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).

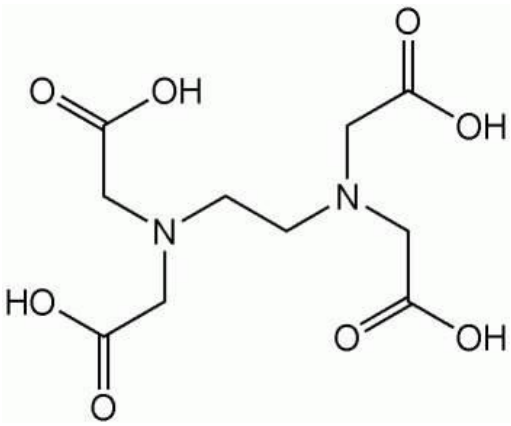

Figure 5.1 Chemical structure of EDTA (ethylenediamine-N,N,N'N'-tetra acetic acid)

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.

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

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**

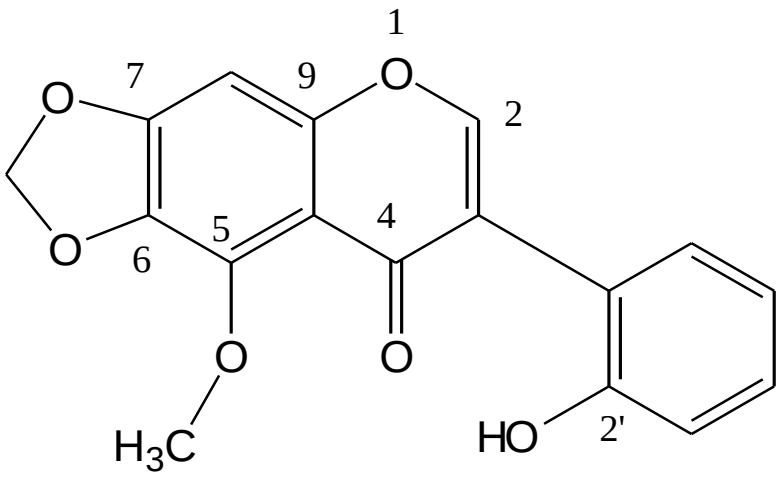

Figure 5.2: Chemical structure of Betavulgarin

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)

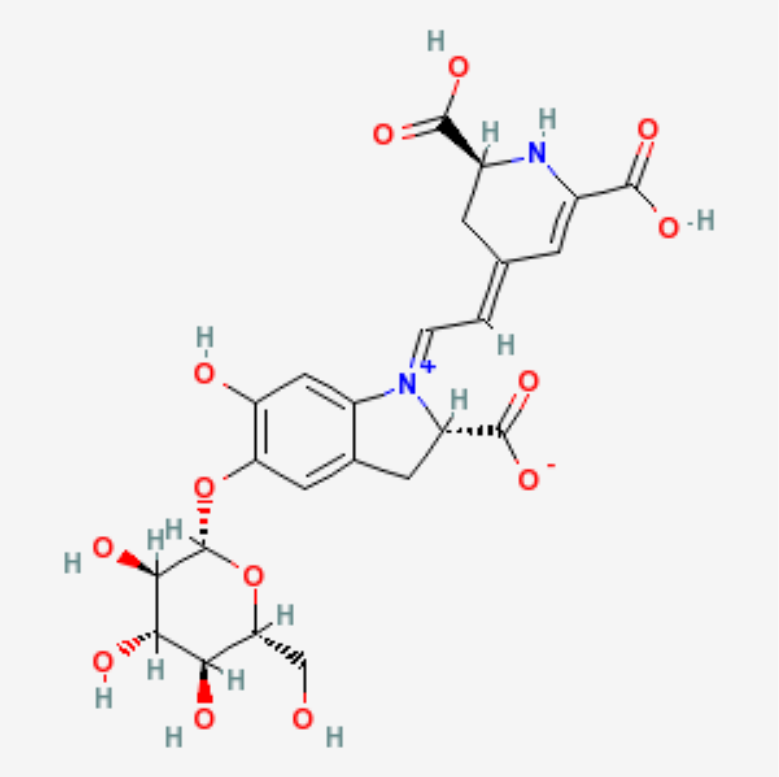

Figure 5.3: Chemical structure of Betanin

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)

