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)

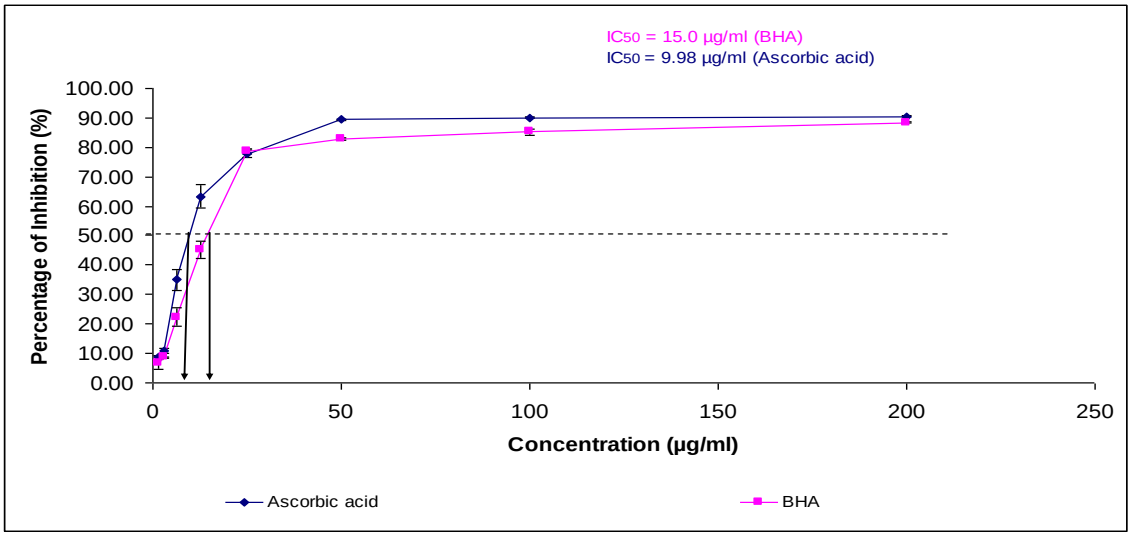


Figure 4.1: The determination of IC_{50} values of ascorbic acid and BHA as positive standard references in DPPH free radical scavenging assay

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)

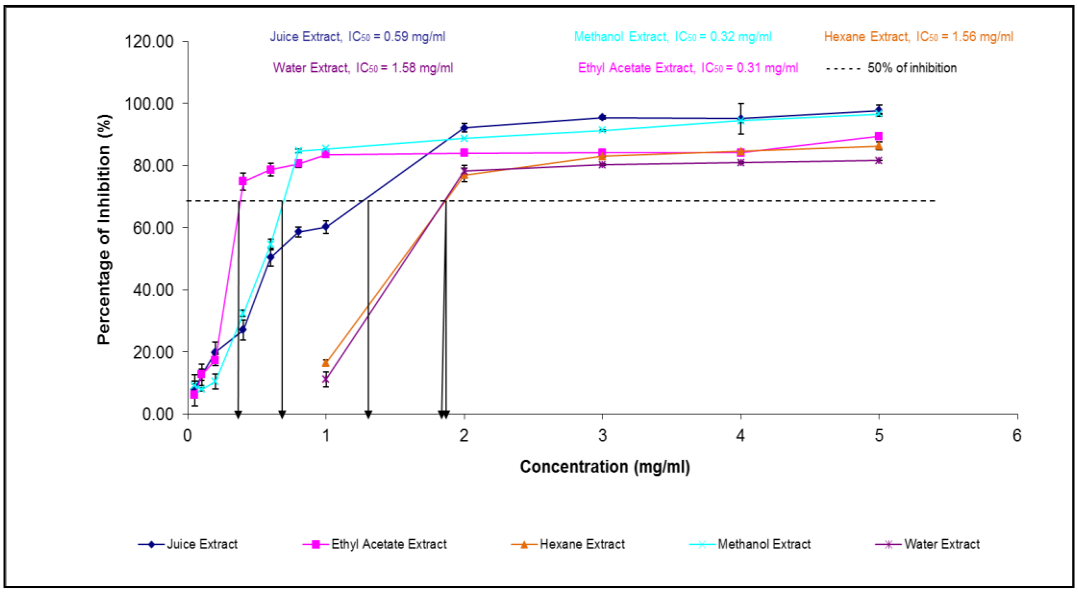


Figure 4.2: The determination of IC_{50} values of the active crude extracts of *Beta vulgaris L.* in the DPPH free radical scavenging assay

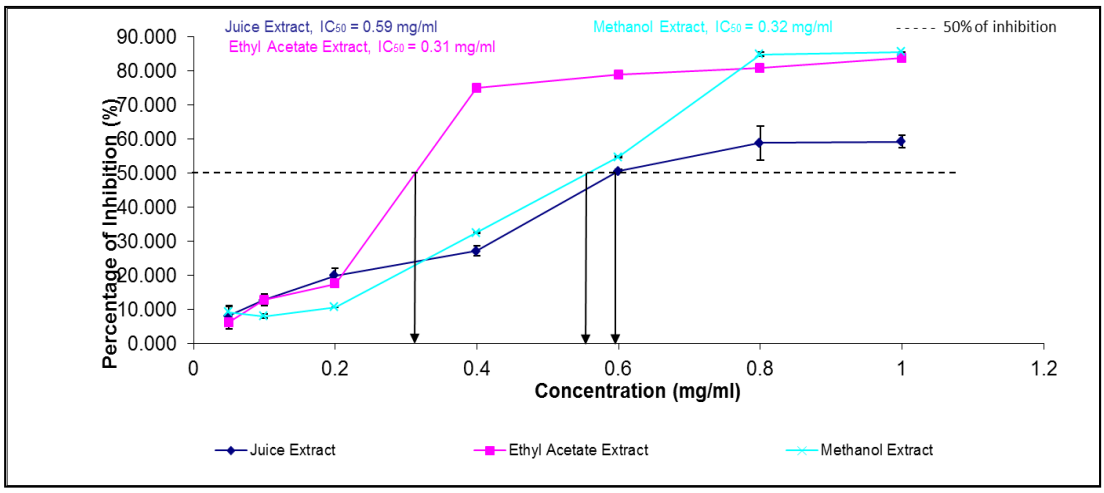


Figure 4.3: The determination of IC_{50} values of active crude extracts of *Beta vulgaris L.* which was tested at lower concentrations in the DPPH free radical scavenging activity assay

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)

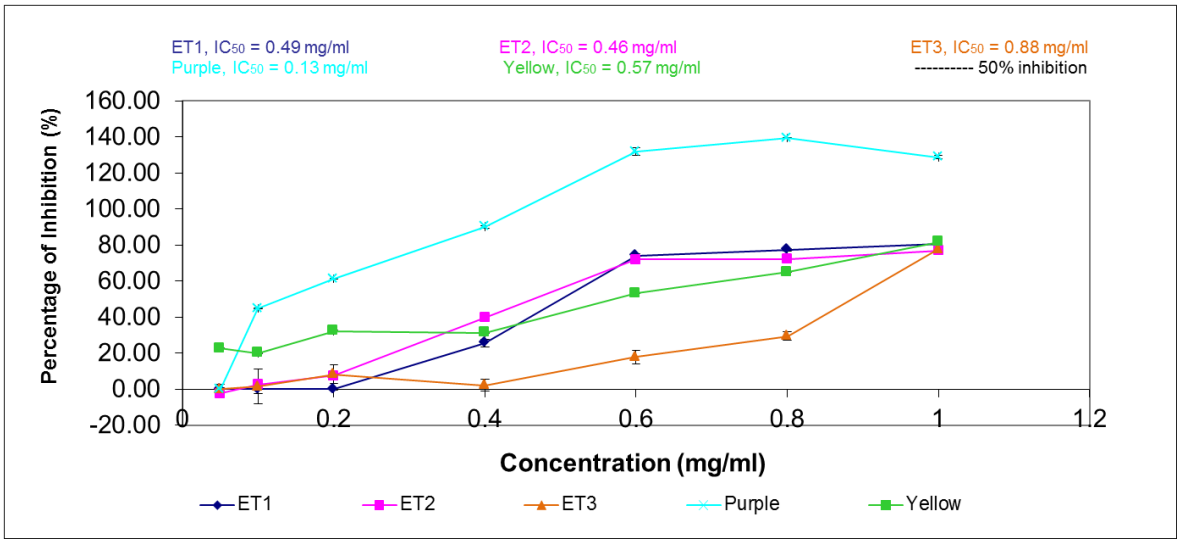


Figure 4.4: The determination of IC_{50} values of sub-fractions of ethyl acetate fraction and juice extracts of *Beta vulgaris L.* in DPPH free radical scavenging assay

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.

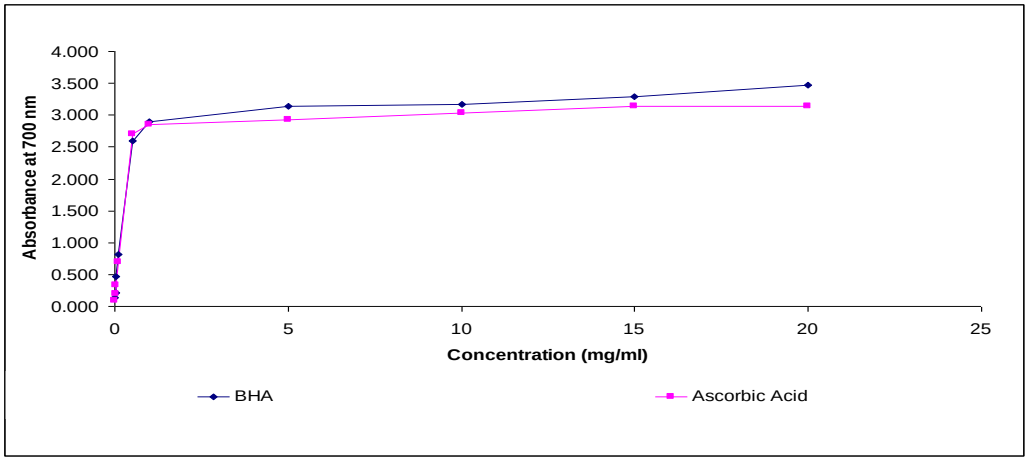


Figure 4.5: Comparison between the reductive ability of BHA and ascorbic acid

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)

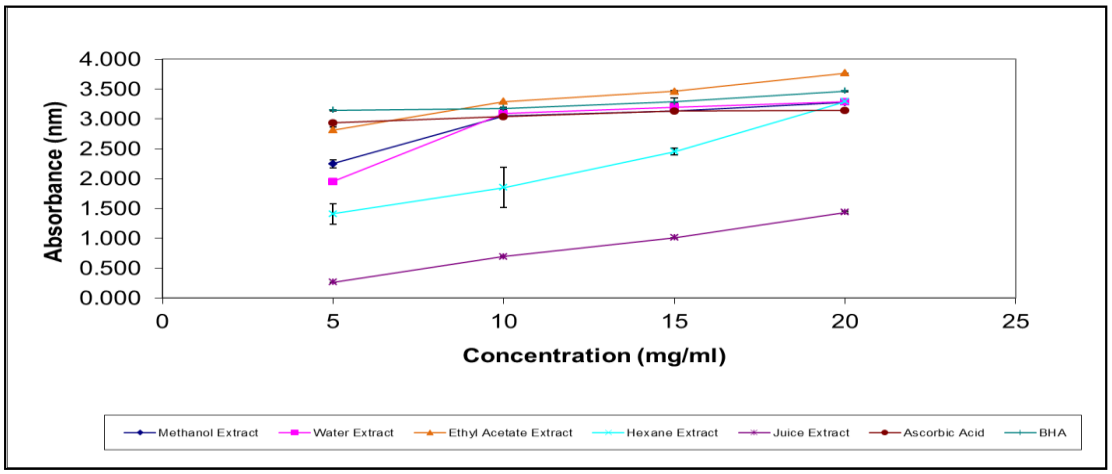


Figure 4.6: Comparison of reducing power activity of various extracts of *Beta vulgaris* and standards

