of triplicate experiments ± standard deviation (SD)

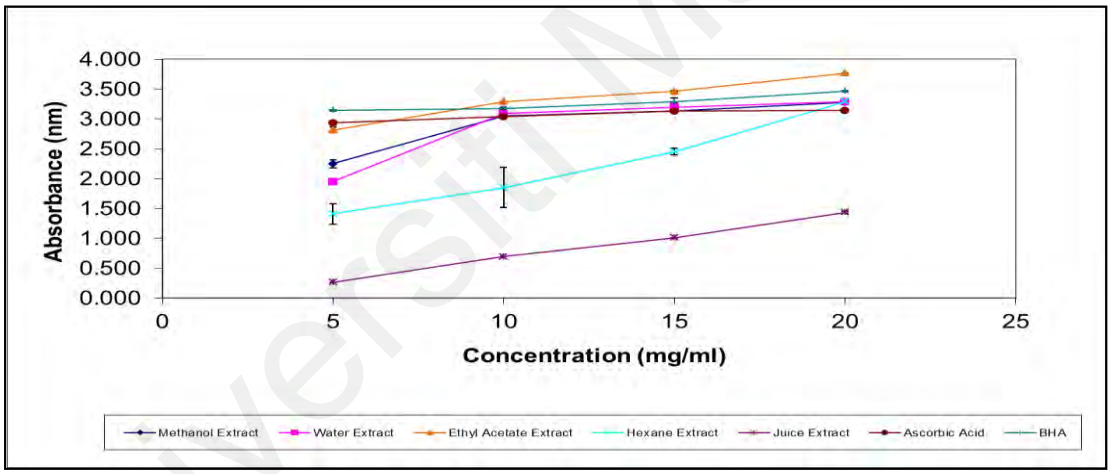


Figure 4.6: Comparison of reducing power activity of various extracts of *Beta vulgaris* and standards

iv) Reducing Power Activity of Sub-fractions

These fractions were tested at a lower concentration compared to the crude extracts.

a) Reducing Power of Fractions Isolated from Ethyl Acetate Fraction and Juice Extracts

The reducing power of the isolated sub-fractions were tabulated in Table 4.14 and portrayed in Figure 4.7. As shown in Figure 4.7, all the sub-fractions exhibited low reducing power at all concentrations (0.010 to 5.000 mg/ml) compared to positive standards

(BHA and ascorbic acid). BHA and ascorbic acid exhibited almost similar reductive activity at low concentrations. Within the extracts, the purple fraction had the highest reducing capacity. The reducing power of the purple fraction increased with increasing concentration i.e. from concentration 0.010 to 5.000 mg/ml, an absorbance value ranging from 0.045A ± 0.001 to 2.633A ± 0.016 was noted. Dose-dependent manner was exhibited by this fraction. Fraction ET1 of ethyl acetate fraction is only slightly lower in its reducing power from the Purple fraction. It exerted a dose dependent pattern in the reducing capabilities with increase from 0.064A ± 0.001 to 2.546A ± 0.006 in the absorbance value. Fraction ET3 and ET2 had almost similar reducing power at lower concentrations (0.01 to 1.000 mg/ml). For concentrations higher than 1.000 mg/ml, ET3 had a higher reducing power than ET2. Both the fractions had dose-dependent reducing power. Lowest reducing power among sub-fractions were exhibited by the yellow fraction. The range of the absorbance value was 0.004A ± 0.001 to 0.263A ± 0.002 for the concentration 0.010 to 5.000 mg/ml. The increase in absorbance value was not drastic for this fraction. However, it exhibited dose-dependent relationship in its reductive capabilities. All the fractions almost had a linear increase in the absorbance value as the concentration increases. This could be viewed clearly in Figure 4.7.

Table 4.14: The reducing power of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.* at absorbance of 700 nm

Concentration of Fractions (mg/ml)	Absorbance at 700 nm*				
	ET1	ET2	ET3	Purple	Yellow
5.000	2.546 ± 0.006	1.257 ± 0.005	2.006 ± 0.002	2.663 ± 0.016	0.263 ± 0.002
1.000	0.786 ± 0.002	0.513 ± 0.001	0.496 ± 0.002	0.911 ± 0.001	0.071 ± 0.001
0.500	0.492 ± 0.004	0.291 ± 0.001	0.274 ±0.001	0.475 ± 0.003	0.043 ± 0.000
0.100	0.147 ± 0.001	0.082 ± 0.000	0.099 ± 0.001	0.127 ± 0.001	0.014 ± 0.001
0.050	0.136 ± 0.001	0.055 ± 0.002	0.049 ± 0.001	0.091 ± 0.001	0.010 ± 0.000
0.025	0.105 ± 0.001	0.035 ± 0.000	0.045 ± 0.001	0.047 ± 0.002	0.008 ± 0.002
0.010	0.064 ± 0.001	0.024 ± 0.002	0.021 ± 0.000	0.045 ± 0.001	0.004 ± 0.001
	-	-	-	-	-

Absorbance value at 700 nm of reducing power assay as a mean of triplicate experiments ± SD

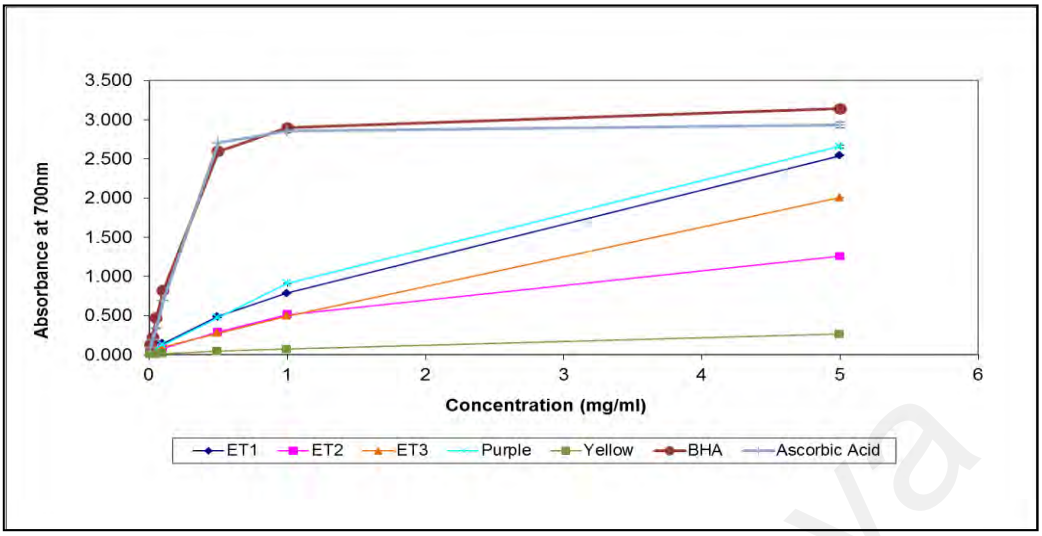


Figure 4.7: Comparison of reductive ability of isolated sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* and standards

4.2.3 β-Carotene Bleaching Assay

β-Carotene bleaching assay tests the ability of a test samples to reduce oxidation towards the β-carotene molecules that is caused by hydroperoxides formed by linoleic acids. In presence of antioxidants in test samples, the oxidation towards β-carotene molecules can be hindered. 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 (Jayaprakasha *et al.,* 2001).

i) BHA and ascorbic acid as positive standards

The inhibition of oxidative damage to β-carotene molecules were evaluated with reference to positive standards BHA and ascorbic acid. The reaction mixture of the standards retained its orange shade without much bleaching to a certain degree at various concentrations. Compounds of higher concentration had higher inhibition towards oxidative damage and could retain its orange shade. There was a decrease in absorbance value if there is no antioxidant present in the reaction system. Table 4.15, 4.16 and Figure 4.8 below shows the β-carotene bleaching at varying concentrations of standard. The inhibition in bleaching

(antioxidant activity) showed an increment with increasing concentrations for both the standards. The antioxidant activity exhibited by BHA was higher than that of ascorbic acid. But at concentration 20 mg/ml, they both had almost the same antioxidant activity. Both the standards exhibited dose-dependent manner antioxidant activity.

a) BHA as the positive standard reference

BHA was tested from a range of concentration from 0.010 to 20.000 mg/ml. BHA had the highest percentage of antioxidant activity at 20.000 mg/ml i.e. 99.760% ± 0.004 and the lowest percentage of antioxidant activity at 0.010 mg/ml (36.470% ± 0.008). Overall BHA had a good inhibition towards bleaching of β-carotene and acts well as the positive standard.

Table 4.15: Antioxidant activity of BHA determined by the β-carotene bleaching assay

Concentration of BHA (mg/ml)	Percentage of Antioxidant Activity*
20.000	99.760 ± 0.004
16.000	94.780 ± 0.071
12.000	92.620 ± 0.086
8.000	91.070 ± 0.000
4.000	90.240 ± 0.004
2.000	87.110 ± 0.004
1.000	80.890 ± 0.003
0.500	76.860 ± 0.005
0.100	69.320 ± 0.018
0.050	49.270 ± 0.014
0.025	46.300 ± 0.006
0.010	36.470 ± 0.008

* Percentage of antioxidant activities of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

b) Ascorbic acid as a positive standard

The degree of inhibition towards oxidation was tested from 0.010 to 5.000 mg/ml for ascorbic acid. It had the highest percentage of antioxidant activity at 20.000 mg/ml (99.610% ± 0.002) and lowest percentage of antioxidant activity at 0.025 mg/ml with

27.990% ± 0.005. At the lowest concentration tested (0.010 mg/ml), 0% antioxidant activity was exhibited.

Table 4.16: Antioxidant activity of ascorbic acid determined by the β-carotene bleaching assay

Concentration of Ascorbic Acid (mg/ml)	Percentage of Antioxidant Activity*
20.000	99.610 ± 0.002
16.000	85.060 ± 0.002
12.000	85.030 ± 0.006
8.000	84.450 ± 0.003
4.000	83.850 ± 0.002
2.000	82.380 ± 0.008
1.000	79.530 ± 0.002
0.500	53.940 ± 0.003
0.100	52.320 ± 0.006
0.050	35.320 ± 0.009
0.025	27.990 ± 0.005
0.010	0

* Percentage of antioxidant activities of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

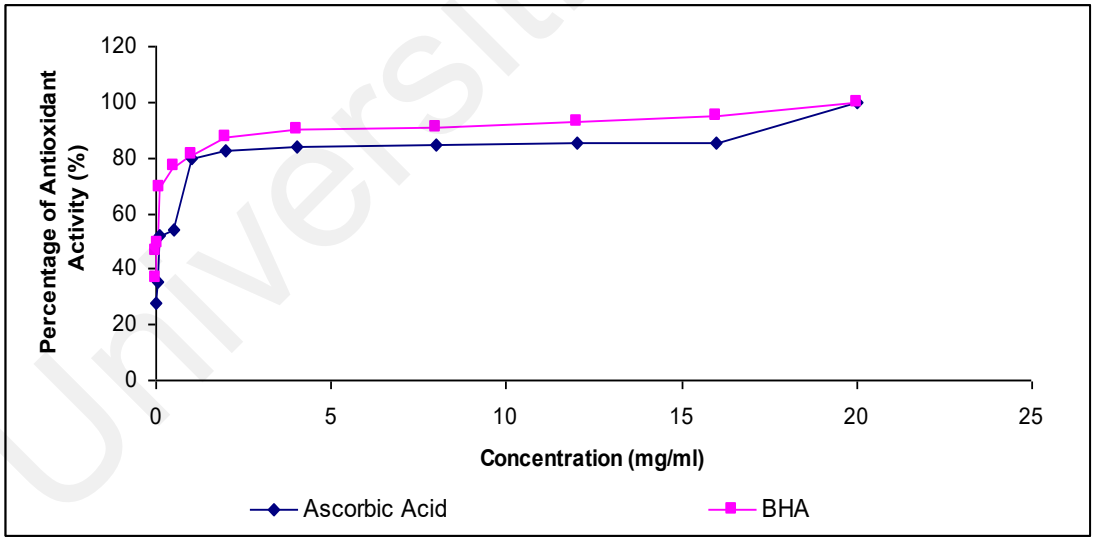


Figure 4.8: Antioxidant activity of ascorbic acid measured by the β-carotene bleaching assay

ii) β-carotene bleaching activity of *Beta vulgaris L.* extracts

The β-carotene bleaching inhibition activity was exhibited in the form of antioxidant activity. The activity is illustrated in Figure 4.9 and tabulated at Table 4.17. Highest

antioxidant activity was exhibited by hexane fraction at concentration 4-16 mg/ml with percentage of antioxidant activity of 132.400% ± 0.010 to 93.240% ± 0.004 for concentration 4.000-20.000 mg/ml. At concentration 20.000 mg/ml, methanol extract had the highest antioxidant activity with a percentage of 106.530% ± 0.011. BHA and ascorbic acid had a percentage of antioxidant activity less compared to those of hexane. Hexane fraction had the percentage of inhibition. Ethyl acetate fraction and BHA (standard) had almost similar antioxidant activity at certain concentrations (8.000, 12.000, 16.000 and 20.000 mg/ml) and lower percentage as compared to standard BHA at concentration 4 mg/ml. For methanol extract, it had a better antioxidant activity than both the standards at higher concentrations (16.000 & 20.000 mg/ml). Juice extract did not show much improvement in the activity as the concentration progresses. Water fraction had the lowest antioxidant percentage as compared to the standards and the extracts.

Table 4.17: The antioxidant activity of extracts and fractions of *Beta vulgaris L.* as determined by the β-carotene bleaching assay

Concentration of Extracts and Fractions (mg/ml)	Antioxidant Activity (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
20.000	106.530 ± 0.011	132.400 ± 0.010	99.020 ± 0.002	71.440 ± 0.002	83.340 ± 0.002
16.000	98.290 ± 0.001	135.320 ± 0.009	91.520 ± 0.006	71.430 ± 0.042	83.250 ± 0.002
12.000	76.260 ± 0.002	134.840 ± 0.001	91.340 ± 0.010	67.170 ± 0.005	81.000 ± 0.002
8.000	76.540 ± 0.002	96.860 ± 0.005	91.060 ± 0.003	66.370 ± 0.003	78.800 ± 0.006
4.000	63.760 ± 0.001	93.240 ± 0.004	74.420 ± 0.009	54.680 ± 0.002	78.950 ± 0.001
Control	-	-	-	-	-

* Percentage of antioxidant activities of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

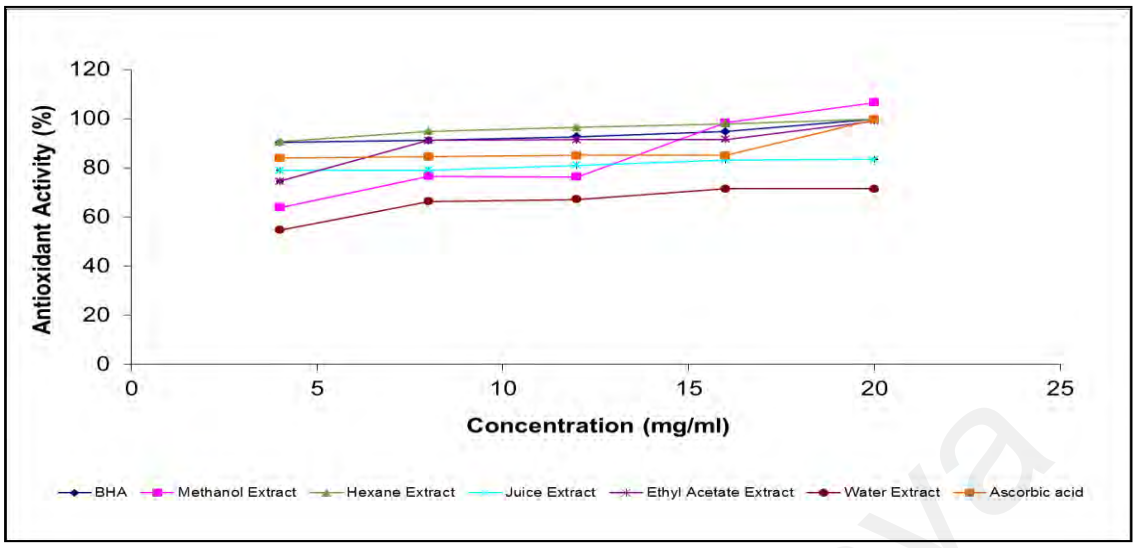


Figure 4.9: Comparison of antioxidant activity in percentage of extracts of *Beta vulgaris L.* extracts determined by the β-carotene bleaching activity assay

iii) β-Carotene bleaching activity of sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.*

The antioxidant activity of isolated sub-fractions are illustrated in Figure 4.10 and tabulated at Table 4.18 below. Sub-fractions were tested at lower concentrations 4.000 – 0.010 mg/ml. The fractions were compared to the positive standards, ascorbic acid and BHA. BHA had a higher antioxidant activity as compared to ascorbic acid. ET1 and ET3 had higher percentage of antioxidant activity than that of BHA and ascorbic acid at lower concentrations. Besides that, BHA had the highest percentage of antioxidant activity. ET1 and ET3 had better antioxidant at concentrations 0.010 – 0.050 mg/ml. ET1 and ET3 had almost similar activity with each other at lower concentrations (0.010 mg/ml to 1.000 mg/ml). ET1 had a higher antioxidant activity as compared to ET3. Fraction purple had a higher percentage of oxidative damage inhibition than fraction yellow at concentration 0.500 mg/ml (43.940% ± 0.010), 1.000 mg/ml (42.960% ± 0.003) and 2.000 mg/ml (48.300% ± 0.017), besides those concentrations, fraction yellow had a higher percentage of antioxidant activity than fraction purple at lower concentration, fraction ET2 had slightly

higher inhibition towards oxidative damage of β-carotene molecule. Overall, ET2 had the lowest β-carotene bleaching inhibition activity.

Table 4.18: The antioxidant activity of sub-fractions of *Beta vulgaris L.* at various concentrations as determined by the β-carotene bleaching assay

Concentration of Sub-fractions (mg/ml)	Antioxidant Activity (%)*				
	ET1	ET2	ET3	Purple	Yellow
4.000	73.140 ± 0.005	18.050 ± 0.032	65.080 ± 0.007	55.440 ± 0.014	56.720 ± 0.006
2.000	67.340 ± 0.007	13.720 ± 0.005	62.060 ± 0.001	48.300 ± 0.017	47.820 ± 0.005
1.000	60.760 ± 0.002	14.320 ± 0.004	59.890 ± 0.003	42.960 ± 0.003	37.290 ± 0.007
0.500	62.400 ± 0.002	12.290 ± 0.004	63.490 ± 0.003	43.930 ± 0.010	35.050 ± 0.005
0.100	64.520 ± 0.002	12.220 ± 0.001	66.300 ± 0.006	15.310 ± 0.004	29.060 ± 0.017
0.050	61.740 ± 0.001	12.810 ± 0.005	64.740 ± 0.001	2.810 ± 0.010	20.450 ± 0.008
0.025	66.370 ± 0.003	9.010 ± 0.008	64.820 ± 0.005	0.000 ± 0.019	8.120 ± 0.010
0.010	54.110 ± 0.002	0.000 ± 0.004	62.610 ± 0.005	0.000 ± 0.005	0.000 ± 0.005
Control	-	-	-	-	-

* Percentage of antioxidant activities of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

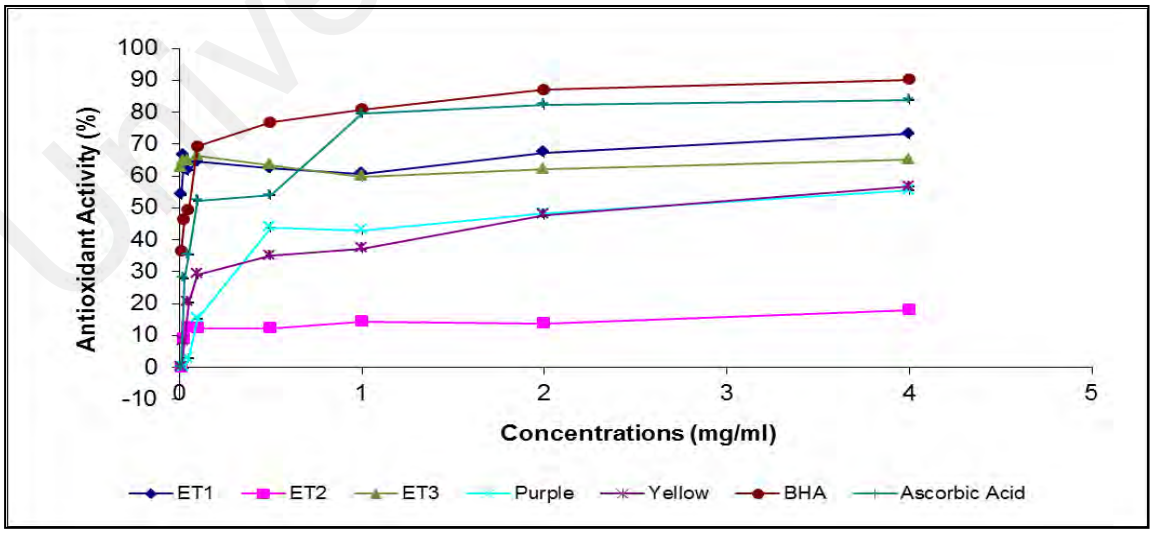


Figure 4.10: Comparison of percentage of antioxidant activity of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.* assessed by the β-carotene bleaching assay.

iv) Oxidation Rate Determination

Oxidation rate determination is the measure of the extent of oxidation that happens to the β-carotene molecule by hydroperoxides from linoleic acid. In the presence of antioxidants in the system, the oxidation to the double bonds of β-carotene molecules would be prevented as the free radicals would be stabilized by the antioxidants via hydrogen transfer mechanism. The oxidation rate value has an inverse relationship with the antioxidant activity as described previously.

a) Reduction of oxidation rate by positive standards BHA and ascorbic acid

The oxidation rate of positive references standards are tabulated in Table 4.19. Based on the results, it was observed that the ascorbic acid has a lower oxidation rate than BHA at concentration 20.000 mg/ml, i.e. 0.004. Ascorbic acid has a higher capacity to hinder the oxidation of the beta carotene molecule. The increase in the oxidation rate for ascorbic acid is higher as the concentration goes lower as compared to BHA. BHA seems to have a better antioxidant property than ascorbic acid as the concentration get lower. At the lowest concentration tested, 0.010 mg/ml, BHA had a lower oxidation rate (0.635) as compared to ascorbic acid (0.720). Hence, BHA had a better oxidation hindering capacity compared to ascorbic acid. The lower the value or oxidation rate, the higher the value of its antioxidant activity.

Table 4.19: The oxidation rate of positive standards, BHA and ascorbic acid, as determined by the β-carotene bleaching assay

Concentration of Standards (mg/ml)	Oxidation rate	
	BHA	Ascorbic Acid
20.000	0.089	0.004
16.000	0.052	0.149
12.000	0.002	0.150
8.000	0.176	0.156
4.000	0.074	0.162
2.000	0.107	0.176
1.000	0.098	0.199
0.500	0.049	0.205
0.100	0.492	0.461
0.050	0.507	0.474
0.025	0.537	0.647
0.010	0.635	0.720
Negative Control	1.000	1.000

* Oxidation rate of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

b) The oxidation rate of extracts of *Beta vulgaris L.* based on β-carotene bleaching assay

The Table 4.20 depicts the oxidation rate of extracts of *Beta vulgaris L.* at various concentrations. From the graph we can see the rate of oxidation that happens to the beta-carotene molecule at various concentrations of extracts of the test sample. From the table, we can see that at concentration 20.000 mg/ml, the methanol extract (-0.065) and hexane fraction (-0.062) has a negative oxidation rate. This means that the extracts completely deter oxidation at this point of concentration. Other than that, ethyl acetate fraction also had a low oxidation rate (0.010). The oxidation rate increases as the concentration of the extract is lower. Hexane fraction had the lowest oxidation rate throughout the tested concentrations (-0.062 – 0.095 for concentrations 20.000 mg/ml to 4.000 mg/ml). Water fraction, on the other hand, had the highest oxidation rate throughout the tested concentrations. At the lowest tested concentration (4.000 mg/ml), water fraction had the highest oxidation rate i.e. 0.495. Hence, the higher the concentration of the extracts, the lower is the oxidation rate towards β-carotene molecule.

Table 4.20: The oxidation rate of extracts of *Beta vulgaris L.* at various concentrations as determined by the β-carotene bleaching assay

Concentration of extracts of *Beta vulgaris L.*	Oxidation rate				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
20.000	-0.065	-0.062	0.010	0.286	0.167
16.000	0.017	0.021	0.085	0.286	0.168
12.000	0.237	0.036	0.087	0.328	0.190
8.000	0.235	0.052	0.089	0.336	0.210
4.000	0.362	0.095	0.256	0.453	0.210
Control	1.000	1.000	1.000	1.000	1.000

* Oxidation rate of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

c) The oxidation rate of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.* based on β-carotene bleaching assay

The oxidation rate of isolated sub-fractions from ethyl acetate fraction and juice extract of *Beta vulgaris L.* is tabulated at Table 4.21. The fractions are tested at a concentration range of 4.000 mg/ml to 0.010 mg/ml. At the highest tested concentration, ET1 had the lowest oxidation rate (0.269) and the highest oxidation rate is exhibited by ET2. A big range in the oxidation rate between lowest and highest concentration was shown by fraction Purple (0.446 to 4.006 at concentration 4.000 mg/ml to 0.010 mg/ml). At lowest concentration tested, i.e. 0.010 mg/ml, fraction purple had the highest oxidation rate 4.006; the oxidation rate of more than 1.000 gives us an idea that this fraction might have the attribute of promoting oxidation towards the beta-carotene molecule. At the lowest concentration tested, fraction ET1 still had a fairly good oxidation inhibition with a lowest oxidation rate among the fraction (0.459).

Table 4.21: The oxidation rate of isolated sub-fractions of ethyl acetate fraction and juice extracts of *Beta vulgaris L.* at various concentrations as determined by the β-carotene bleaching assay

Concentration of isolated sub-fractions of *Beta vulgaris L.*	Oxidation rate				
	ET1	ET2	ET3	Purple	Yellow
4.000	0.269	0.819	0.349	0.446	0.433
2.000	0.327	0.863	0.379	0.517	0.522
1.000	0.392	0.857	0.401	0.570	0.627
0.500	0.376	0.877	0.365	0.561	0.649
0.100	0.355	0.878	0.337	0.847	0.709
0.050	0.383	0.872	0.353	0.972	0.795
0.025	0.336	0.910	0.352	1.622	0.919
0.010	0.459	1.281	0.374	4.006	1.155
Control	1.000	1.000	1.000	1.000	1.000

* Oxidation rate of β-carotene bleaching assay as a mean of triplicate experiments ± standard deviation (SD)

4.2.4 Metal Chelating Assay

This assay measures the capabilities of a sample to act as a chelating agent of ferrous ions. Chelating agents acts as antioxidants because they reduce the redox potential of metal ions and stabilizes the oxidized form. Ferrozine salt generally has high affinity towards ferrous ion (Fe^{2+}) that is present in the system. Combination of ferrozine salt and ferrous ion gives a red color complex. In presence of antioxidants, the formation of ferrous ion-ferrozine complex would be disrupted as the antioxidants would now chelate with the ferrous ions. Hence, the red color complex would not form and there would be reduction in the red color intensity of the reaction mixture.

i) EDTA as the positive standard

The activity of EDTA is illustrated at Figure 4.11 and tabulated at Table 4.22. EDTA is well known for its excellent chelating ability. The percentage inhibition at various concentrations (5.000-0.010 mg/ml) was measured and compared to test samples of *Beta vulgaris L*. Highest percentage inhibition was exhibited at 5.000 mg/ml (99.000% ± 0.775) and lowest percentage inhibition at concentration 0.010 mg/ml (-24.070% ± 1.579). The

reaction mixture had almost a clear solution when tested at concentrations ranging 5.000-1.000 mg/ml and only had a slight pinkish hue at lower concentrations. Almost all the complex formation between ferrozine and ferrous ion was inhibited. The concentration when there was 50% inhibition (IC_{50} value) was 0.040 mg/ml. EDTA exhibited a dose-dependent response on metal chelating activity.

Table 4.22: The percentage inhibition of metal chelating activity by positive standard (EDTA)

Concentration of EDTA (mg/ml)	Percentage Inhibition*
5.000	99.000 ± 0.775
4.000	97.230 ± 0.343
3.000	95.230 ± 0.272
2.000	92.510 ± 0.236
1.000	90.510 ± 0.438
0.500	87.600 ± 0.136
0.100	83.110 ± 0.491
0.050	79.020 ± 0.360
0.025	13.760 ± 1.547
0.010	0

* Percentage inhibition activities of metal chelating assay as a mean of triplicate experiments ± standard deviation (SD)

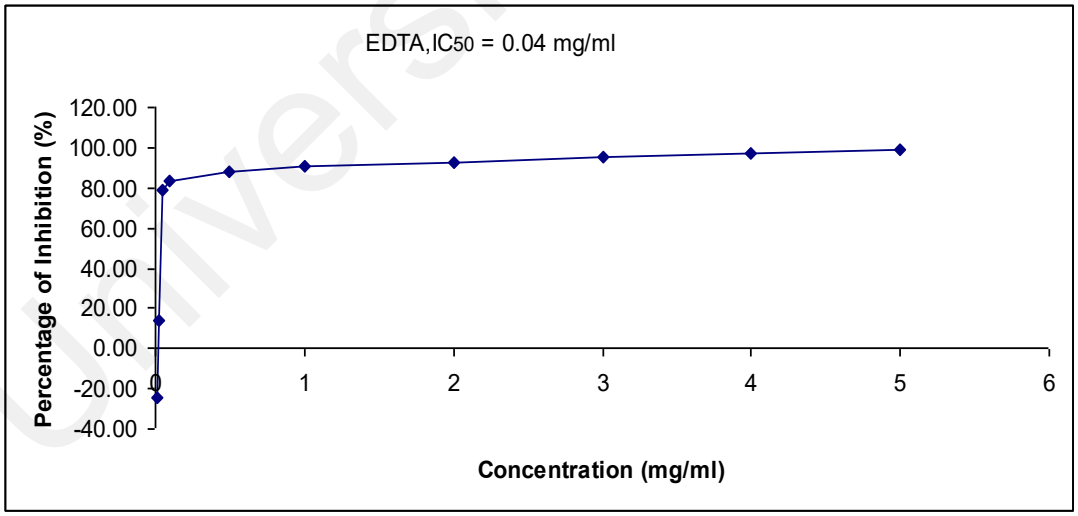


Figure 4.11: Percentage inhibition of metal chelating activity by standard EDTA

ii) Metal chelating activity of extracts and fractions of *Beta vulgaris L.*

The metal chelating activities of the extracts and fractions were illustrated at Figure 4.12 and Table 4.23. Extracts and fractions of *Beta vulgaris L.* were tested for its metal chelating ability from concentration 5.000 mg/ml to 0.500 mg/ml. EDTA, the positive standard had the highest chelating ability whereas all the extracts and fractions could not measure up to the standard. Within the extracts, highest chelating ability was exerted by methanol extract. Hexane fraction exceeded methanol extract on its chelating ability at concentrations 1.000 mg/ml (36.180% ± 1.135) and 2.000 mg/ml (52.820% ± 0.624). Ethyl acetate fraction and juice extract had moderate chelating ability. Almost similar activities to each other were noted. Water fraction had the lowest chelating ability among the extracts. At the highest tested concentration (5.000 mg/ml), methanol extract had the highest chelating activity (93.530% ± 0.666) and the lowest activity was exhibited by water fraction (25.540% ± 0.275) at the particular concentration. At the lowest concentration tested (0.500 mg/ml), hexane fraction had the highest inhibition in ferrozine + ferrous ion formation with percentage of inhibition 20.640% ± 1.645 and the lowest percentage of inhibition is exerted by ethyl acetate fraction with a percentage of 2.420% ± 2.521. The reaction mixture had various shades of red color based on the intensity of the metal chelating ability that is present in the sample.

