CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction yield of *P. oleracea*

Extraction of *P. oleracea* was conducted as described in Section 3. The result of extraction using methanol is shown in Table 4.1. The moisture content in the whole plant of *P. oleracea* was 95.01 %. The percentage yield of crude methanol extract was calculated based on the weight of dried and ground plant materials. The crude methanol extracts were further fractionated into hexane, ethyl acetate and water fractions. The percentage yield of fractionated extracts was based on the weight of crude methanol extract. The water fraction was the most abundant fraction (43.22 %) among the fractionated extract. Whilst, the ethyl acetate fraction gave the lowest yield which is 5.65 %. Table 4.2 shows the yield of extracts obtained after fractionation of crude methanol extract.

Table 4.1: Yield of methanol extracts of *P. oleracea*

Plant	Samples/ Extracts	Weight (g)	Percentage (%)
P. oleracea	Fresh samples	45000.00	-
	Dried and ground plant sample	2250.00	4.99
	Methanol extract	218.00	9.71

Table 4.2: Yield of extracts fractionated from *P. oleracea* methanol extract

Plant	Extracts (Fractions)	Yield of extracts (g) (extracted from 218 g of methanol extract)	Percentage (%)
P. oleracea	Hexane	54.42	24.96
	Ethyl acetate	12.31	5.65
	Water	94.22	43.22

The crude methanol extract and its fractions were subjected to treatment with activated charcoal to remove chlorophyll. Table 4.3 shows the yield of methanol extracts after treatment with activated charcoal. Whilst, Table 4.4 shows the yield of fractionated extracts after treatment with activated charcoal. The water fraction was the most abundant fraction (43.13 %) among the fractionated extracts. Meanwhile, the ethyl acetate fraction gave the lowest yield (5.75 %). After the treatment with activated charcoal, the yields of extracts markedly decreased indicating that some components were also adsorbed by the charcoal.

Table 4.3: Yield of methanol extracts of *P. oleracea* (treated with activated charcoal)

Plant	Samples/ Extracts	Weight (g)	Percentage (%)
P. oleracea	Fresh samples	10000.00	-
	Dried and ground plant sample	489.00	4.89
	Methanol extract	8.23	1.68

Table 4.4 : Yield of extracts fractionated from *P. oleracea* methanol extract (treated with of activated charcoal)

Plant	Extracts (Fractions)	Yield of extracts (g) (extracted from 8 g of methanol extract which treated with activated charcoal)	Percentage (%)
P. oleracea	Hexane	2.43	25.00
	Ethyl acetate	0.46	5.75
	Water	3.45	43.13

Flavonoids isolation and antioxidant investigation have been reported for methanol extracts of *P. oleracea* (Abas *et al*., 2006; Sakai *et al.,* 1996). Lim and Quah (2007) found that methanol can effectively extract all the polyphenols from *P. oleracea*. Thus, methanol is used as the extraction solvent in this study.

4.2 Antioxidant activity of *P. oleracea* extracts

Free radicals are chemical species that contained one or more unpaired electron in the outermost shell. Free radicals are highly reactive because they require an electron to attain the stable diamagnetic molecule. Oxidative stress occurs when there is an imbalance between productions of reactive oxygen species (exogenous sources or endogenous sources) against antioxidant protection mechanism (enzymatic and non-enzymatic) in an organism. The excess free radicals will attack DNA, proteins and lipid and lead to cell or tissue injury. Therefore antioxidant supplement from fruits and vegetables are strongly needed to reduce the oxidative damage caused by free radicals.

Antioxidant capacities of test samples can be assessed by various antioxidant assays such as DPPH radical scavenging, reducing power and β-carotene bleaching assays.

4.2.1 Scavenging activity of *P. oleracea* extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The DPPH radical scavenging assay was used to evaluate free radical scavenging ability of *P. oleracea* extracts because this method is simple, accurate, convenient, produce reliable result, inexpensive, required only small amount of sample and required only relatively short time compared to other methods (Soares *et al*., 1997). DPPH radicals are not affected by side chain, like enzyme inhibition and metal ion chelating. Besides, DPPH radical only recognizes free-radical scavenging effects and not pro-oxidant activity.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay is based on a single electron transfer reaction principle. DPPH radical scavenging assay provide the mechanism with the reaction taking place in an oxidizing system, such as auto-oxidation of lipid or other unsaturated substances. 1,1-Diphenylpicrylhydrazyl (DPPH) is a stable free radical which do not dimerise due to the virtue of the delocalization of the spare electron over the molecule as a whole. The free radical DPPH is deep violet in color and possesses a characteristic absorption at 520 nm. DPPH radical accepts an electron to become a stable diamagnetic molecule. The deep violet color of DPPH gradually changes to pale yellow (reduced form) when DPPH is exposed to free radical scavengers (substance that can donate electron on antioxidants). The decrease in absorbance at 520 nm induced by antioxidants determines the reduction capability on the DPPH radicals. The remaining DPPH radicals measured after a certain