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

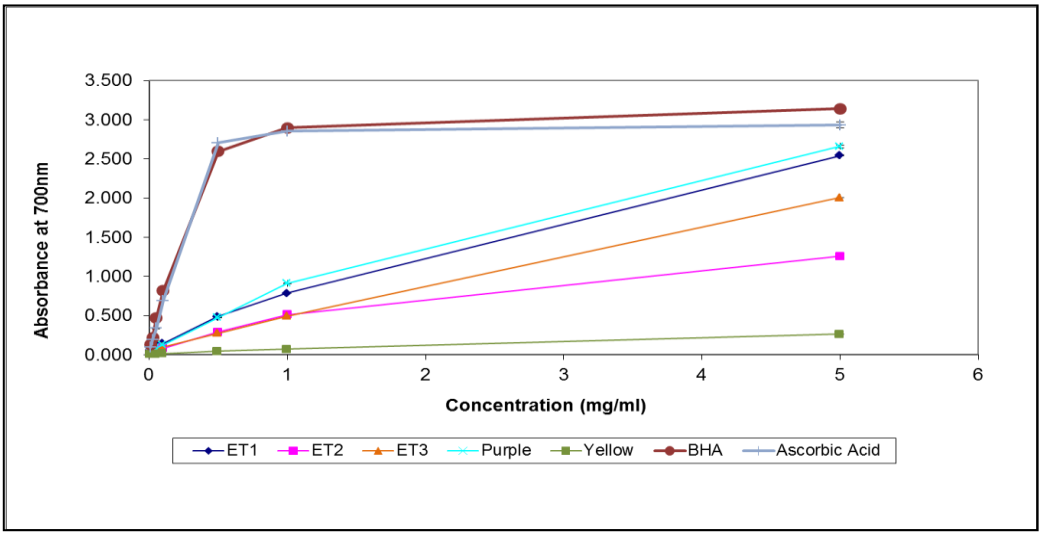


Figure 4.7: Comparison of reductive ability of isolated sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* and standards

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)

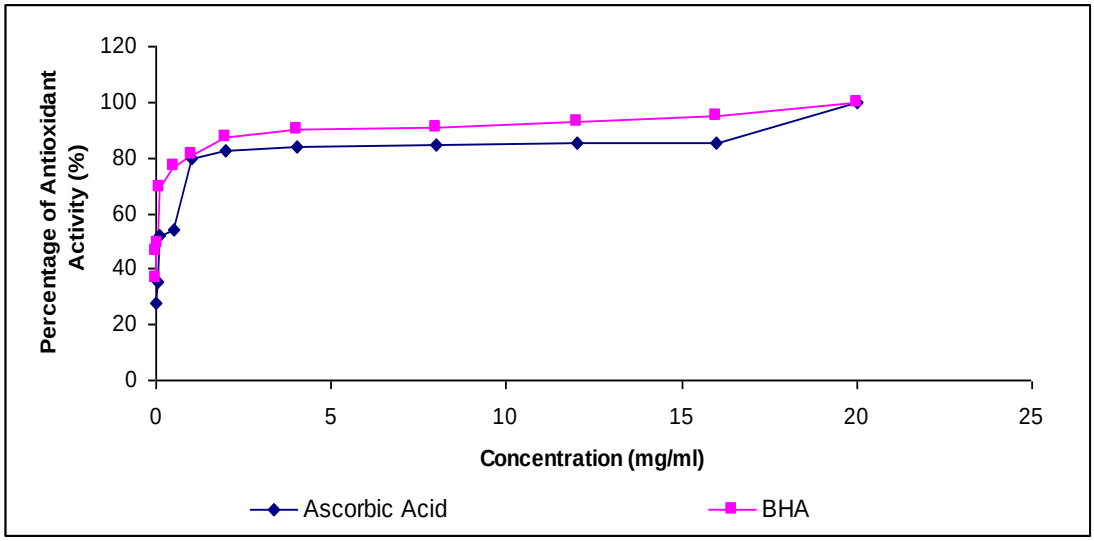


Figure 4.8: Antioxidant activity of ascorbic acid measured by the β-carotene bleaching assay

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)

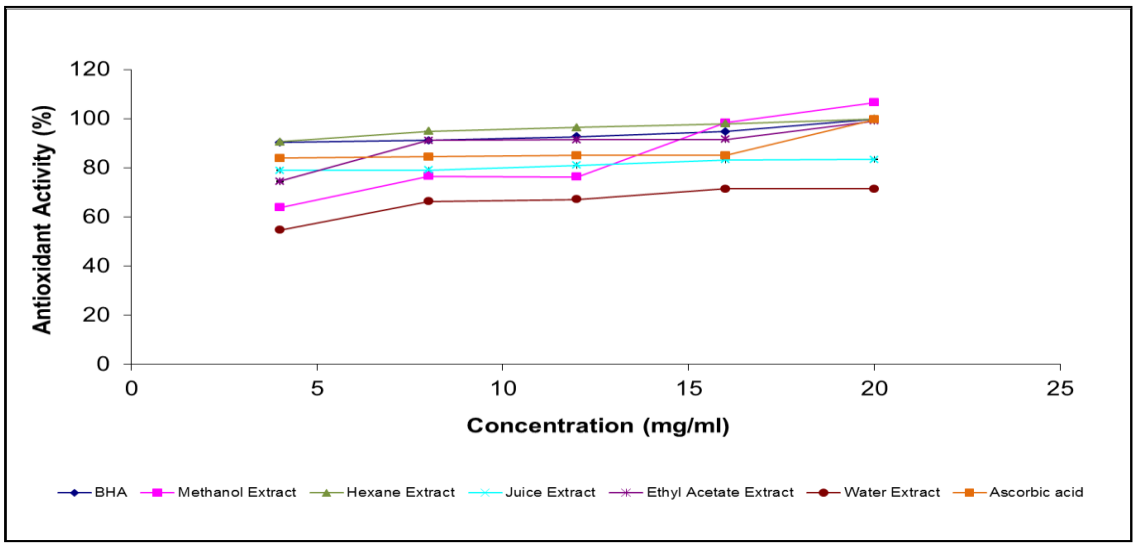


Figure 4.9: Comparison of antioxidant activity in percentage of extracts of *Beta vulgaris L.* extracts determined by the β-carotene bleaching activity assay

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)

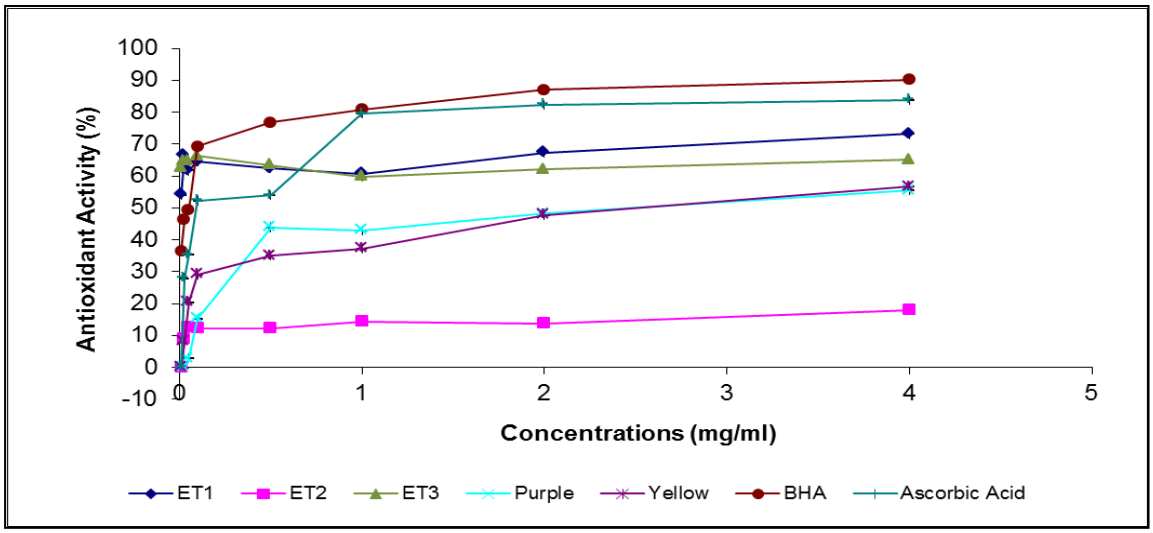


Figure 4.10: Comparison of percentage of antioxidant activity of sub-fractions of ethyl acetate and juice extract of *Beta vulgaris L.* assessed by the β-carotene bleaching assay.

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)

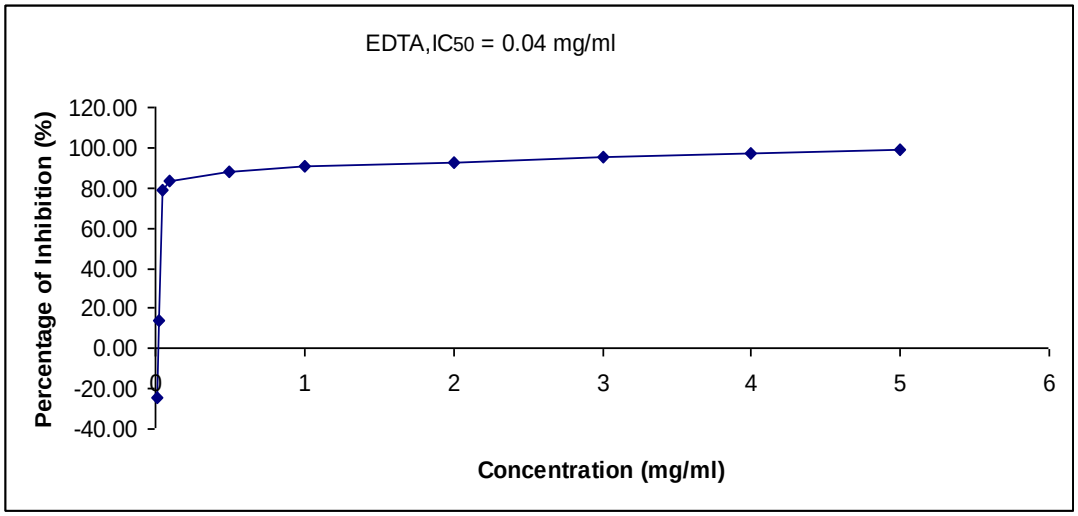


Figure 4.11: Percentage inhibition of metal chelating activity by standard EDTA

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)

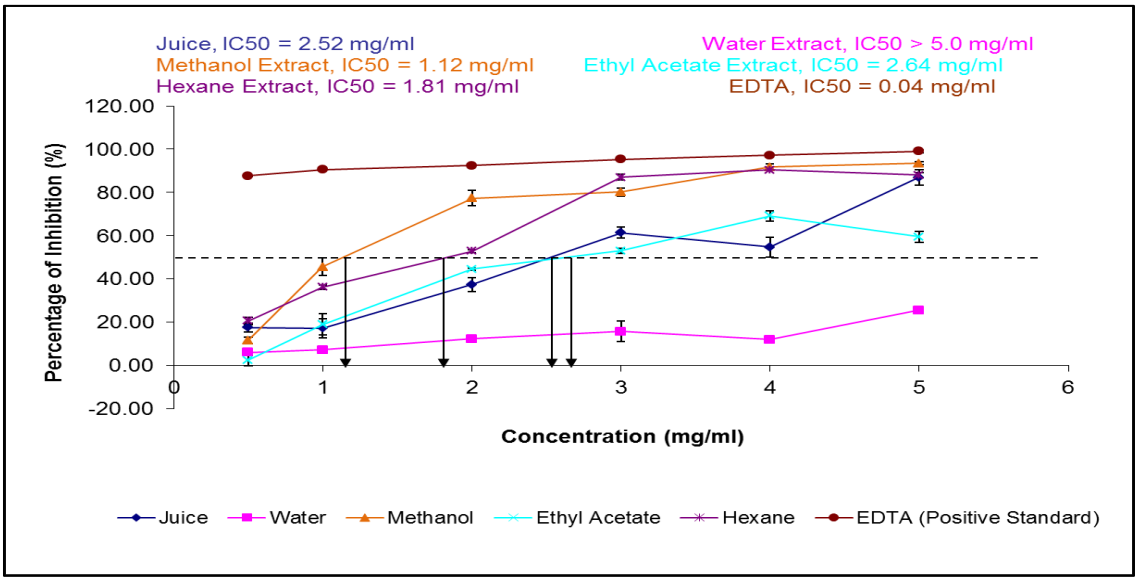


Figure 4.12: Comparison of metal chelating activity of crude extracts and fractions of *Beta vulgaris L.* with standard EDTA at various concentrations and determination of IC_{50} values

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)

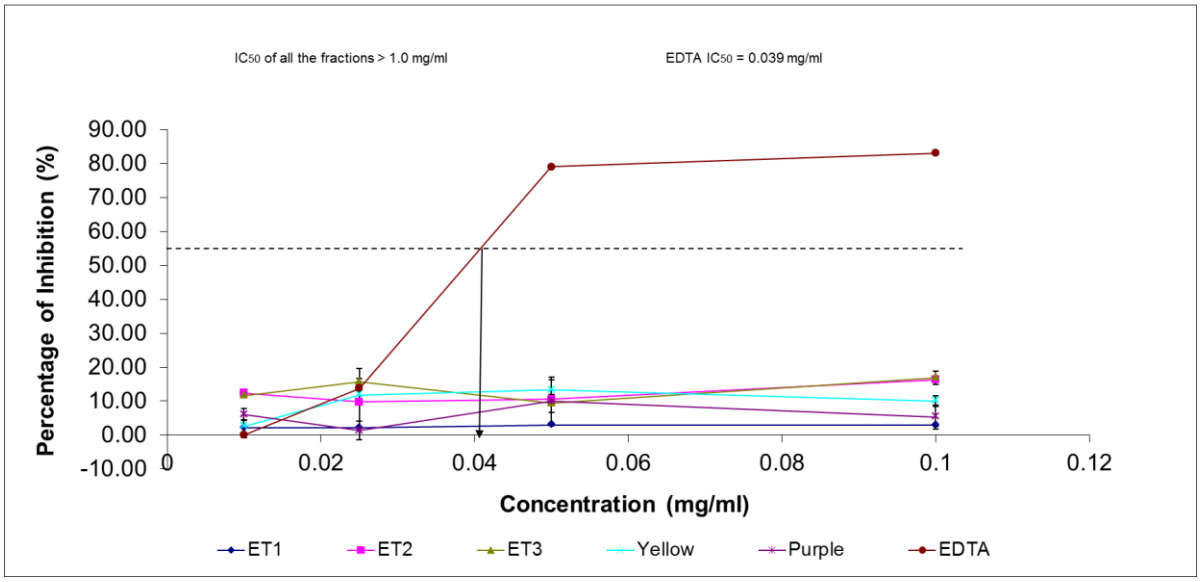


Figure 4.13: Comparison of metal chelating activity of isolated sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard EDTA