Determination of the IC_{50} value of the chelating activity of the extracts of *Beta vulgaris L.* was based on Figure 4.12

Table 4.23: The metal chelating activity of extracts of *Beta vulgaris L.* at various concentrations

Concentration of Extract (mg/ml)	Percentage Inhibition*				
	Methanol Extract	Hexane Fraction	Ethyl acetate Fraction	Water Fraction	Juice Extract
5.000	93.530 ± 0.666	88.010 ± 1.171	59.520 ± 2.497	25.540 ± 0.275	86.950 ± 3.697
4.000	91.730 ± 1.391	90.350 ± 0.229	69.200 ± 2.336	11.920 ± 1.560	54.770 ± 4.631
3.000	80.130 ± 1.787	87.010 ± 1.344	53.000 ± 1.227	15.740 ± 4.681	61.450 ± 2.582
2.000	77.370 ± 3.463	52.820 ± 0.624	44.580 ± 0.458	12.350 ± 0.459	37.400 ± 3.324
1.000	45.640 ± 4.008	36.180 ± 1.135	18.970 ± 4.951	7.210 ± 1.652	17.180 ± 4.478
0.500	11.600 ± 1.469	20.640 ± 1.645	2.420 ± 2.521	6.090 ± 1.431	17.550 ± 1.948
Control	-	-	-	-	-

* Percentage inhibition of metal chelating activity as a mean of triplicate experiments ± standard deviation (SD)

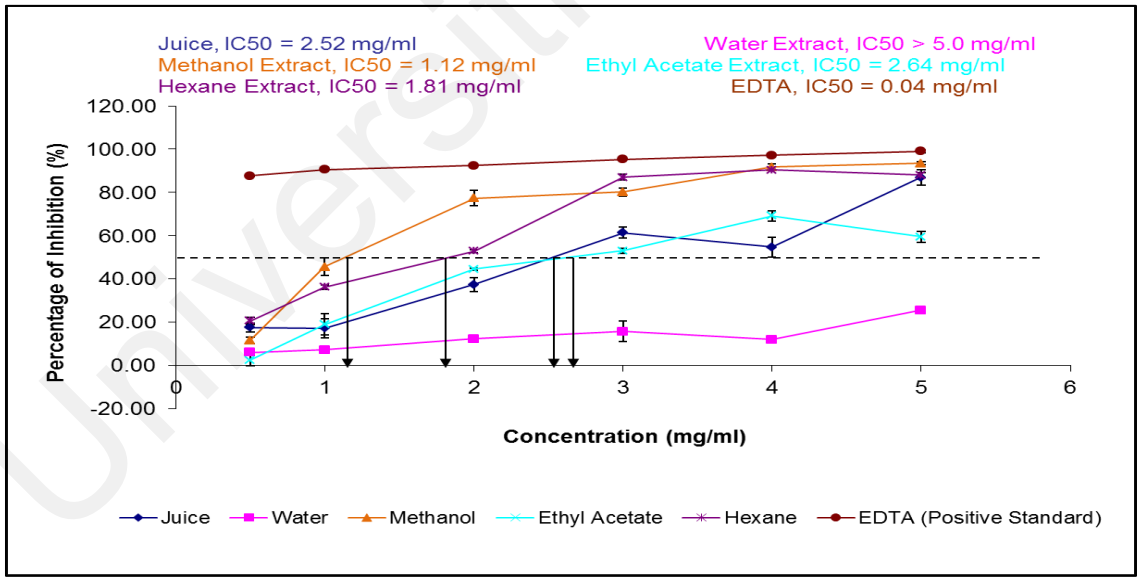


Figure 4.12: Comparison of metal chelating activity of crude extracts and fractions of *Beta vulgaris L.* with standard EDTA at various concentrations and determination of IC_{50} values

iii) Metal chelating activity of isolated sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.*

The sub-fractions were tested at lower concentrations. The fractions were tested at a range of concentrations (1.000 – 0.010 mg/ml). At the highest concentration tested, 1.0 mg/ml, ET1 had the lowest percentage inhibition with a percentage of 3.000% ± 0.264, whereas ET2 had the highest percentage inhibition (20.720% ± 0.061), at the particular concentration. At the lowest concentration tested, 0.010 mg/ml, ET2 had the highest percentage inhibition in metal chelating activity with 9.710% ± 1.992 and lowest metal chelating activity was at ET1 with percentage inhibition 2.100% ± 0.555. EDTA, positive reference standard, had the highest chelating activity against metal as compared to all other sub-fractions. EDTA had an IC_{50} value of 0.040 mg/ml. The chelating ability exhibited by all the fractions is seemingly unstable and not dose-dependent.

All the fractions had an IC_{50} value of more than 1.000 mg/ml. The lowest chelating ability was exhibited by ET1 with percentage inhibition at a range of 3.000% ± 0.264 to 2.100 ± 0.555 from concentration 1.000 mg/ml to 0.010 mg/ml. Highest chelating ability was exerted by ET2 with percentage of inhibition 20.720% ± 0.061 to 9.710% ± 1.992 for the concentration 1.000 mg/ml to 0.010 mg/ml. The reaction mixture had various shades of red color based on the intensity of the metal chelating ability that was present in the sample.

Table 4.24: The metal chelating activity of sub-fraction of ethyl acetate fraction and juice extract of *Beta vulgaris L.*

Concentration of Sub-Fractions (mg/ml)	Percentage Inhibition (%)*				
	ET1	ET2	ET3	Purple	Yellow
1.000	3.000 ± 0.264	20.270 ± 0.061	19.570 ± 1.973	17.890 ± 3.543	19.690 ± 1.651
0.500	3.070 ± 0.264	16.320 ± 0.473	18.060 ± 2.671	16.790 ± 7.198	14.140 ± 2.977
0.100	2.870 ± 0.160	15.900 ± 0.951	16.950 ± 0.996	5.310 ± 2.703	10.000 ± 7.802
0.050	2.940 ± 2.472	12.370 ± 0.320	15.650 ± 0.436	9.950 ± 1.738	13.290 ± 3.289
0.025	2.100 ± 0.555	10.520 ± 0.121	11.640 ± 2.202	1.350 ± 8.962	11.840 ± 8.575
0.010	2.100 ± 0.555	9.710 ± 1.992	9.360 ± 1.271	6.000 ± 4.155	2.550 ± 7.634
Control	-	-	-	-	-

* Percentage of inhibition of metal chelating activity as a mean of triplicate experiments ± standard deviation (SD)

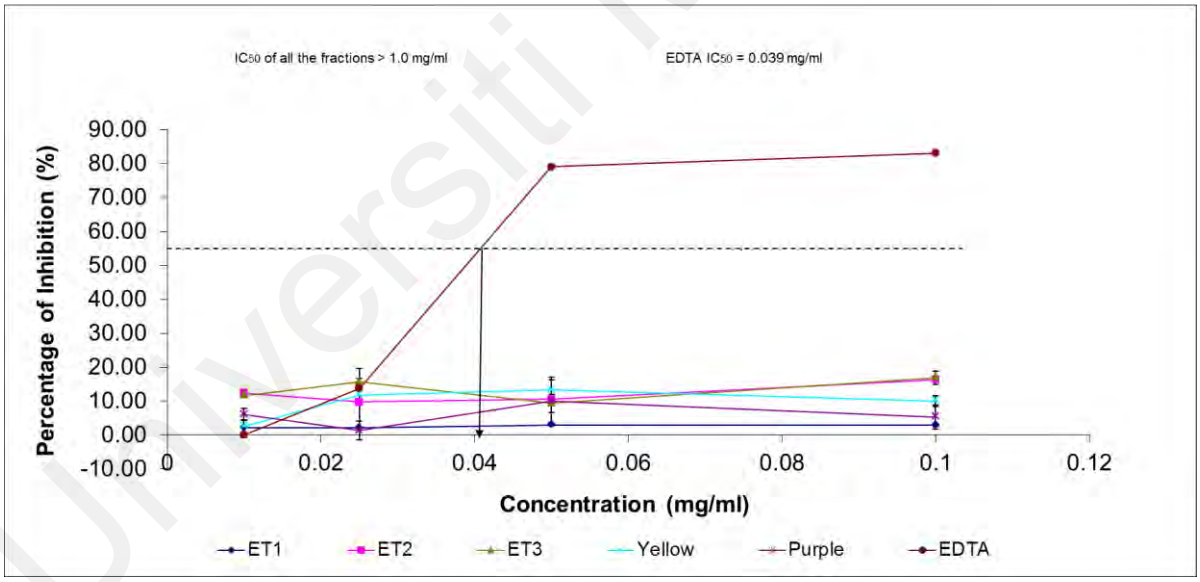


Figure 4.13: Comparison of metal chelating activity of isolated sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard EDTA

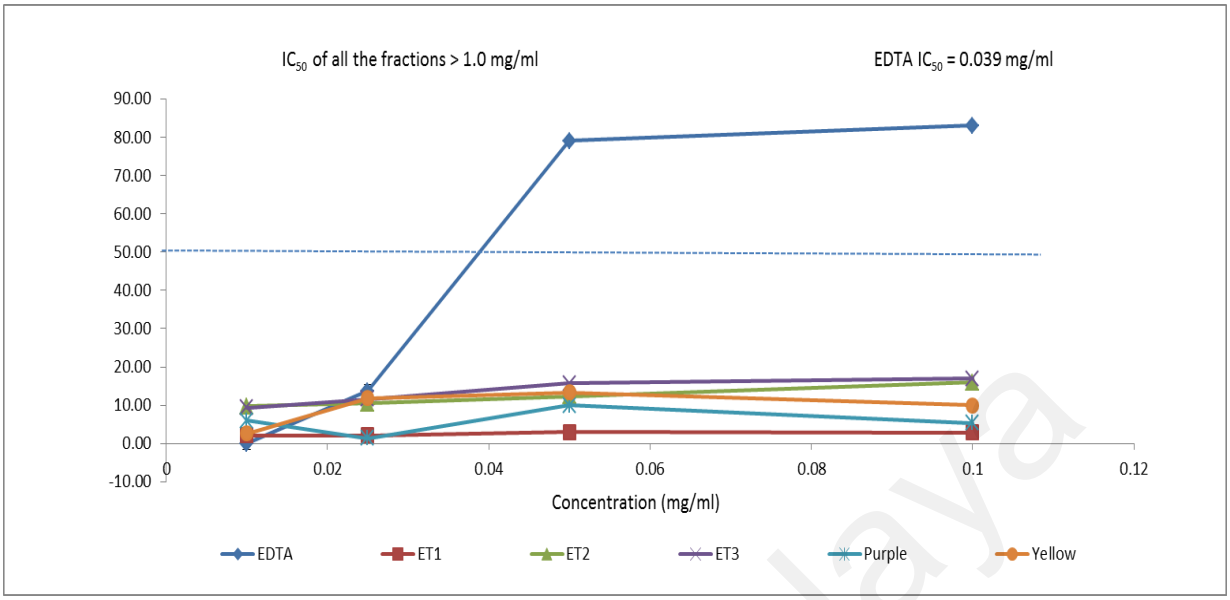


Figure 4.14: Magnification of lower concentration on comparison of metal chelating activity of sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard

iv) Comparison of the IC_{50} values of the extracts, fractions and isolated sub-fractions of *Beta vulgaris L.* with standard EDTA

A comparison of the concentration of samples of *Beta vulgaris L.* at 50% inhibition (IC_{50} value) shows that, EDTA which acts as the positive standard had the lowest IC_{50} value (0.040 mg/ml) and hence the highest metal chelating activity (antioxidant activity). Within the extracts of *Beta vulgaris L.*, methanol extract had the lowest IC_{50} value (1.120 mg/ml) and hence the highest antioxidant activity among the extracts. Hexane fraction, ethyl acetate fraction and juice extract had a moderate IC_{50} value with 1.810 mg/ml, 2.640 mg/ml and 3.000 mg/ml respectively. Water fraction had the highest IC_{50} value (>5.000 mg/ml) and had lowest chelating ability with ferrous ion. As for the sub-fractions of ethyl acetate and juice extracts, all of them exhibited poor metal chelating activity as compared to

standard EDTA with an IC_{50} value >1.000 mg/ml. The IC_{50} values are tabulated at Table 4.25.

Table 4.25: The IC_{50} values of samples of *Beta vulgaris L.* and standard EDTA

Extract/Fractions	IC_{50} (mg/ml)
Methanol	1.120
Ethyl Acetate	2.640
Water	>5.000
Hexane	1.810
Juice	3.000
Isolated fraction	>1.00
Standard (EDTA)	0.040

4.2.5 SOD (Superoxide Dismutase) Activity Assay

Superoxide dismutase is an antioxidant enzyme that catalyzes the dismutation of superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) and O_2 molecule. For this particular assay, a kit has been developed by Fluka. SOD assay kit-WST is a convenient SOD determination method as it utilizes the 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 formazan dye upon reduction by a superoxide anion. In high presence of SOD like materials, less superoxide anion will be present in the reaction mixture and hence less formazan dye (yellow color) was produced. SOD activity was quantified by measuring the decrease in color development at 450 nm.

i) BHA as the positive reference standard

The SOD activity of BHA as the positive reference standard is shown in Table 4.26 and Figure 4.15. The range of concentration tested was 5.000 – 0.010 mg/ml. At concentration 5.000 mg/ml, a very high percentage inhibition, 102.040% ± 0.871 was observed. At the lowest concentration tested (0.010 mg/ml), 73.740% ± 0.907 inhibitions was exhibited. It was observed that, the reaction solution was almost clear during the experiment. Almost no formazan dye was formed. Hence, BHA had very high SOD activity in inhibiting the xanthine oxidase (XO). IC_{50} value <0.010 mg/ml.

Table 4.26: The SOD activity represented in percentage inhibition on BHA (positive reference standard)

Concentration of BHA (mg/ml)	Percentage Inhibition*
5.000	102.040 ± 0.871
4.000	93.400 ± 1.823
3.000	90.100 ± 0.558
2.000	89.080 ± 3.199
1.000	76.720 ± 5.617
0.500	88.940 ± 0.426
0.100	80.900 ± 1.571
0.050	80.150 ± 0.907
0.025	77.830 ± 0.483
0.010	73.740 ± 0.907

* Percentage inhibition activities of SOD as a mean of triplicate experiments ± standard deviation (SD)

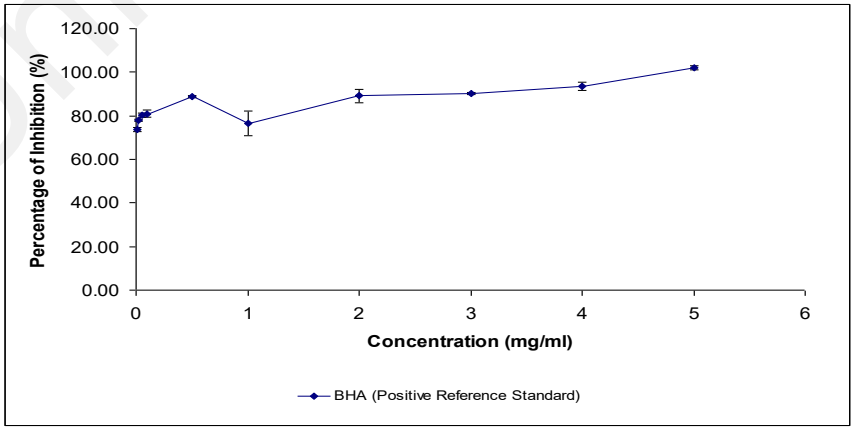


Figure 4.15: The SOD activity of BHA (positive reference standard) over varying concentrations

ii) SOD activity of extracts and fractions of *Beta vulgaris L.*

The SOD activity is shown in Table 4.27 and Figure 4.16 & 4.17. The reaction mixture turns from a clear color to various shades of yellow in the system that lacks SOD/SOD-like materials. Extracts of *Beta vulgaris L*. was tested on a concentration range of 1.000-5.000 mg/ml; only the ethyl acetate fraction exhibited percentage of inhibition higher than 50% at the lowest concentration (1.000 mg/ml). The ethyl acetate fraction was re-tested at lower concentrations (1.000-0.010 mg/ml).

BHA which acts as the positive standard had the highest activity as compared to all the extracts. Among the extracts the ethyl acetate fraction showed the highest XO inhibition. It even had a better inhibition than the standard BHA at concentration 2.000 mg/ml (91.430% ± 1.767) and 3.000 mg/ml (91.170% ± 0.403). The methanol extract possessed good SOD activity at lower concentrations but the percentage inhibition dropped at higher concentrations. The water and juice extracts had moderate yet stable inhibition activity. Among all the extracts, the hexane fraction had the lowest SOD activity.

At the highest concentration tested; 5.000 mg/ml, ethyl acetate fraction had the highest percentage inhibition (84.450% ± 5.447) followed by the water fraction and juice extract with 82.430% ± 1.025 and 80.940% ± 0.351 respectively. The methanol extract had a moderate activity (69.900% ± 2.560). The hexane fraction had the lowest inhibition (24.610% ± 8.677).

At the lowest concentration (1.000 mg/ml), ethyl acetate fraction had the highest inhibition value (68.350% ± 1.508) and the lowest inhibition was noted by juice extract with 6.460% ± 0.658 percentage. Methanol extract, hexane fraction and water fraction had a moderate activity with 34.280% ± 4.245, 24.100% ± 6.695 and 16.110% ± 4.762 respectively.

Table 4.27: The SOD activity represented in percentage inhibition on extracts of *Beta vulgaris L.*

Concentration of Extracts (mg/ml)	Percentage Inhibition (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
5.000	69.600 ± 2.560	24.610 ± 8.677	84.450 ± 5.447	82.430 ± 1.028	80.940 ± 0.351
4.000	55.940 ± 5.434	30.050 ± 3.695	73.550 ± 1.954	70.580 ± 2.178	62.880 ± 0.624
3.000	57.890 ± 1.893	23.820 ± 5.295	91.170 ± 0.403	57.150 ± 0.639	43.080 ± 0.510
2.000	54.450 ± 1.536	22.340 ± 5.033	91.430 ± 1.767	38.600 ± 1.766	19.640 ± 1.028
1.000	34.280 ± 4.245	24.100 ± 6.695	68.350 ± 1.508	16.110 ± 4.762	6.460 ± 0.658
Control	-	-	-	-	-

* Percentage inhibition of SOD as a mean of triplicate experiments ± standard deviation (SD)

iii) Investigations of SOD activity for ethyl acetate fractions at lower concentrations

The ethyl acetate fraction was tested at lower concentrations in order to be able to determine the IC_{50} value of it. At concentration 0.010 mg/ml, the inhibition dropped to below 0 (-9.310% ± 0.351), Figure 4.28. The IC_{50} value was 0.710 mg/ml for this extract.

Table 4.28: The SOD activity represented in percentage inhibition on ethyl acetate fraction of *Beta vulgaris L,* tested at lower concentrations

Concentration of Ethyl acetate fraction (mg/ml)	Percentage Inhibition (%)*
1.000	68.350 ± 1.508
0.500	35.910 ± 1.330
0.100	10.160 ± 0.793
0.050	4.720 ± 1.917
0.025	1.240 ± 1.608
0.010	0.000
Control	-

* Percentage inhibition of SOD activity as a mean of triplicate

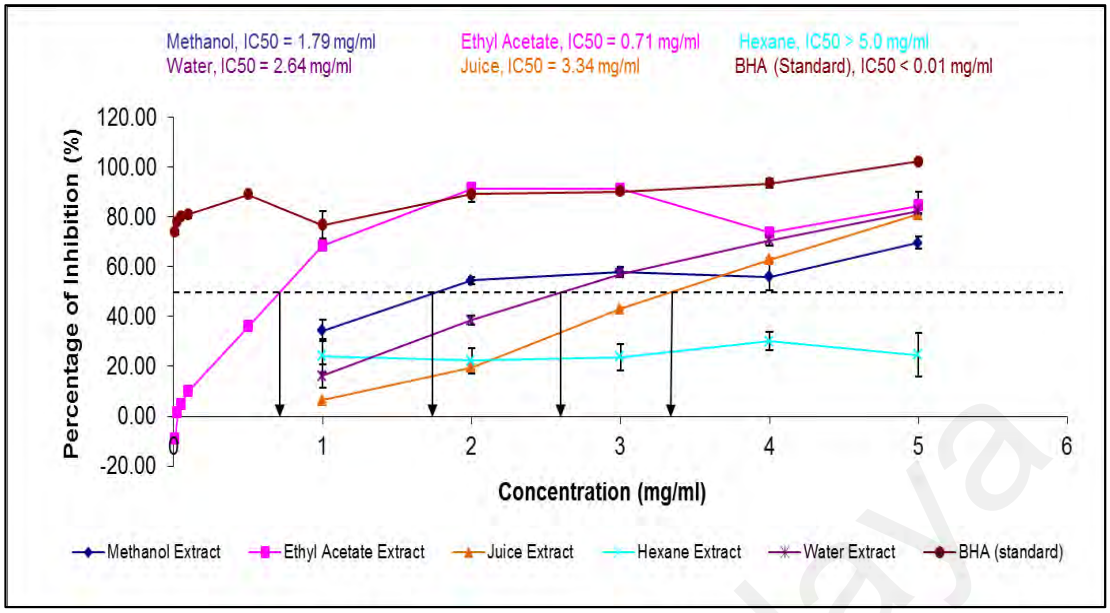


Figure 4.16: Comparison of SOD inhibition activity between extracts of *Beta vulgaris L.* and standard (BHA) at various concentrations

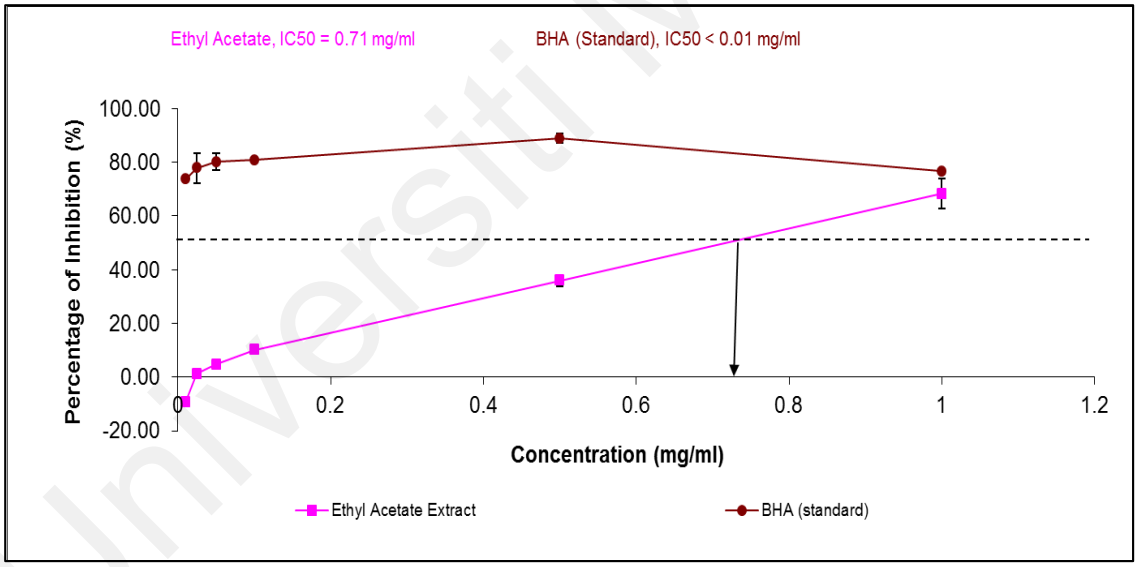


Figure 4.17: Investigations of ethyl acetate fraction of *Beta vulgaris L.* and BHA at lower concentrations (0-1.0 mg/ml) for SOD activity assay.

iv) SOD activity of sub-fractions from ethyl acetate fraction and juice extracts of *Beta vulgaris L.*

The SOD activities of the sub-fractions are shown in Table 4.29 and Figure 4.18 & 4.19.

BHA was used as the positive reference standard. ET2 had a remarkably high SOD activity

at concentration 1.000 mg/ml with a percentage inhibition 177.820% ± 2.329. ET1 (91.680% ± 0.768) and fraction purple (88.570% ± 0.871) too had a better activity compared to standard at 1.000 mg/ml. At lower concentrations (<1.000 mg/ml), BHA had a better SOD activity as compared to the sub-fractions. Overall, comparison of SOD activity within the fractions showed that ET2 had the highest activity followed by ET1. Fraction purple had moderate activity while fraction ET3 and fraction yellow had the lowest activity. At concentration 1.000 mg/ml, the highest concentration, fraction ET2 had the highest SOD activity (177.820% ± 2.329), while fraction yellow had the lowest SOD activity (9.140% ± 0.966). At the lowest concentration, 0.010 mg/ml, ET2 had the highest percentage of inhibition 9.830% ± 0.564 and ET3 had the lowest (0) percentage inhibition on SOD activity assay.

Table 4.29: The SOD activity represented in percentage inhibition on extracts of *Beta vulgaris L.*

Concentration of Sub-fractions (mg/ml)	Percentage Inhibition (%)*				
	ET1	ET2	ET3	Purple	Yellow
1.000	91.680 ± 0.768	177.820 ± 2.329	27.870 ± 1.893	88.570 ± 0.871	9.140 ± 0.966
0.500	77.970 ± 3.665	94.160 ± 2.244	15.920 ± 0.793	44.970 ± 1.555	5.510 ± 1.107
0.100	39.440 ± 3.494	28.200 ± 2.304	0.630 ± 0.418	19.360 ± 0.949	-0.200 ± 2.130
0.050	14.620 ± 0.279	12.010 ± 0.528	-5.130 ± 0.242	15.040 ± 1.476	-1.270 ± 0.290
0.025	7.090 ± 1.089	13.970 ± 0.852	-6.990 ± 0.290	10.390 ± 0.688	-1.600 ± 0.564
0.010	1.280 ± 1.766	9.830 ± 0.564	-8.200 ± 0.448	7.370 ± 0.503	-4.340 ± 2.063
Control	-	-	-	-	-

* Percentage inhibition of SOD as a mean of triplicate experiments ± standard deviation (SD)

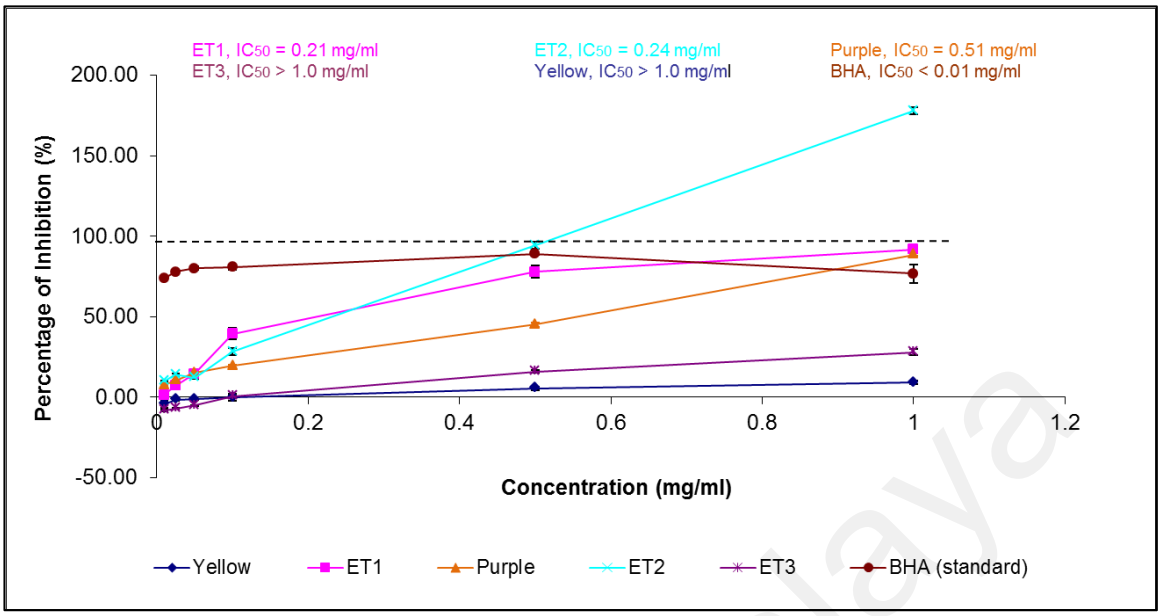


Figure 4.18: Comparison of SOD inhibition activity of isolated compounds from ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard BHA at various concentrations

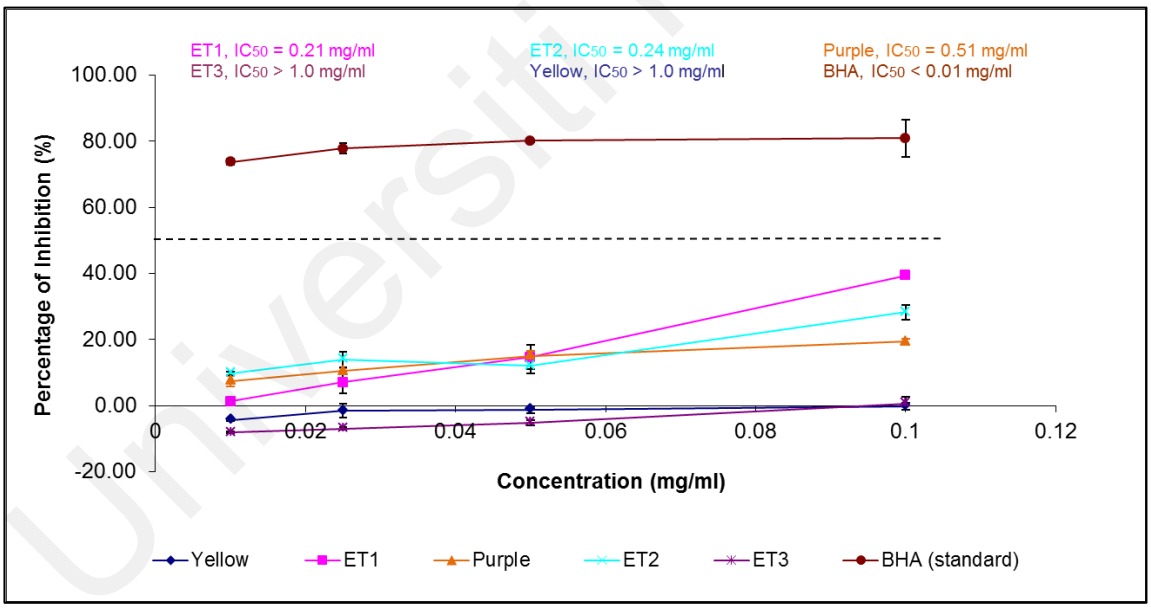


Figure 4.19: Magnification of activity at lower concentrations of SOD inhibition activity of isolated compounds from ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard BHA at various concentrations

v) Comparison of IC_{50} values of extracts and fractions of *Beta vulgaris L.*

All the extracts were compared to positive standard (BHA). The standard BHA has an IC_{50} value of <0.010 mg/ml. It has the highest SOD activity. Within the extracts, ethyl acetate fraction has the lowest IC_{50} value (0.710 mg/ml) and the highest SOD activity. Hexane fraction had the lowest activity of SOD with an IC_{50} value >5.000 mg/ml. Methanol extract, water fraction and juice extract had a moderate IC_{50} value of 1.790, 2.640 and 3.340 mg/ml respectively.