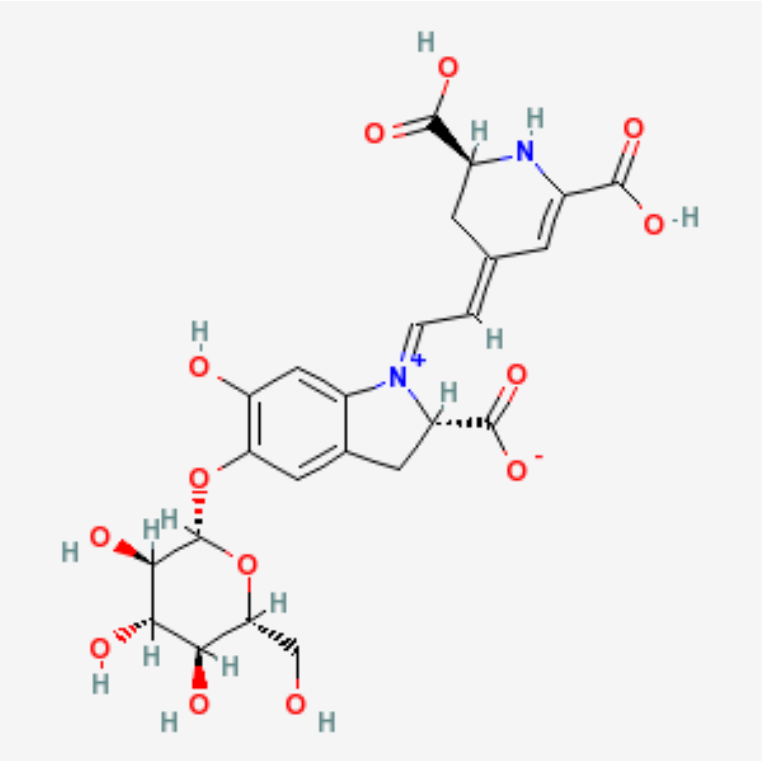

Figure 5.4: Chemical structure of Isobetanin

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**

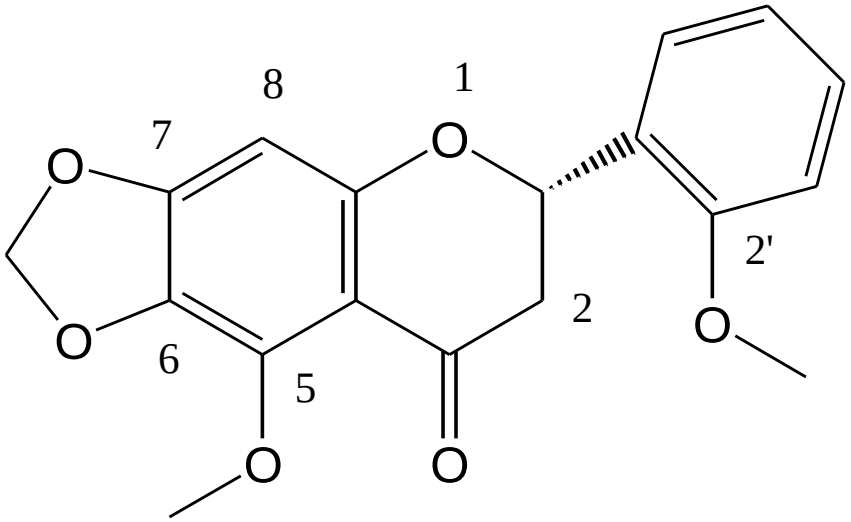

Figure 5.5: Chemical Structure of Betagarin

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**

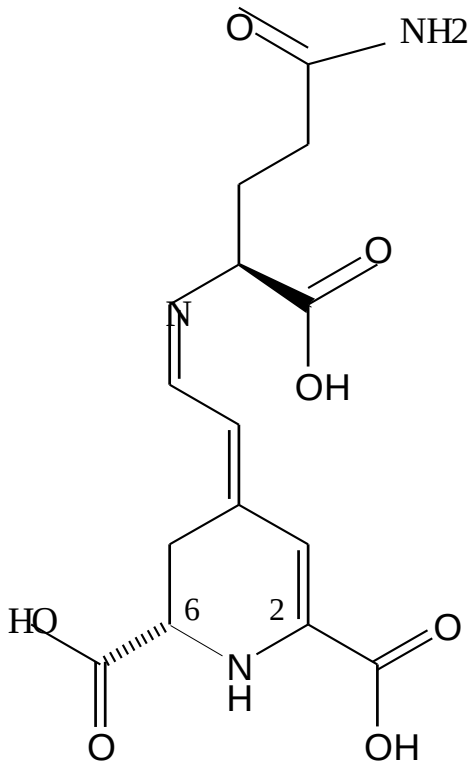

Figure 5.6: Chemical structure of Vulgaxanthine I