time corresponds inversely to the radical scavenging activity of the antioxidant (Blois, 1958).

Extracts of *P. oleracea* contained active substances that were capable of scavenging DPPH radicals (Figure 4.2). In the DPPH assay, the scavenging activity of *P. oleracea* extracts were investigated through reaction with the stable DPPH radicals. Two synthetic antioxidants namely butylated hydroxyanisol (BHA) and ascorbic acid were used as positive controls. The scavenging activity of *P. oleracea* extracts and the positive reference standards on DPPH are shown in Figure 4.2 and Figure 4.1. The scavenging activities of extracts on DPPH increases with increase in concentration of extracts and were expressed as the efficient concentration (EC_{50}). EC_{50} is the amount of antioxidant needed to decrease the initial DPPH concentration by 50 %. Lower EC_{50} value indicates higher antioxidant activity. Table 4.5 shows the scavenging activity (EC_{50} values) of *P. oleracea* extracts and positive reference standards on DPPH radicals.

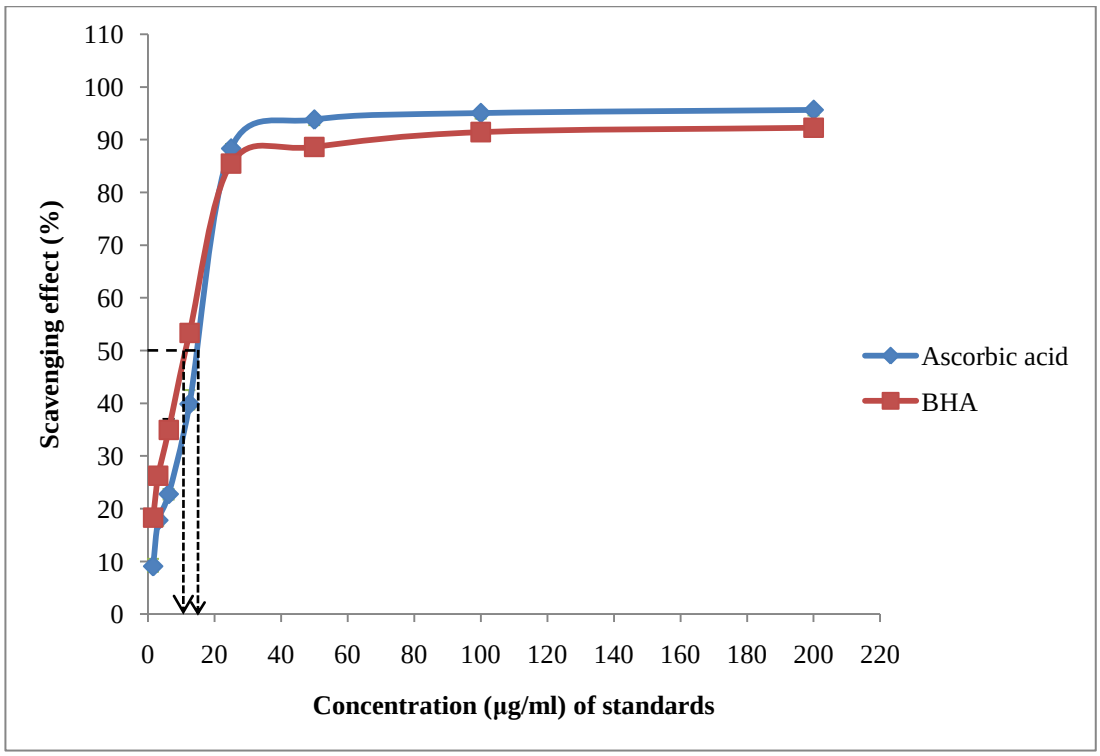


Figure 4.1: Scavenging effect of positive reference standards (BHA and ascorbic acid) on DPPH radicals. Each value is expressed as mean ± standard deviation of three measurements