Table 4.30: Comparison of IC_{50} values of SOD activity assay of extracts of *Beta vulgaris L.* with positive standard (BHA)

Extracts of *Beta vulgaris L.*	IC_{50} (mg/ml)
Methanol extract	1.790
Ethyl Acetate fraction	0.710
Hexane fraction	>5.000
Water fraction	2.640
Juice extract	3.340
BHA (standard)	<0.010

vi) Comparison of IC_{50} values of sub-fractions of ethyl acetate and juice extract with standard BHA

BHA had the highest SOD activity with an IC_{50} value <0.010 mg/ml. ET1 and ET2 had a remarkable SOD activity as well with an IC_{50} value of 0.210 and 0.240 mg/ml respectively. The purple fraction had a moderate IC_{50} value and SOD activity with 0.540 mg/ml. Fraction ET3 and yellow had a high IC_{50} value and low SOD activity with IC_{50} value of >1.000 mg/ml each.

Table 4.31: Comparison of IC_{50} values of sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* with positive standard (BHA) on SOD assay

Sub-fractions of *Beta vulgaris L.*	IC_{50} (mg/ml)
ET1	0.210
ET2	0.240
ET3	>1.000
Purple	0.540
Yellow	>1.000
BHA (standard)	<0.010

4.2.6 Thiobarbituric Acid Reactive Substances (TBARS)/ Lipid Peroxidation

TBARS assay measures the product malonyldehyde (MDA) that is the end product formed during the lipid peroxidation of polyunsaturated fatty acids of egg yolk. MDA would bind with thiobarbituric acid to form a red color complex. This complex could be measured at 532 nm. In the presence of antioxidants that hinder the lipid peroxidation from occurring. MDA-TBA complex would be decreased and a less red/pinkish hue would be formed. TBARS assay is a useful and easy screening method to evaluate lipid peroxidation in biological systems. The TBARS reaction was standardized by analysis of tetraetoxypropane (TEP) standard solutions. BHA and ascorbic acid was used as the positive reference standard. The lower the formation of MDA on a reaction system, the better the sample acts as an antioxidant in inhibiting the lipid peroxidation.

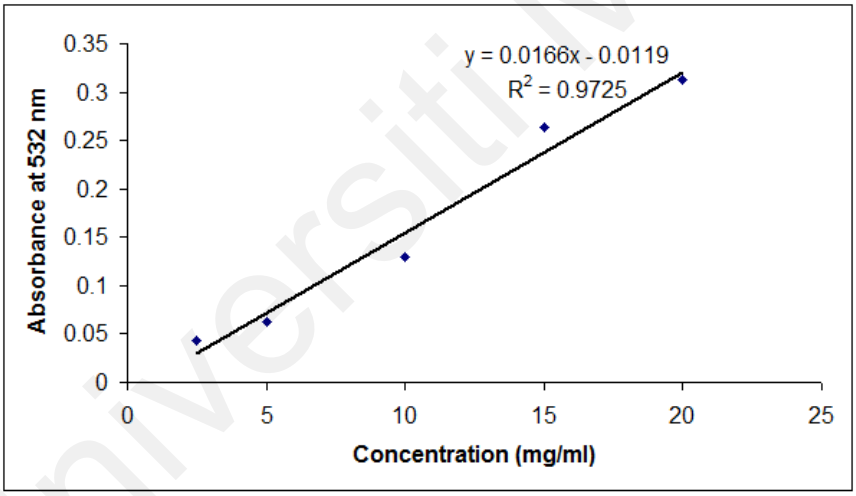


Figure 4.20: TEP (tetraethoxypropane) calibration graph

i) BHA and Ascorbic acid as the positive standards

The TBARS or lipid peroxidation capacity of BHA and ascorbic acid were tabulated in Table 4.32 and portrayed in Figure 4.21. It was observed that both the standards had almost the same lipid peroxidation inhibition capacity. Ascorbic acid (1.010 ± 0.040) had slightly lower production of TBARS in the reaction system and thus it was better at inhibiting lipid peroxidation as compared to BHA (1.040 ± 0.040).

Table 4.32: Lipid peroxidation capacity of positive standards

Standard (Positive References)	Lipid Peroxidation Capacity* (mg of TEP/ g of extracts)
BHA	1.040 ± 0.040
Ascorbic acid	1.010 ± 0.080

* Lipid peroxidation inhibition activity expressed as mean of triplicate experiments ± standard deviation (SD)

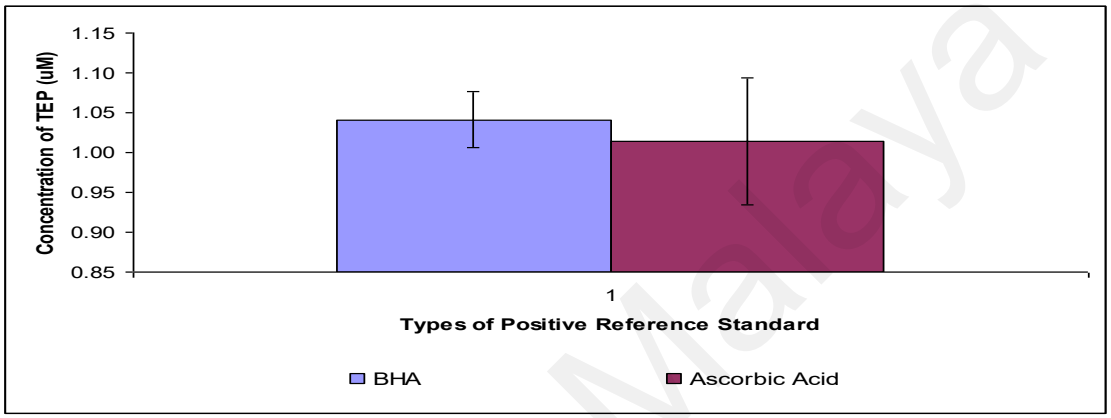


Figure 4.21: Lipid peroxidation capacity of positive standard

ii) Lipid peroxidation inhibition of extracts of *Beta vulgaris L.*

The result of TBARS assay in the presence of extracts of *Beta vulgaris L*. is shown in Table 4.33. Lipid peroxidation capacity was measured in mg of TEP formed per gram of extracts. The lower the concentration of TEP per gram, the lower the lipid peroxidation that occurred and hence the higher the antioxidant activity (lipid peroxidation inhibition activity) possessed by the extract. The results were tabulated at Table 4.33 and shown graphically in Figure 4.22. It is observed that the methanol extract had the highest ability to inhibit the lipid peroxidation with a value of (0.061 ± 0.063 mg of TEP per gram of extract). The activity was even better than that exhibited by both the positive standards. This value was followed by hexane fraction, juice extract with a value of 0.150 ± 0.024 and 0.154 ± 0.019 mg of TEP per gram of extracts respectively. The water fraction and ethyl acetate fraction

possessed the least capacity to inhibit lipid oxidation in comparison to the standard and other extracts.

Table 4.33: Lipid peroxidation capacity of extracts of *Beta vulgaris L.*

Extracts of *Beta vulgaris L.*	Lipid Peroxidation Capacity* (mg of TEP/ g of extracts)
Methanol	0.061 ± 0.063
Hexane	0.150 ± 0.024
Ethyl Acetate	0.338 ± 0.028
Water	0.281 ± 0.016
Juice	0.154 ± 0.019

* Lipid peroxidation inhibition activity expressed as mean of triplicate experiments ± standard deviation (SD)

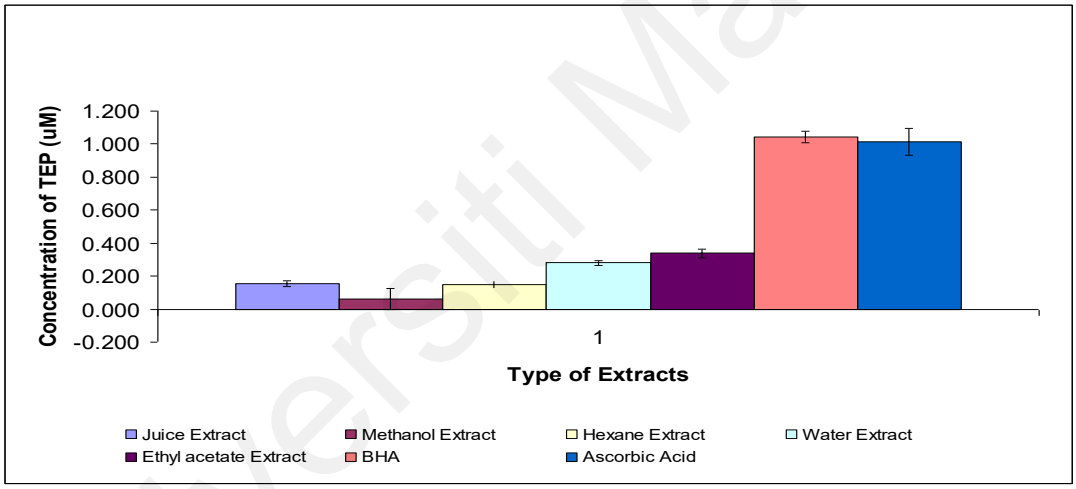


Figure 4.22: Comparison of inhibition of lipid peroxidation of extracts of *Beta vulgaris L.* with positive standards (BHA and ascorbic acid)

iii) Lipid peroxidation inhibition capacity of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.*

The lipid peroxidation inhibition capacity as assessed by the TBARS assay was conducted on the sub-fractions of the ethyl acetate and juice extract of *Beta vulgaris L.* The reaction mixture had a range of shade from pink to orange color. The reaction mixture that had high lipid peroxidation has the pinkish color due to the formation of MDA-TBA complex, whereas the ones which had lower antioxidant capacity to inhibit lipid peroxidation

possessed a more orange shade. The results were tabulated at Table 4.34 and graphically represented in Figure 4.23. The results showed that yellow fraction exhibited the best capacity to inhibit the lipid peroxidation by having the least amount of TEP per g of sample (0.508 ± 0.711). ET2, ET3 and fraction Purple showed moderate inhibition towards lipid peroxidation. ET3 had the lowest antioxidant capacity with a value of 2.713 ± 0.518 mg of TEP equivalent to per gram of sample. Hence, only fraction Yellow displayed the highest inhibition towards lipid oxidation even better than the standards (Figure 4.23)

Table 4.34: Lipid peroxidation capacity of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.*

Sub-fractions of *Beta vulgaris L.*	Lipid Peroxidation Capacity* (mg of TEP/ g of sample)
ET1	2.540 ± 0.576
ET2	1.563 ± 0.208
ET3	2.713 ± 0.518
Purple	2.417 ± 0.431
Yellow	0.508 ± 0.711

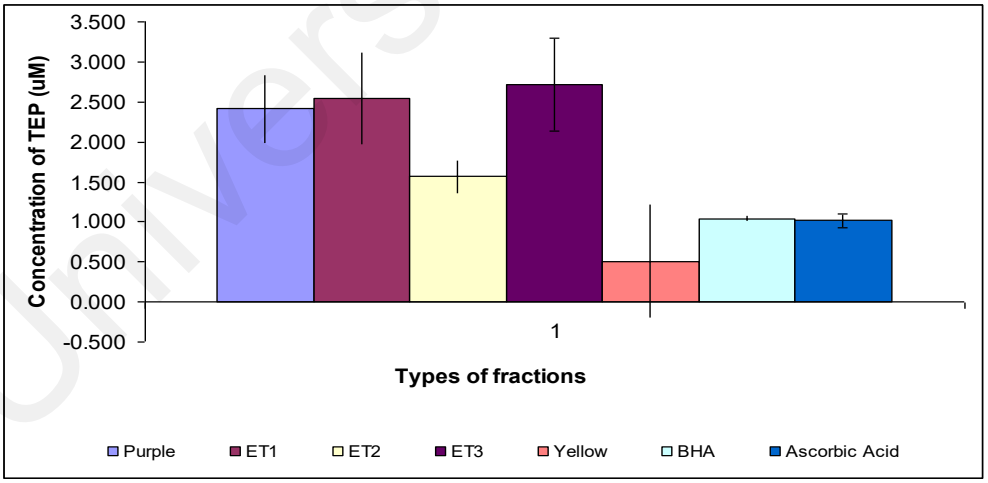


Figure 4.23: Comparison of inhibition of lipid peroxidation of sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* with positive standards

4.2.7 Tyrosinase Inhibitory Assay

In this assay, two substrates were used i.e. L-tyrosine and L-Dopa whilst tyrosinase is the enzyme. The concept of the experiment is to inhibit the enzyme. Tyrosinase is responsible for browning in plants and melanization in animals (Kubo and Kinst-Hori, 1999). Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used because it is readily available (Kubo and Kinst-Hori, 1999). All of the solutions were prepared with phosphoric acid buffer solution (pH 6.8). Kojic acid was used as positive control (Liu *et al.,* 2009). In the monophenolase activity, tyrosinase catalyzes the conversion of tyrosine to 3,4-dihydroxy phenylalanine (DOPA). The oxidation of DOPA into DOPA quinine is known as diphenolase activity, which are the initial steps in the pathway. The highly reactive quinines spontaneously evolve through non-enzymatic coupling to brown pigments of high molecular weight. Tyrosinase inhibitors (antioxidants) usually either chelate the copper ion within the tyrosinase active site, obstructing the substrate-enzyme interaction, or prevent oxidation via an electrochemical process (Jeong *et al.,* 2009). Dopachrome formation was measured at 490 nm (Huey-Ko *et al.,* 2008). The higher the tyrosinase inhibitors present in the test sample, the lesser the dopachrome formation (brownish to black color pigment).

i) L-Tyrosine as substrate

This part of the experiment measured the ability of the test samples to inhibit the monophenolase activity of the tyrosinase enzyme that catalyzes the conversion of tyrosine to DOPA.

a) Positive Standard (Kojic Acid)

Kojic acid which was used as the positive standard, exhibited a remarkable activity in inhibition of tyrosinase enzyme. At concentration 20.000 mg/ml, the highest percentage

inhibition was observed (97.950% ± 1.524). The lowest percentage inhibition was observed at concentration 0.01 mg/ml with -31.380% ± 0.293. There was a drastic drop in the percentage inhibition at concentration 0.100 mg/ml. The percentage inhibitions at various concentrations were shown in Table 4.35. The IC_{50} value as determined from Figure 4.24 is 0.345 mg/ml.

Table 4.35: Tyrosinase inhibitory activity by using L-tyrosine as substrate on kojic acid (positive standard references)

Concentration of Kojic Acid (mg/ml)	Percentage Inhibition*
20.000	97.950 ± 1.524
15.000	95.700 ± 0.339
10.000	96.770 ± 0.000
5.000	96.580 ± 0.169
4.000	95.890 ± 0.000
3.000	96.290 ± 0.169
2.000	96.190 ± 0.508
1.000	89.150 ± 0.000
0.500	87.680 ± 1.016
0.100	0.000
0.050	0.000
0.025	0.000
0.010	0.000

* Percentage inhibition of tyrosinase expressed as mean of triplicate experiments ± standard deviation (SD)

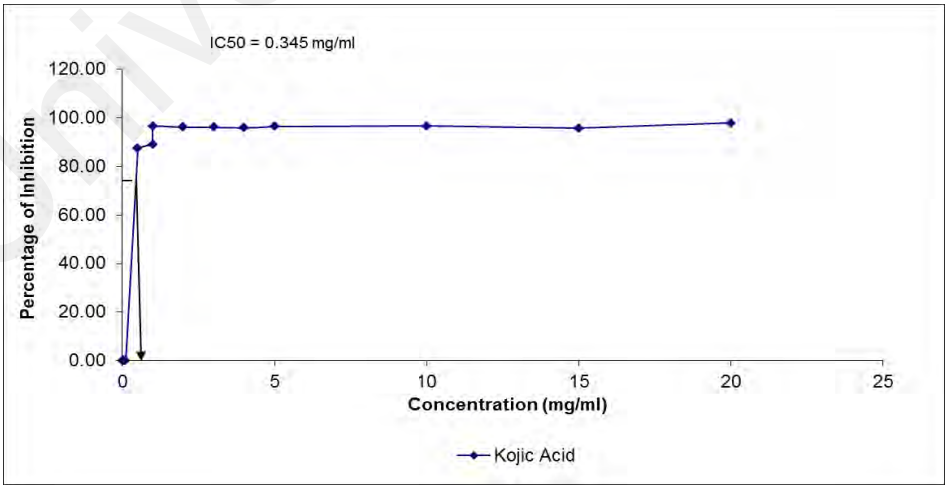


Figure 4.24: Tyrosinase inhibitory activity by using L-tyrosine as substrate and kojic acid (positive reference standard)

b) Tyrosinase inhibitory activity (monophenolase activity) of *Beta vulgaris L.* extracts

The results were tabulated in Table 4.36 and graphically showed in Figure 4.25. Based on the results, the monophenolase activity inhibition was assessed at various concentrations. At the highest concentration tested, 20.000 mg/ml, the ethyl acetate fraction had the highest inhibition with a percentage of 73.200% ± 9.818. The lowest inhibition at the highest concentration was shown by water fraction with 8.720% ± 4.286. At the lowest concentration tested, 1.000 mg/ml, the ethyl acetate fraction again exhibited the highest percentage of inhibition (22.440% ± 0.492) whereas methanol extract had the lowest percentage of inhibition (-17.400% ± 0.738). A comparison of the extracts with the positive standard (kojic acid) showed that kojic acid had far more remarkably good activity as compared to all the extracts. The extracts were not comparable to the positive standard. Overall among the extracts, the ethyl acetate fraction possessed the highest inhibition towards tyrosinase enzyme for monophenolase activity whilst the water fraction displayed the lowest tyrosinase inhibition.

The lowest IC_{50} value and the highest tyrosinase inhibition towards L-tyrosine were exhibited by positive standard, kojic acid with IC_{50} value 0.345 mg/ml. Among the extracts, ethyl acetate fraction had the highest activity with IC_{50} value 8.020 mg/ml. Methanol extract had a moderate inhibition with IC_{50} value of 19.250 mg/ml. Hexane, water fraction and juice extract had poor inhibition value with IC_{50} value more than 20.000 mg/ml respectively.

Table 4.36: Tyrosinase inhibitory activity by using L-tyrosine as substrate on extracts of *Beta vulgaris L.*

Concentration of Extract (mg/ml)	Percentage Inhibition*				
	Methanol Extract	Hexane Fraction	Ethyl acetate Fraction	Water Fraction	Juice Extract
20.000	51.120 ± 1.110	43.280 ± 1.281	73.200 ± 9.818	8.720 ± 4.286	22.970 ± 3.932
15.000	44.090 ± 0.587	38.540 ± 0.568	65.630 ± 1.848	4.460 ± 2.638	7.850 ± 1.776
10.000	41.840 ± 1.768	21.690 ± 1.863	61.460 ± 0.591	3.590 ± 2.638	5.520 ± 4.513
5.000	-5.570 ± 2.214	21.690 ± 1.476	31.720 ± 0.820	1.740 ± 4.341	1.740 ± 3.202
1.000	-17.400 ± 0.738	6.160 ± 1.136	22.440 ± 0.492	-9.880 ± 6.289	1.360 ± 1.678
Control	-	-	-	-	-

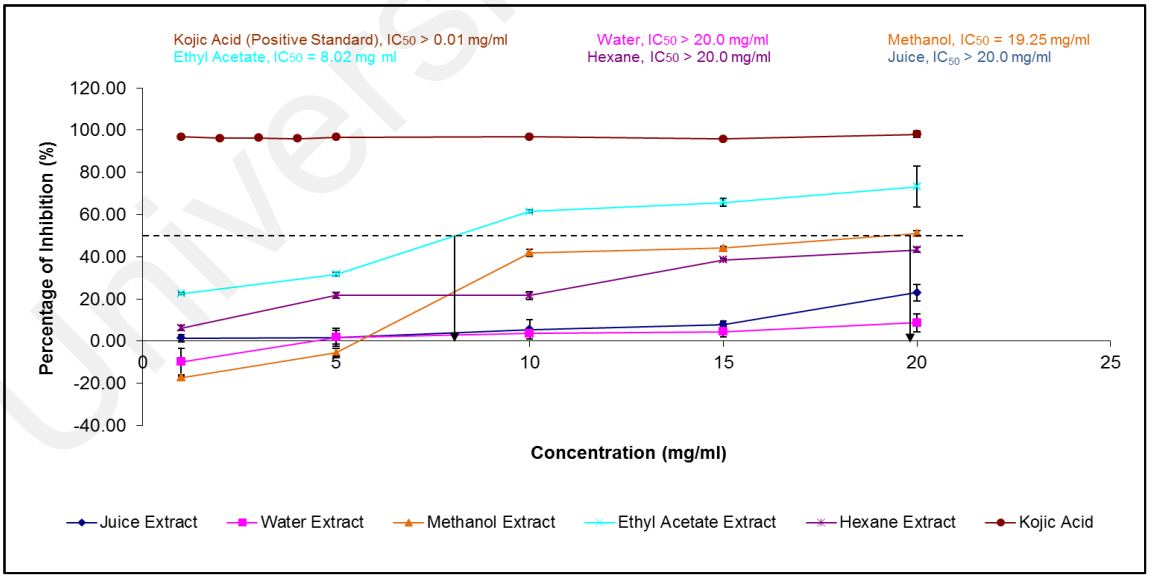


Figure 4.25: Comparison of tyrosinase inhibitory activity using L-tyrosine as substrate between extracts of *Beta vulgaris L.* and standard

c) Tyrosinase inhibitory activity (monophenolase activity) of sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.*

The results were expressed in Table 4.39 and Figure 4.26 & 4.27. The fractions were tested at a concentration ranging from 1.000 mg/ml to 0.010 mg/ml. At concentration 1.000 mg/ml, fraction purple had a remarkably good activity with percentage inhibition, 468.220% ± 4.767. ET1 had the lowest inhibition at the above concentration (39.880% ± 2.152). At the lowest tested concentration, 0.010 mg/ml, fraction ET3, yellow and purple had percentage inhibition more than 50.000%. ET1 and ET2 had lower inhibition -3.960% ± 2.152 and -3.790% ± 4.185, respectively. Based on Figure 4.26, we could clearly see that ET3, purple and yellow fraction had inhibition better than the positive standard, kojic acid. Among these, the purple fraction had the highest activity. ET1 and ET2 had lower inhibition as compared to kojic acid.

The IC_{50} values of sub-fractions were compared with that of positive standard (kojic acid). Fraction ET3, Purple and Yellow had the lowest IC_{50} value i.e. <0.010 mg/ml which is even lower than kojic acid. ET2 had a moderate IC_{50} value i.e. 0.980 mg/ml. ET1 had a high IC_{50} value (>1.000 mg/ml), and hence low inhibition towards tyrosinase.

Table 4.37: Comparison of IC_{50} values of monophenolase tyrosinase inhibitory activity between positive reference standard and extracts of *Beta vulgaris L.*

Extracts of *Beta vulgaris L.*	IC_{50} (mg/ml)
Methanol extract	19.250
Ethyl Acetate fraction	8.020
Hexane fraction	>20.000
Water fraction	>20.000
Juice extract	>20.000
Kojic Acid (Positive Standard)	0.345

Table 4.38: Comparison of IC_{50} values of monophenolase tyrosinase inhibitory activity between positive reference standard and sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.*

Sub-fractions of *Beta vulgaris L.*	IC_{50} (mg/ml)
ET1	>1.000
ET2	0.980
ET3	<0.010
Purple	<0.010
Yellow	<0.010
Kojic Acid (Positive Standard)	0.345

Table 4.39: Tyrosinase inhibitory activity by using L-tyrosine as substrate on sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.*

Concentration of Fractions (mg/ml)	Percentage Inhibition*				
	ET1	ET2	ET3	Purple	Yellow
1.000	39.880 ± 2.152	51.080 ± 3.508	174.940 ± 1.343	468.220 ± 4.767	291.820 ± 7.671
0.500	35.490 ± 3.061	36.950 ± 0.258	176.230 ± 3.046	310.940 ± 2.685	226.700 ± 3.379
0.100	35.830 ± 3.061	33.160 ± 0.149	160.120 ± 1.469	129.800 ± 2.787	143.580 ± 2.980
0.050	34.800 ± 0.298	34.800 ± 0.149	162.880 ± 4.795	107.840 ± 5.360	133.510 ± 2.995
0.025	32.130 ± 0.395	9.990 ± 0.298	174.850 ± 6.117	74.500 ± 9.571	133.760 ± 5.628
0.010	-3.960 ± 2.152	-3.790 ± 4.185	155.990 ± 4.056	77.000 ± 5.149	115.760 ± 8.073
Control	-	-	-	-	-

* Percentage inhibition of enzyme tyrosinase expressed as mean of triplicate experiments ± standard deviation (SD)

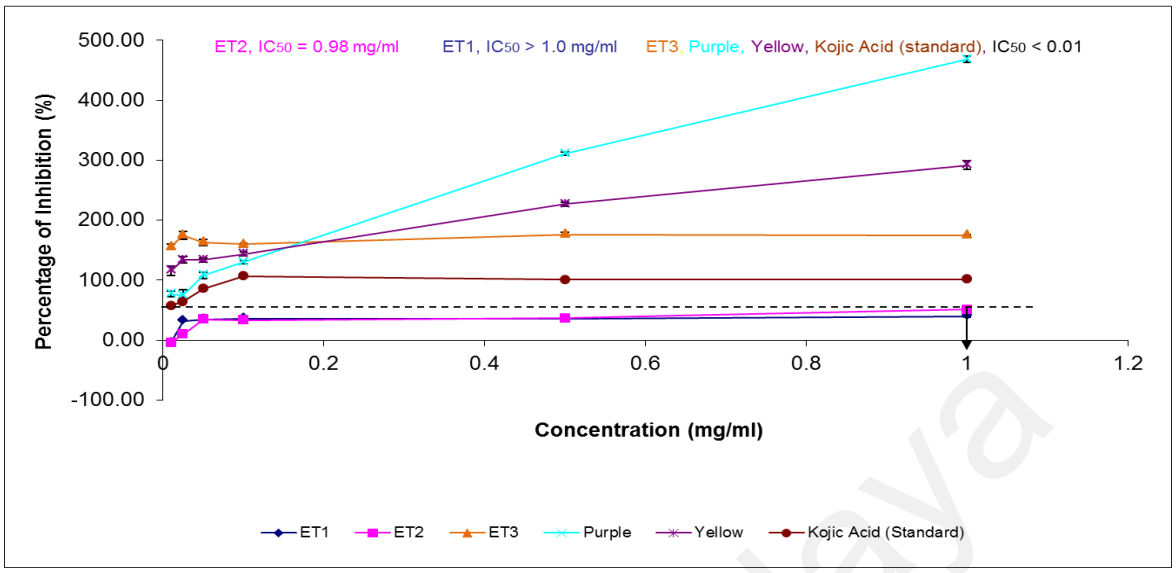


Figure 4.26: Comparison of tyrosinase inhibitory activity, between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-tyrosine as substrate.