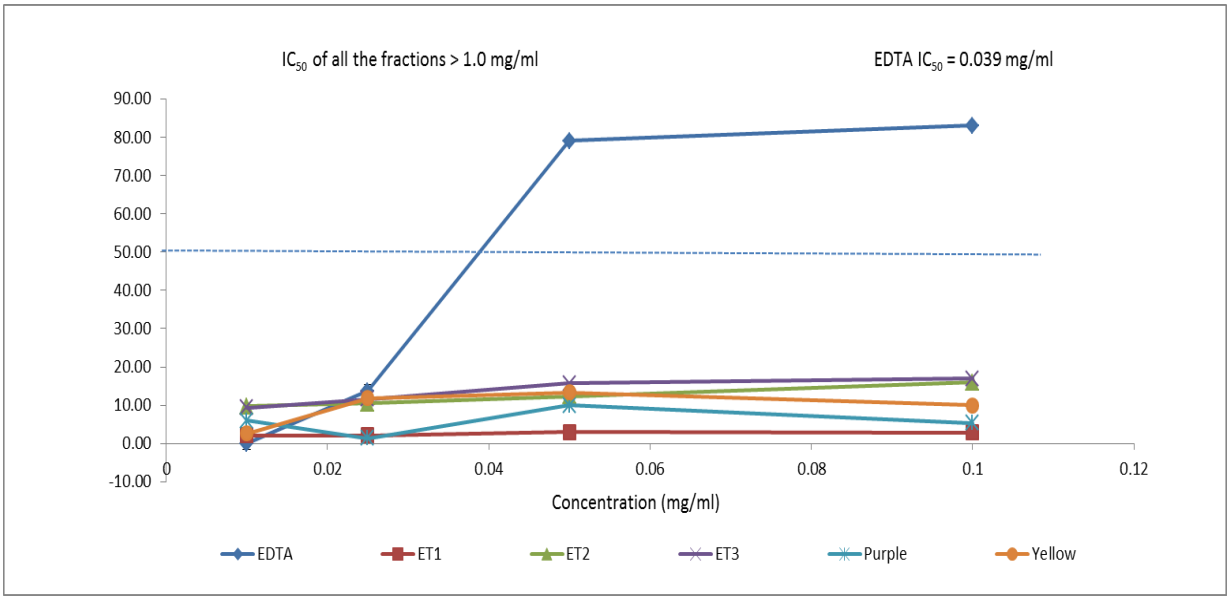


Figure 4.14: Magnification of lower concentration on comparison of metal chelating activity of sub-fractions of ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard

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-Iodophenyl)-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)

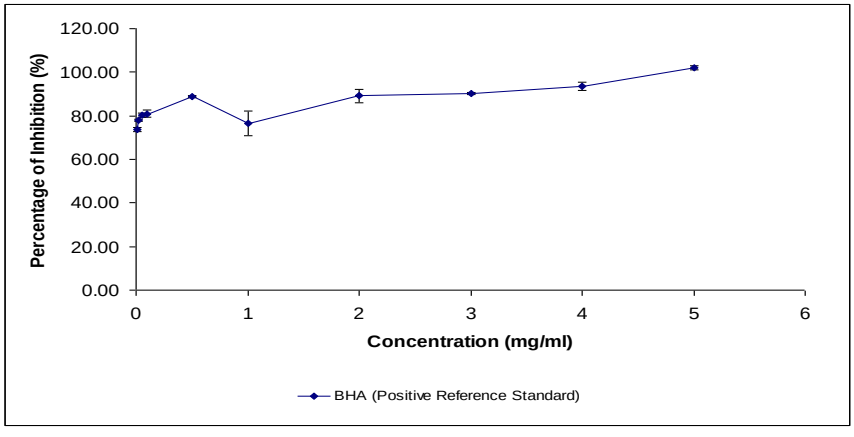


Figure 4.15: The SOD activity of BHA (positive reference standard) over varying concentrations

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

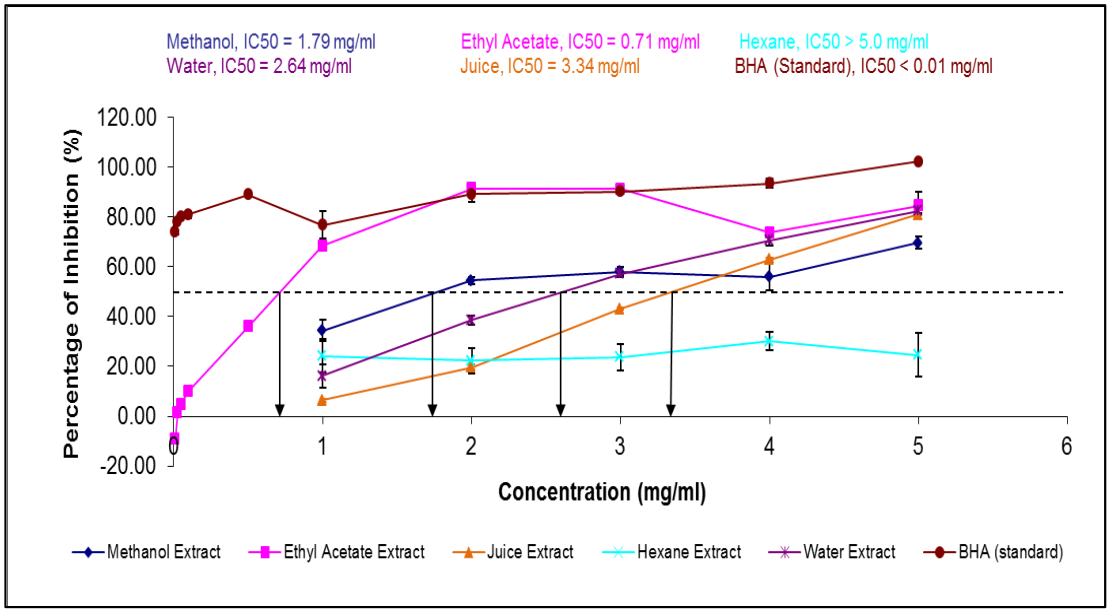


Figure 4.16: Comparison of SOD inhibition activity between extracts of *Beta vulgaris L.* and standard (BHA) at various concentrations

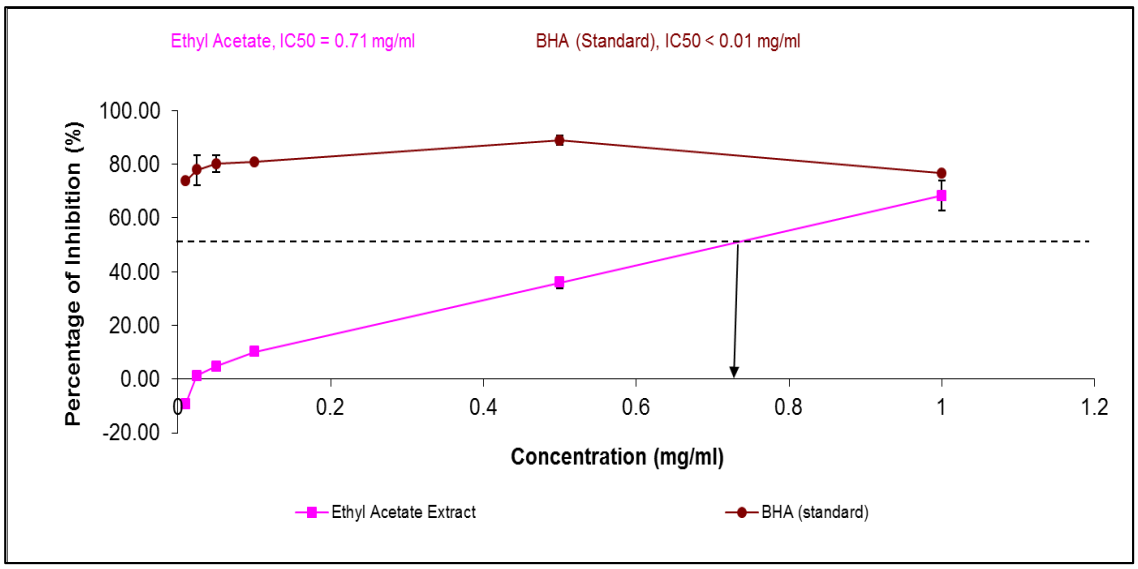


Figure 4.17: Investigations of ethyl acetate fraction of *Beta vulgaris L.* and BHA at lower concentrations (0-1.0 mg/ml) for SOD activity assay.

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)

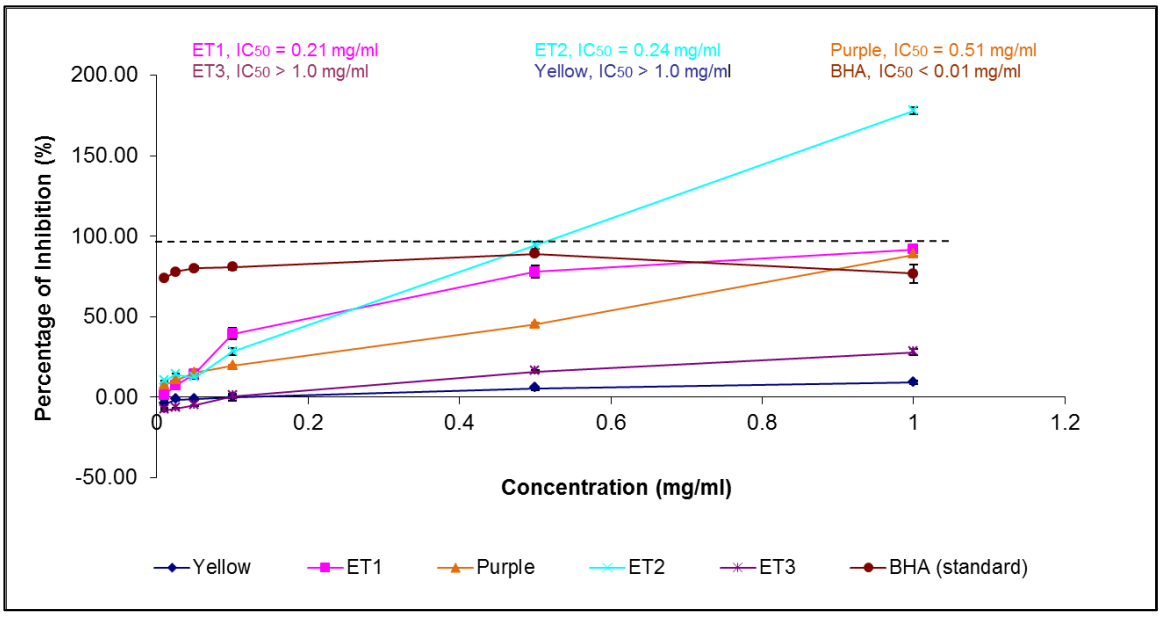


Figure 4.18: Comparison of SOD inhibition activity of isolated compounds from ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard BHA at various concentrations

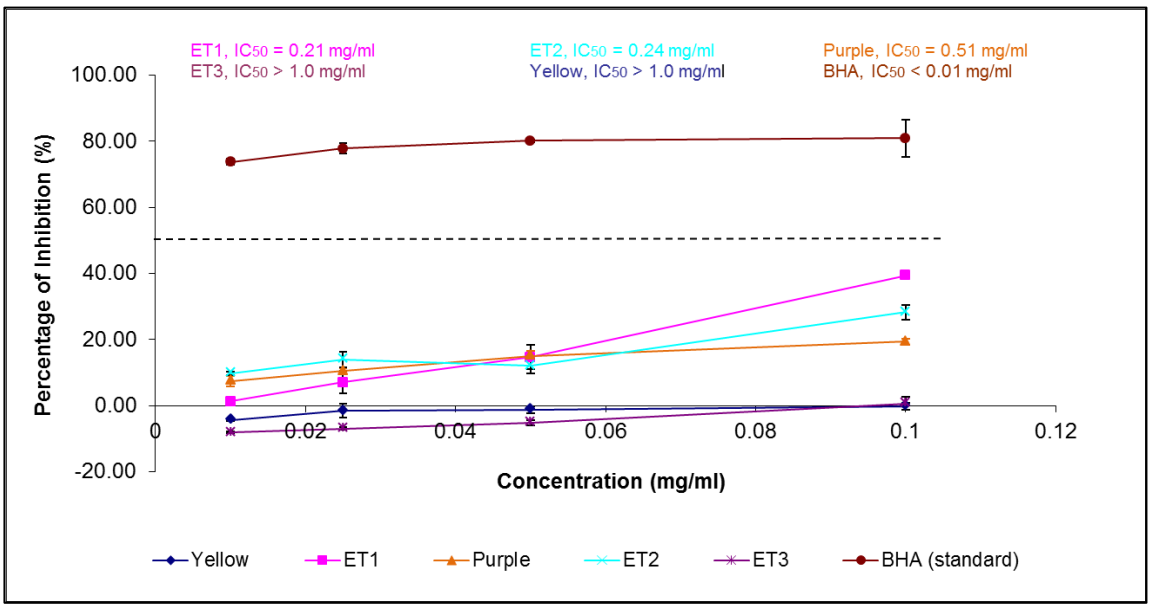


Figure 4.19: Magnification of activity at lower concentrations of SOD inhibition activity of isolated compounds from ethyl acetate fraction and juice extract of *Beta vulgaris L.* with standard BHA at various concentrations

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.