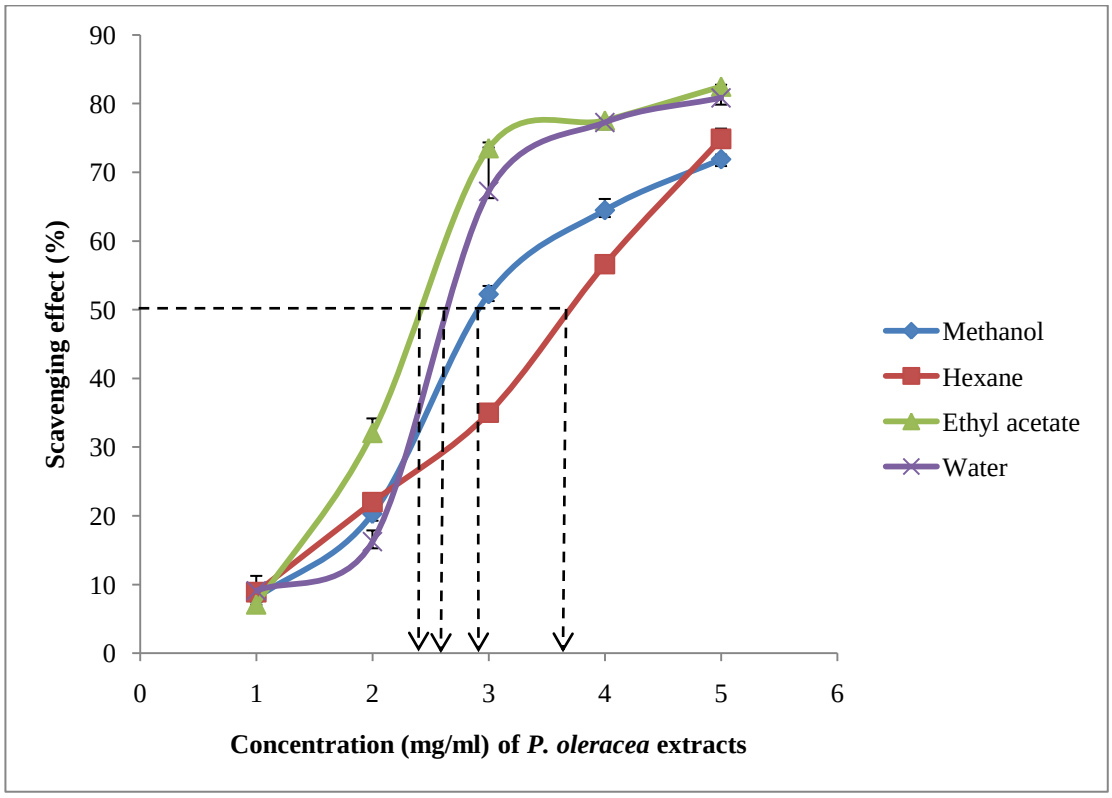


Figure 4.2: Scavenging effect of *P. oleracea* extracts on DPPH radicals. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.5: The scavenging activity (EC_{50} values) of *P. oleracea* extracts and positive reference standard on DPPH radicals

Plant/ Standard	Extract/ Fractions	EC_{50} (µg /ml)
P. oleracea	Methanol	2920 ± 0.06
	Hexane	3710 ± 0.01
	Ethyl acetate	2400 ± 0.05
	Water	2600 ± 0.01
Positive reference standard	Ascorbic acid	15.97 ± 0.56
	BHA	11.98 ± 0.01

Generally, in comparison to both positive reference standards, ascorbic acid (EC_{50} 15.97 ± 0.56 µg/ml) and BHA (EC_{50} 11.98 ± 0.01 µg/ml), all the extracts displayed weak radical scavenging activity. Among the extracts, the ethyl acetate fraction of *P. oleracea* exhibited the best DPPH scavenging capacity with the lowest EC_{50} value of 2.40 ± 0.05 mg/ml followed by water (EC_{50} 2.60 ± 0.01 mg/ml), methanol (EC_{50} 2.92 ± 0.06 mg/ml) and hexane (EC_{50} 3.71 ± 0.01 mg/ml). The highest antioxidant activity shown by the ethyl acetate fraction in the DPPH scavenging assay indicated that compounds with the strongest radical scavenging activity in *P. oleracea* are of medium polarity.