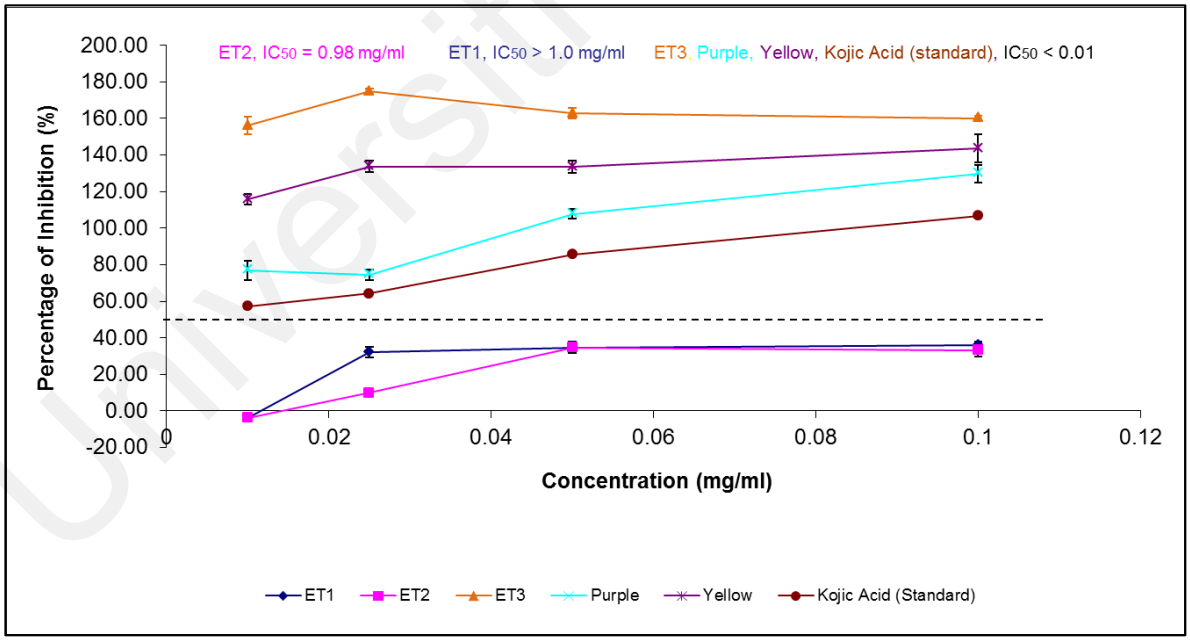


Figure 4.27: Magnification of activity at lower concentrations for of tyrosinase inhibitory activity, between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-tyrosine as substrate.

ii) L-DOPA as substrate

This part of the experiment measured the ability of the test samples to inhibit the diphenolase activity of the tyrosinase enzyme that catalyzed the conversion of DOPA to DOPA quinine.

a) Positive Standard (Kojic Acid)

Kojic acid which was used as the positive standard, exhibited a remarkable activity in inhibition of tyrosinase enzyme. At concentration 20.000 mg/ml, the highest percentage of inhibition observed was 93.230% ± 0.541. The lowest percentage of inhibition was observed at concentration 0.010 mg/ml with -12.710% ± 1.542. After concentration 0.500 mg/ml, there was a drastic drop at the percentage inhibition at concentration 0.100 mg/ml. The percentages inhibitions at various concentrations were shown in Table 4.40. The IC_{50} value for kojic acid is 0.460 mg/ml (Fig. 4.28).

Table 4.40: Tyrosinase inhibitory activity of kojic acid (positive standard) using L-Dopa as substrate

Concentration of Kojic Acid (mg/ml)	Percentage Inhibition*
20.000	93.230 ± 0.541
15.000	91.250 ± 0.651
10.000	90.630 ± 0.180
5.000	85.830 ± 0.180
4.000	86.670 ± 0.541
3.000	83.850 ± 0.541
2.000	82.080 ± 0.477
1.000	72.810 ± 0.180
0.500	55.000 ± 0.827
0.100	0.000
0.050	0.000
0.025	0.000
0.010	0.000

* Percentage inhibition of enzyme tyrosinase as a mean of triplicate experiments ± standard deviation (SD)

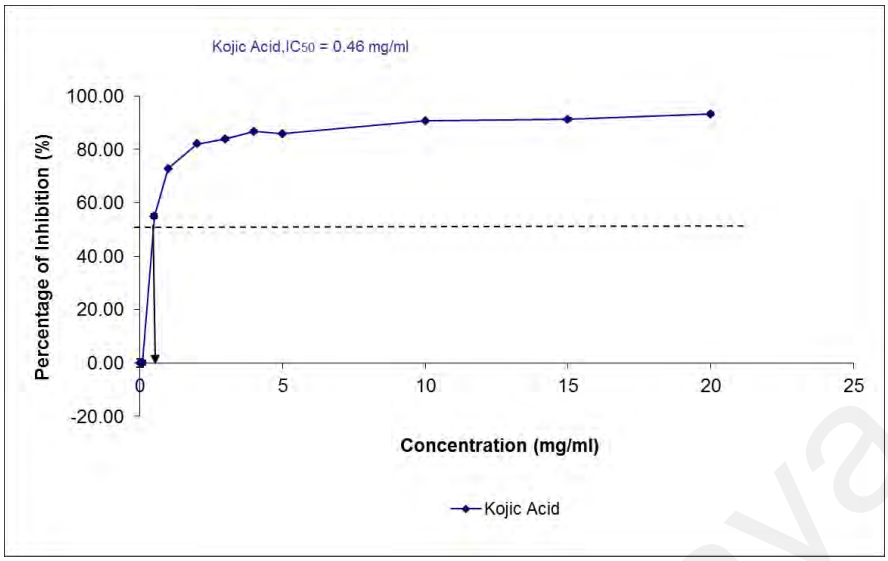


Figure 4.28: Tyrosinase inhibitory activity of kojic acid using L-Dopa as substrate

b) Tyrosinase inhibitory activity (diphenolase activity) of extracts of *Beta vulgaris L.*

The result from this experiment is shown in Table 4.41 and 4.42 and graphically shown in Figure 4.29 & 4.30. The diphenolase activity inhibition was assessed at various concentrations. At the highest concentration tested, 20.000 mg/ml, juice extract had the highest inhibition with a percentage of 201.040% ± 5.871. The lowest inhibition at the highest concentration was shown by methanol extract with 35.100% ± 0.651. At the lowest concentration tested, 1.000 mg/ml, ethyl acetate fraction again had the highest percentage of inhibition (51.250% ± 3.780) whereas methanol extract had the lowest percentage of inhibition (9.900% ± 4.311). Comparison of the extracts with the positive standard (kojic acid) showed that kojic acid at higher concentration (15.000 mg/ml and 20.000 mg/ml), water fraction had a better activity than kojic acid. Juice extract had a remarkably good activity at 20.000 mg/ml, with a percentage inhibition 201.040% ± 5.871, this activity was even better than the standard and also the highest inhibition towards tyrosinase in diphenolase activity was noted. Among the extracts, methanol extract had the lowest inhibition. Ethyl acetate fraction had inhibitions higher than 50.000% at the lowest

concentration tested i.e. 1.000 mg/ml. Hence tests were further continued at lower concentrations to determine the IC_{50} value.

The

inhibition value of ethyl acetate fraction at lower concentrations was tabulated at Table 4.42.

Table 4.41: Tyrosinase inhibitory activity of extracts of *Beta vulgaris L.* by using L-Dopa as substrate

Concentration of Extract (mg/ml)	Percentage Inhibition*				
	Methanol Extract	Hexane Fraction	Ethyl acetate Fraction	Water Fraction	Juice Extract
20.000	35.100 ± 0.651	99.250 ± 1.736	89.580 ± 2.261	101.660 ± 1.793	201.040 ± 5.871
15.000	30.420 ± 3.802	45.360 ± 1.565	84.790 ± 2.127	100.210 ± 1.563	77.430 ± 2.510
10.000	16.560 ± 1.909	35.340 ± 2.417	69.480 ± 4.865	84.680 ± 0.621	35.610 ± 5.968
5.000	18.230 ± 0.651	29.070 ± 3.858	68.960 ± 3.789	51.350 ± 3.421	42.440 ± 4.825
1.000	9.900 ± 4.311	27.820 ± 6.739	51.250 ± 3.780	43.890 ± 4.969	28.570 ± 3.187
Control	-	-	-	-	-

* Percentage inhibition of enzyme tyrosinase expressed as mean of triplicate experiments ± standard deviation (SD)

Table 4.42: Tyrosinase inhibitory activity of ethyl acetate fraction tested at lower concentrations using L-Dopa as substrate

Concentration of Ethyl Acetate Fraction (mg/ml)	Percentage Inhibition (%)*
1.000	51.250 ±3.780
0.500	37.190 ± 6.156
0.100	7.290 ± 11.835
0.050	24.900 ± 1.183
0.025	40.940 ± 7.147
0.010	29.270 ± 2.526
Control	-

* Percentage inhibition of enzyme tyrosinase as a mean of triplicate experiments ± standard deviation (SD)

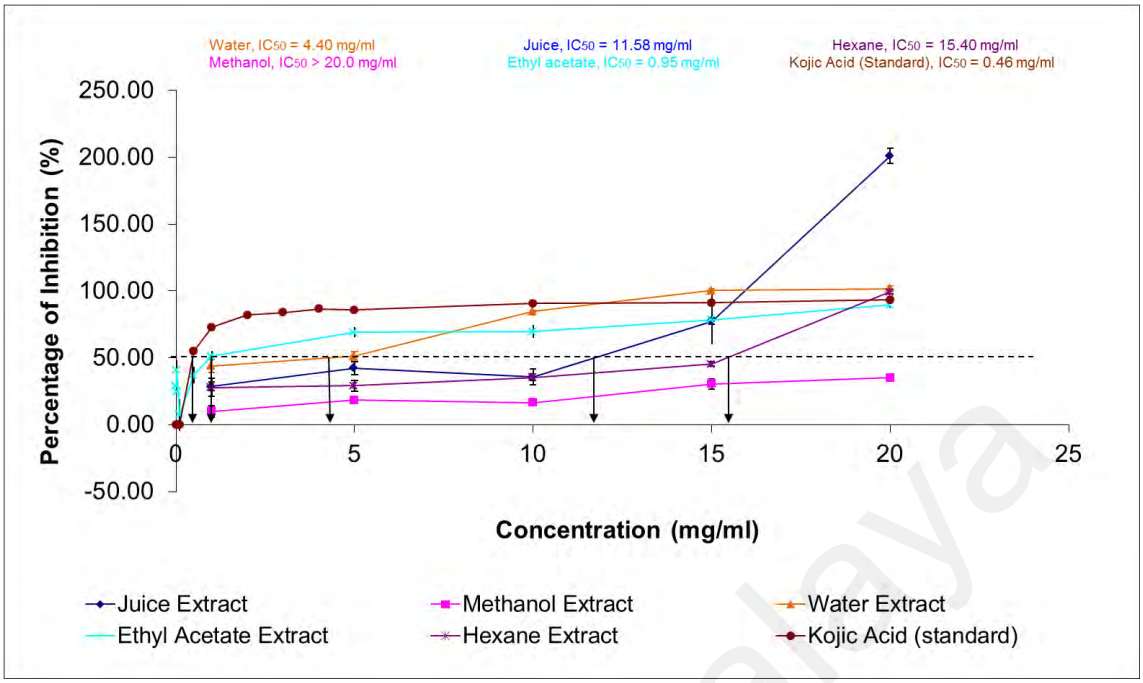


Figure 4.29: Comparison of tyrosinase inhibitory activity between extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate

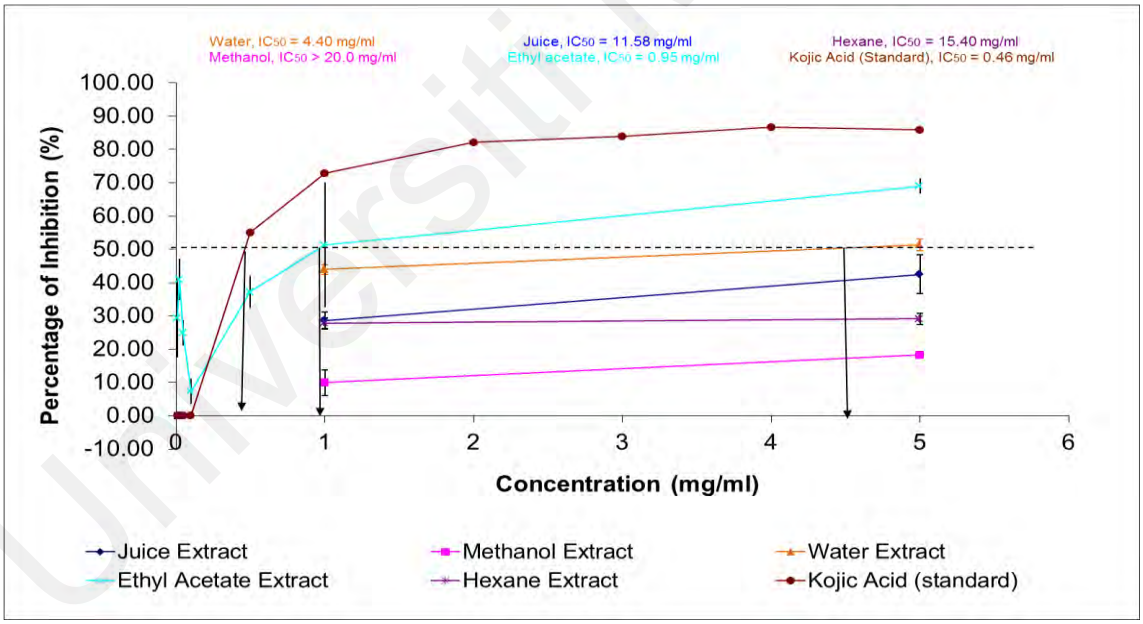


Figure 4.30: Magnification of readings at lower concentration for tyrosinase inhibitory activity between extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate

c) Tyrosinase inhibitory activity (diphenolase activity) of sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.*

The results were expressed in Table 4.43 and Figure 4.31 & 4.32. The fractions were tested at a concentration ranging from 1.000 mg/ml to 0.010 mg/ml. At concentration 1.000 mg/ml, the yellow fraction had remarkably good activity with percentage of inhibition, 297.100% ± 4.136. The purple fraction had the lowest inhibition among the tested fractions at the particular concentration (96.270% ± 1.643). At the lowest tested concentration, 0.010 mg/ml, all the tested fractions had percentage inhibition more than 50.000%. ET1 and ET2, ET3, purple and yellow had percentage inhibition 82.820% ± 3.187, 54.870% ± 4.136, 79.090% ± 0.359, 66.870% ± 0.717 and 51.970% ± 3.744 respectively. Based on Figure 4.26, all the fractions had inhibition better than the positive reference standard, kojic acid. Among these, the yellow fraction had the highest activity.

Table 4.43: Tyrosinase inhibitory activity of sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* by using L-Dopa as substrate

Concentration of Fractions (mg/ml)	Percentage Inhibition*				
	ET1	ET2	ET3	Purple	Yellow
1.000	98.550 ± 5.020	98.340 ± 3.639	106.830 ± 5.590	96.270 ± 1.643	297.100 ± 4.136
0.500	95.860 ± 3.126	94.620 ± 3.993	97.720 ± 5.172	92.750 ± 7.199	140.990 ± 2.846
0.100	85.920 ± 5.636	82.820 ± 8.106	94.620 ± 2.181	89.030 ± 7.199	67.080 ± 7.453
0.050	85.710 ± 2.846	81.370 ± 0.621	87.780 ± 5.968	84.060 ± 4.136	47.620 ± 3.795
0.025	83.440 ± 0.949	55.690 ± 2.801	87.370 ± 3.126	68.530 ± 5.020	45.760 ± 2.510
0.010	82.820 ± 3.187	54.870 ± 4.136	79.090 ± 0.359	66.870 ± 0.717	51.970 ± 3.744
Control	-	-	-	-	-

* Percentage inhibition as a mean of triplicate ± standard deviation (SD)

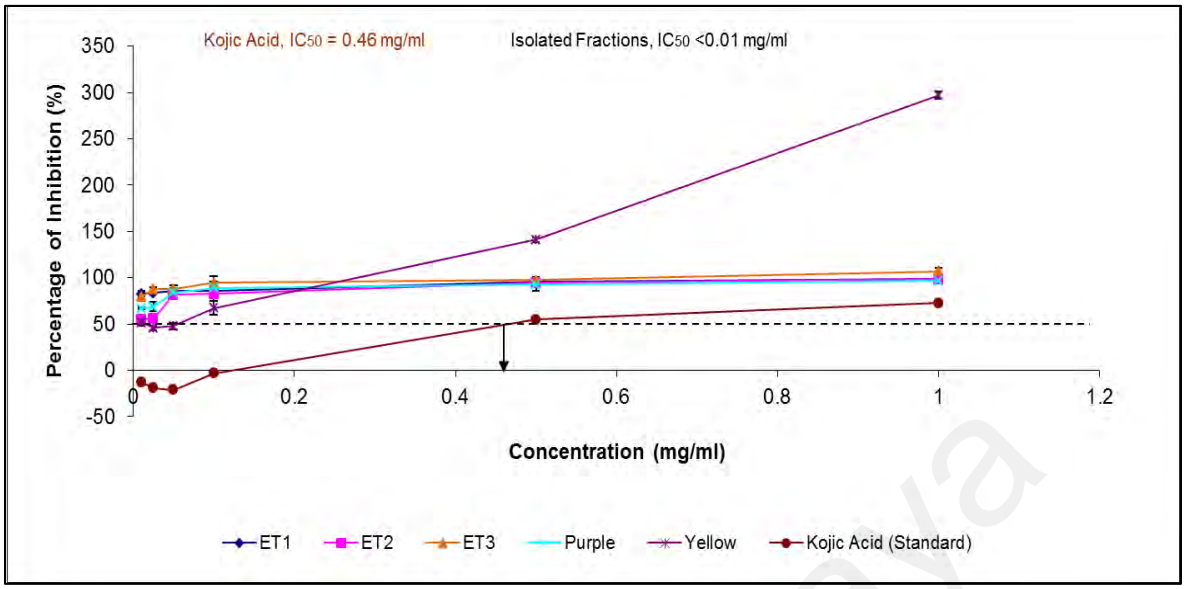


Figure 4.31: Comparison of tyrosinase inhibitory activity between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate.

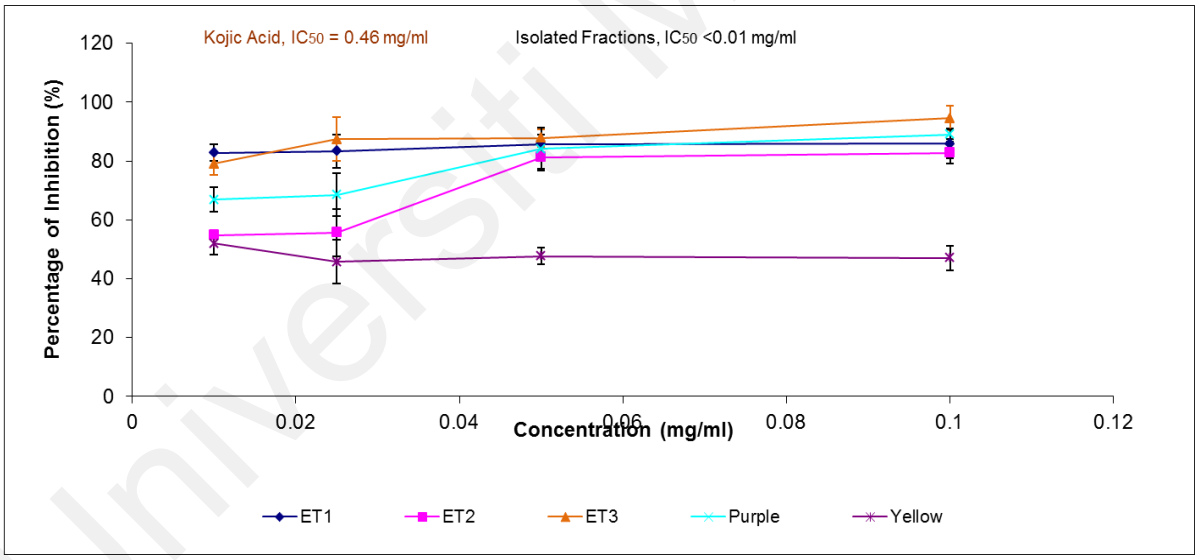


Figure 4.32: Magnification of lower concentration of tyrosinase inhibitory activity between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate.

d) Comparison of IC_{50} values between positive standard and extracts of *Beta vulgaris L.*

The lowest IC_{50} values and the highest tyrosinase inhibition towards L-Dopa were exhibited by positive standard, kojic acid with IC_{50} value 0.460 mg/ml. Among the extracts, ethyl acetate fraction had the highest activity with IC_{50} value 0.950 mg/ml. Water fraction had a moderate inhibition with IC_{50} value of 4.400 mg/ml. Juice, hexane fraction and methanol extract had poor inhibition value with IC_{50} values 11.580, 15.400 and >20.000 mg/ml respectively.

Table 4.44: Comparison of IC_{50} values of diphenolase tyrosinase inhibitory activity between positive standard and extracts of *Beta vulgaris L.*

Extracts of *Beta vulgaris L.*	IC_{50} (mg/ml)
Methanol extract	>20.000
Ethyl Acetate fraction	0.950
Hexane fraction	15.400
Water fraction	4.400
Juice extract	11.580
Kojic Acid (Positive Standard)	0.460

e) Comparison of IC_{50} values between positive standard and sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.*

The IC_{50} values of sub-fractions were compared with that of positive standard (Kojic Acid). All the fractions had an inhibition towards tyrosinase in diphenolase activity which is better than the standard, kojic acid. The IC_{50} values were <0.010 mg/ml respectively. Kojic acid had an IC_{50} value of 0.460 mg/ml.

Table 4.45: Comparison of IC_{50} values of diphenolase tyrosinase inhibitory activity between positive standard and sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.*

Sub-fractions of *Beta vulgaris L.*	IC_{50} (mg/ml)
ET1	<0.010
ET2	<0.010
ET3	<0.010
Purple	<0.010
Yellow	<0.010
Kojic Acid (Positive Standard)	0.460

4.2.8 Total Phenolic Content (Folin-Ciocalteu Assay)

The amount of total phenolic was determined according to the method of Velioglu *et al.* (1998). This assay gained its popularity as it is commonly known as total phenolic assay. The assay in reality measures the sample's reducing capacity but it is not shown in the name of the assay. The test samples that had reductones would turn into a blue color shade. The absorbance value of the reaction mixture was taken at 725 nm. The standard calibration curve was a plot using gallic acid. This assay is a convenient, simple and reproducible assay and is a routine assay that is used in studying phenolic antioxidants (Huang *et al.,* 2005). The extracts were expressed as mg of gallic acid equivalent per gram of extracts.

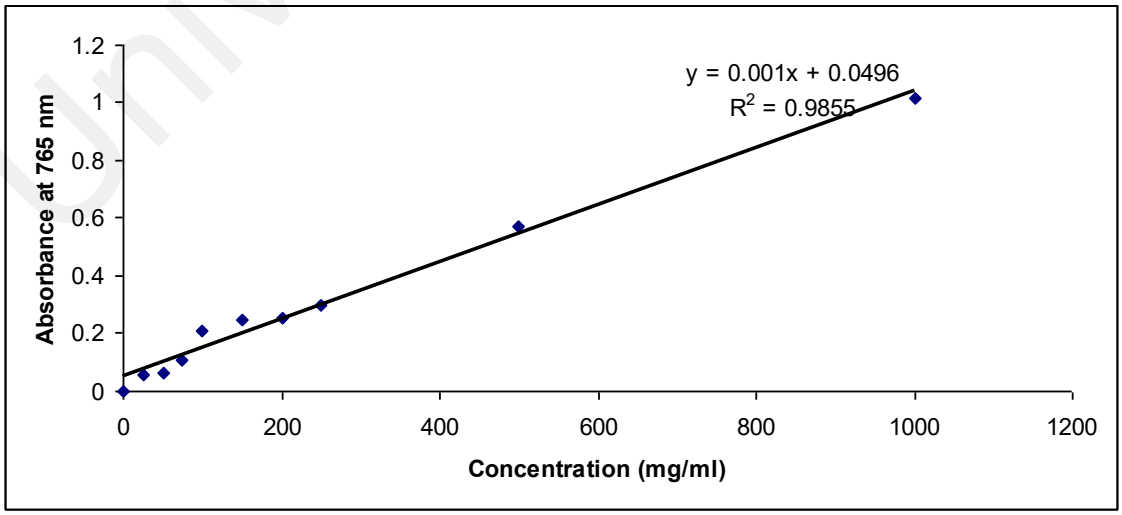


Figure 4.33: The gallic acid calibration graph

i) The reducing capacity of extracts of *Beta vulgaris L.* and positive reference standard (BHA) based on total phenolic content assay

The reducing capacity of extracts of *Beta vulgaris L*, determined from regression quotation of calibration curve (y=0.001x + 0.0496, R2 = 0.9855, Figure 4.33) were expressed as mg of GAE per gram of extracts. The absorbance value of the test extract after subtraction of control was translated into reducing capacity [mg/l of gallic acid equivalents (GAEs)] using the gallic acid calibration plot with the following formula:

$$\text{Reducing capacity (mg/ml of GAEs)} = \frac{(y-0.0496)}{0.001}$$

As for the extracts, the ethyl acetate fraction had the highest reducing capacity (58.860 ± 0.566 mg of GAE/ g of extract). This was the highest value among the extracts. Hexane and methanol extract had moderate reducing capacity with 24.190 ± 0.243 and 20.380 ± 0.821 mg of GAE/ g of extract. Water fraction and juice extract had quite low reductive capacity with 17.180 ± 0.198 and 10.800 ± 0.293 mg of GAE/ g of extract. However, the highest reducing capacity was exhibited by positive standard with a remarkable value of 247.020 ± 1.871 mg of GAE/ g of sample. Figure 4.34 show the comparison of the reducing capacity of extracts of *Beta vulgaris L*. with the positive standard BHA.

Table 4.46 Reducing capacity of extracts of *Beta vulgaris L.* in Folin-Ciocalteau assay (total phenolic content assay)

Extracts of *Beta vulgaris L.*	Reducing Capacity* (mg of GAEs/ g of extracts)
Methanol	20.380 ± 0.821
Hexane	24.190 ± 0.243
Ethyl Acetate	58.860 ± 0.566
Water	17.180 ± 0.198
Juice	10.800 ± 0.293
BHA (positive standard)	247.020 ± 1.871

* Reducing capacity as a mean of triplicate experiments ± standard deviation (SD)

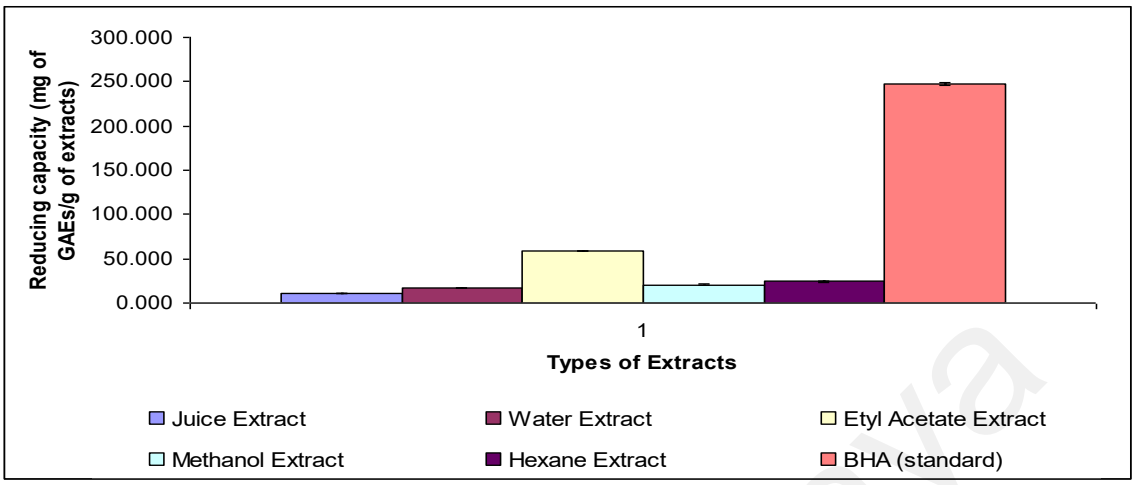


Figure 4.34: Comparison of reducing capacity between extracts of *Beta vulgaris L.* and standard on Folin-Ciocalteu assay

4.3 Cytotoxicity Assay

4.3.1 *In vitro* Neutral Red Cytotoxicity Assay on extracts of *Beta vulgaris L.*

i) The cytotoxicity of extracts of *Beta vulgaris L.* on CasKi cell line

The cytotoxicity of extracts of *Beta vulgaris L.* were tabulated in Table 4.47 and graphically shown in Figure 4.35. Based on the results, we could see that all the extracts did not exhibit high cytotoxic activity. The highest activities among the extracts were expressed by ethyl acetate fraction and the lowest activity was exhibited by hexane fraction. Juice extract, methanol extract and water fraction had a moderate activity in comparison within the extracts. Highest inhibition as noted at 100.000 µg/ml of ethyl acetate fraction (43.450% ± 0.136) and lowest inhibition was expressed by hexane fraction at a concentration 1.000 µg/ml with a percentage of (-48.170% ± 0.086).