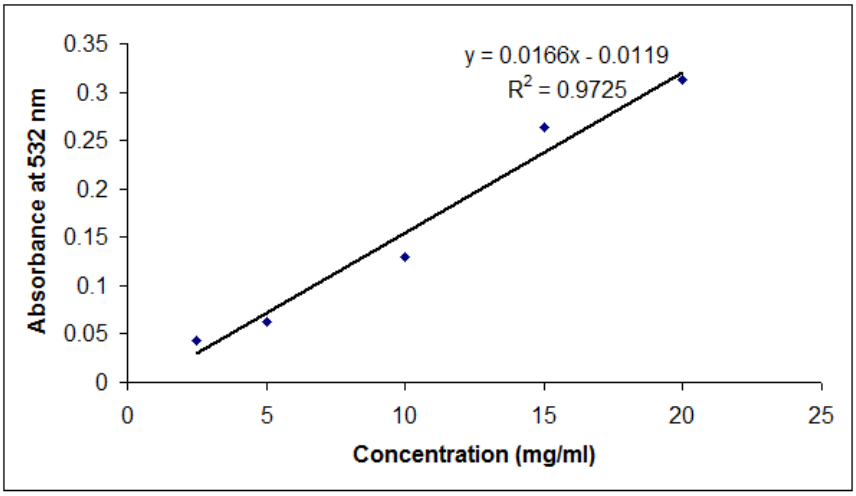


Figure 4.20: TEP (tetraethoxypropane) calibration graph

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)

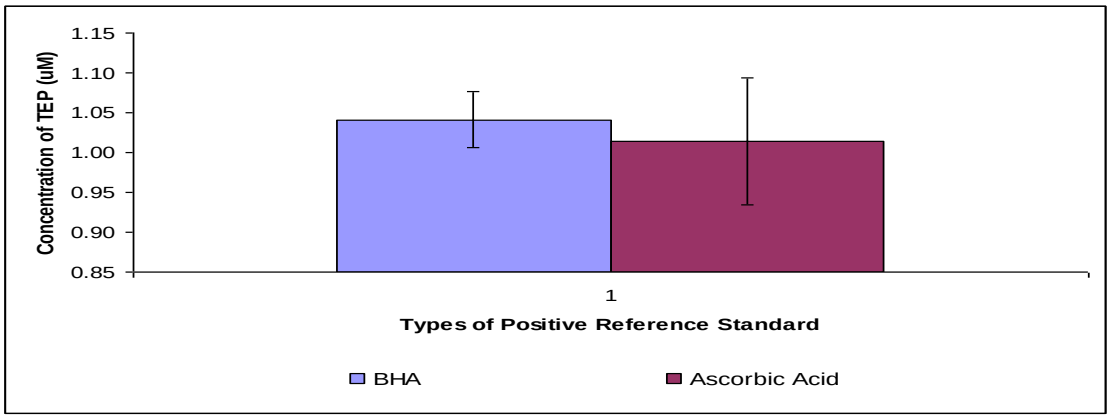


Figure 4.21: Lipid peroxidation capacity of positive standard

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)

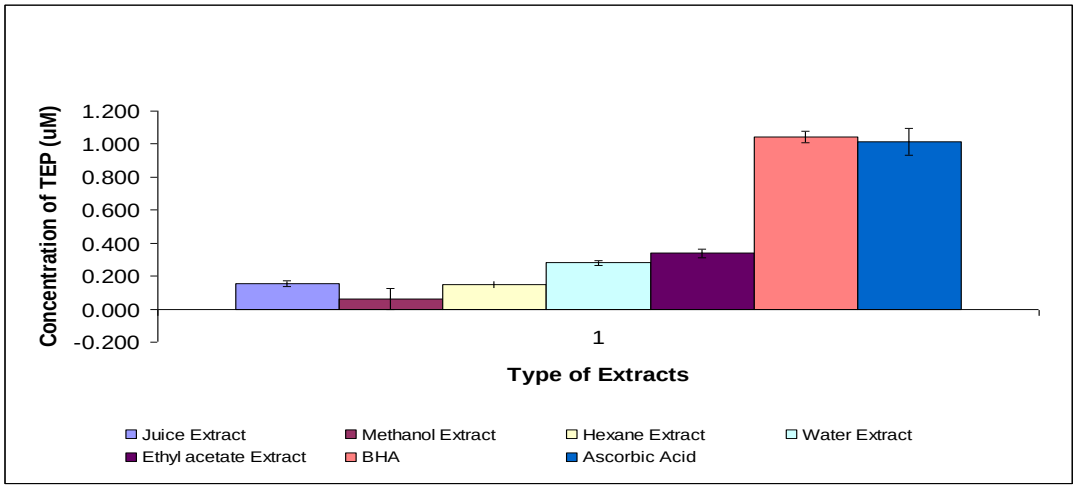


Figure 4.22: Comparison of inhibition of lipid peroxidation of extracts of *Beta vulgaris L.* with positive standards (BHA and ascorbic acid)

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

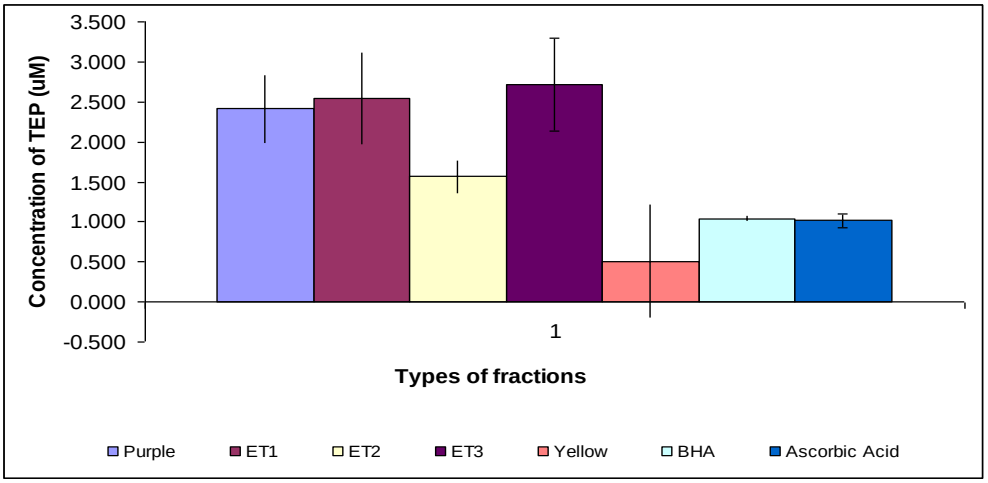


Figure 4.23: Comparison of inhibition of lipid peroxidation of sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* with positive standards

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)

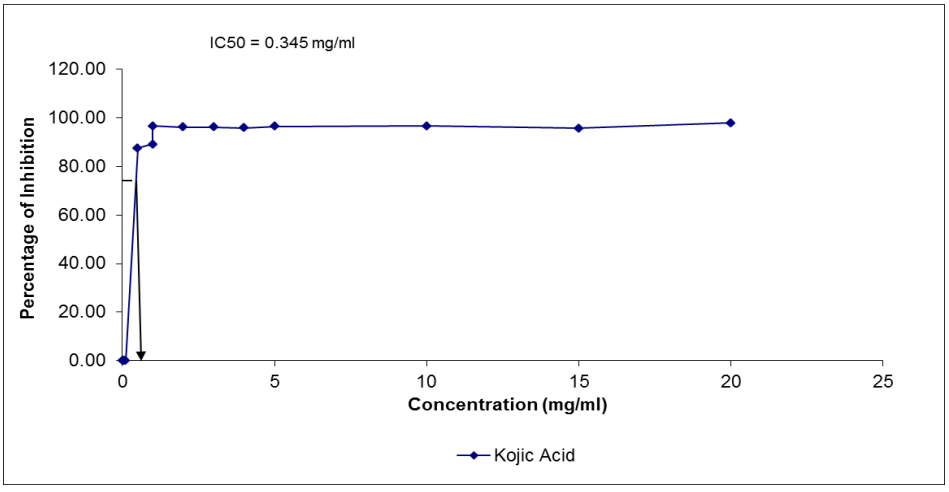


Figure 4.24: Tyrosinase inhibitory activity by using L-tyrosine as substrate and kojic acid (positive reference standard)

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	-	-	-	-	-

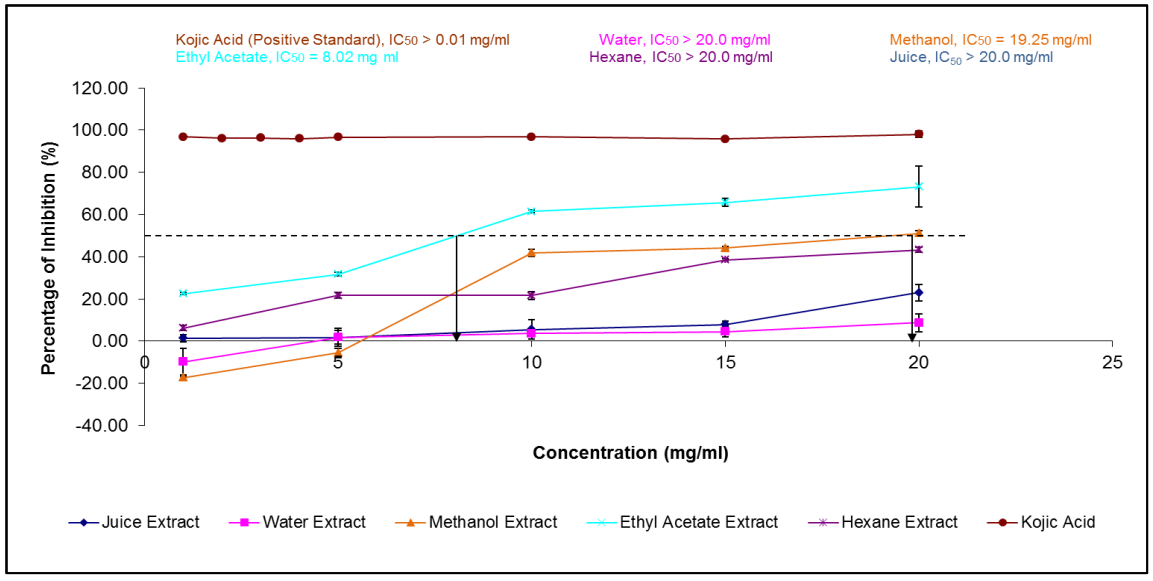


Figure 4.25: Comparison of tyrosinase inhibitory activity using L-tyrosine as substrate between extracts of *Beta vulgaris L.* and standard

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)

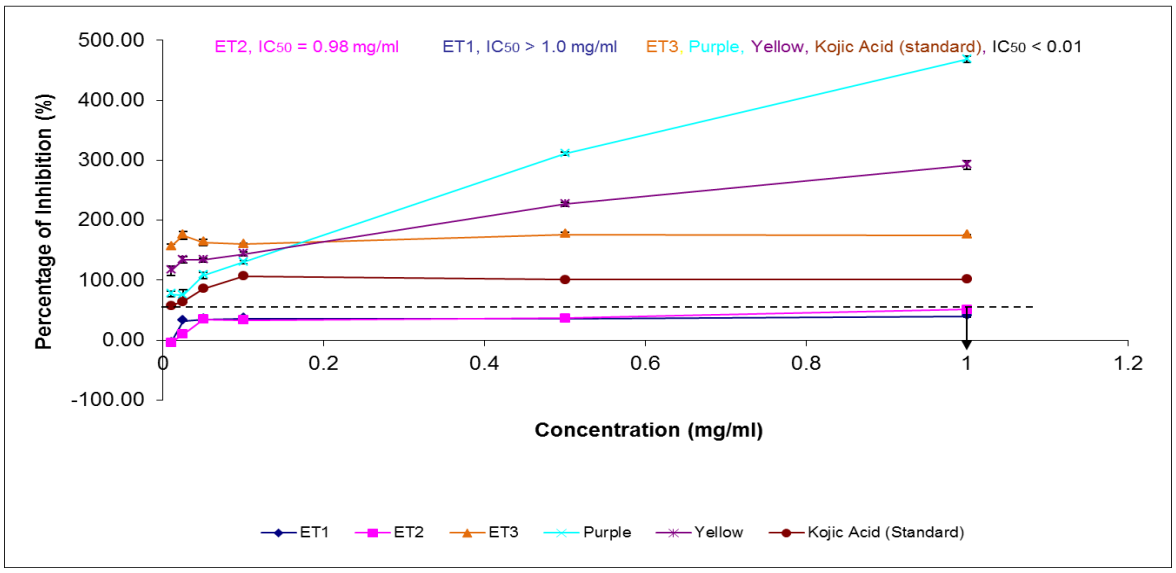


Figure 4.26: Comparison of tyrosinase inhibitory activity, between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-tyrosine as substrate.