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 4°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

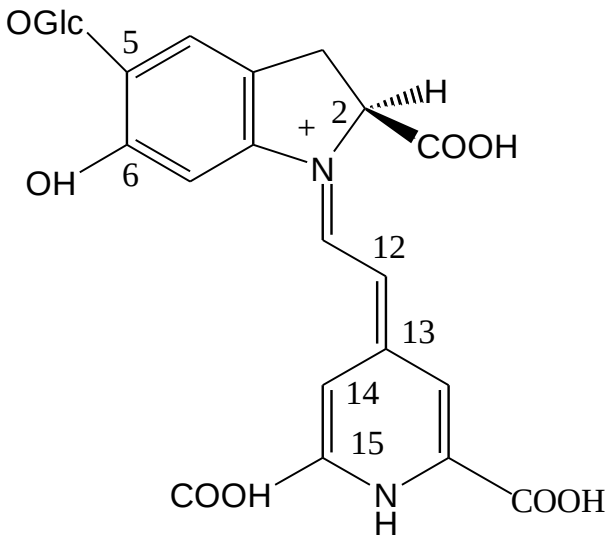

Figure 5.7: Chemical Structure of Neobetanin

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

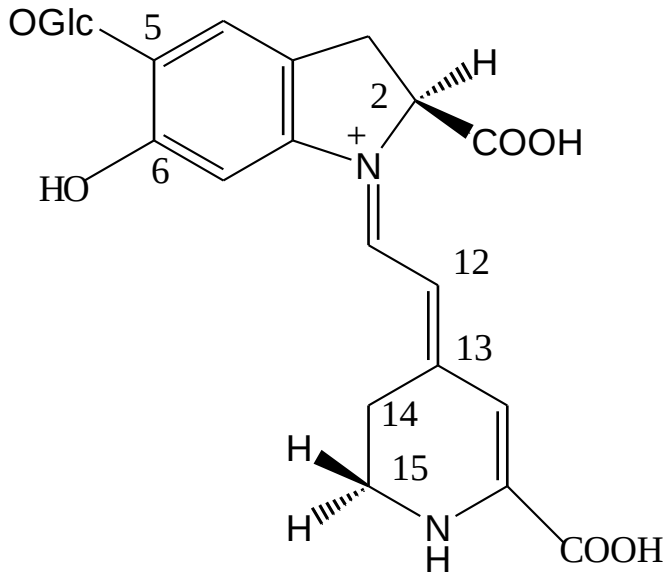

Figure 5.8: Chemical structure of decarboxylated betanin

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.