Table 4.47: Percentage inhibition of extracts and fractions of *Beta vulgaris L.* on CasKi cell line (*in vitro*)

Concentration of extracts and fractions (µg/ml)	Percentage Inhibition (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
100.000	17.260 ± 0.000	18.690 ± 5.306	43.450 ± 0.136	6.360 ± 0.228	5.490 ± 2.746
75.000	8.880 ± 1.033	-15.330 ± 1.456	37.530 ± 2.490	8.800 ± 0.000	6.900 ± 0.000
50.000	7.260 ± 0.000	-12.020 ± 2.303	11.960 ± 0.679	3.690 ± 0.057	5.730 ± 1.361
25.000	4.630 ± 0.365	-27.300 ± 0.257	6.390 ± 0.679	3.360 ± 0.343	-5.970 ± 2.377
10.000	7.300 ± 2.487	-31.110 ± 0.086	4.470 ± 0.118	0.030 ± 0.057	-8.800 ± 0.070
1.000	9.330 ± 0.486	-48.170 ± 0.086	-3.020 ± 2.513	0.000 ± 0.000	-10.770 ± 0.991
Control	-	-	-	-	-

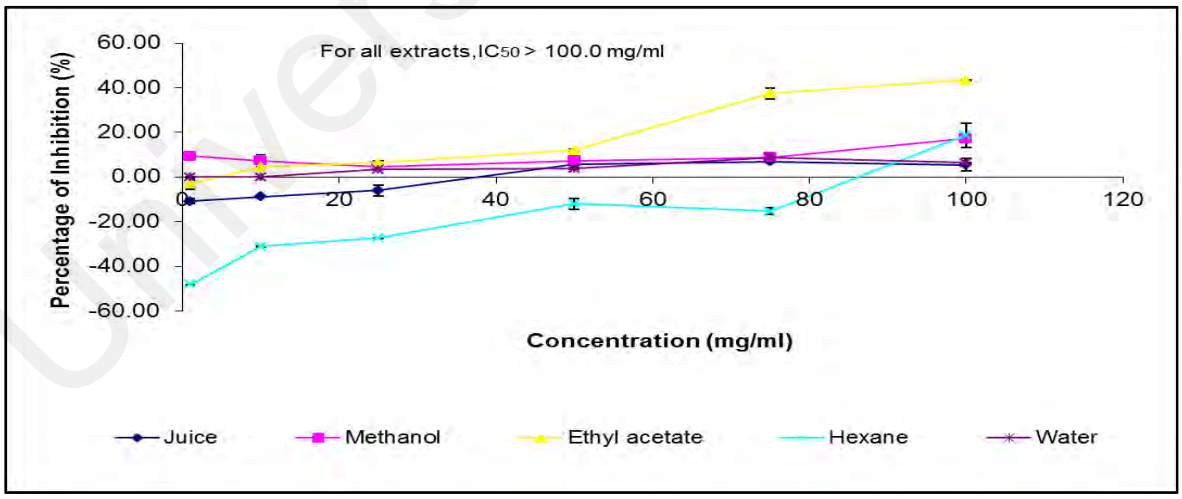


Figure 4.35: The *in vitro* growth inhibitions of CasKi cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

IC_{50} values of *Beta vulgaris L.* on Neutral Red Cytotoxicity assay based on CasKi cell line

All the extracts of *Beta vulgaris L*. exhibited IC_{50} values more than the value 100.000 µg/ml (Table 4.48).

Table 4.48: IC_{50} values of *Beta vulgaris L.* extracts on CasKi cell line

Extracts of *Beta vulgaris L.*	IC_{50} (µg/ml)
Methanol extract	>100.000
Ethyl acetate fraction	>100.000
Hexane fraction	>100.000
Water fraction	>100.000
Juice extract	>100.000

ii) The cytotoxicity of *Beta vulgaris L*. extracts on A549 cell line

The cytotoxic activity of extracts of *Beta vulgaris L.* on A549 is shown in Table 4.49 and Figure 4.36. Based on the results, we can see that almost all the extracts have very low or no inhibition at all towards the lung carcinoma cell. Only juice extract and ethyl acetate fraction had some inhibition towards the higher concentration of extracts. At the highest concentration (100.000 µg/ml), highest inhibition exhibited by ethyl acetate fraction (42.050% ± 1.413) and this is the highest inhibition among the tested extracts at various concentrations. Almost all the extracts had a negative inhibition value. Methanol extract and hexane fraction had the lowest inhibition against this cell line.

Table 4.49: Percentage inhibition of *Beta vulgaris L.* extracts on A549 cell line (*in vitro*)

Concentration of Extracts (µg/ml)	Percentage Inhibition (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
100.000	0.140 ± 1.036	6.590 ± 5.904	42.050 ± 1.413	-17.120 ± 3.612	4.790 ± 0.297
75.000	-33.740 ± 1.426	-24.000 ± 2.428	37.220 ± 0.540	-14.640 ± 4.073	2.510 ± 0.494
50.000	-40.190 ± 5.112	-34.080 ± 14.318	27.440 ± 1.338	-12.610 ± 0.975	1.710 ± 1.199
25.000	-51.440 ± 0.412	-48.380 ± 3.107	15.310 ± 0.204	-13.180 ± 8.282	-6.680 ± 0.593
10.000	-60.080 ± 4.115	-73.510 ± 2.585	-19.200 ± 4.418	-13.180 ± 4.645	-4.570 ± 1.909
1.000	-61.870 ± 3.688	-85.950 ± 4.309	-26.030 ± 7.793	-10.920 ± 1.861	-6.790 ± 1.985
Control	-	-	-	-	-

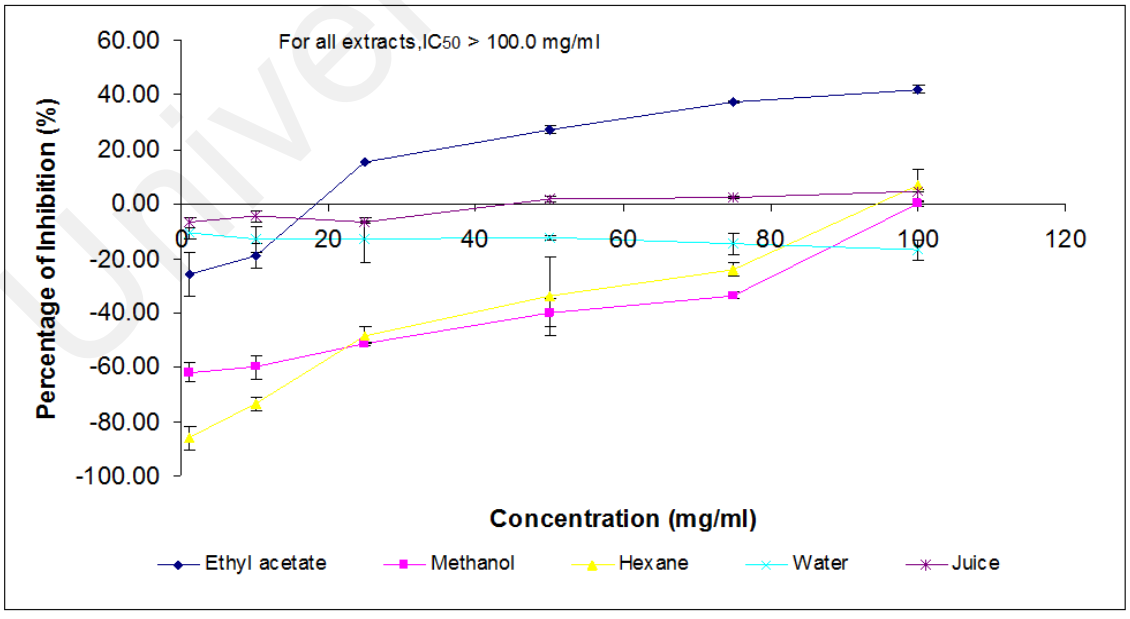


Figure 4.36: The *in vitro* growth inhibitions of A549 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

IC_{50} values of *Beta vulgaris L.* extracts on A549 cell line

All the extracts of *Beta vulgaris L*. exhibited IC_{50} values more than the value 100.000 µg/ml (Table 4.50).

Table 4.50: IC_{50} values of *Beta vulgaris L.* extracts on A549 cell line

Extracts of *Beta vulgaris L.*	IC_{50} (µg/ml)
Methanol extract	>100.000
Ethyl acetate fraction	>100.000
Hexane fraction	>100.000
Water fraction	>100.000
Juice extract	>100.000

iii) The cytotoxicity of *Beta vulgaris L.* extracts on MCF7 cell line

The cytotoxicity of extracts of *Beta vulgaris L.* is tabulated at Table 4.51 and portrayed at Figure 4.37. All the extracts had a positive inhibition at all the concentrations towards this cell line. At the highest concentration tested (100.000 µg/ml), ethyl acetate fraction and juice extract had moderate inhibition with a percentage inhibition of 59.330% ± 4.423 and 41.830% ± 1.680 respectively. Hexane fraction had the lowest inhibition (6.880% ± 0.000). Juice extract exhibited a stable inhibition that is dose dependent from the low to high concentration tested (41.830% ± 1.680 to 3.130% ± 2.173). At the lowest concentration tested, 1 µg/ml, juice extract had the lowest percentage inhibition 3.130% ± 2.173.

Table 4.51: Percentage inhibition of extracts of *Beta vulgaris L.* on MCF7 cell line (*in vitro*)

Concentration of Extracts/Fractions (µg/ml)	Percentage Inhibition (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
100.000	10.920 ± 3.278	6.880 ± 0.000	59.330 ± 4.423	13.940 ± 4.621	41.830 ± 1.680
75.000	12.750 ± 0.933	3.340 ± 0.613	26.450 ± 3.645	15.480 ± 2.943	34.730 ± 4.598
50.000	9.260 ± 0.838	5.800 ± 2.801	24.100 ± 4.086	13.490 ± 2.259	25.000 ± 0.000
25.000	7.700 ± 1.496	5.210 ± 2.552	13.540 ± 3.862	13.830 ± 1.432	11.470 ± 0.850
10.000	8.050 ± 1.496	2.780 ± 0.954	14.190 ± 1.172	15.900 ± 0.406	6.000 ± 1.000
1.000	9.460 ± 0.658	8.250 ± 0.170	8.950 ± 4.108	15.260 ± 4.621	3.130 ± 2.173
Control	-	-	-	-	-

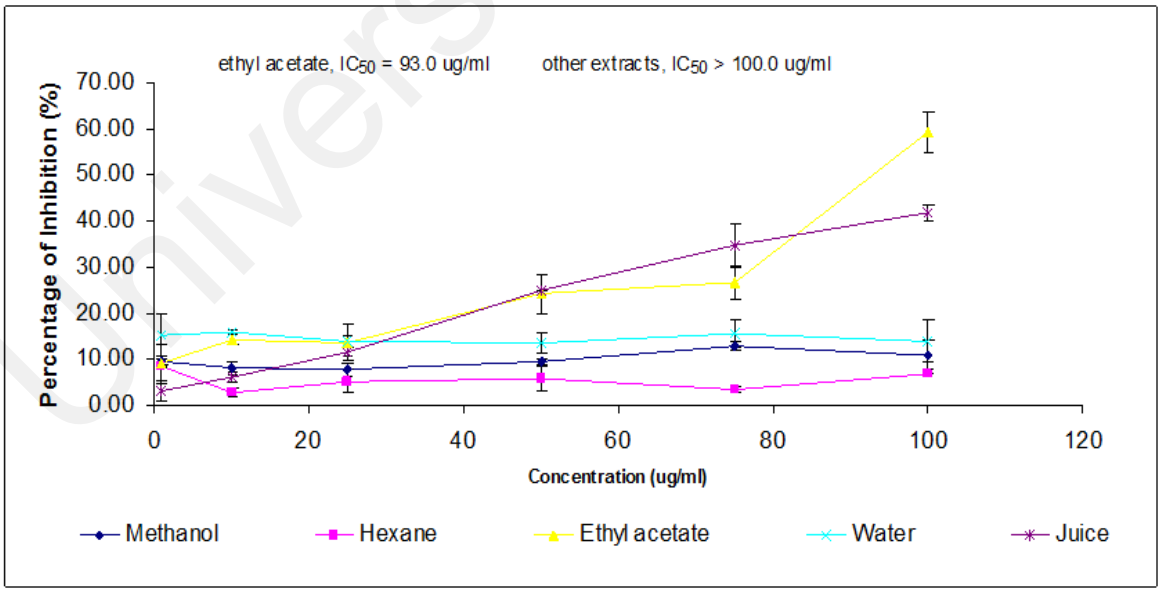


Figure 4.37: The *in vitro* growth inhibitions of MCF7 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

IC_{50} values of *Beta vulgaris L.* on Neutral Red Cytotoxicity assay based on MCF7 cell line

Ethyl acetate fraction exhibited the lowest IC_{50} value among the extracts i.e. 93.000 µg/ml. All the other extracts had an IC_{50} value more than 100.000 µg/ml. Hence, ethyl acetate fraction had better inhibition towards MCF7 as compared to all the other extracts.

Table 4.52: IC_{50} values of *Beta vulgaris L.* extracts on MCF7 cell line

Extracts of *Beta vulgaris L.*	IC_{50} (µg/ml)
Methanol extract	>100.000
Ethyl acetate fraction	93.000
Hexane fraction	>100.000
Water fraction	>100.000
Juice extract	>100.000

iv) The cytotoxicity of *Beta vulgaris L.* extracts on HT-29 cell line

The results were represented in the Table 4.53 and Figure 4.38. It was obvious that the ethyl acetate fraction had the highest inhibition towards this cell line whereas juice extract had the lowest inhibition towards this cell. At the highest concentration tested (100.000 µg/ml), ethyl acetate fraction expressed a very good inhibition value of 82.930% ± 3.675 but on the other hand, juice extract had the lowest inhibition (7.370% ± 1.637) at this concentration. At the lowest concentration tested, (1.000 µg/ml), methanol extract had the highest inhibition (13.650% ± 0.939) whereas, juice extract had the lowest inhibition (-12.480% ± 1.150). Other extracts, such as methanol extract, water fraction and hexane fraction had a moderate inhibition.

Table 4.53: Percentage inhibition of extracts of *Beta vulgaris L.* on HT-29 cell line (*in vitro*)

Concentration of Extracts (µg/ml)	Percentage Inhibition (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
100.000	17.140 ± 1.020	24.610 ± 0.912	82.930 ± 3.675	18.610 ± 0.000	7.370 ± 1.637
75.000	13.500 ± 1.975	25.300 ± 1.083	58.880 ± 0.000	9.640 ± 3.339	-1.510 ± 0.000
50.000	12.360 ± 0.765	20.500 ± 0.114	18.160 ± 1.591	10.410 ± 0.000	-3.970 ± 0.000
25.000	16.810 ± 0.701	15.730 ± 0.317	21.940 ± 0.000	8.800 ± 2.064	-5.860 ± 0.000
10.000	3.130 ± 0.446	10.760 ± 0.171	19.590 ± 0.714	9.640 ± 1.153	-10.020 ± 2.947
1.000	13.650 ± 0.939	-8.030 ± 3.660	-4.830 ± 2.415	6.660 ± 0.971	-12.480 ± 1.150
Control	-	-	-	-	-

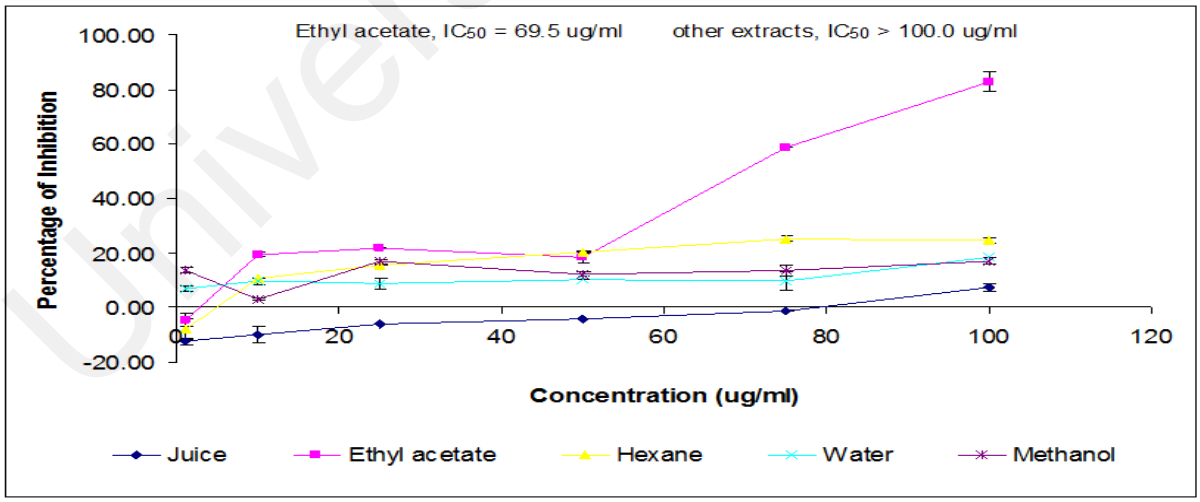


Figure 4.38: The *in vitro* growth inhibitions of HT29 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

IC_{50} values of *Beta vulgaris L.* on Neutral Red Cytotoxicity assay based on HT-29 cell line

Ethyl acetate fraction exhibited the lowest IC_{50} value among the extracts i.e. 69.500 µg/ml. In fact, this was the lowest IC_{50} value recorded among all the extracts throughout the whole cytotoxicity study. All the other extracts had an IC_{50} value more than 100.000 µg/ml. Hence, ethyl acetate fraction had better inhibition towards HT-29 as compared to all the other extracts.

Table 4.54: IC_{50} values of *Beta vulgaris L.* extracts on HT-29 cell line

Extracts of *Beta vulgaris L.*	IC_{50} (µg/ml)
Methanol extract	>100.000
Ethyl acetate fraction	69.500
Hexane fraction	>100.000
Water fraction	>100.000
Juice extract	>100.000

v) The cytotoxicity of *Beta vulgaris L.* extracts on HTC-116 cell line

The cytotoxicity of extracts of *Beta vulgaris L.* towards HCT-116 cell line in various concentrations is represented in Table 4.55 and Figure 4.39. From the data obtained, we can see that the ethyl acetate fraction had the highest percentage inhibition (55.680% ± 0.000 to 5.430% ± 2.664). The lowest percentage inhibition was by water and juice extracts. At concentration 100.000 µg/ml, the highest concentration tested, ethyl acetate fraction had the highest percentage inhibition with 55.680% ± 0.000 whereas the lowest inhibition was exhibited by juice extract (25.850% ± 1.650). At the lowest concentration tested, 1.000 µg/ml, hexane fraction had the highest inhibition (21.220% ± 2.439), whereas the lowest inhibition was by water and juice extract with a percentage inhibition of -19.730% ± 2.098 each.

Table 4.55: Percentage inhibition of extracts of *Beta vulgaris L.* on HCT-116 cell line (*in vitro*)

Concentration of Extracts (µg/ml)	Percentage Inhibition (%)*				
	Methanol	Hexane	Ethyl Acetate	Water	Juice
100.000	36.490 ± 0.936	35.560 ± 0.000	55.680 ± 0.000	32.400 ± 0.000	25.850 ± 1.650
75.000	29.680 ± 2.700	26.780 ± 1.262	50.970 ± 1.374	14.900 ± 0.000	15.410 ± 0.873
50.000	24.090 ± 1.692	26.580 ± 0.000	41.530 ± 0.000	-12.170 ± 0.125	2.300 ± 1.562
25.000	17.640 ± 0.000	15.040 ± 1.159	37.130 ± 0.583	-7.420 ± 0.125	-7.420 ± 0.125
10.000	12.320 ± 0.396	8.620 ± 0.000	24.800 ± 0.000	-19.870 ± 3.965	-20.520 ± 2.857
1.000	15.020 ± 2.916	21.220 ± 2.439	5.430 ± 2.664	-19.730 ± 2.098	-19.730 ± 2.098
Control	-	-	-	-	-

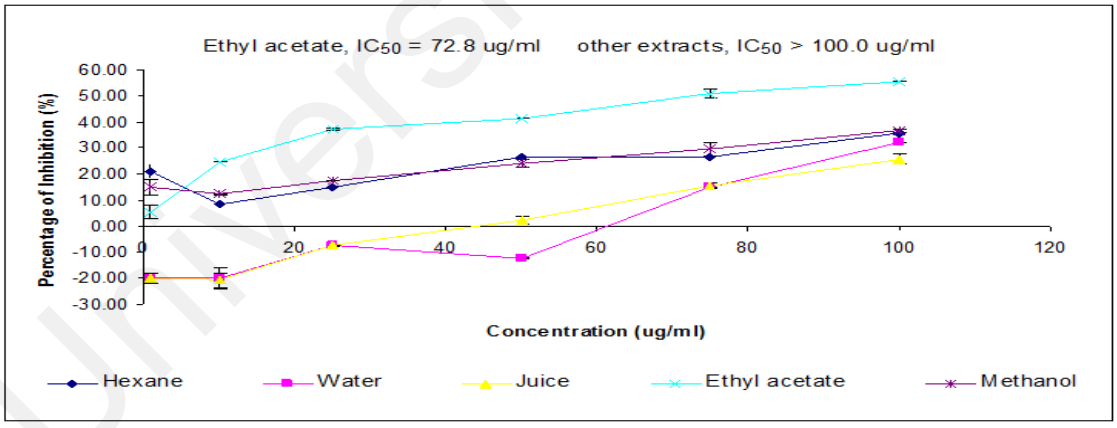


Figure 4.39: The *in vitro* growth inhibitions of HCT-116 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay.

IC_{50} values of *Beta vulgaris L.* on Neutral Red Cytotoxicity assay based on HCT-116 cell line

Ethyl acetate fraction exhibited the lowest IC_{50} value among the extracts i.e. 72.800 µg/ml. All the other extracts had an IC_{50} value more than 100.000 µg/ml. Hence, ethyl acetate fraction had better inhibition towards HCT-116 as compared to all the other extracts.

Table 4.56: IC_{50} values of *Beta vulgaris L.* extracts on HCT-116 cell line

Extracts of *Beta vulgaris L.*	IC_{50} (µg/ml)
Methanol extract	>100.000
Ethyl acetate fraction	72.800
Hexane fraction	>100.000
Water fraction	>100.000
Juice extract	>100.000

4.4 Isolation of Chemical Compounds/ Fractions

4.4.1 Isolation via High Performance Liquid Chromatography (HPLC)

i) Isolation via HPLC on ethyl acetate fraction

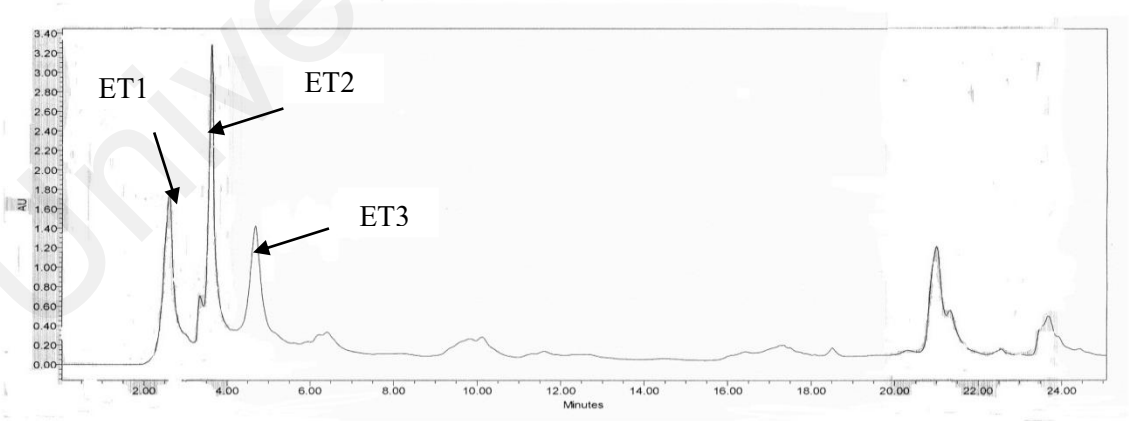


Figure 4.40: Chromatogram at wavelength 280nm of the HPLC separation of ethyl acetate fraction at certain conditions

a) ET1 Fraction

Weight of the fraction obtained was about 32.20 mg from a starting material of 250.00 mg extract of ethyl acetate fraction. Hence, the percentage of yield would be 12.88%.

ET1 had a brownish orange color. It did not have any smell. It stuck to the walls of the round bottom flask that was used to rotary evaporate to remove the solvent and was difficult to be separated until unless it was dissolved again with the dissolving solvent i.e. distilled water. It partially dissolved in methanol and did not dissolve in chloroform. It was dissolved completely in distilled water. The sample was stored in the freezer at -20ºC to ensure the stability of it.

ET1 was re-injected into HPLC using the same condition and detected to determine the purity of the sample. The chromatogram is shown in the Figure 4.41 below.

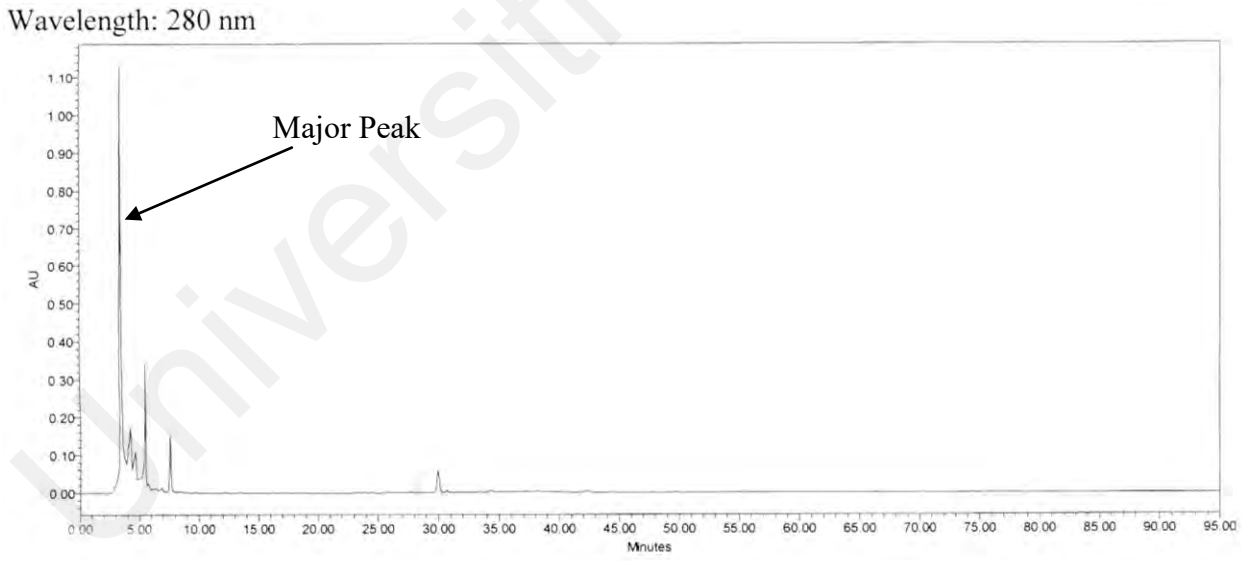


Figure 4.41: HPLC Chromatogram at wavelength 280 nm

From the chromatogram, we could see that the fraction ET1 that was isolated had one major peak and several minor peaks. Hence, it was not a pure compound, but it was a fraction.

b) ET2 Fraction

Weight of the fraction obtained from semi preparative method of HPLC from the starting material of 250.00 mg/ml of ethyl acetate fraction was 29.50 mg. The percentage of yield was thus 11.80 %.

ET2 had a brownish orange color. It did not have any smell. It stuck thinly to the walls of the round bottom flask after rotary evaporation process and could only be separated properly from the flask by using the dissolving solvent i.e. water. It dissolved about 90% in methanol and did not dissolve in chloroform. It dissolved completely in distilled water. The sample was stored in the freezer at -20ºC to ensure the stability of it.

ET2 was re-injected into HPLC using the same condition and detected to determine the purity of the sample. The chromatogram is shown in the Figure 4.42 below

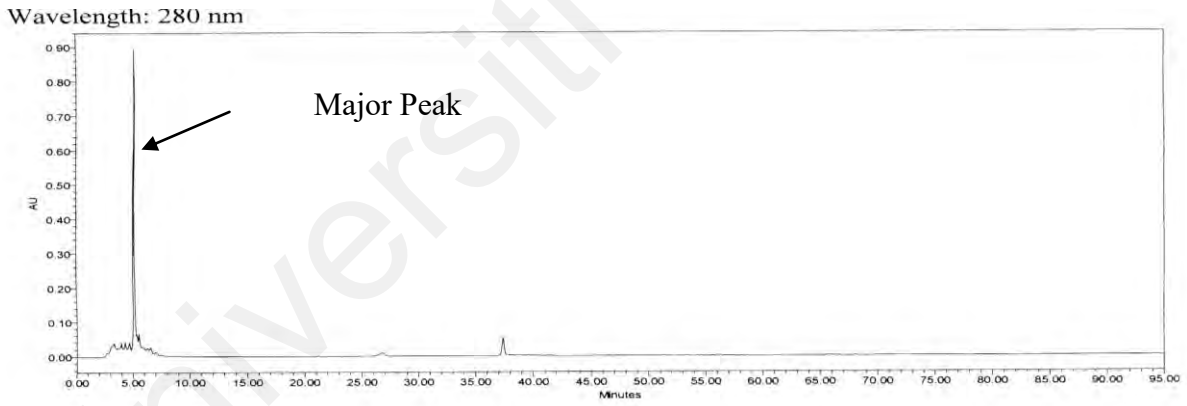


Figure 4.42: HPLC chromatogram at wavelength 280 nm

From the chromatogram obtained, we can say that the fraction ET2 had one major peak and some minor peaks that are very small. Hence, this fraction was considered to be almost pure compound. This fraction was further analyzed by LC-MS-MS to identify the chemical constituents.

c) ET3 Fraction

Weight of the fraction obtained from semi preparative method of HPLC from the starting material of 250.00 mg/ml of ethyl acetate fraction was 27.20 mg. The percentage of yield was thus 10.88 %.

ET3 had a light yellowish color. It had a very sweet smell. It dissolved about 40% in methanol and did not dissolve in chloroform. It dissolved completely in distilled water. The sample was stored in the freezer at -20ºC to ensure the stability of it.