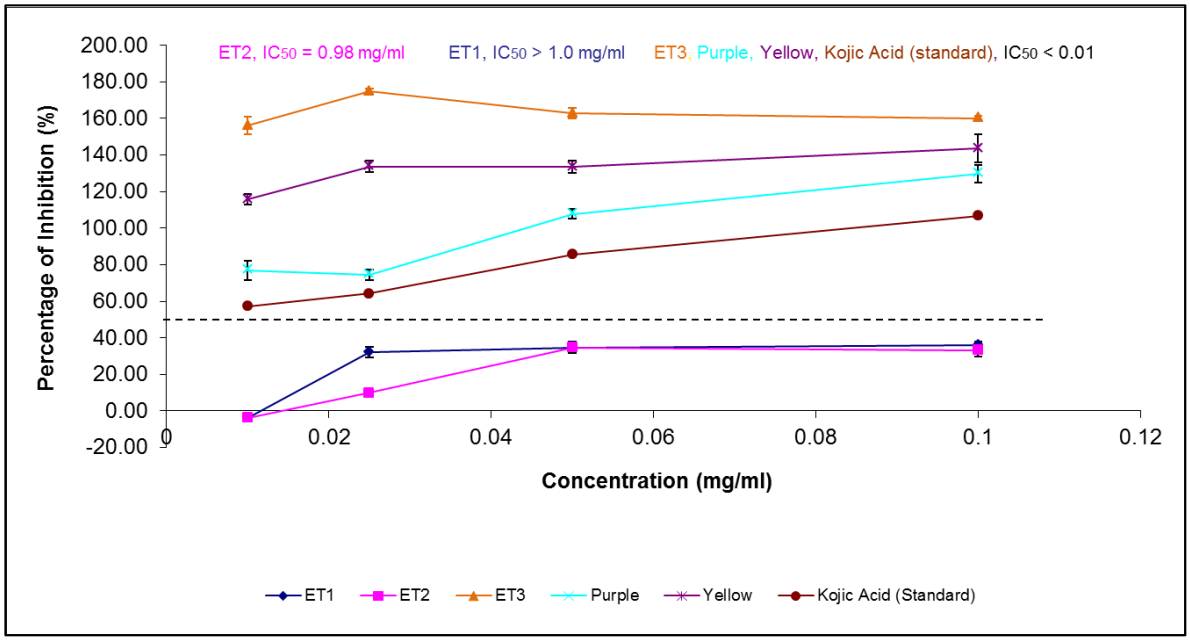


Figure 4.27: Magnification of activity at lower concentrations for of tyrosinase inhibitory activity, between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-tyrosine as substrate.

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)

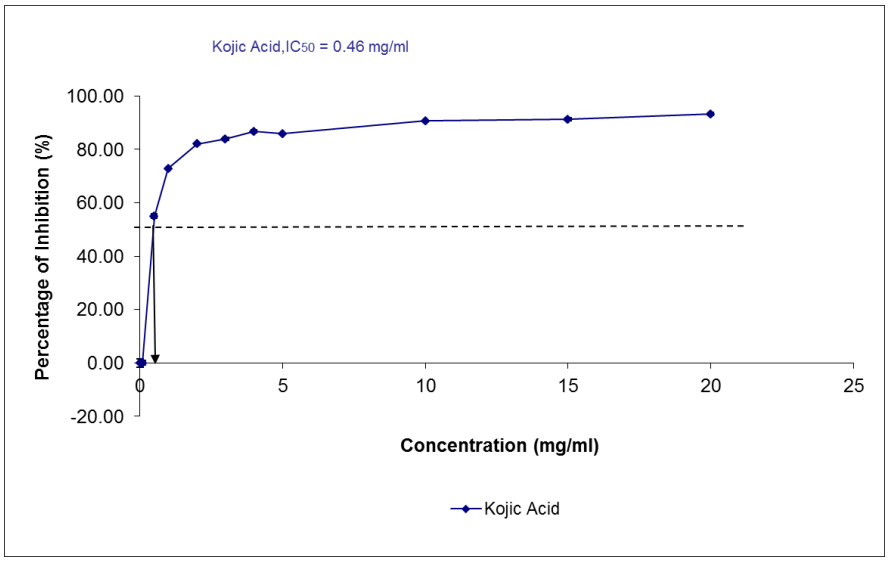


Figure 4.28: Tyrosinase inhibitory activity of kojic acid using L-Dopa as substrate

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)

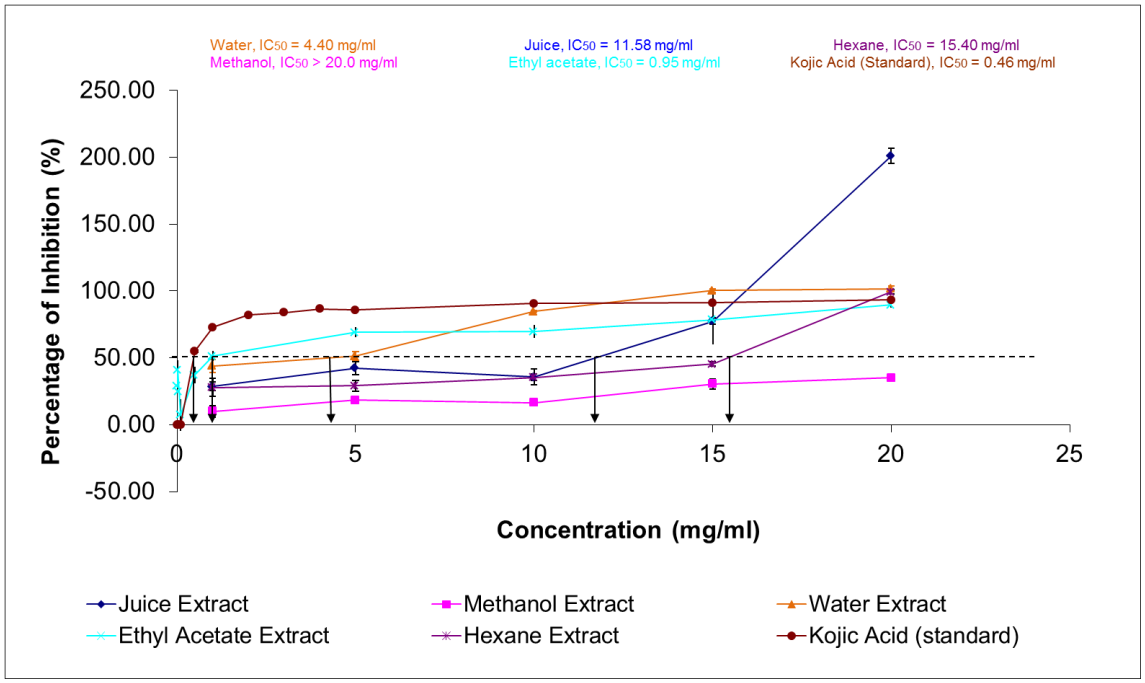


Figure 4.29: Comparison of tyrosinase inhibitory activity between extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate

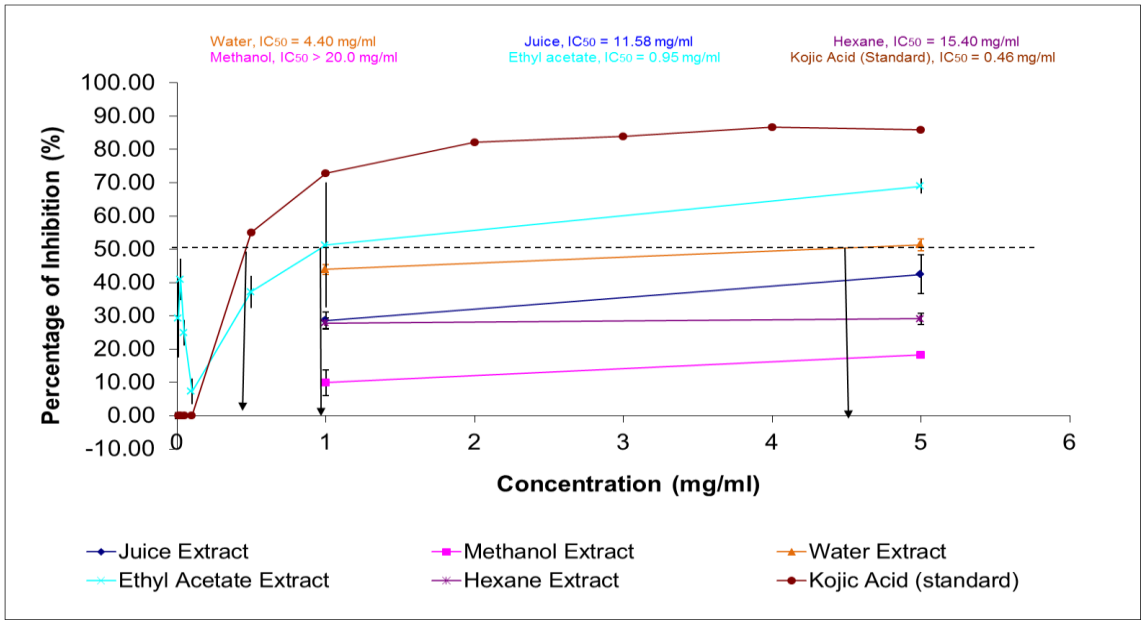


Figure 4.30: Magnification of readings at lower concentration for tyrosinase inhibitory activity between extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate

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)

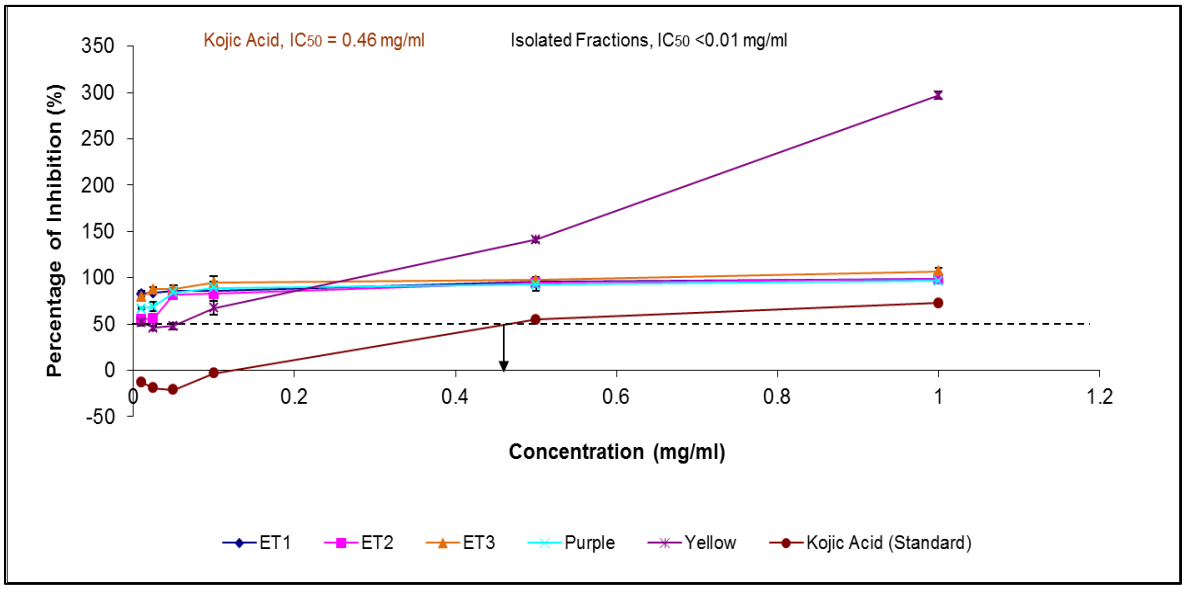


Figure 4.31: Comparison of tyrosinase inhibitory activity between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate.