According to Sanja *et al*. (2009), Lim and Quah (2007) and Ercisli *et al*. (2008), the EC_{50} value of methanolic extract in DPPH assay was 12.67 ± 1.2 µg/ml, 3.41 ± 0.41 mg/ml and 54.33 ±1.26 µg/ml respectively. The difference in cultivar of the plants may produce different EC_{50} value. A comparative study among the different cultivars of *P. oleracea* has been done by Lim and Quah (2007). They found that the total phenolic content varies in different cultivars. The total phenolic content in a plant usually are well correlated with the antioxidant activities, thus different EC_{50} values were observed.

Besides that, the difference in plant growth stages also affects the antioxidant activity. The components in plant tissues change with maturity. The total phenolic content in the developing plant is slightly higher than the mature plant. Plants at developing stage are more metabolically active as they need higher concentration of essential compounds for growth. Meanwhile, the lower total phenolic content in mature plants could be due to oxidative stress as the plant is dying off (Witzell *et al*., 2003). Thus the difference in plant growth stages will produce different antioxidant activity. Furthermore, the growing environment of plant also affected the antioxidant activity of the plant. Sunlight, content of soil and moisture content affect the constituents in plants. Olivera *et al*. (2009) found that samples of *P. oleracea* collected in different locations of Bragança have different content of organic acid and moisture, fat and total phenolic content. The difference in plant constituents will surely produce different antioxidant activity.

The scavenging activity (EC_{50} values) of *P.oleracea* methanol extract obtained from the study was consistent with that reported by Lim and Quah (2007) whose samples were also obtained from Malaysia. The ability of the extracts to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (Rekka and Kourounakis, 1991).

4.2.2 Reducing power assay of *P. oleracea* extracts

The reducing power assay measures the ability of antioxidants to reduce the ferric 2, 4, 6-tripyridyl-s-triazine complex [Fe (III)-(TPTZ)2]$^{3+}$ to the intensely blue colored ferrous complex [Fe (II)-(TPTZ)2]$^{2+}$ (Benzie and Strain,1996) . The presence of reductants (i.e. antioxidants) in *P. oleracea* extracts causes reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form. The yellow colour of the test solution changes into various shades of green and blue. Thus, the concentration of Fe^{2+} can be monitored

by measuring the formation of Pearl's Prussian blue at 700 nm. The reducing power is indicated by the increase in absorbance value.

Two commercial antioxidants namely butylated hydroxyanisol (BHA) and ascorbic acid were used as positive reference standard in this assay. The reducing power of ascorbic acid, BHA and *P. oleracea* extracts are shown in Table 4.6. Whilst, Figure 4.3 shows a comparison of the reducing power of ascorbic acid, BHA and extracts of *P. oleracea* at various concentrations.

The reducing power of all the extracts increases with increase of the extracts concentration. The positive standards, ascorbic acid and BHA showed very high capacity of reducing the ferric complex to ferrous form. The highest absorbance for ascorbic acid and BHA were 3.154 and 3.456 respectively at the concentration of 20 mg/ml. Meanwhile, at the concentration of 5 mg/ml, the absorbance value for the ascorbic acid and BHA were 2.802 and 3.151 respectively. BHA showed higher reducing power as compared to ascorbic acid.

The ethyl acetate fraction showed the best reducing potential followed by the hexane fraction, methanol extract and water fraction. The ethyl acetate fraction showed the highest absorbance value ranging from 2.038 – 3.468 for the concentration of 5 mg/ml – 20 mg/ml in comparison to hexane fraction (1.724 - 2.255), methanol extract (0.997 - 2.339) and water fraction (0.630 - 1.712). At concentration ranging from 5 - 15 mg/ml, BHA showed the highest absorbance value among all the studied samples, but at the concentration of 20 mg/ml, the ethyl acetate fraction showed the highest reducing power. Thus, the ethyl acetate fraction has the highest reducing power among all the fractions / extract at concentration 20 mg/ml even better than shown by ascorbic acid and BHA. The ethyl acetate fraction showed stronger reducing power compared to the crude methanol extract was probably due to the partial purification process.