ET3 was re-injected into HPLC using the same condition and detection to determine the purity of the sample. The chromatogram obtained is shown in the Figure 4.43 below

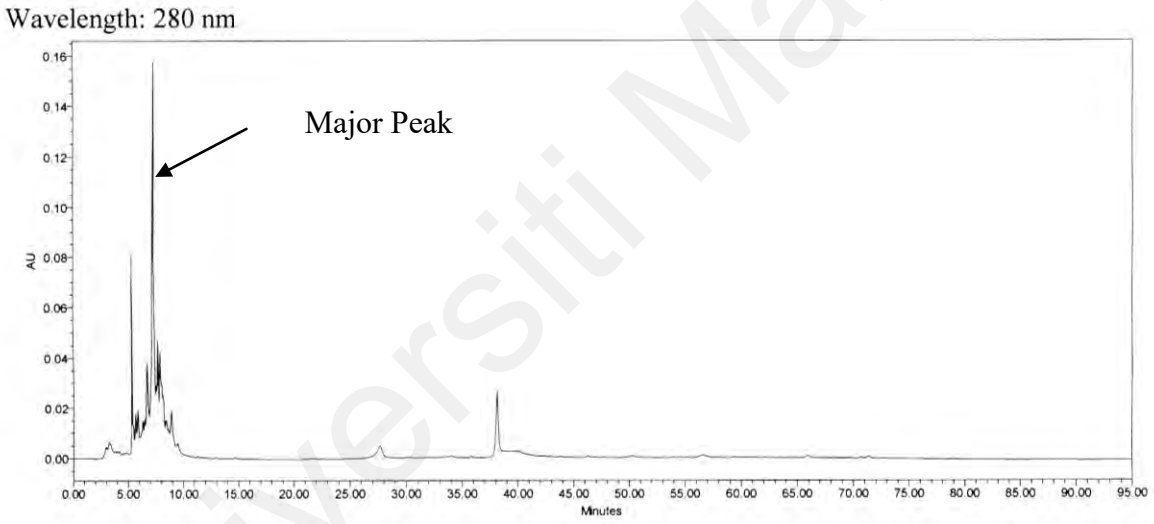


Figure 4.43: HPLC chromatogram at wavelength 280 nm

From the chromatogram obtained, we can say that the fraction ET3 had one major peak and some minor peaks. Hence, this fraction was considered to be not a pure sample and a fraction of few compounds. Further elucidation was done using LC-MS-MS.

ii) Isolation using HPLC from juice extract

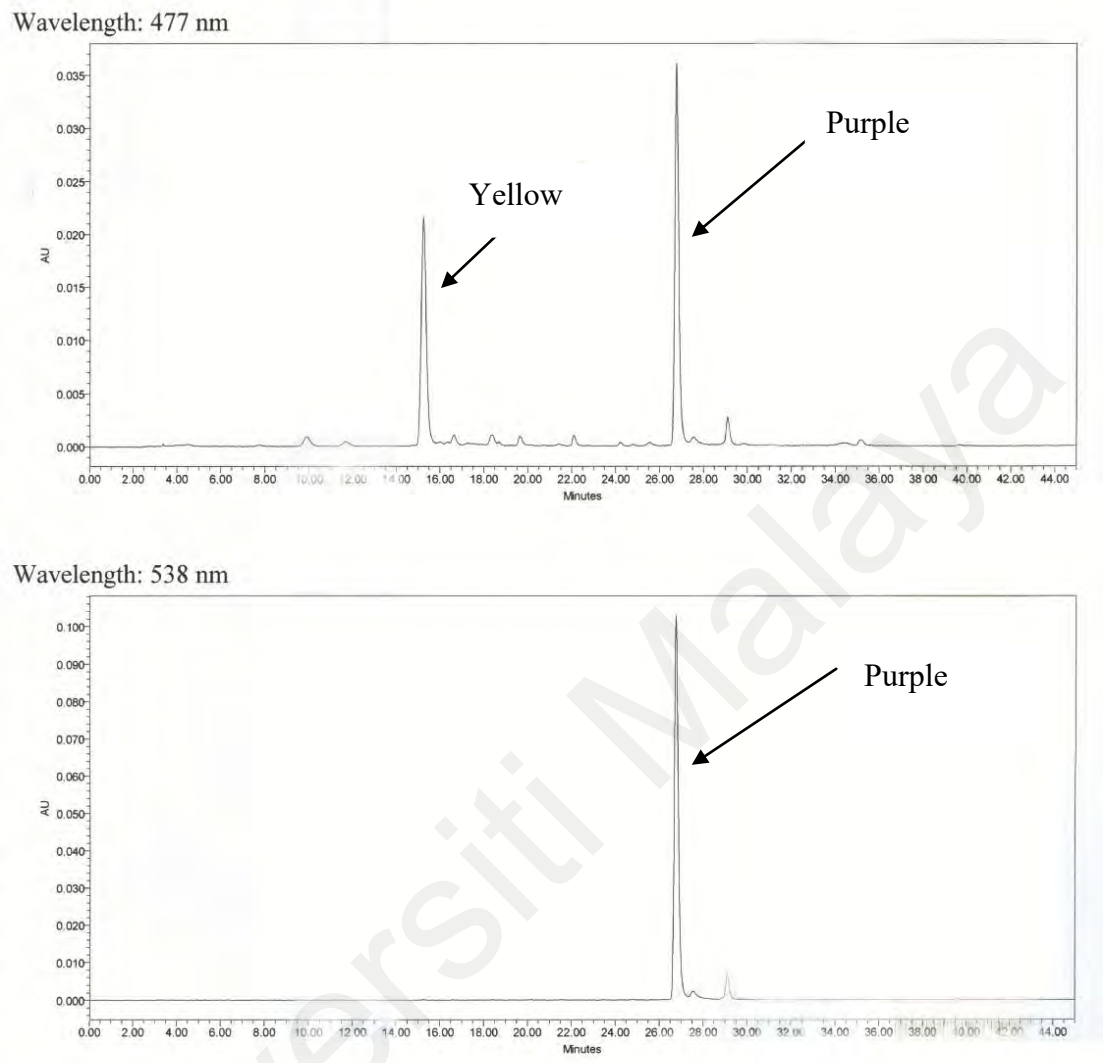


Figure 4.44: HPLC chromatogram at wavelength 477 nm and 538 nm

A starting material of 1400.00 mg of juice extract was used for isolation. Peak Yellow and Purple were isolated using the semi preparative method. It has been collected in Duran bottles and has been evaporated at a reduced pressure. It was then further re-injected into the HPLC again to determine the purity of the sample and also been identified using the LC-MS-MS method to determine the components of the fraction and also further used in antioxidant testing.

a) Yellow Fraction

The yellow fraction was obtained as yellow solid (10.00 mg, 0.714%). The amount obtained was very small even though repeated isolation procedure has been done. As the name of the fraction, the color of the fraction was bright yellow. It was odorless and it had characteristics of a pigment. It only dissolved in distilled water and was stable if kept in freezer (-20ºC).

The yellow fraction was re-injected into the HPLC with the same condition as used for initial separation and the peak obtained were observed. The chromatogram below shows the peaks formed upon injection with fraction Yellow sample.

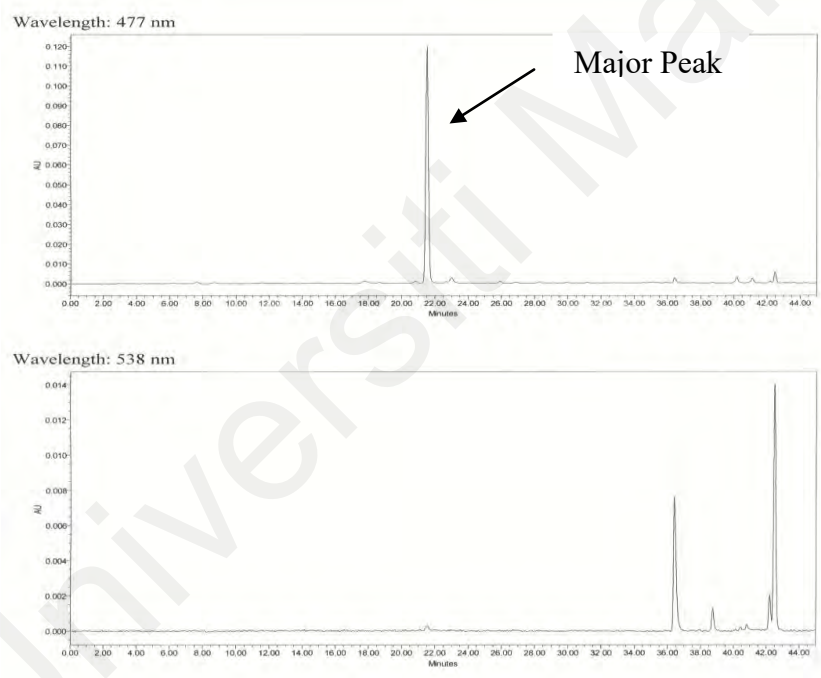


Figure 4.45: Chromatogram at wavelength 477nm and 538 nm of the HPLC separation of Yellow fraction at certain conditions

From the chromatogram we could see that the yellow fraction consists of 2 major and some minor peaks in HPLC. Hence it was not a pure compound but a mixture of compounds and

hence it was a fraction. Further isolation of the compounds could be done. But, due to limited amount of the sample, isolation could not be proceeded.

Figure 4.46: Image showing the morphology of the yellow fraction in the round bottomed flask

b) Purple Fraction

The amount of sample obtained from 1400.00 mg was approximately about 13.00 mg, just slightly higher than the yellow fraction. The percentage of yield was thus, 0.93%. The amount obtained was very small even though repeated isolation procedure has been done. As the name of the fraction, the color of the fraction was deep purple. It was odorless and it had characteristics of a pigment. It only dissolved completely in distilled water but was very unstable. To stabilize the fraction, a stabilizing procedure based on Reynoso *et al.,* 1997 was done.

It also was covered with aluminium foil to avoid contact with light and stored in -20ºC. These steps were taken to ensure the chemical composition of the fraction is maintained. For antioxidant assays with the purple fraction, a blank consisting of distilled water that was adjusted to pH 5.5 and added with 0.10% ascorbic acid crystals was done to avoid false positive results.

The purple fraction was re-injected into the HPLC with the same condition as used for initial separation and the peak obtained were observed. The chromatogram below shows the peaks formed upon injection with fraction Purple sample.

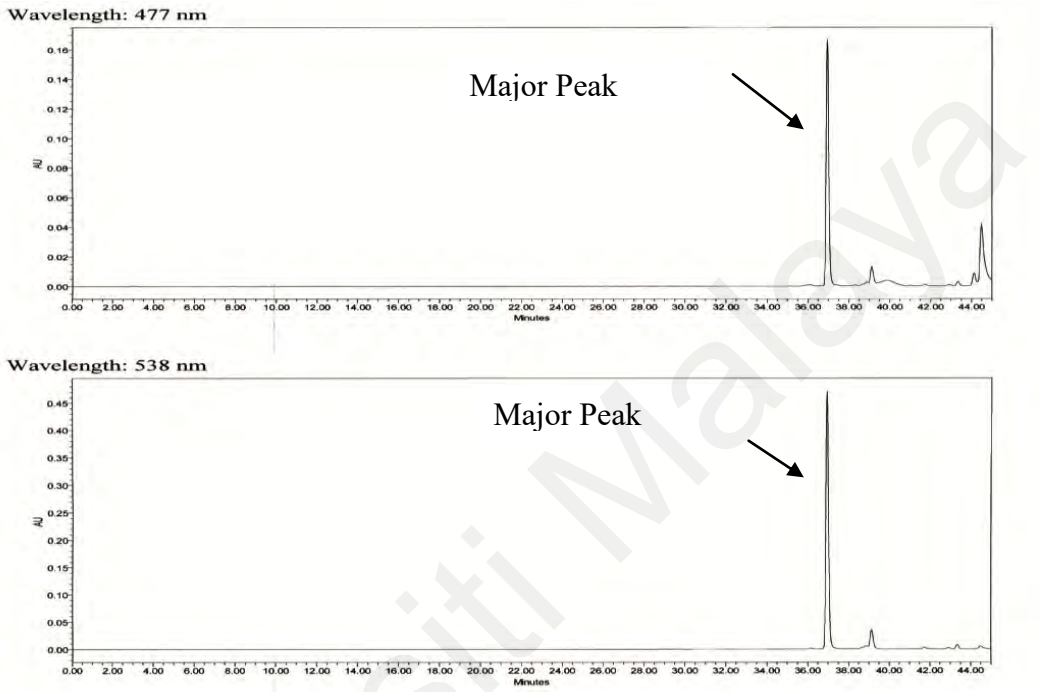


Figure 4.47: HPLC chromatogram at wavelength 477 nm and 538 nm

From the chromatogram we could see that the purple fraction consists of one major peak in HPLC. It seems to be quite pure and further identification using LC-MS-MS method was done.

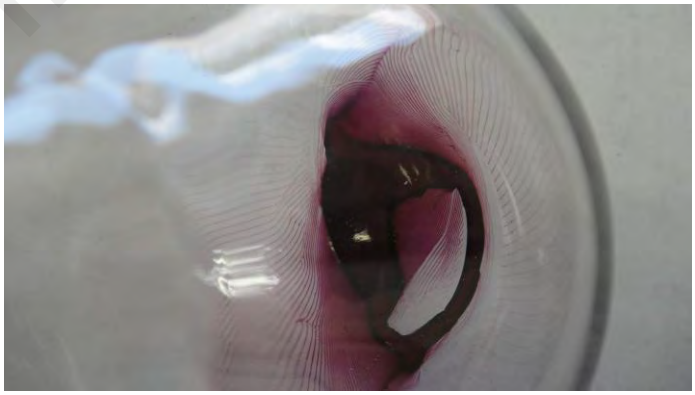
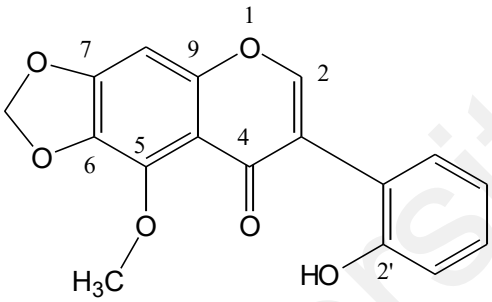


Figure 4.48: Image of Purple fraction in the round bottomed flask

4.4.2 Identification of the sub-fractions via Liquid Chromatography-Mass Spectroscopy-Mass Spectrometry (LC-MS-MS)

i) ET1 Fraction

In this fraction, four (4) potential active components were identified. The mass of the [M+H] ion for the four peaks were 180, 213, 313 and 550. The major peaks corresponds to [M+H] 313 and 550 identified as betavulgarin and betanin/isobetanin respectively. This was based on the comparison of molecular ion with previous work by Kujala *et al.* (2002). Fragmentation pattern of the components were not attached. Other peaks could not be identified and further work on identification needs to be carried out. The total ion chromatogram and mass spectral data obtained from LC-MS-MS analysis on ET1 is attached at the Appendix 4 (Figure 1-4).



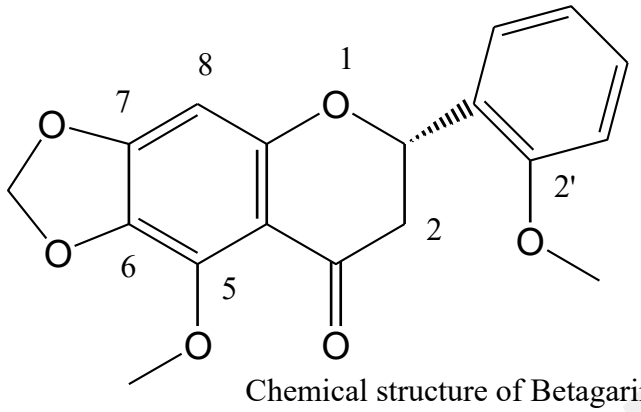
Chemical Structure of Betavulgarin

Chemical structure of betanin/isobetanin

ii) ET2 Fraction

In this fraction, 7 potential active components were identified. The m/z is at 180, 222, 256, 329, 365, 663 and 708. The peak with m/z 329 was identified as betagarin with molecular weight 328.12 g by comparison with molecular ions as reported in an earlier report by Kujala *et al.* (2002). Fragmentation pattern were not attached in the report. Other peaks could not be identified. Further identification using other techniques need to be done to

confirm the presence of this components and also to identify the components that could not be identified. The total ion chromatogram and mass spectral data obtained from LC-MS-MS on ET2 is attached at the Appendix 4 section (Figure 5-11).



Chemical structure of Betagarin

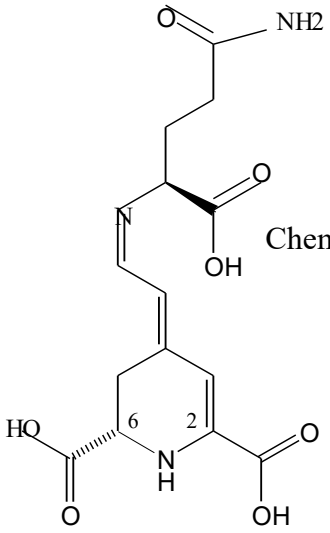
iii) ET3 Fraction

This fraction had 6 potential active components. The m/z was at 220, 231, 284 and 329. There was an observation of an identical nominal mass for both 220 and 231 at two different retention times. This could be due to potentially stereo/isomeric compounds. Nominal mass 328 could be possibly being betagarin based on comparison of molecular ion as reported by Kujala *et al.* (2002). Other components could not be identified. Further identification procedure need to be carried out to confirm the components. The total ion chromatogram and mass spectral data obtained from LC-MS-MS on ET3 is attached at the Appendix 4 section (Fig. 12-17).

iv) Yellow Fraction

In this fraction, 8 potential active components were observed corresponding to peaks at retention time 0.878, 1.074, 3.024, 6.345, 7.430, 8.120 and 8.515 and 9.302 min were

identified. The $[M+H]^+$ for these peaks are 166, 315, 340, 359 and three same m/z at three different retention times for 599 respectively. This situation might be due to the presence of stereo or isomeric compounds. The $[M+H]^+$ peak of 340 corresponds to betaxanthine compound i.e vulgaxanthine I. Vulgaxanthine I is a component in the betaxanthine pigment group. Other components could not be identified and further identification should be done. The total ion chromatogram and mass spectral data obtained from LC-MS-MS on Yellow fraction is attached at the Appendix 4 section (Figure 26-33).

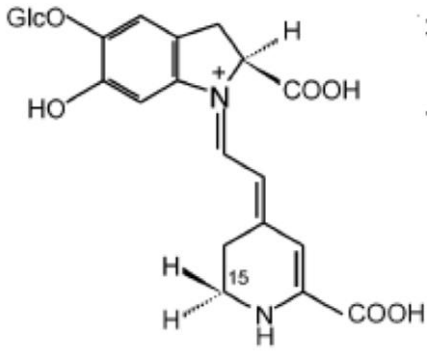


Chemical Structure of vulgaxanthine I

v) Purple Fraction

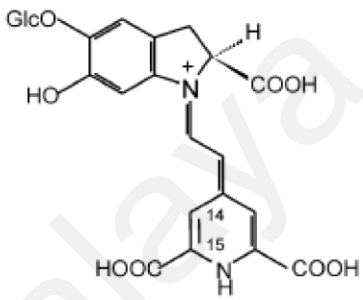
This fraction seems to have the most number of components as compared to the other components. It had 11 potential active components. The m/z values were 188, 315, 346, 507, 549 and 551. The peak at retention time 0.780 min corresponds to $[M+H]^+$ 551.1 has fragmentation pattern consistent with that of betanin or isobetanin. Whereas $[M+H]^+$ 549 corresponds to neobetanin. These are common pigments present in the *Beta vulgaris L*. It belongs to the pigment betacyanin group. The $[M+H]^+$ 507 is consistent with decarboxylated betanin. Other peaks could not be identified and have to be isolated and

identified by other techniques. The total ion chromatogram and mass spectral data obtained from LC-MS-MS on the purple fraction is attached in the Appendix section (Fig: 18-25).



Decarboxylated Betanin

$[M+H]^+$ 507



Neobetanin

[M+H]+ 549

CHAPTER 5

DISCUSSION

5.1 Plant Extraction

Two methods of extraction were employed in the present study. One was the normal infusion (cold extraction) method and the other was through juice extraction.

The plant samples were initially dried in an oven at a temperature not exceeding 50ºC. This step was done to ensure that during the drying process, the active ingredients were not damaged. A higher temperature might interfere with the chemical content of the plant sample and hence low temperature was maintained. Results in Table 4.1 show that the yield of dried sample was only 9.52%. This is due to the fact that *Beta vulgaris L.* is a very succulent root vegetable with high water content. Hence, the yield of dried sample was low.

In the cold extraction method, the dried ground samples were then soaked in methanol. Methanol is the most appropriate initial extracting solvent as it is polar in nature and most chemical components can be extracted. According to Lim and Quah, 2007, methanol is the most suitable solvent in the extraction of polyphenolic compounds from plant tissue, due to its ability to inhibit the action of polyphenol oxidase that causes the oxidation of polyphenols and its ease of evaporation compared to water. Extraction process was carried out at the temperature 25ºC and placed in shaker with 200 rpm. Shaking is required to ensure that the solvent mixes well with the dried ground plant sample and extraction could take place optimally. After 3 days, the solvent (methanol) was decanted off and a new batch of solvent was added to the dried ground leaves. This process was repeated twice to ensure that the compounds in the samples were extracted maximally. The solvent containing extract was filtered to remove plant debris that might be present. Anhydrous

natrium sulphate was added to the filtrate to remove water present in the filtrate. The excess solvent were evaporated under reduced pressure using a rotary evaporator at a temperature of 40-50ºC to give a reddish, gummy extract. During the rotary evaporation, sudden boiling might occur due to bumping of the chemical components in the round bottomed flask, and hence, might lead to loss of the extract through overshooting of extract in the condenser part of the rotary evaporator. This could be avoided by placing anti-bumping granules in the solvent (Houghton and Raman, 1998). To ensure that all the solvents were removed completely from the extract, the extracts were placed in the oven at a low temperature of about 40-50ºC for a period of 2-3 days until the weight was constant. The extracts of *Beta vulgaris L.* were kept in a refrigerator at 4ºC whereas the sub-fractions from ethyl acetate fraction and juice extract was kept at freezer (-20ºC) until further usage for bioassays. This was to ensure that there were no changes in the phytochemical constituents that could be caused by heat. Solvent extraction is the most common method used in sample preparations from plants. The extraction yield depends on extraction solvent, time and temperature of extraction as well as on the chemical nature of the sample. At the same time, temperature of extraction, the solvent used and the chemical property of sample are the two most important factors (Sun and Ho, 2005).

The 2nd method of extraction involved extracting the juice of beetroot evaporating at a reduced pressure using a rotary evaporator. The extraction method was conducted to ensure that all water soluble compounds like pigments betacyanin and betaxanthine were extracted. These pigments are water soluble components so there was concern that these chemical components might not be extracted if methanol was used as the initial solvent. The yield of extract from this method was high (50.00 ml juice = 5.00 g extract).

Methanol was used as the extracting solvent. This is due to its polarity and its ability to extract compounds like flavonoids, phenolics and polar compounds (Velioglu *et al.,* 1998). Methanolic extract consist of both polar and non-polar compounds. Fractionation was done to further partition the crude methanol extract to non-polar and polar components using different solvents. Hexane fraction consists of only the non-polar part of the extract. Ethyl acetate and water on the other hand, consists of the polar compounds. The non-polar solvents will extract non-polar compounds (oil and fatty acids) and polar solvents will extract polar compounds (phenolic compounds and flavonoids) (Houghton and Raman, 1998).

5.2 Antioxidant activity of extracts, fraction and sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.*

Increasing evidences show that consumption of fruits and vegetables can reduce the risk of various pathological events such as cancer and cardio- and cerebro-vascular diseases. This is attributed to the presence of antioxidants in the fruits and vegetables, for example, vitamin C, E, lycopenes, carotenoid, betacyanins and flavonoids. They function to prevent free radical damages. Research has been largely focused on the investigations of antioxidants that can scavenge ROS, especially natural antioxidants, phenolic and flavonoids from plants (Semiz and Sen, 2007). Antioxidant activities need to be assessed using different methods to understand the mechanism or the method of action of the extract as antioxidant. There is no single testing method which is sufficient to estimate antioxidant activity of test samples (Huang *et al.,* 2005). In our studies, eight different assays were tried on the extracts and sub-fractions of *Beta vulgaris L.*

By these assays, measurement of the first line, second line and third line defence defense of antioxidant is possible. We are protected against free radical mediated oxidative

stress by a battery of defense system. The first line of defense would be the preventive antioxidants such as peroxidases and metal chelating proteins that stop the generation of free radical. These would act as the first line defense. The radical scavenging antioxidants such as vitamin C and vitamin E scavenge the radicals and thus inhibit chain initiation and break the chain propagation. This would act as the second line defense antioxidants. The third line defense is denoted by the repair and *de novo* enzymes. Examples are lipases, proteases, DNA repair enzymes and transferases (Niki, 2005). By conducting the different assays, we can understand the mechanism of action of the antioxidant that is present in the plant tested.

5.2.1 DPPH (1,2-diphenyl -2-picrylhydrzyl) Free Radical Scavenging Activity

Radical scavenging activity is very important, due to the deleterious role of free radicals in foods and in biological systems (Bursal and Koksal, 2010). DPPH assay is a sensitive, easy to perform and offers a rapid way to screen radical scavenging activity of the isolated natural components, crude plant extracts and foods. Unlike laboratory-generated free radicals, such as, hydroxyl radical and superoxide anion, DPPH radical has the advantage of being unaffected by certain side reactions such as the metal ion chelation and enzyme inhibition brought about by various additives. It is also important to note that the DPPH test only recognizes free-radical scavenging effects and not pro-oxidant activity (Amarowicz *et al*., 2004).

The free radical DPPH is reduced to the corresponding hydrazine when it reacts with hydrogen donors (antioxidants). This ability is evaluated using electron spin resonance spectroscopy on the basis that the DPPH signal intensity is inversely related to the test antioxidant concentration and to the reaction time, but the more frequently used technique is the discoloration assay, which evaluates the absorbance decrease at 515–528 nm

produced by the addition of the antioxidant to a DPPH solution in ethanol or methanol (Moreno, 2002).

The absorbance for the extracts that are coloured, is measured differently from the conventional way. Absorbance at 520 nm is taken for the mixture of the extract with methanol. Absorbance is taken again at wavelength 520 nm after addition of DPPH radical and incubated for 30 minutes. The purpose of taking the absorbance value without addition of the DPPH solution is to find the absorbance that is contributed by pigment in the extract. This first absorbance value would be deducted from the second value to get a more accurate reading and to avoid interference from the pigment.

Methanol extract of *Beta vulgaris L.* showed highest inhibition at 5.00 mg/ml (96.54% ± 0.08) and lowest at 1.00 mg/ml with quite a high percentage of inhibition i.e. 85.41% ± 0.04. The percentage inhibition increased with increasing concentration. As for hexane fraction, highest percentage inhibition was 86.31% ± 1.22 at 5.00 mg/ml and lowest percentage inhibition was at 1.00 mg/ml with 16.41% ± 1.07. There was quite a big change between the percentage of inhibition at 5.00 mg/ml and 1.00 mg/ml. The ethyl acetate fraction exhibited the highest percentage of inhibition (85.06% ± 1.11) at 5.00 mg/ml. At 1.00 mg/ml, a percentage of 83.57% ± 0.90 was obtained. There was not much change in the percentage of inhibition at the highest and the lowest concentration of the assay. For water fraction, highest percentage of inhibition was 81.69% ± 0.07 at 5.00 mg/ml and lowest inhibition was at 1.00 mg/ml (11.19% ± 2.49). There was a drastic drop from 2.00 mg/ml to 1.00 mg/ml in the percentage of inhibition. Juice extract exhibited the highest percentage of inhibition at 5.00 mg/ml with 97.72% ± 1.91 and the lowest percentage of inhibition at 1.00 mg/ml with a percentage of 60.24% ± 2.02. The drop of inhibition was quite big from 2.00 mg/ml to 1.00 mg/ml. The drop in inhibition observed from 5.00 mg/ml

to 2.00 mg/ml was not very big. Inhibition observed was dose dependent for all the extracts.

Comparison of inhibition among the extracts showed that at 5.00 mg/ml, the highest percentage of inhibition was exhibited by juice extract (97.72% ± 1.91) and the lowest inhibition at the concentration was by water fraction (81.69% ± 0.07). From concentration 5.00 mg/ml to 1.00 mg/ml, the percentage of inhibition dropped as the concentration decreases for all the extracts. At 1.00 mg/ml, lowest percentages of inhibition among the tested concentration were observed. Within the extracts, methanol had the highest percentage of inhibition with 85.41% ± 0.04, followed by ethyl acetate fraction (83.57% ± 0.90), juice extract (60.24% ± 2.02), whereas hexane fraction (16.47% ± 1.07) and water fraction (11.19% ± 2.49) had the lowest percentages of inhibition. As methanol, ethyl acetate and juice extract had a percentage of inhibition higher than 50% at 1.00 mg/ml, they were subjected for further testing at lower concentrations.

Based on Table 4.8, the extracts of *Beta vulgaris L.* has lower DPPH radical scavenging capacity as compared to standards BHA (15.00 µg/ml) and ascorbic acid (9.80 µg/ml). Among the extracts, the ethyl acetate fraction had the lowest IC_{50} (0.31 mg/ml) value and hence the highest radical scavenging capacity. The components present in the ethyl acetate fraction could be responsible for the scavenging activity of the fraction. Water fraction had the lowest capacity to scavenge DPPH radical with the highest IC_{50} value of 1.58 mg/ml. Since the DPPH molecule has no similarity towards peroxyl radical that is involved in lipid peroxidation, some antioxidants that react quickly towards peroxyl radicals might react slowly or may also be inert towards DPPH (Huang *et al.,* 2005). This could be one of the reasons why the extracts had a lower activity compared to the standards.

The sub-fractions from the ethyl acetate fraction had lower scavenging activity towards DPPH radical compared to positive standard references, BHA and ascorbic acid (Table 4.9). Among the sub-fractions, the purple fraction had the highest radical scavenging activity (IC_{50} = 0.13 mg/ml). This could be due to the presence of betacyanin pigments (betanin, isobetanin and neobetanin in Fig: 5.2, 5.3 and 5.6) in this fraction. Fraction ET3 had the lowest scavenging activity (IC_{50} = 0.88 mg/ml) as compared to other isolated fraction.

On the study it was observed that the sub-fractions have remarkably good activity as compared to the crude extracts. This is due to the reason where in crude form, the presence of certain active chemical components are in low concentration but in the purified form, they are in higher concentration and could exhibit high radical scavenging activity.