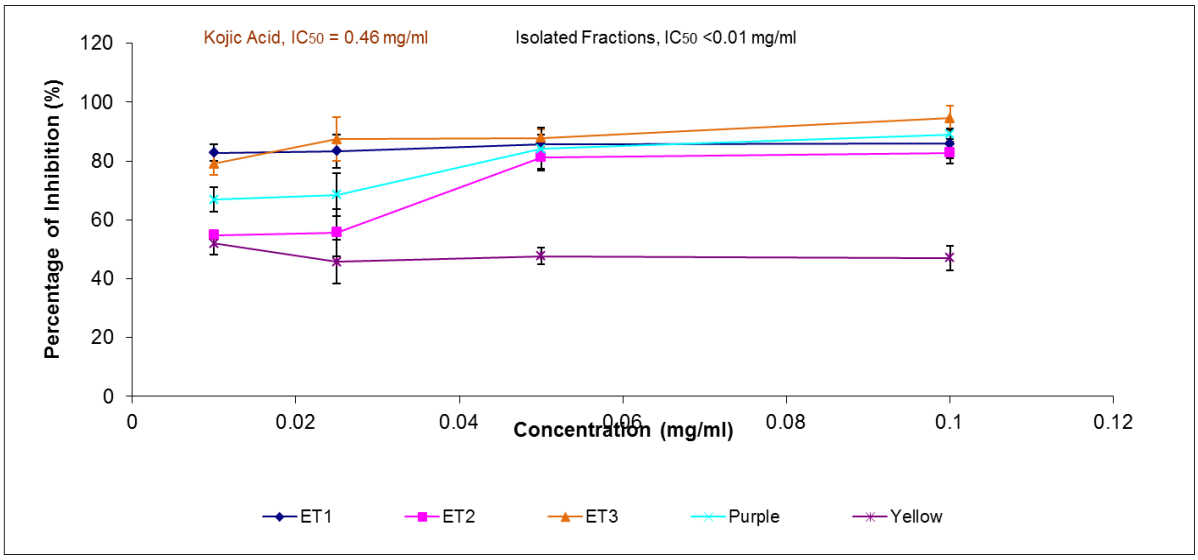


Figure 4.32: Magnification of lower concentration of tyrosinase inhibitory activity between sub-fractions of ethyl acetate and juice extracts of *Beta vulgaris L.* and standard using L-Dopa as substrate.

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.

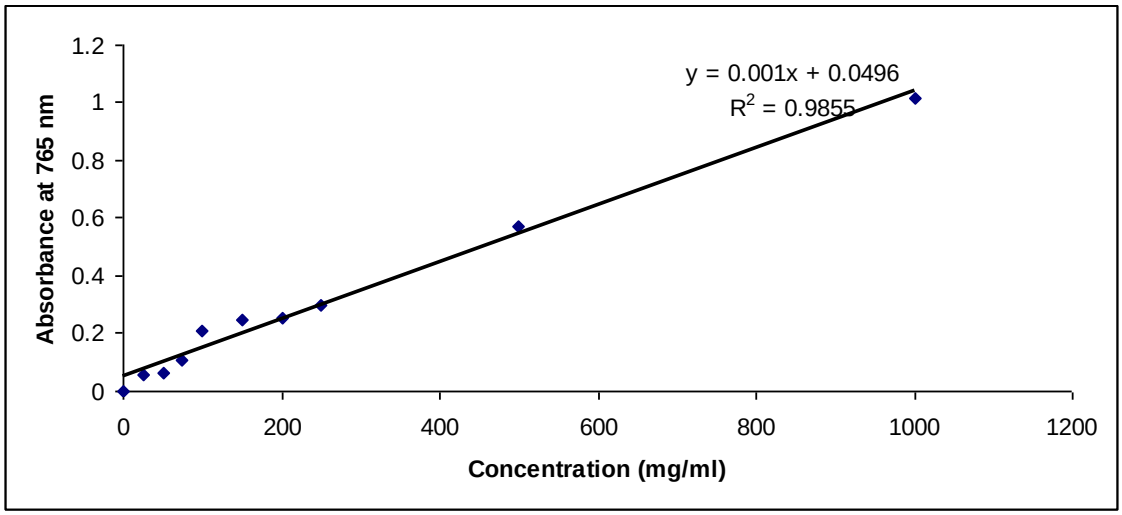


Figure 4.33: The gallic acid calibration graph

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)

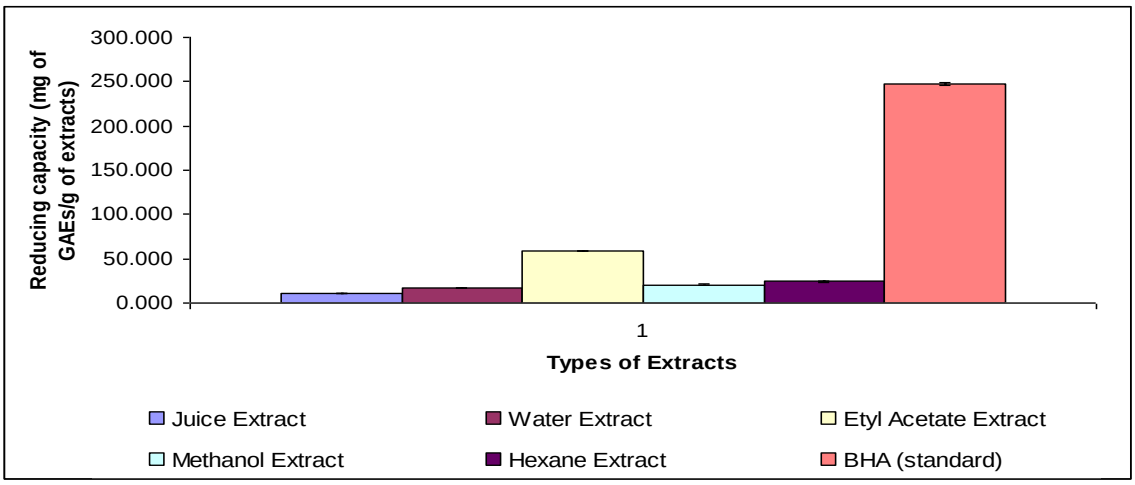


Figure 4.34: Comparison of reducing capacity between extracts of *Beta vulgaris L.* and standard on Folin-Ciocalteu assay

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	-	-	-	-	-

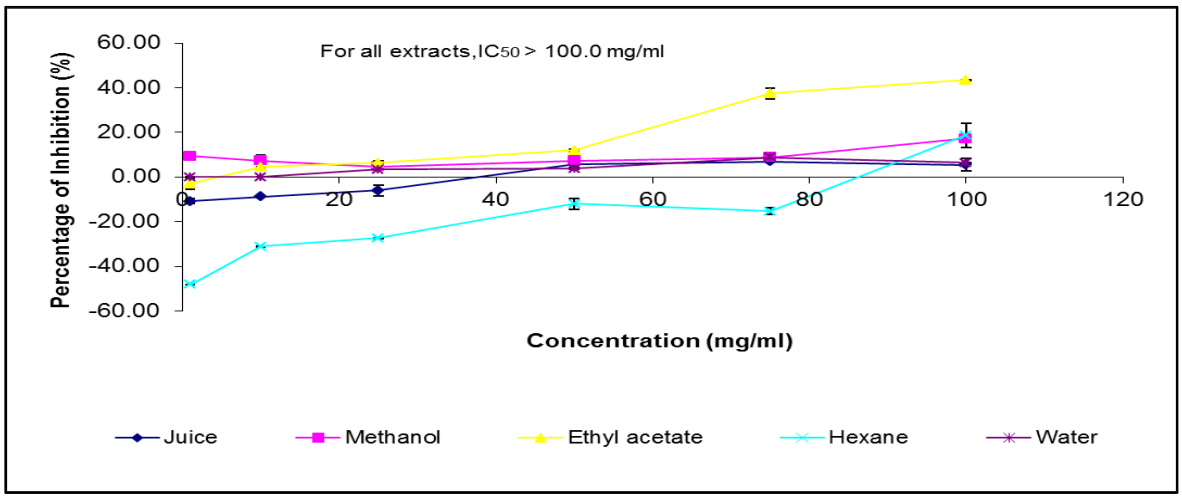


Figure 4.35: The *in vitro* growth inhibitions of CasKi cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

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	-	-	-	-	-

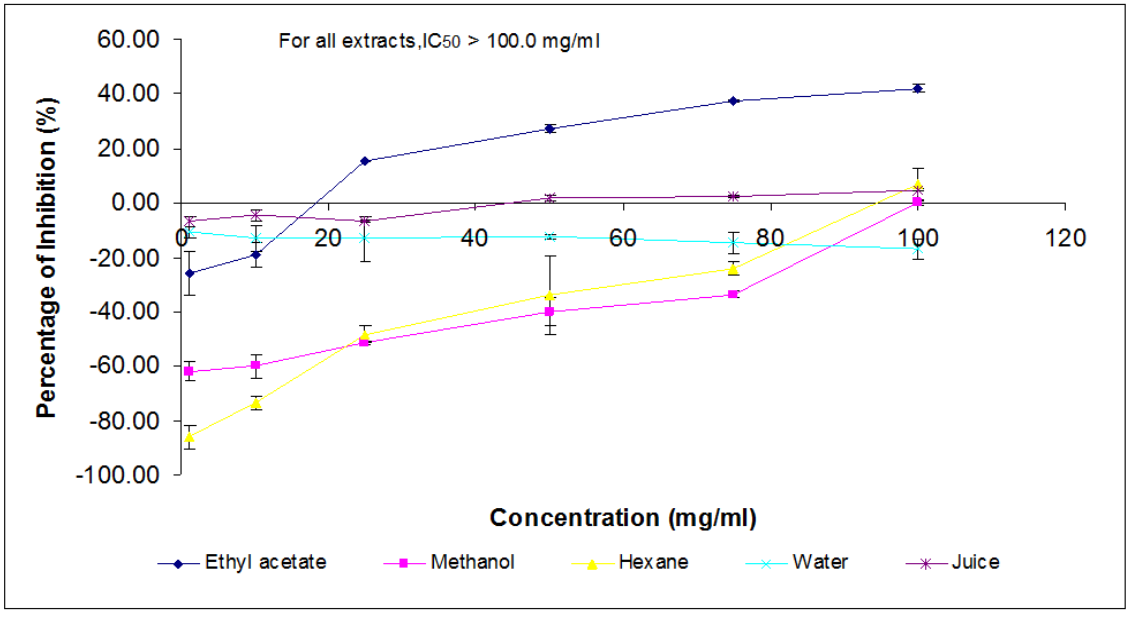


Figure 4.36: The *in vitro* growth inhibitions of A549 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

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	-	-	-	-	-

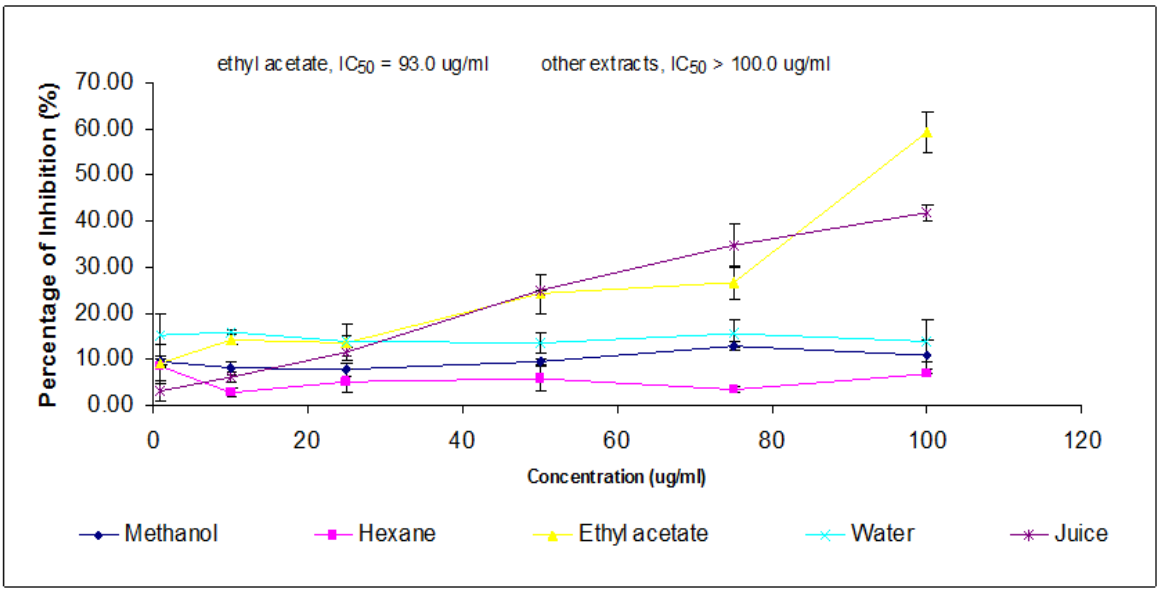


Figure 4.37: The *in vitro* growth inhibitions of MCF7 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