The reducing ability of a compound is generally dependent on the presence of reductones (Pin- Der-Duh, 1998). The reductones will exert antioxidant activity by breaking the free radical chain through donating an electron. Reductones will react with certain precursors of peroxide and prevent the formation of peroxide (Jayaprakasha *et al*., 2001). The antioxidant activity of *P. oleracea* extracts may be related to their reductive activity. The ethyl acetate fraction of *P. oleracea* showed the highest reducing ability of all other fractions tested indicated that compounds with strongest reducing power in reducing power assay were of medium polarity. Higher reducing power indicates that more reductones are present in the fraction which affects the reducing capacities. These compounds may act as reductones by donating electrons. The compounds will react with free radicals and convert them to a more stable products and thus eliminating the lipid peroxidation process. These compounds can act as primary and secondary antioxidants (Yen and Chen 1995).

Table 4.6: Reducing power of *P. oleracea* extracts and positive reference standards at various concentrations

Plant/ Standard	Extract/ Fractions	Concentrations of extracts (mg/ml)			
		5	**10**	**15**	**20**
P. oleracea	Methanol	0.997 ±0.02	1.839 ±0.01	2.052 ±0.01	2.339 ±0.01
	Hexane	1.724 ±0.01	1.891 ±0.01	2.022 ±0.01	2.255 ±0.01
	Ethyl acetate	2.038 ±0.01	2.470 ±0.03	2.925 ±0.01	3.468 ±0.02
	Water	0.630 ±0.01	1.248 ±0.01	1.446 ±0.02	1.712 ±0.01
Positive reference	Ascorbic acid	2.802 ±0.01	2.853 ±0.16	2.933 ±0.03	3.154 ±0.05
standard	BHA	3.151 ±0.01	3.160 ±0.01	3.280 ±0.02	3.456 ±0.01

Absorbance values expressed are means ±standard deviation of triplicate measurements.

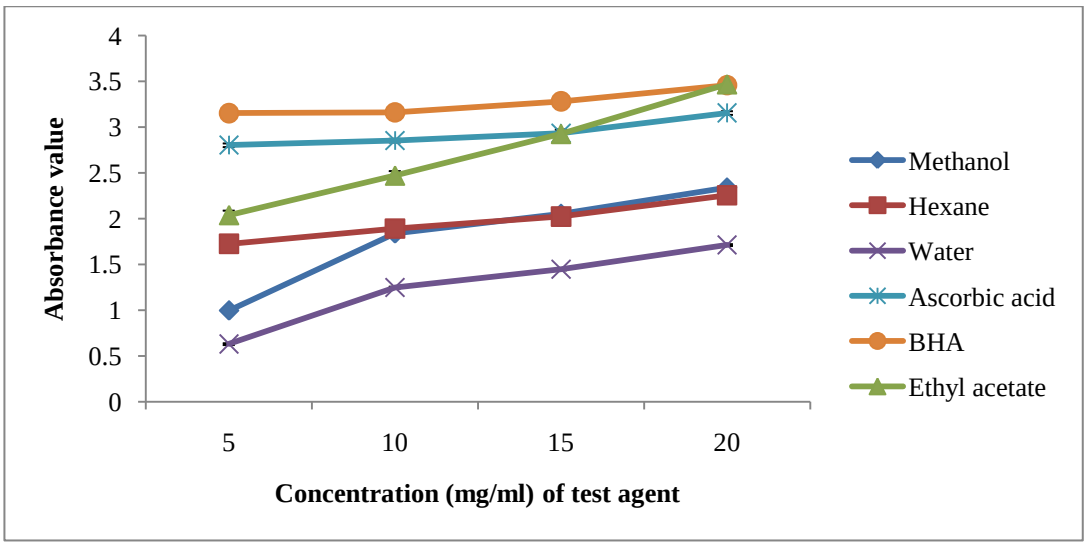


Figure 4.3 : Comparison of reducing power of ascorbic acid, BHA and extracts of *P. oleracea* at various concentrations. Each value is expressed as mean ± standard deviation