As for comparison of result with other vegetables, *Melicope ptelofolia* or commonly known as tenggek burung was chosen, according to Naidu (2008) activity of the plant on DPPH assay, showed that methanolic extract had an IC_{50} value of 0.211 mg/ml, ethyl acetate fraction had IC_{50} value of 0.221 mg/ml, hexane fraction had IC_{50} value of 0.255 mg/ml and water fraction had IC_{50} value of 0.795 mg/ml. All values obtained showed better activity as compared to the extracts of *Beta vulgaris L.*

DPPH is a light sensitive component. It could be degraded if it is exposed to the light. So, the whole assay and the preparation of solvent must be done in a dark room under very careful condition to obtain an accurate result (Hani, 2007).

The extracts/fractions that have high scavenging activity might have beneficial effect towards human health. This assay is based on second line of antioxidant defense mechanism where antioxidant would scavenge free radical before the free radicals initiate and start a chain reaction (Vimala, 2003).

5.2.2 The Reducing Power Assay

Electron donating capacity reflects the reducing power of bioactive compounds, and is associated with antioxidant activity. Antioxidants can be reductants, and inactivation of oxidants by reductants can be explained as redox reactions where one reaction species is reduced at the expense of the oxidation of the other. In the reducing power assay, the presence of reductants, such as antioxidant substances in test samples, causes the reduction of the Fe3+/ferricyanide complex to the ferrous form (Ak and Gulcin, 2008). The reducing power assay therefore measures the ferric reducing ability of samples and it assesses the antioxidant effects of non-enzymatic antioxidants which are known as reductants. It provides an index of antioxidant ability to resist the oxidative damage that could be caused by oxidative stress and its effects (Vimala, 2003). This assay indicates how easily a given antioxidant donates electrons to reactive free radicals species, and promoting the termination of free radical chain reactions (Lue *et al.,* 2010)

Samples that exhibited good activity in the reducing power assay provide a good second line of antioxidant defense. The methanolic extract, hexane fraction, ethyl acetate fraction, water fraction, juice extract and sub-fractions were assessed for their reducing capacity using the reducing power assay. BHA and ascorbic acid was used as the positive standard. The plot of absorbance reading at 700nm of reducing power assay for BHA and ascorbic acid showed an increase of absorbance with increase in concentrations from 5 mg/ml to 20 mg/ml. This shows a dose-dependent relationship.

It is interesting to learn that the ethyl acetate fraction showed a very high reducing power activity (3.767A ± 0.002) and it showed the highest reducing power activity as compared to the standards (3.465A ± 0.008 for BHA and 3.142A ± 0.017 for ascorbic acid) and other extracts at the concentration of 20 mg/ml. It also showed a very high reducing

power as compared to standards and other extracts in other concentrations as well. The water fraction and methanol extract showed a steep increase in the reducing power from 5 mg/ml to 10 mg/ml, however methanolic extract showed a slightly higher reducing power compare to water fraction. Juice extract, on the other hand, showed the lowest reducing power activity in comparison to all the standards and the extracts. As for the standards, BHA showed a higher reducing power activity as compared to ascorbic acid.

The good reducing power capacity that is exhibited by ethyl acetate fraction could be due to the presence of reductones such as betanin, betavulgarin, isobetanin, betagarin and others. Antioxidants are generally compounds that could donate a single electron or hydrogen atom for reduction. Reports show that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom and this could prevent oxidative stress related tissue damage (Shimada, Fujikawa, Yahara & Nakamura, 1992, Vimala, 2003). Juice extract had the lowest reducing capacity among the extracts. This might be due to the fact that there are too many components present in the extract and the compound that is responsible for the reducing capabilities is only present in trace amount.

As for the sub-fractions of ethyl acetate and juice extract, Figure 4.7 shows that, all the fractions had a lower reducing capacity as compared to the positive standard. This could be due to the fact that the comparison what made with a pure compound whereas our sub-fractions are still in the form of purified fractions but not as pure compounds. Further purification of the sub-fractions to separate the pure compounds and retesting with the antioxidant test could perhaps give a better result. Hence, the activity exerted might not be

appropriate to be compared with a pure compound that has definitely got a high reducing power capacity. Within the fractions, the purple fraction had the highest reducing power as compared to all the other fractions. This could highly be due to the presence of pigment betacyanins (isobetanin, betanin and neobetanin) in this fraction which has high reducing power capacity. Fraction ET1 had almost equally good reductive capabilities as the purple fraction, and this could be due to the presence of the same components of betacyanin and also the presence of betavulgarin (Fig. 5.1), a phenolic compound, in the fraction. Other phenolic compounds identified in ET1 are betanin (Fig. 5.2) and isobetanin (Fig. 5.3). The compounds identified in the purple fraction are betanin, isobetanin neobetanin and decarboxylated betanin (Fig. 5.2, 5.3, 5.6 and 5.7). Decarboxylated betanin is not a phenolic compound. Hence these compounds might have electron donating capacity and could react with free radicals to convert them to a more stable product and terminate the chain reaction (Duh *et al.,* 2004). The yellow fraction had the lowest reducing power and this shows that pigment betaxanthine (Fig. 5.5) does not have high electron donating capacity.

As for comparison with other vegetable, a famous malay ‗ulam' *Melicope ptelofolia* was chosen. According to reports by Naidu (2008), in the reducing power assay, methanol extract had absorbance value of 1.041-1.826A for a concentration range of 5.00 mg/ml to 20.00 mg/ml. Hexane fraction, ethyl acetate fraction and water fraction had an absorbance value of 1.143-1.758A, 1.721-1.332A and 1.335-1.092A respectively for the same concentration range as described for methanol extract. As for Beta vulgaris, methanol extract, hexane fraction, ethyl acetate fraction and water fraction had an absorbance value of 3.275-2.249A, 3.290-1.408A, 3.767-2.816A and 3.287-1.949A respectively for the same

concentration range. We can generally see that *Beta vulgaris L.* has a better reducing power capacity as compared to the famous malay vegetable.

Ferric reducing power and total phenolic content are related with each other. Fe (III) reduction is usually used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Bursal and Koksal, 2010).

5.2.3 β-carotene Bleaching assay

β-Carotene shows strong biological activity and is an important physiological compound, if it is decomposed before its intake, the biological function would be lost. However, its 11 pairs of double bonds are extremely sensitive to free-radical mediated oxidation, and it is discolored easily with oxidation of linoleic acid (Zhang *et al.,* 2003). In the current study, the antioxidant capacity was determined from the ability of samples to inhibit β-carotene bleaching caused by free radicals generated by linoleic acid present in the peroxidation (Kubola and Siriamornpun, 2008). 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). Antioxidant capacity is classified as high (>70%), intermediate (40–70%) or low (<40%) levels of oxidation inhibition (O.I.)/antioxidant capacity (Rufino *et al.,* 2010). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reaction (Chanwitheesuk *et* al., 2005). β-Carotene is one of the best quenchers for singlet oxygen, and can act as chain-breaking antioxidant. This spectrophotometric method is based on the ability of different extracts to decrease oxidative losses of β-carotene/ linoleic acid emulsion. β-Carotene loses its orange colour when reacting with the radicals (malondialdehyde (MDA) and conjugated dienes). β-Carotene

bleaching method is suitable for lipophilic compounds (Rufino *et al.,* 2010). The presence of a phenolic antioxidant can hinder the extent of β-carotene destruction by ―neutralizing" the linoleate free radical and any other free radical formed within the system. Hydrophobic antioxidants are reported to perform more efficiently than hydrophilic antioxidants in the β-carotene bleaching test by orienting themselves in the lipid phase and the lipid–water interface, thus directly combating lipid radical formation and β-carotene oxidation (Miraliakbari and Shahidi, 2008).

From the result obtained (Figure 4.9), the hexane fraction has the highest antioxidant activity in the β-carotene bleaching assay. This could be due to the reason where this assay is suitable for lipophilic compounds. Hexane fraction is known to have non-polar components which could be oils and fatty acids which are lipophilic thus, the assay is suitable to exhibit the antioxidant activity of this extract. This result is consistent with those obtained by Son and Lewis, 2002, in which hydrophobic antioxidants tend to exhibit better antioxidative activity in the emulsion system. This is the highest activity noted among the extracts and the standards. At the highest concentration tested (20.00 mg/ml), methanol extract had an exceptional antioxidant activity of (106.53% ± 0.011). It had even higher antioxidant activity compared to standard BHA. Ethyl acetate fraction also had good antioxidant activity as its activity was only slightly lower than standard BHA. However, at lower concentrations methanol extract, water fraction and juice extract had lower antioxidant activity as compared to the standard BHA. Other antioxidant assay showed remarkably good antioxidant activity for ethyl acetate and juice extract (polar fractions) but this was not the case for this assay as it showed hexane fraction to be the highest in antioxidant activity. This could be due to an interesting phenomenon called ―polar paradox' which has been reported in earlier studies (Ahmadi *et al.,* 2007). The polar

antioxidants remaining in the aqueous phase of the emulsion are more diluted in the lipid phase and are, thus, less effective in protecting the linoleic acid. It explains that hydrophilic antioxidants are more effective than lipophilic antioxidants in bulk oil, whereas lipophilic antioxidants show greater activity in emulsions (Kulisic *et al.,* 2004).

Sub-fractions of ethyl acetate fraction and juice extract were also subjected to this assay. The results revealed that all the fractions have lower activities as compared to standard BHA at the tested concentrations. This could be due to the fact that all these fractions are from polar fraction and they have high polarity based on isolation process on HPLC.

As for comparison with other vegetables, a report by Hassimoto *et al,* (2005) was chosen. This report antioxidant acvitity of methanolic extracts of various vegetables at concentration 50 μM based on β-carotene bleaching assay. The highest antioxidant activities were shown by watercress, and arugula (>70% inhibition), followed by wild chicory, white cabbage, red cabbage, sweet red pepper, and red lettuce, with intermediate activities (40-70% inhibition), and sweet green pepper (<40% inhibition),with low activity. Methanolic extract showed antioxidant activity of 106.530% to 63.760% for a concentration range of 20.000 mg/ml to 4.000 mg/ml. Thus we can clearly view that methanol extract of *Beta vulgaris L.* showed very high antioxidant activity as compared to other vegetables.

However, based on the scale given by Rufino *et al.* (2010), all the crude extracts exhibited high antioxidant activity (>70%) at tested concentrations, except for water fraction that exhibited moderate antioxidant activity (40-70%). Sub-fractions, on the other hand, had moderate to low antioxidant activity (<70%).

The strong antioxidant activity of *Beta vulgaris L*. in this assay suggests to us that it is suitable to be used as antioxidant food supplement to prevent oxidative related symptoms and degenerative diseases. This assay shows the third line defense antioxidant, where the antioxidants such as vitamins and enzymes act in reducing the impact of oxidative cellular damage. Since they are sensitive and unstable, dose response activity is hard to achieve (Vimala, 2003).

Among the standards, BHA possessed excellent antioxidant activity compared to ascorbic acid. Ascorbic acid might not be effective in showing its activity in this assay as it displays pro-oxidant activity in the β-carotene system. Pro-oxidant activity has previously been reported for ascorbic acid when using the β-carotene bleaching method or the liposome method. The pro-oxidant nature of ascorbic acid appears to be due to the formation of ascorbyl radicals during oxidation (Rufino *et al.,* 2010).

Oxidation rate ratio bears an inverse relationship with antioxidant activity index (Kubola and Siriamornpun, 2008). This parameter measures the ratio of oxidation by free radicals formed from linoleic acid towards β-carotene molecule.

β-carotene assay is used to measure the ability of the antioxidant to prevent the oxidative deterioration of lipids and fatty acids. Therefore, the higher antioxidant activity of the extracts and sub-fractions in this assay, there is high possibility biological functionality in preventing the oxidative degradation of membrane lipids (Ahmadi *et al.,* 2007).

5.2.4 Metal Chelating Assay

Elemental species such as ferrous ion (Fe^{2+}) can cause the production of ROS. The ability of a substance to chelate iron can be a valuable antioxidant property. Iron can be found either in ferrous (Fe^{2+}) or ferric ion (Fe^{3+}) form in nature. Ferric ion is predominant

in foods. Ferrous chelation may render important antioxidative effects by retarding metal-catalysed oxidation (Ak and Gulcin, 2008).

The metal ion chelating capacity plays an important role in the antioxidant mechanism because it prevents oxyradical generation and the consequent oxidative damage (Srivastava *et al.,* 2006). The production of highly ROS such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals is also catalysed by free iron through Haber–Weiss reaction ($O_2^{-}\bullet + H_2O_2 \rightarrow O_2 + OH^{-} + {\cdot}OH$)

Iron is known to be the most important lipid oxidation pro-oxidant due to its high reactivity. Therefore, ferrous ion chelators could also possibly protect against oxidative damage by removing iron that could take part in ·HO generating reactions such as Fenton reactions as described below.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {\cdot}OH$$

Ferric ions also produce radicals from peroxides although the rate is slower than ferrous ion (Ak and Gulcin, 2008).

EDTA is a strong metal chelator; hence, it is used as standard metal chelator agent in this study (Gulcin, 2006). Ferrozine could form complexes with Fe2+, quantitatively. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of color reduction therefore allows estimating the metal chelating activity of the coexisting chelator. Lower absorbance indicates higher metal chelating activity (Gulcin, 2006).

Extracts of *Beta vulgaris L*. and sub-fractions were tested for its ability to compete with ferrozine (a salt that has high affinity towards ferrous ion), to chelate the ferrous ion in the solution. Based on the results shown in Figure 4.12, it showed that the extracts of *Beta vulgaris L.* had lower chelating capability compared to positive standard EDTA. This is

possibly due to the reason where the extracts do not have components that can form chelating ligand with the ferrous ion (Chen *et al.,* 2009). Hence, the extracts might not be a good material to stop free radical reactions that is initiated by metal ions (Kim *et al.,* 2005). Generally, compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, $–PO_3H_2$, C=O, $–NR_2$, –S– and –O– in a favorable structure–function configuration will have chelation activity (Yuan *et al.,* 2005). However, among the extracts, methanol extract had the highest chelating ability with IC_{50} of 1.12 mg/ml and the lowest chelating capacity was exhibited by water fraction with IC_{50} value of more than 5.00 mg/ml.

As for the sub-fractions of ethyl acetate and juice extract, Figure 4.13 shows that the fractions had low metal chelating ability as compared to standard EDTA. All the fractions had IC_{50} value of more than 1.00 mg/ml.

Based on literature search, there was no previous report on beets for metal chelating assay. As comparison with other reports, the results were compared with a report from Valvi *et al.* (2011) which studied the antioxidant potential of some wild edible plants. The plant studied were *Cipadessa baccifera, Bridelia scandens* and *Mimusops elengi.* The extracts that were studied were aqueous extract, methanol extract, ethanol extract and hexane extract. The results showed that all the extracts of the plants had percentage of inhibition between 65.00-40.00%. *Beta vulgaris L.* had better chelating activity (percentage of inhibition) as compared to the reports of Valvi *et al.* (2011).

The reason why EDTA had remarkably high activity as compared to extracts and sub-fractions is that EDTA complexes with pro-oxidative metal ions, such as copper (Cu^{2+}) and iron (Fe^{2+}). There are six lone pairs of electrons from two nitrogen atoms and four negatively charged oxygen atoms in EDTA, creating a hexadentate ligand to complex with

metal ions (Fig. 5.1). This may be the reason that Fe^{2+} chelating capacities of EDTA were significantly greater than of test samples (Chen *et al.,* 2009).

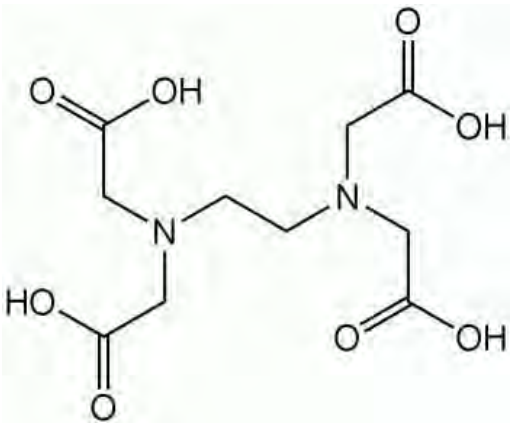


Figure 5.1 Chemical structure of EDTA (ethylenediamine-N,N,N'N'-tetra acetic acid)

Metal chelating capacity is an important property since it reduces the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Gulcin, 2006).

5.2.5 SOD (Superoxide dismutase) Enzyme Activity Assay

Superoxide is biologically quite toxic and is used by the immune system to kill invading microorganisms. It is an oxygen-centred radical which has selective reactivity. The biological toxicity of superoxide is due to its capacity to inactivate enzymes containing iron–sulfur cluster, which are critical in a wide variety of metabolic pathways, thereby liberating free iron in the cell, which can undergo Fenton-chemistry and generate the highly reactive hydroxyl radical. It can also reduce certain iron complex such as cytochrome c.

Superoxide anions could initiate the production of active free radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage. It

has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Also, superoxide has been observed to directly initiate lipid peroxidation. In addition, it has been reported that antioxidant properties of some flavonoids are effective mainly through scavenging of superoxide anion radicals. Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Gulcin *et al.,* 2009). Superoxide radical is usually formed first, and its effects could be bigger because it produces other kinds of free radicals and oxidizing agents (Liu *et al.,* 2007).

SOD is an important cellular antioxidant enzyme, which converts superoxide radical into H_2O_2 and O_2 (Kaviarasan *et al.,* 2007). ($O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$) (McCord and Fridovich,1969). Fenton reaction could catalyse the hydrogen peroxide and oxygen molecule to highly ROS such as hydrogen peroxide and hydroxyl radical. These products could have variety of effects on tissue macromolecules that could cause diseases.

The results of SOD activity of extracts of *Beta vulgaris L*. is summarized in Figure 4.15. From the graph, the standard BHA had the highest inhibition activity. At certain concentrations (2.00 mg/ml and 3.00 mg/ml), the ethyl acetate fraction exhibited comparable activity to BHA. Within the extracts, ethyl acetate fraction had the highest activity with IC_{50} value of 0.71 mg/ml. Hence, polar components of *Beta vulgaris L.* has the capabilities to inhibit the action of xanthine oxidase (XO) which produces superoxide anions or act like superoxide dismutase in neutralizing the superoxide anion. The lowest activity among the extracts was exhibited by the hexane fraction with IC_{50} value more than 5.00 mg/ml. This clearly shows that non-polar components do not have SOD activity.

Figure 4.16 showed the SOD activity of the sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.* with standards. The results show that ET2 at higher concentrations (>0.5 mg/ml) had SOD activity higher than that of the standard BHA. It had a remarkably good activity as compared to standard BHA at concentration 1.00 mg/ml. At the highest concentration tested, 1.00 mg/ml, fraction ET1, ET2 and Purple had activity higher than standard. Overall ET1 had the highest activity with IC_{50} of 0.21 mg/ml whereas fraction. Based on LC-MS-MS analysis, the possible components in this fraction are betavulgarin, isobetanin and betanin. These components might have SOD like activity. The yellow fraction had the lowest IC_{50} which is more than 1.00 mg/ml; hence, this is most probably due to presence of vulgaxanthine I that might have SOD like activity.

Since extracts of *Beta vulgaris L.* and the purple fraction had activity better than standards, thus *Beta vulgaris L.* has high superoxide anion scavenging activity, it shows its capacity as radical scavenging antioxidant and acts as a second line antioxidant defense where it stops the chain initiation and breaks chain propagation caused by the free radicals (Vimala, 2003) and it may have health benefits especially for prevention or treatment of free radical-related diseases. Xanthine oxidase inhibitors also have been found to be defensive agents against oxidative stress and effective in the prevention of skin carcinogenesis (Huey Ko *et al.,* 2008). According to Calixto *et al*. (2003), superoxide scavenging antioxidants could prevent inflammatory arthritis in which they scavenge the excess free radicals during inflammation.

5.2.6 TBARS (Thiobarbituric Acid Reactive Substances) Assay

The free radical-mediated peroxidation of lipids has received a great deal of attention in connection with oxidative stress *in vivo* (Niki *et al.,* 2005). This reaction starts a

free radical chain reaction that takes place in human body that produces highly reactive secondary products. These secondary products would react with other biological components, such as DNA, proteins and amino acids. Lipid peroxidation can cause lysosomal destabilization, apoptosis and cellular changes, change in membrane structure and cellular necrosis and death (Vimala, 2003). Lipid peroxidation has been associated in various diseases and aging, including atherosclerosis, cataract, rheumatoid arthritis, and neurodegenerative disorders (Niki *et al.,* 2005).

A modified TBARS assay was used to measure the potential antioxidant capacity of extracts and sub-fractions using egg yolk homogenates as lipid rich media (Kulisic *et al.,* 2004). The thiobarbituric acid reactive species (TBARS) assay, involves the spectrophotometric measurement of the pink pigment [(an adduct between TBA and MDA (malonaldehyde) in acidic condition)] and other secondary lipid peroxidation product. MDA is very reactive due to its bifunctional aldehyde (Duh *et al.,* 2004). MDA, formed from the breakdown of polyunsaturated fatty acids, serves as an easy index to measure extent of lipid peroxidation (Liu *et al.,* 1997). This is an important assay as it shows the first line of antioxidant defense of the test sample, in which preventative antioxidants stops the formation of free radicals. This could stop the damage towards fatty acids and prevent various diseases (Vimala, 2003).

The extracts and sub-fractions were tested for their inhibitions towards lipid peroxidation expressed as equivalence of mg of TEP (tetraethoxypropane) per gram of extract/sub-fractions. TEP was the external standard (Jayakumar *et al.,* 2008). The higher the value of equivalence towards TEP, the lower the ability of the extract/fraction to inhibit the lipid peroxidation. TEP is equivalent to MDA which is a by product of lipid peroxidation. Hence if there is no lipid peroxidation, there would not be formation of TEP.

From the results obtained in Figure 4.19, all the extracts of *Beta vulgaris L*. have inhibition of lipid peroxidation better than the positive standards BHA and ascorbic acid. Among the extracts, methanol extract had an excellent capacity to oppose lipid peroxidation. Hexane fraction was next best in inhibiting lipid peroxidation. This result complements the result of β-carotene bleaching assay where hexane fraction had the highest inhibition towards lipid peroxidation. Hence, besides methanol extract, hexane fraction was proven to have high lipid peroxidation inhibition capacity in both TBARS assay and β-carotene bleaching assay. Ethyl acetate fraction displayed the lowest ability to inhibit lipid peroxidation compared to other extracts/fractions.

Figure 4.20 shows the inhibition of lipid peroxidation of sub-fractions of ethyl acetate and juice extract. From the graph it was observed that only the yellow fraction had inhibition higher than positive standard (BHA and ascorbic acid). All the other fractions could not inhibit lipid peroxidation better than the positive standards. Based on results of lipid peroxidation assay conducted by Reddy *et al.* (2005), betanin had 71% inhibition, but we did not observe any significant inhibition by ET1 or the purple fraction which had the component betanin. This difference could be due to the presence of other compounds in the fraction which may have a negative effect on the assay.

For comparison with other vegetables, report by Kuppusamy *et al.* (2002) was used. According to Kuppusamy *et al.* (2002) the antioxidant activity of extracts of various fruits, vegetables and whole plants at a final concentration of 0.42–4.17 mg/ml, using the lipid peroxidation of buffered egg yolk, as in this study. The IC_{50} value was 0.3–3.97 mg/ml and the lipid peroxidation inhibitory potency decreased in the order: curry leaf > ginger> okra > Chinese parsley > Chinese kale > spearmint > cabbage > Chinese mustard > spinach > Chinese radish.

Hence, from the results obtained, it is clear that *Beta vulgaris L.* has first line antioxidant defense to prevent the formation of free radicals. Isolated yellow fraction which contains vulgaxantine I showed high inhibition towards lipid peroxidation, indicating contribution of vulgaxanthine I towards the activity. Consumption of this plant could prevent the occurrence of various diseases as described earlier.

5.2.7 Folin-Ciocalteau Assay (Total Phenolic Content Assay)

According to Huang *et al.* (2005), the Folin-Ciocalteau Reagent (FCR) based assay gained popularity and is commonly known as the total phenols (or phenolic) assay. This assay in reality measures a sample‘s reducing capacity, but this is not shown in the name ―total phenolic assay". Numerous publications applied the total phenols assay by FCR and an ET-based antioxidant capacity assay (e.g., FRAP, TEAC, etc.) and often found excellent linear correlations between the ―total phenolic assay" and ―the antioxidant activity". This is due to the similarity of chemistry between the two assays.

The real chemical mechanism of the FC reagent is still unknown, but it is believed to contain heteropolyphosphotunstates-molybdates. One- or two-electron reduction reactions lead to blue species, which possibly is $(PMoW_{11}O_{40})^{4-}$. It is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI). The reaction can be simplified as below.

$$Mo\ (VI) + e \rightarrow Mo\ (V) \qquad \text{(Huang } et\ al.,\ 2005\text{)}$$

Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Javanmardi *et al.,* 2003). Total phenolic content (TPC) were analysed

by the Folin–Ciocalteau colorimetric method using gallic acid as a standard (Liu *et al.,* 2007). Gallic acid is a water soluble phenolic (Miraliakbari and Shahidi, 2008). BHA was used as the positive standard and had remarkably high total phenolic content/reducing capability, which is 247.02 ± 1.871 mg of gallic acid equivalence to per gram of BHA.

The ethyl acetate fraction had the highest total phenolic content/reducing capability, as compared to all the other extracts, expressed in equivalence to mg of gallic acid per gram of extract. The extract with the lowest total phenolic content/reducing capability was juice extract. These results corresponds to the reducing power assay in Figure 4.6, in which ethyl acetate fraction had the highest reducing power and juice extract had the lowest reducing capacity. These results complements with the statement made by Huang *et al.* (2005) where the assay would have same results with reducing power assay or other assay that involves measuring the reducing capability of the sample. According to a study by Vinson *et al.* (1998), it was found that beets had the highest total phenolic content, followed by red onion, broccoli and kidney beans.

The Folin–Ciocalteau assay gave an estimation of the total phenolic compounds present in an extract. This estimation is not specific to polyphenols, but many interfering compounds may react with the reagent, giving a result of higher than real phenolic concentrations (Prior, Wu, & Schaich, 2005). Various phenolic compounds respond differently in this assay, depending on the number of phenolic groups they have, and total phenolics content does not incorporate all the antioxidants that may be present in an extract (Tawaha *et al.,* 2007). The total phenols assay by FCR is convenient, simple, and reproducible (Huang *et al.,* 2005).

5.2.8 Tyrosinase Inhibitory Assay

This assay measures the third line antioxidant defense. The third line defense involves sacrifice of the antioxidants to arrest oxidative degenerations of large molecules such as enzymes and vitamins in the human body (Vimala, 2008).

Inhibitory activity on tyrosinase was evaluated by IC_{50} values obtained through extrapolation from standard curve. All measurements were carried out in triplicate. All of the solutions were prepared with phosphoric acid buffer solution (pH 6.8). Kojic acid was used as positive control (Liu *et al.,* 2009). Based on extensive literature search done, there were no previous reports of this assay on the samples of beetroot and thus comparison could not be made. The findings in this research could be novel. Masuda *et al.* (2005) observed that seashore plant species, which are exposed to full sunlight, possess strong antioxidant activity and high tyrosinase inhibition ability.

i) L-Tyrosine as substrate

As described earlier, the use of L-tyrosine as substrate measures the monophenolase activity of tyrosinase enzyme.

Based on Figure 4.22, it can be observed that extracts of *Beta vulgaris L.* has lower tyrosinase inhibitory activity as compared to positive reference standard (Kojic acid). Among the extracts, ethyl acetate fraction had the highest inhibitory with an IC_{50} value of 8.02 mg/ml and water fraction had the lowest inhibitory with IC_{50} value of more than 20.00 mg/ml. Hence ethyl acetate fraction may consist of polar components which might be responsible for the inhibitory activity of the tyrosinase enzyme.

The sub-fractions of ethyl acetate and juice extract were also tested for its tyrosinase inhibitory capacity. Based on the results shown in 4.23, the purple fraction had extremely

good activity in inhibition of the monophenolase activity. This is followed by fraction ET1 and Yellow fraction. These 3 fractions had activity better than the positive reference standard, kojic acid. Fraction ET2 and ET3 had lower activity as compared to the positive reference standard. The fractions show remarkably good activity as compared to the crude extracts. This could be due to the nature of the chemical components where they could exert their activity better in the purified form. In the purified form, the active components could be present in higher concentration as compared to in the crude form. Thus, this could be the reason that we could see the excellent tyrosinase inhibitory activity. Based on the LC-MS-MS results, ET1 contain betavulgarin, isobetanin, and betanin. Fraction Purple contains betanin, isobetanin, neobetanin and decarboxylated betanin. The yellow fraction contains betaxanthine pigment i.e. vulgaxanthine I. These components could be responsible for the activity exhibited by the fractions. Based on the literature search conducted, there have been no previous reports on the activity of the assay of the compounds above except that the betacyanins are derivatives of tyrosinase enzyme (Steiner *et al.,* 1999). The betaxanthines, are reported as physiological substrates of betaxanthine (betaxanthine-tyrosine is also known as portulacaxanthin II) (Gandia-Herrero *et al.,* 2005). The involvement of tyrosinase on the biosynthesis of betalains have been suggested (Piatteli, 1981 and Streiner *et al.,* 1999). However, the correlation of the activity of the fractions with these earlier findings was not understood.

ii) L-Dopa as substrate

As described earlier, this part of the assay measures the diphenolase activity of the enzyme tyrosinase.

Based on Figure 4.25, kojic acid had the lowest IC_{50} value (0.46 mg/ml). At higher concentrations, water fraction and ethyl acetate fraction had higher inhibition towards

diphenolase activity of tyrosinase as compared to the kojic acid. Among the extracts/fractions, ethyl acetate fraction had the best inhibitory activity with IC_{50} value of 0.96 mg/ml. The lowest activity was exhibited by methanol extract with IC_{50} of more than 20.00 mg/ml. From this result, methanol possessed the lowest inhibitory activity because it is in the crude form where the active components might be in low concentrations. Ethyl acetate fraction which originates from methanol extract has undergone certain extend of purification, thus it might contain higher concentration of the active components and exerts a higher inhibitory activity. Since hexane fraction which contains non-polar components exhibited low inhibitory activity, it can be concluded that polar components are responsible for the tyrosinase inhibitory activity with L-Dopa as substrate.