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	-	-	-	-	-

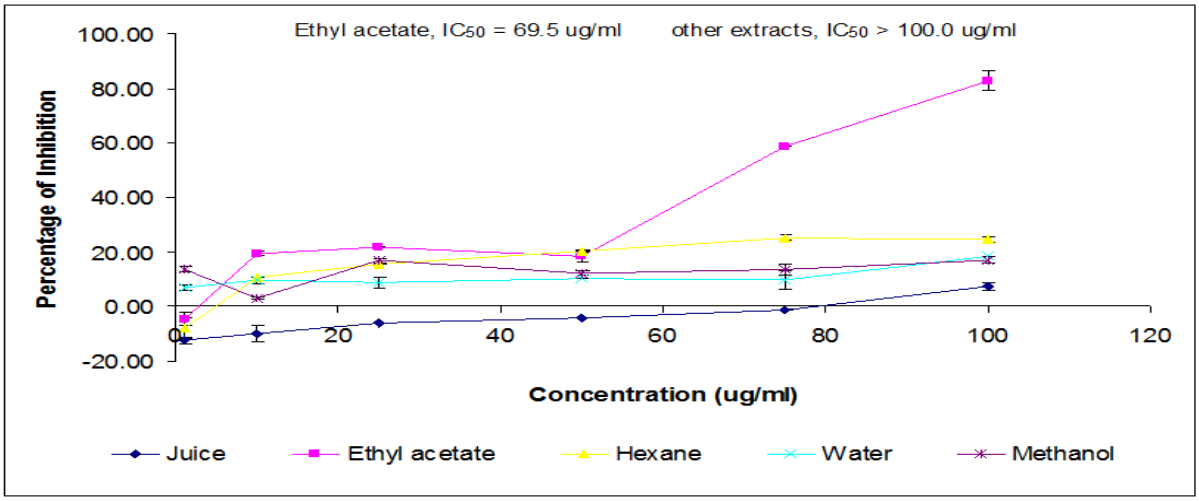


Figure 4.38: The *in vitro* growth inhibitions of HT29 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay

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	-	-	-	-	-

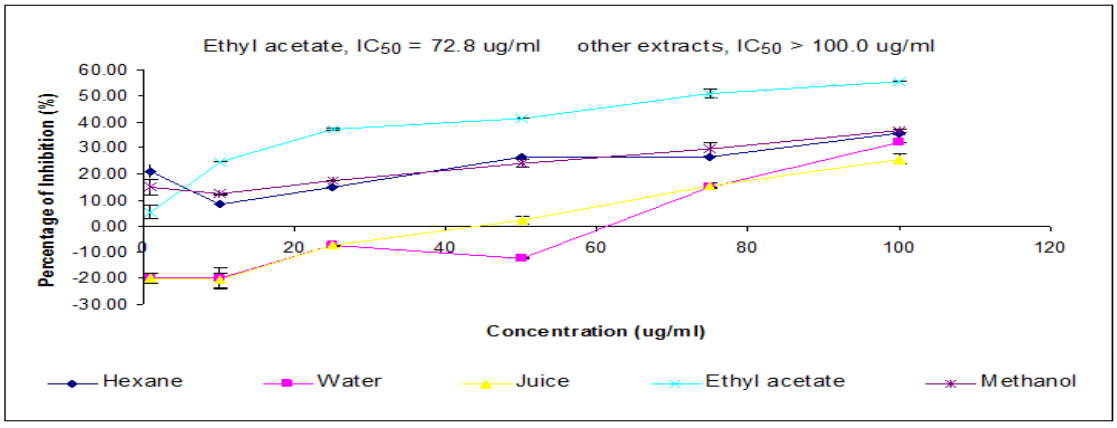


Figure 4.39: The *in vitro* growth inhibitions of HCT-116 cells by extracts of *Beta vulgaris L.* determined by using neutral red cytotoxicity assay.

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

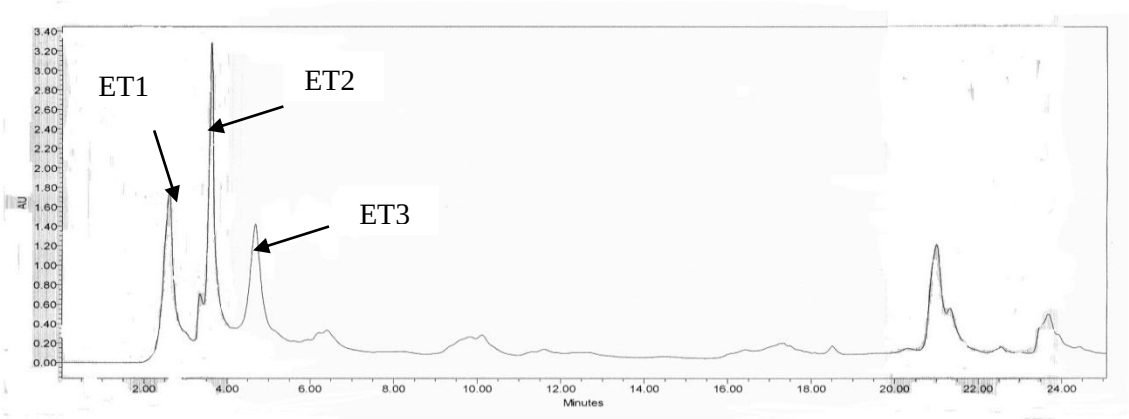


Figure 4.40: Chromatogram at wavelength 280nm of the HPLC separation of ethyl acetate fraction at certain conditions

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.