Figure 4.26 shows the tyrosinase inhibitory activity (diphenolase activity) of fractions isolated from ethyl acetate fraction and juice extract of *Beta vulgaris L.* The graph showed that all sub-fractions exhibited an activity higher than positive standard, kojic acid. Among these fractions, ET1 exhibited remarkably high inhibition at higher concentration. These results suggest that the components that are present in these fractions could be having high inhibition towards tyrosinase enzyme. These fractions which have been partially purified from the crude extract could exhibit their true potential in inhibition as compared to the crude extracts. This could be the reason why the crude extracts didn‘t show excellent activity but vice versa for the sub-fractions. The components in the sub-fractions could be further researched so that it could be incorporated in the cosmetics industry, food industry and insect control.

5.3 Comparison of antioxidant activities

5.3.1 Comparison of antioxidant activity of extracts of *Beta vulgaris L.* between different antioxidant assays

Among the various antioxidant assays that the extracts were tested on, certain extracts shows better activity as compared to the tested positive standards. Among the assays that noted such activity are reducing power assay, β-carotene bleaching assay, SOD activity assay and TBARS assay.

TBARS assay shows the first line of defense of preventive antioxidants. In this study, extracts of *Beta vulgaris L*. (methanol extract and hexane fraction) shows very high lipid peroxidation inhibition capacity in the TBARS assay. Hence, the regular consumption of the plant could bring benefit in preventing the diseases stated above.

The second line of defense of the antioxidant works as to stops the chain reaction caused by free radicals and breaks chain propagation. Superoxide activity assay and DPPH radical scavenging assay could be classified as specific free radical scavenging assay while the reducing power assay comes under non-specific free radical scavenging assay (Vimala, 2008). Whilst SOD assay specifically scavenges superoxide anion radical and in DPPH assay, it is the DPPH radical that scavenged. In the reducing power assay, there is no specificity on the scavenged radical. It measures the reducing capacity of a certain test sample. The ethyl acetate fraction of *Beta vulgaris L.* shows remarkably good activity in the SOD activity assay at higher concentrations. DPPH assay showed moderately good results but could not be compared to the positive standards. Hence, extracts shows moderate activity as second line defense antioxidant mechanism. Reducing power activity also corresponds to the second line defense antioxidant mechanism. Since certain extracts of *Beta vulgaris L.* has higher reducing capacity as compared to the positive standard, hence it

could play its role in resisting oxidative damage by reducing oxidative stress and its effects if it is regularly consumed.

The third line of defense antioxidant could be evaluated by the β-carotene bleaching assay and the tyrosinase inhibitory assay. As for the β-carotene bleaching assay, certain extracts had better activity as compared to positive standard but in the tyrosinase inhibitory assay, the crude extracts did not show higher activity than kojic acid, except that the juice and water fractions had higher activity at the highest tested concentration (20 mg/ml) than kojic acid in diphenolase activity. This concludes that, the plant might have some third line defense mechanisms.

Besides that, we can also view the results based on the mechanism of each antioxidant assay. Although the results were good for reducing power assay, the β-carotene bleaching assay did not show excellent result for DPPH assay, it can be concluded that the extracts may inhibit the action of free radical by electron transfer (high reducing power activity) and hydrogen transfer mechanism (high antioxidant activity in β-carotene bleaching assay). SOD activity assay also showed high activity in some extracts of the plant and this shows the presence of SOD like components that could act as enzymatic antioxidant. TBARS assay also showed excellent activity of the plant to inhibit lipid peroxidation and hence the plant could have ability to resist oxidation towards lipid. Metal chelating assay did not exhibit excellent result. This could be due to the reason the sample did not have components that could chelate ferrous ions. Tyrosinase assay also did not mark a good activity in the crude extracts of the plant. Hence, the crude extracts might not have ability to inhibit melanin pigment biosynthesis that is caused by tyrosinase enzyme.

5.3.2 Comparison of antioxidant activity of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.* between different antioxidant assays

The sub-fractions ET1, ET2, ET3, Yellow and Purple showed different antioxidant activities in different assays.

In the DPPH assay which monitors single electron transfer, the purple fraction had the highest radical scavenging activity with a significantly low IC_{50} value of 0.13 mg/ml. This is possibly due to compounds such as betanin, isobetanin and neobetanin present in this fraction. According to Georgiev *et al.,* 2010, the antioxidant activity of betanin was related to its electron donating ability. The withdrawal of an electron from betacyanins is relatively easy. This is because the electron would be from the phenolic oxygen. The betacyanin radical that has lost its electron to the DPPH radical would be stabilized by the delocalization of the unpaired electron through the aromatic ring (Escribano *et al.,* 1998).

In the reducing power assay, the purple fraction showed the highest activity among the tested fractions, but showed lower activity in comparison to the standards, BHA and ascorbic acid. The high reducing capacity of the purple fraction (all belonging to the betacyanin group) might be due to components like betanin, neobetanin and isobetanin that are present that could donate electron easily. Dose-dependent activity was observed in all the tested fractions. According to Cai *et al.,* 2003, although betalain compounds are not flavonoids, they possess a monoglucosylated *O*-diphenol group and a cyclic amine group, the partial structure of which resembles that of ethoxyquin, a very strong antioxidant. Betalain molecules were very good electron donors (Cai *et al.,* 2003).

The results shown by both the DPPH and reducing power assay correlated with the research done by Escribano *et al.,*1998 in which betacyanins showed greater antiradical activity than betaxanthine. In both the assays, the yellow fraction did not show extremely good results. This could be explained in reference to its structure where the withdrawal of

an electron from betacyanins is relatively easy as the electron would be one from the phenolic oxygen. The betacyanin radicals that are formed from the donation of the electron could be stabilized by the delocalization of the unpaired electron through the aromatic ring. In the case of betaxanthine, the electron abstracted could only be from the conjugated π-orbitals, this loss is hindered by the positive charge that is present in the nitrogen atom. Hence, it is easier to abstract an electron from the betacyanin molecules (Escribano *et al.,* 1998). The structure of betanin, isobetanin and vulgaxanthine I (a betaxanthine compound) are shown in Figure 5.2, 5.3 and 5.5.

The β-carotene bleaching assay showed that all the fractions did not have good hydrogen atom donating capacity to stop the radical attack towards the β-carotene molecule as compared to the positive standards. This could be due to the structure of the components present which have electron donation capacity rather than hydrogen transfer.

Sub-fractions also did not have the ability to chelate metal ions that would cause oxidation. The results in Figure 4.13 clearly depicts that all the fractions are not metal ion chelators.

In the SOD activity assay, some compounds such as ET1, ET2 and purple fraction has higher activity as compared to the standard BHA at higher concentrations. This shows that the fractions have SOD like materials that are able to stop the action of XO and also to neutralize superoxide anions.

In the lipid peroxidation assay (TBARS assay), the yellow fraction showed highest inhibition towards lipid oxidation. This could be due to the presence of the betaxanthine pigment (Vulgaxanthine I). According to previous reports, betaxanthine was found to have high antioxidant activity (Georgiev *et al.,* 2010). A research that was carried out by Gandia-Herrero *et al,* (2009), found that the biological property as above mentioned are not due to

the presence of hydroxy groups or aromaticity, but are enhanced by the presence of phenolic hydroxyl groups in their structure. This compound might be able to suppress the formation of free radicals and thus prevent the oxidative damage in the unsaturated fatty acids. This result also corresponds to Zakharova and Petrova, (1998) in which the antioxidant activity of vulgaxanthine (betaxanthine) was higher than that of isobetanin/betanin (betacyanin) in inhibition of lipid peroxidation.

Sub-fractions showed high ability to inhibit tyrosinase enzyme in monophenolase (L-tyrosine) and also in diphenolase activity (L-Dopa). ET3, purple and yellow fraction showed high inhibition on monophenolase activity even higher than kojic acid. Diphenolase activity was inhibited excellently by all the fractions as all of them had higher inhibition as compared to kojic acid. This could be due to the presence of betacyanin and betaxanthine pigments and also the presence of betagarin and betavulgarin. These components have the ability to block the oxidation of L-Dopa to dopaquinone that would eventually form melanin. Tyrosinase was found to be involved in the betacyanin biosynthetic pathway which involved conversion of tyrosine to betanin (Strack *et al.,* 2003). There might be a link with this fact that causes the components to have high inhibition towards diphenolase activity of tyrosinase. Hence, these components might be a potent inhibitor of tyrosinase enzyme and could be used in the cosmetic industry and others, but further research has to be done to confirm the mechanism of the inhibition.

Based on all the results obtained in the various assays, it can be concluded that the isolated fraction (Yellow fraction) have first line antioxidant defense basing on TBARS assay. The sub-fractions also possess second line antioxidant assay as they have remarkably good activity in the SOD assay (Purple, ET1 and ET2) and DPPH assay (Purple). The sub-fractions also show third line antioxidant defense mechanism as they showed excellent

activity in the diphenolase activity of tyrosinase inhibitory assay (Purple, Yellow and ET3). The difference may be due to the presence of different components in each fractions.

5.4 Cytotoxicity (Neutral Red Cytotoxicitiy Assay)

5.4.1 Cytotoxic Activity of extracts of *Beta vulgaris L.*

In *in vitro* toxicology studies, cytotoxicity assays have been widely used. It is a rapid, standardized, sensitive and inexpensive method to measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death (Wilson, 1986). In this study, the experiment that was conducted to determine the cytotoxic activity of the plant was Neutral Red Assay. This assay measures the availability of viable cells to incorporate and bind Neutral Red (NR) dye. NR is a weak cationic supravital dye that readily penetrates cell membranes by non-ionic diffusion and predominantly accumulates intracellularly in lysosomes. Alteration of the cell surface or the sensitive lysosomal membrane by plant extracts lead to lysosomal fragility and other changes that gradually become irreversible. Such changes produced by toxic substances will lead to a decrease in the uptake and binding of NR, making it possible to distinguish between viable, damaged or dead cells via spectrophotometric measurements.

The result obtained showed that *Beta vulgaris L.* only exhibits mild to weak inhibition against cancer cell lines. The best activity shown by the plant was from ethyl acetate fraction which showed IC_{50} of 69.50 µg/ml with HT-29 colon carcinoma cell line. Besides that, IC_{50} for other extracts with various cancer cell lines showed only weak activity with 90-100 µg/ml. This shows that the plant does not have high cancer inhibiting activity.

According to a previous study by Kapadia *et al.* (1996), betanin, which is a component that is isolated via HPLC, showed inhibition of TPA induced promotion of mice skin tumors and also lung tumor in ICR mice and a conclusion was made that beetroot is a useful cancer preventive vegetable based on the study (Kapadia *et al.,* 2003). Another study by Reddy *et al.* (2005), showed that betanin has excellent growth inhibition of breast, colon, stomach, CNS, and lung cancer cell lines with IC_{50} values of 162,142,158,164 and 147 μg/ml respectively based on MTT assay. Polar, aqueous extractable component in beetroot tissue was found to be an active phase II inducer in murine hepatoma. Maintanence of high levels of phase II enzymes in bodily tissues could be cancer chemopreventive defense against highly reactive electrophiles (Wettasinghe *et al.,* 2002). Betanin pigment was also found to induce apoptosis in human chronic myeloid leukemia cell line (K562) (Sreekanth *et al.,* 2007). However, our results were not promising on the cytotoxicity assay. This could be due to difference in the nature of the assay used and difference in the response of the cells used in the assay.

5.5 Chemical Analysis

5.5.1 Isolation (using HPLC) and identification (using LC-MS-MS) on ethyl acetate and juice extract of *Beta vulgaris L.*

Chemical investigation of chemical components was directed to the ethyl acetate fraction because it showed high activity in many of the tested antioxidant assays. It also showed moderate activity in HT-29 cell line in Neutral Red cytotoxicity assay. Important active chemical components are targeted to be isolated from these fractions.

The juice extract was focused for isolation because of the presence of the important pigment of beetroot which is the betalains which consists of betacyanins and betaxanthines.

Hence, isolation was targeted to isolate these components and test further on the antioxidant activities.

i) Isolation of components in the ethyl acetate fraction

Isolation of this extract gave us 3 main isolated sub-fractions which are ET1, ET2 and ET3.

a) ET1

Three components found present in the sub-fraction were betavulgarin, isobetanin and betanin.

1. Betavulgarin

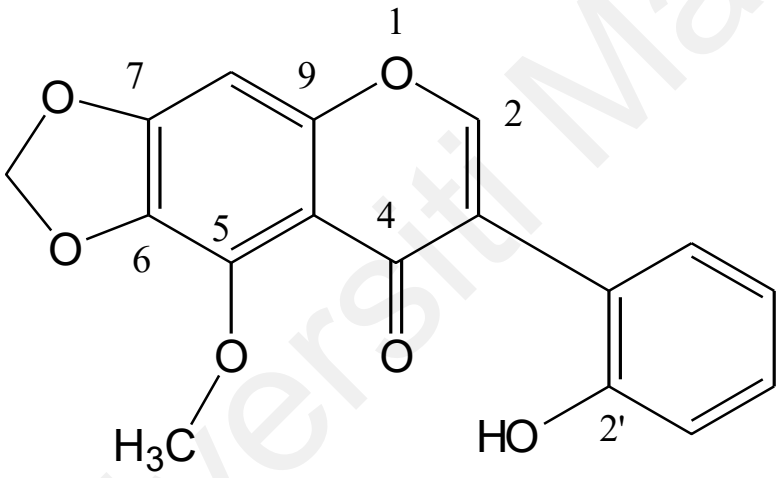


Figure 5.2: Chemical structure of Betavulgarin

The systematic name for betavulgarin is 2'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone (Martin, 1989). The compound has a molecular weight of 312.27. Betavulgarin was found to have anti-fungal activity and was considered to be phytoalexins (compounds that fight fungal attack to the plant) (Martin, 1977). It is a flavonoid (isoflavone) that is commonly found in *Beta vulgaris L.*

2. Betanin and Isobetanin (Betacyanin)

Figure 5.3: Chemical structure of Betanin

The compound has molecular weight of 550.46 and molecular formula of $C_{24}H_{26}N_2O_{13}$.

Isobetanin is a C15 epimer of betanin (Gliscyznska-Swiglo *et al.,* 2006).Betanin and isobetanin is well known for its antixodant property (Escribano *et al.,* 1998). Its antioxidant capacity has been studied extensively. The antioxidant capacity is associated with the ability of the molecule to donate electron (Gandia-Herrero *et al.,* 2009). Betanin has also been reported to have inhibition towards cell proliferation of a variety of tumor cells (Reddy *et al.,* 2005). Betanin was also found to induce apoptosis in human chronic myeloid leukemia cell line-K562 (Sreekanth *et al.,* 2007)

Figure 5.4: Chemical structure of Isobetanin

The compound has a molecular weight similar to betanin 550.46 and molecular formula $C_{24}H_{26}N_2O_{13}$.

b) Fraction ET2

The major peak at retention time 7.53 mins gave molecular ion 255 consistent with 2,15,17-tridecarboxy betanin and another peak at $[M+H]^+$ 329 was identified as betagarin by comparison of its mass fragmentation pattern with the literature Kujala *et al.,* 2002. The fraction dissolves completely in distilled water and hence it showed that the compounds present in this fraction are very polar in nature.

1. **Betagarin**

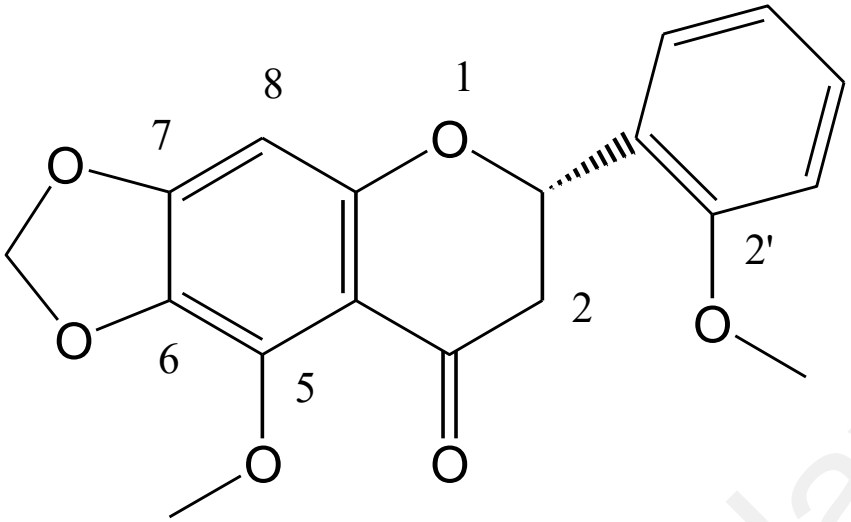


Figure 5.5: Chemical Structure of Betagarin

The molecular formula of this compound is $C_{18}H_{16}O_6$. It is also known as 5,2' -dimethoxy-6,7-methylenedioxyflavanone. It is categorized as flavonoids (flavone). Betagarin was found to have anti-fungal activity and are considered to be phytoalexins (compounds that fight fungal attack to the plan) (Martin, 1977).

c) Fraction ET3

The HPLC chromatogram of fraction ET3 in Fig 4.36 showed one major peak and several minor peaks. Although most of the components in this fraction could not be identified, a component which has molecular weight 328 was found to be possibly betagarin based on comparison with the $[M+H]^+$ of compounds identified by Kujala *et al.,* 2002. The data that was compared to that of high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) by Kujala *et al.,* 2002, which shows the $[M+H]^+$ ion corresponding to 329. Betagarin has been described in ET2. There is an observation of an

identical nominal mass for both 220 and 231 at two different retention times. This could be due to potentially stereo/isomeric compounds. NMR could not be conducted as the sample contains many components and it is not a pure compound. The fraction dissolved completely in distilled water and hence it has high polarity.

ii) Isolation of juice extract

Isolation of this extract gave us 2 main sub-fractions which are yellow and purple fraction. They were named such based on the morphology of the isolated fraction. The eluent from HPLC for the Yellow fraction had a bright yellow color while the eluent from HPLC for the Purple fraction had a deep purple colour.

a) Yellow Fraction

The LC-MS-MS chromatogram showed several major peaks. The peak at retention time 0.878 min, was identified as Vulgaxanthine I. This fraction dissolves completely in distilled water and hence the compounds present in this fraction could be polar compounds. The yield of the yellow fraction is very low (0.71%).

1. **Vulgaxanthine I**

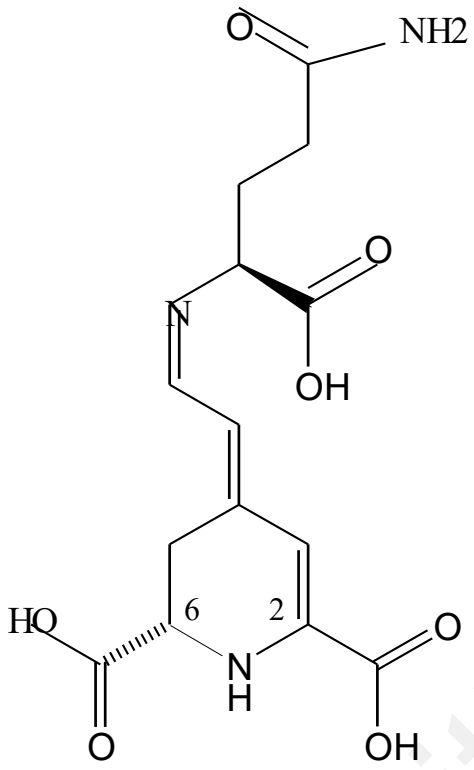


Figure 5.6: Chemical structure of Vulgaxanthine I

Vulgaxanthine I has a molecular weight of 339.30 and a molecular formula of $C_{14}H_{17}N_3O_7$. Vulgaxanthine I and Vulgaxanthine II belong to the betaxanthine pigment class which is also known as yellow pigment. It normally co-occur in beetroot (Kujala *et al.,* 2001). According to Cai et al., 2005, common beets usually contains both red betacyanins (consisting of 75–95% betanin) and yellow betaxanthins (95% vulgaxanthine I), in various ratios depending on cultivar. This explains why vulgaxanthine II was not detected in our studies. According to Czapski *et al.,* 2009, betaxanthine was found to have antioxidant activities but is dependent on the red pigments (betacyanins) to exert its high antioxidant capacity. In the current study, results showed that the isolated fraction containing

vulgaxanthine I had high ability to inhibit lipid oxidation and also to scavenge DPPH free radicals quite well (IC_{50} = 0.57 mg/ml). It was also found that the fraction containing the compound had high inhibition of tyrosinase activity on both the monophenolase and diphenolase activity. Betaxanthine sources are much little worldwide as compared to carotenoids which is a common yellow color food colorant (low water solubility), betaxanthine's water solubility could propel their application as yellow-orange food colourants in situations when the water solubility is crucial (Azerado, 2009). Betaxanthine is more stable compared to betacyanins for storage purposes and it has excellent antioxidant property (Cai *et al.,* 2005). According to Delgado-Vargas *et al.,* 2000, betaxanthine could be used as introducing essential amino acids in the diet.

b) Purple Fraction

It has a deep purple color and very sensitive to various conditions such as light, air, temperature and etc. The fraction was stabilized before storage to ensure the chemical stability of the compounds in it is maintained. Identification was done using LC-MS-MS and a few compounds were identified. Among the identified compounds were betanin, isobetanin, neobetanin and decarboxylated betanin. Since betanin and isobetanin has been discussed earlier, neobetanin and decarboxylated betanin will be discussed here.

1. Chemical stabilitzation of the Purple fraction

Betalains are compounds which have molecular weight ranging between 400-500 and are compounds with quartenary amino group. It has attractive red colour. The pigment has poor color stability and has been a hinderance in industrial applications. The stability of betalains was reported to be strongly dependent on oxygen, pH, temperature, light and water activity (Reynoso *et al.,* 1997). The pigments have stability at certain conditions and hence the

storage conditions were altered to achieve maximum stability for a longer period of time. pH 5.5 was found to be the optimal pH for the storage of the pigment with minimum percentage of degradation. As for the temperature, betanin was found to degrade at a faster rate at higher temperature and this degradation is further promoted by presence of oxygen (Reynoso *et al.,* 1997). At pH 5.5 and storage temperature of C, deactivation half life time of more than 1 year was determined (Castellar *et al.,* 2003). Betalains are 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. It was found that the addition of ascorbic acid to the pigment extract protected their color stability. Besides that, presence of metal ions could act as prooxidant in promoting the degration of the pigment by causing attack to the electrophilic centre of betalains and thus losing its color. Ascorbic acid also protects the compound in presence of metal ions such as iron and chromium (Reynoso *et al.,* 1997).

2. Neobetanin

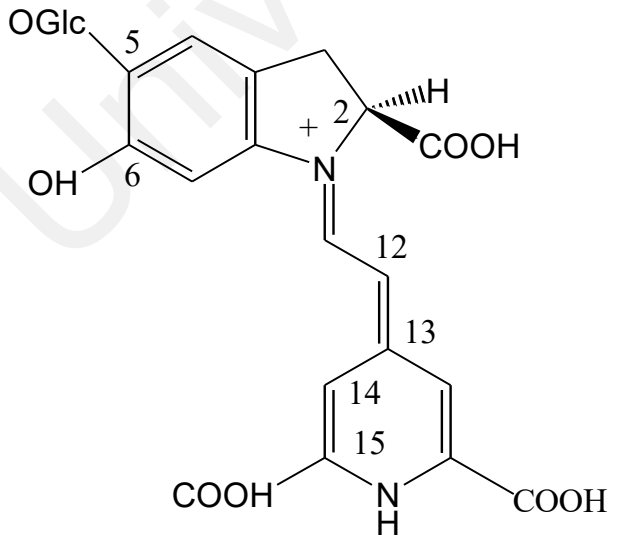


Figure 5.7: Chemical Structure of Neobetanin

It is also known as 5-*O*-β-D-glucopyranosylneobetanidin. It is an orange colored water soluble pigment (Alard *et al.,* 1985). Neobetanin was also found to occur as a minor constituent in petals of *Opuntia ficusindica, Portulaca grandiflora, Zygocactus truncatus* and in fruits of *Phytolacca bogotensis* and *Rhipsalis warmingiana* (Alard *et al.,* 1985). The molecular weight of the molecule is 548.

3. Decarboxylated betanin

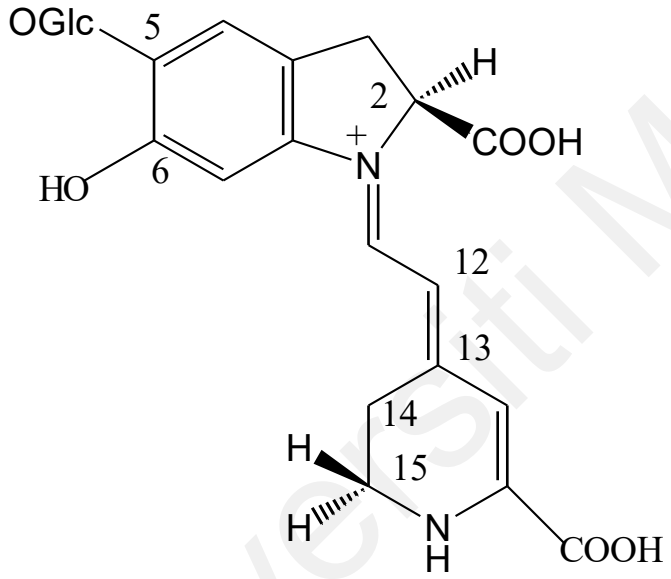


Figure 5.8: Chemical structure of decarboxylated betanin

This compound was identified based on LC-MS-MS report. It is one of the degradation products of betanin besides isobetanin, betalamic acid and cyclodopa glucoside (Kujala *et al.,* 2001).

CHAPTER 6

CONCLUSION

This research has brought about several beneficial discoveries.

The crude extracts of *Beta vulgaris L*. were subjected to 8 different antioxidant assays. Some of the extracts tested showed better activity compared to the tested positive standards on assays such as the reducing power assay, β-carotene bleaching assay, SOD activity assay and TBARS assay.

Overall, the ethyl acetate fraction showed high antioxidant activity as compared to other extracts/fractions in most of the assays but hexane fraction showed good inhibition of the lipid peroxidation in the β-carotene bleaching assay and TBARS assay. Hence *Beta vulgaris L.* has first, second and third line antioxidant defense mechanism. It could act as reductones to donate electron to stop the free radical chain reaction. It could also work in the mechanism of hydrogen atom transfer to quench radicals. The plant also has SOD like materials to inhibit XO enzyme and could prevent lipid oxidation.

Hexane fraction act best as first (based on TBARS assay) and third (based on β-carotene bleaching assay) line of defense whereas ethyl acetate fraction exhibited second (based on SOD, reducing power, Folin-Ciocalteu and DPPH assay) and third (based on tyrosinase inhibitory assay) line of antioxidant defense mechanism.

In metal chelating activity (first line of antioxidant defense), methanol extract exhibited the best activity among other extracts.

In this study the ethyl acetate fraction exhibited good antioxidant capacity in DPPH assay, reducing power assay and SOD activity assay, chemical investigations was thus directed to the ethyl acetate fraction and juice extract. Juice extract was also subjected subfractionation to isolation as there was some concern on non-presence of betalain

pigment in the ethyl acetate fraction. From the subfractionation, pure compounds could not be obtained but partially purified fraction that has important compounds was isolated. From the identification using LC-MS-MS, we found that ET1 has betavulgarin, betanin and isobetanin and other unidentified compounds. ET2 has 2,15,17-tridecarboxybetanin and betagarin and other unidentified compounds. ET3 was found to have betagarin and other unidentified compounds. Fraction yellow was found to have vulgaxanthine I and other unidentified compounds. Fraction purple was found to have betanin, isobetanin, neobetanin, decarboxylated betanin and other unidentified compounds.

The sub-fractions from ethyl acetate fraction (ET1, ET2 & ET3) and fractions of juice extract (Yellow and Purple) were tested on 8 different antioxidant assays. Based on all the results obtained in various assays, it can be concluded that the yellow fraction has first line antioxidant defense basing on excellent activity at TBARS assay. The sub-fractions also possess second line antioxidant assay as they have remarkably good activity in SOD assay (Purple, ET1 and ET2 fractions) and DPPH radical scavenging assay (Purple fraction). The sub-fractions also show third line antioxidant defense mechanism as it showed excellent activity in the diphenolase activity of tyrosinase inhibitory assay (Purple, Yellow and ET3 fractions). The difference in action of the sub-fractions might be due to the presence of different components in each fraction. Previously there have been reports that betalains show antioxidant and radical scavenging activities. Betalains as natural antioxidants may have protection against oxidative stress-related disorders (Gliszczynska-Swiglo *et al.,* 2006). These results obtained in our studies strengthened the previous results and also added a few new additional findings. There has been no previous report on antioxidant activities of various crude extracts and sub-fractions that contains certain important chemical components, by assessing on the antioxidant assays that was done in

this study. The results on SOD assay, tyrosinase inhibitory assay, metal chelating assay, total phenolic content, TBARS assay and β-carotene bleaching assay are new findings in the research.

In conclusion, as a combined result of the crude extracts and sub-fractions of the plant, the plant has various antioxidant capacities such as electron and hydrogen donating capacity, has SOD like materials which can quench superoxide anions and also inhibit XO enzyme, has lipid peroxidation inhibition capacity and also tyrosinase diphenolase inhibition activity. The findings also show that the plant does not have excellent metal chelating ability.

Cytotoxicity test did not show excellent results of inhibition for all the human carcinoma cell lines tested on the extracts of *Beta vulgaris L.* Only the ethyl acetate fraction showed moderate inhibition towards HT29 (IC_{50} = 69.5 µg/ml). Some components that are present in the ethyl acetate fraction (which were not identified and tested in our study) could be responsible for this.

Finally, from all the results obtained, we can conclude that, *Beta vulgaris L.* is a plant that has chemopreventive property but it does not show excellent anti-cancer property. Beetroot can be recommended to be consumed on a regular basis to maintain good health and prevent diseases that are caused by free radicals such as cancer, cardiovascular diseases, Alzheimer‘s disease and others.

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