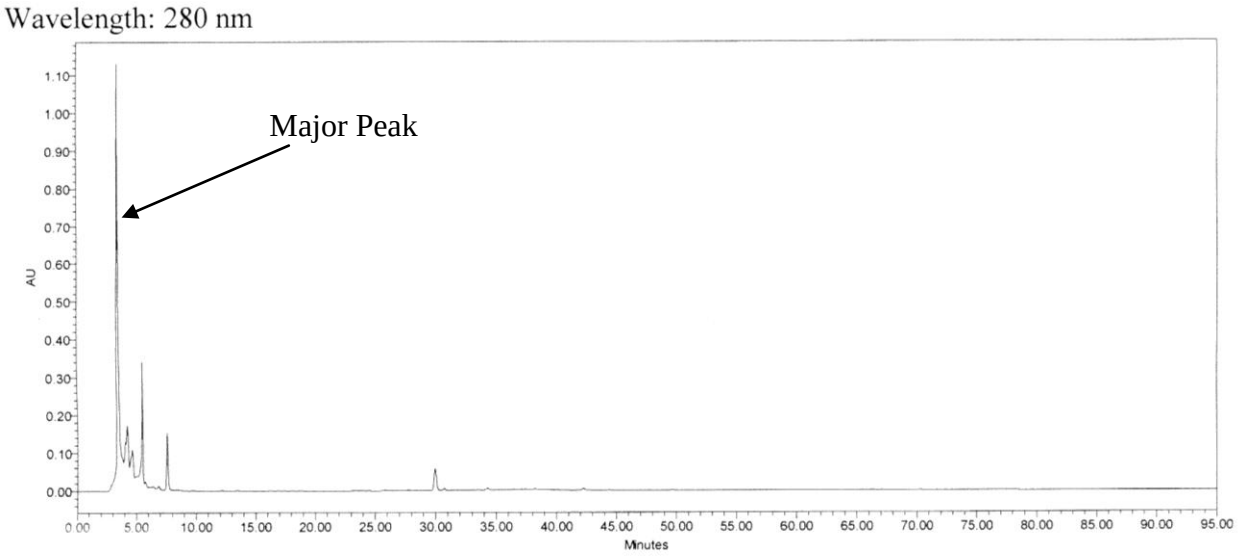


Figure 4.41: HPLC Chromatogram at wavelength 280 nm

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

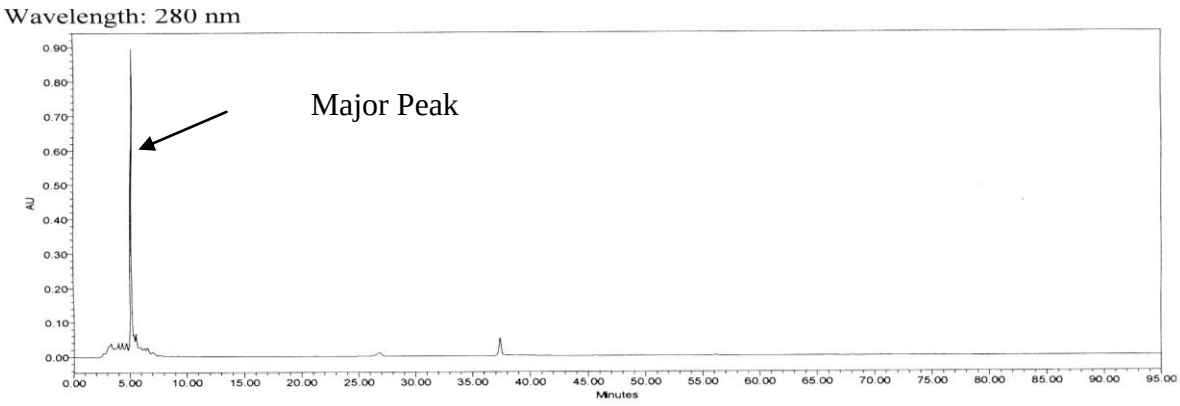


Figure 4.42: HPLC chromatogram at wavelength 280 nm

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

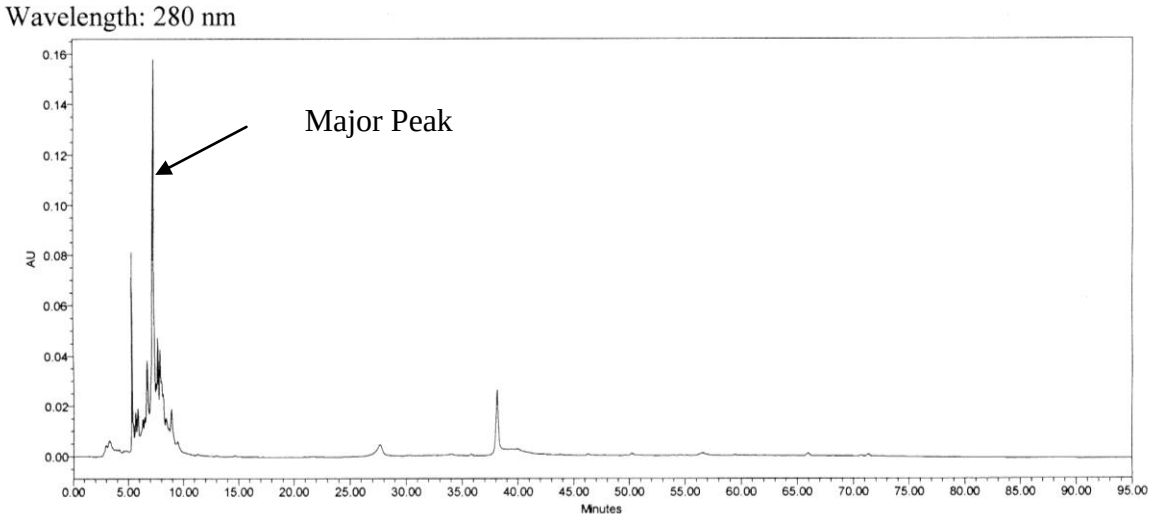


Figure 4.43: HPLC chromatogram at wavelength 280 nm

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

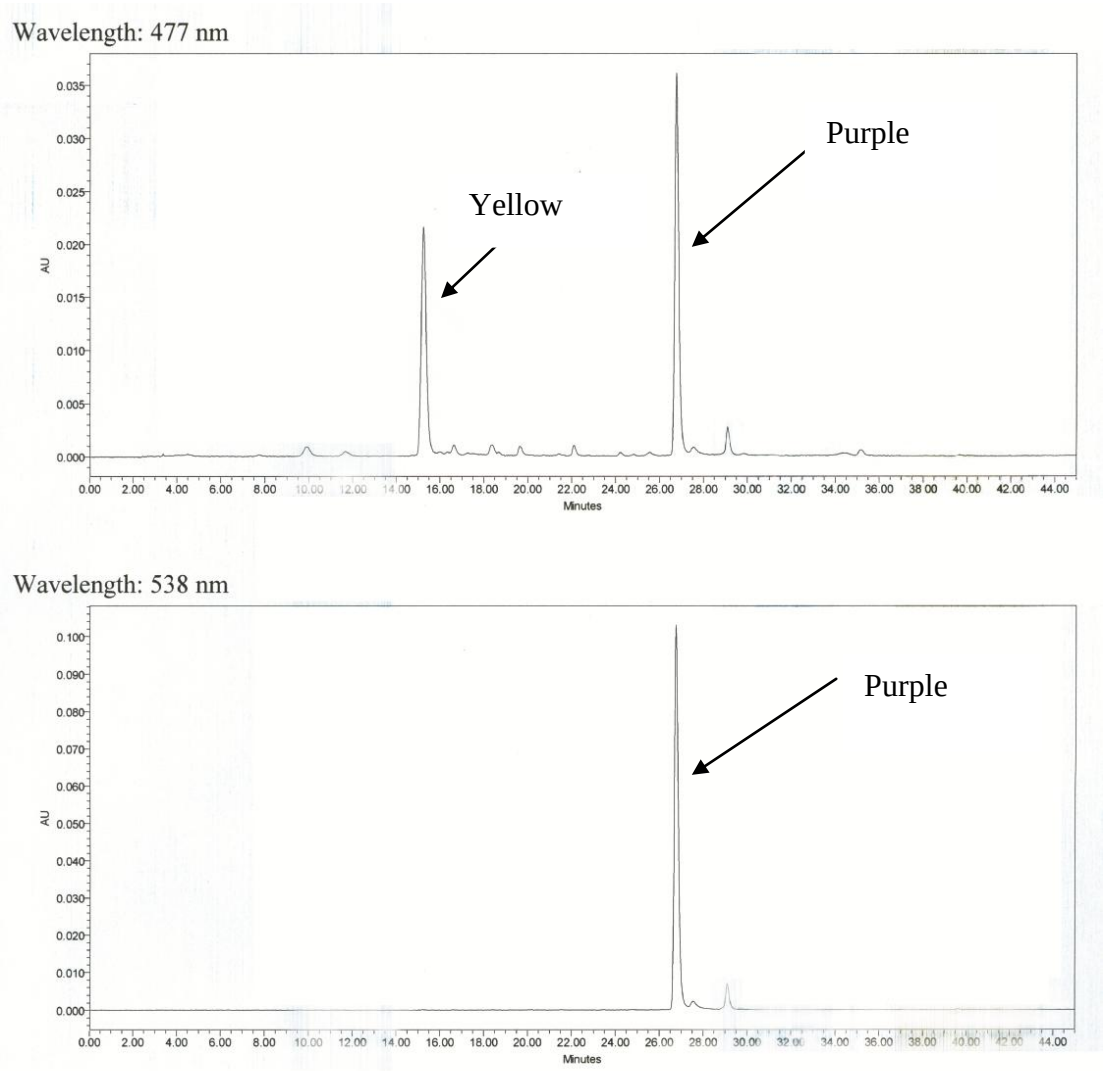


Figure 4.44: HPLC chromatogram at wavelength 477 nm and 538 nm

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.

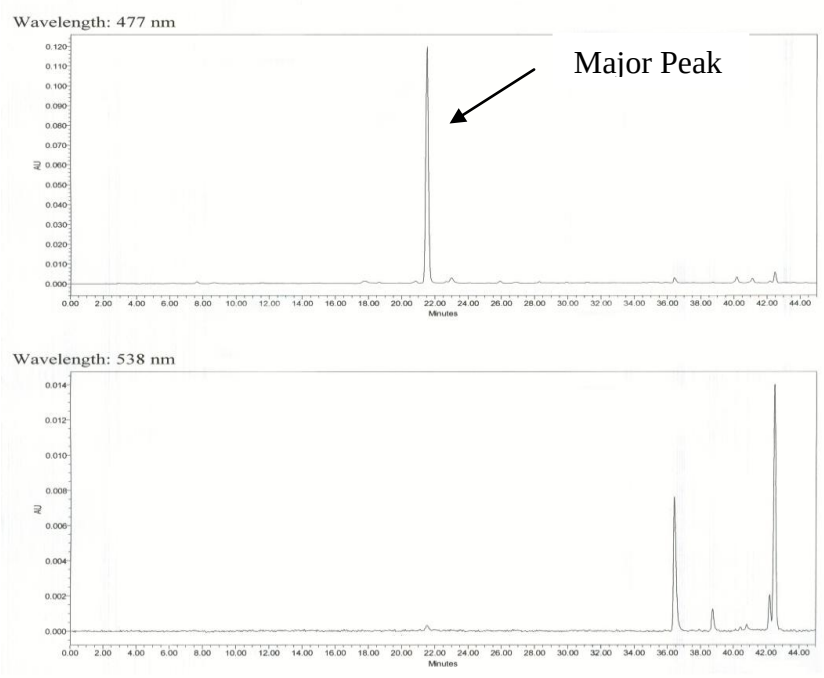


Figure 4.45: Chromatogram at wavelength 477nm and 538 nm of the HPLC separation of Yellow fraction at certain conditions

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.

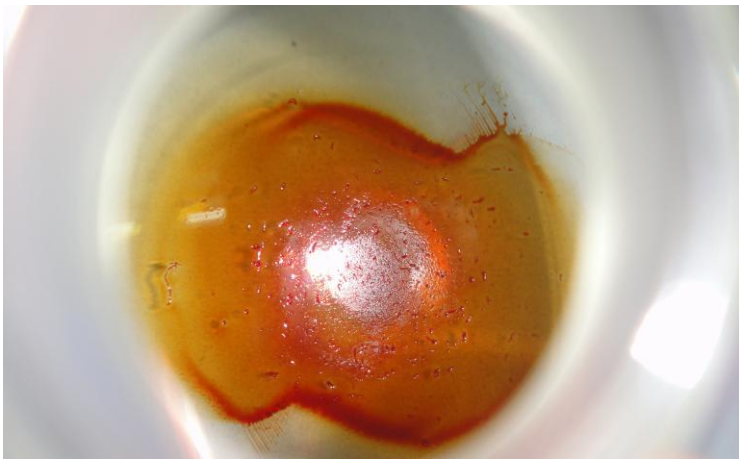


Figure 4.46: Image showing the morphology of the yellow fraction in the round bottomed flask

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.

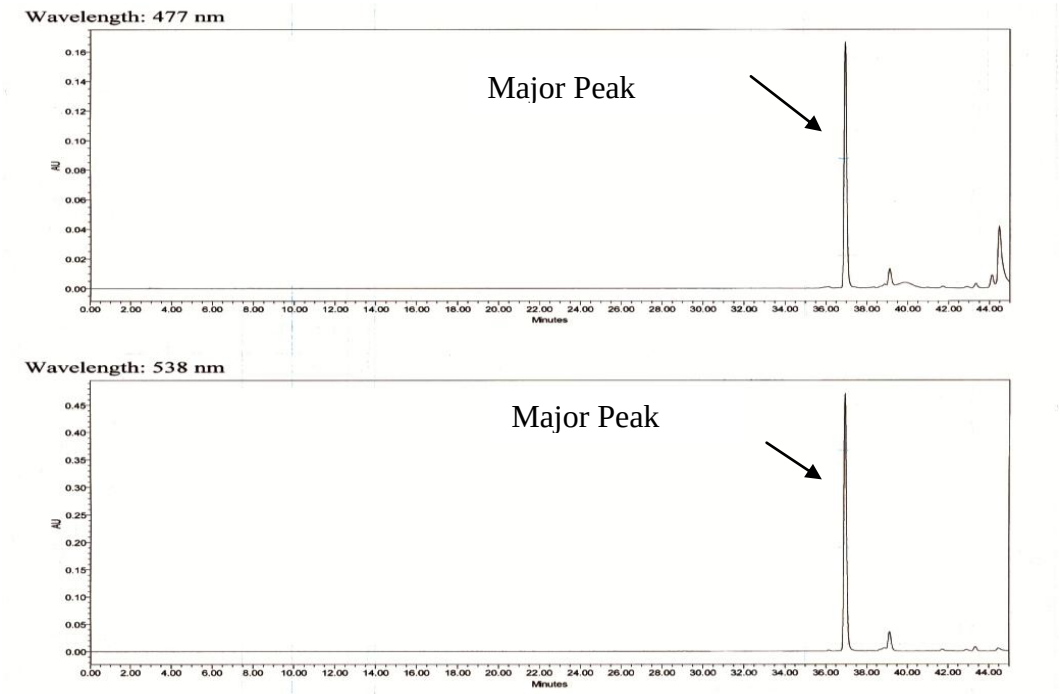


Figure 4.47: HPLC chromatogram at wavelength 477 nm and 538 nm

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.

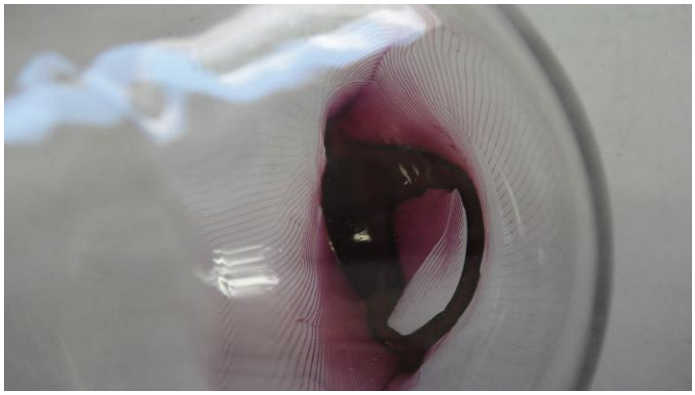
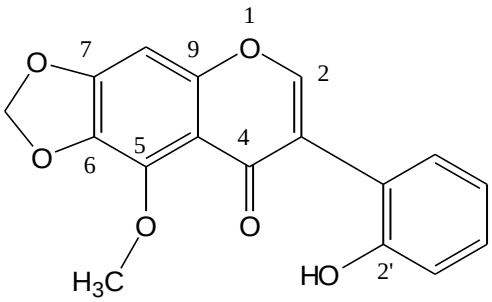


Figure 4.48: Image of Purple fraction in the round bottomed flask

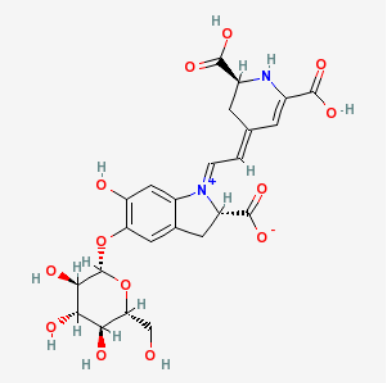
4.4.2 Identification of the sub-fractions via Liquid Chromatography-Mass Spectroscopy-Mass Spectrometry (LC-MS-MS)

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

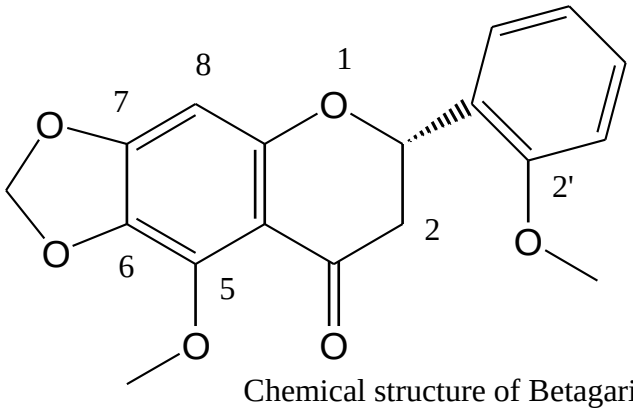


Chemical structure of betanin/isobetanin

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

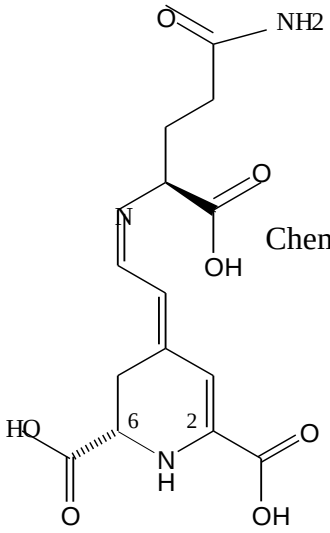
iii) ET3 Fraction

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).

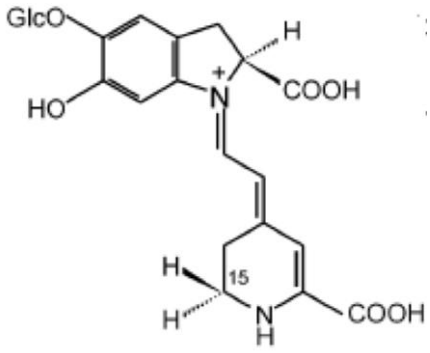


Chemical Structure of vulgaxanthine I

v) Purple Fraction

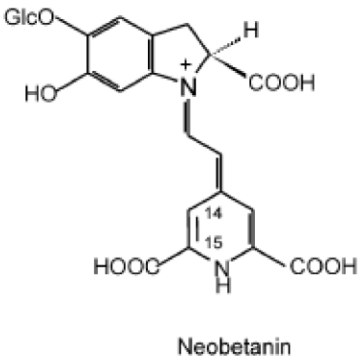
This fraction seems to have the most number of components as compared to the other components. It had 11 potential active components. The m/z values were 188, 315, 346, 507, 549 and 551. The peak at retention time 0.780 min corresponds to $[M+H]^+$ 551.1 has fragmentation pattern consistent with that 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