4.2.3 β-carotene bleaching activity of *P. oleracea* extracts

β-Carotene bleaching assay is based on hydrogen atom transfer (HAT) reaction. HAT reaction normally occurs between antioxidants and peroxyl radicals. The mechanism of bleaching of β -carotene is a free-radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid (Jayaprakasha *et al*., 2001). β-Carotene is an orange coloured compound. In this model system, the absence of an antioxidant will make β-carotene undergoes rapid discoloration. The linoleic acid radicals are formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules (refer to Figure 2.1, in Section 2.2.3 (iii)). Thus, β-carotene will undergoes oxidation; β-carotene molecule losses its chromophore and orange colour characteristic. This can be monitored spectrophotometrically. Antioxidants are able to slow down the rate of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals that

are formed in the system. After the two hours incubation period (120 minutes), the non-availability of linoleic acid will stop the formation of peroxides. This fact is used to determine the antioxidant activity of the *P. oleracea* extracts in comparison to the synthetic antioxidant, BHA.

Table 4.7 shows the antioxidant activities of the *P. oleracea* extracts (methanol, hexane, ethyl acetate and water) and positive reference standard with the coupled oxidation of β-carotene. Figure 4.4 shows the antioxidant activity (%) of *P. oleracea* extracts and BHA measured by the β-carotene bleaching method. Each value is expressed as mean ± standard deviation.

BHA showed the highest antioxidant activity as compared to the *P. oleracea* extracts. BHA exhibited antioxidant activity in a concentration- dependent manner. There is a decrease in absorbance reading of β-carotene in the presence of BHA and plant extracts. This is due to the ability of extracts to compete with β-carotene to react with the linoleate radicals. The antioxidant activity of *P. oleracea* extracts gradually increases with increase of the extracts concentration. The antioxidant activity of all the extract increases with increase of extracts concentration. The water fraction showed the highest β-carotene bleaching activity followed by the ethyl acetate fraction, crude methanol extract and finally the hexane fraction. The water fraction exhibited the highest antioxidant activity, which is 74.87 % at a concentration 20 mg/ml. Whilst, at the concentration of 4 mg/ml, the antioxidant activity for the water fraction was only 64.19 %. For the ethyl acetate fraction, the antioxidant activity ranges from 54.47 % - 68.92 % for concentration of extract ranging from 4 - 20 mg/ml. For the methanol and hexane fractions, the antioxidant activity ranges from 12.14 – 29.46 % and 5.85 – 26.05 % respectively for concentration of extract ranging from 4 – 20 mg/ml. The highest antioxidant activity among the fractions was observed in the water extract while the

hexane fraction showed the lowest antioxidant activity. This indicated that the antioxidative components were very polar and water soluble in nature.

The water-soluble antioxidant compounds of *P. oleracea* showed better antioxidant activity in the β-carotene bleaching assay in comparison to the compounds present in methanol, ethyl acetate and hexane fraction.

Some previous studies indicated that polar compounds did not show any antioxidant activity in the β-carotene bleaching assay. This is due to the 'polar paradox' phenomenon (Frankel *et al*., 1994; Koleva *et al*., 2002; Porter, 1993), based on the assumption that polar antioxidant remaining in the aqueous phase of the emulsion, do not protect the linoleic acid very well because linoleic acid is soluble in lipid phase. However, in this study, it was found that inhibition of β-carotene bleaching by water fraction was slightly higher than the ethyl acetate fraction but much higher than the other fractions. According to Koleva *et al* (2000), a complex composition of the extract could provoke certain interactions (synergistic, additive or antagonistic effects) between their components and or within the medium. Probably this is the reason why the same fractions may vary remarkably in their antioxidant activity.

Table 4.7: Antioxidant activity (%) of *P. oleracea* extract / fractions and positive reference standards at various concentrations by β-carotene bleaching assay

Plant/ Standard	Extract / Fractions	Concentrations of extracts (mg/ml)			
		4	**8**	**16**	**20**
P. oleracea	Methanol	12.14 ±1.19	15.48 ±0.77	20.18 ±0.60	29.46 ±0.58
	Hexane	5.85 ±0.75	10.31 ±0.57	14.39 ±0.82	26.05 ±0.92
	Ethyl acetate	54.47 ± 0.56	59.89 ±0.87	62.62 ±1.25	68.92 ±0.74
	Water	64.19 ±0.74	65.02 ±0.61	68.31±0.28	74.87 ±0.68
Positive reference standard	BHA	70.44 ±0.69	79.51 ±0.67	88.70 ±0.94	92.62 ±0.40

Antioxidant activity (%) expressed are means ±standard deviation of triplicate measurements

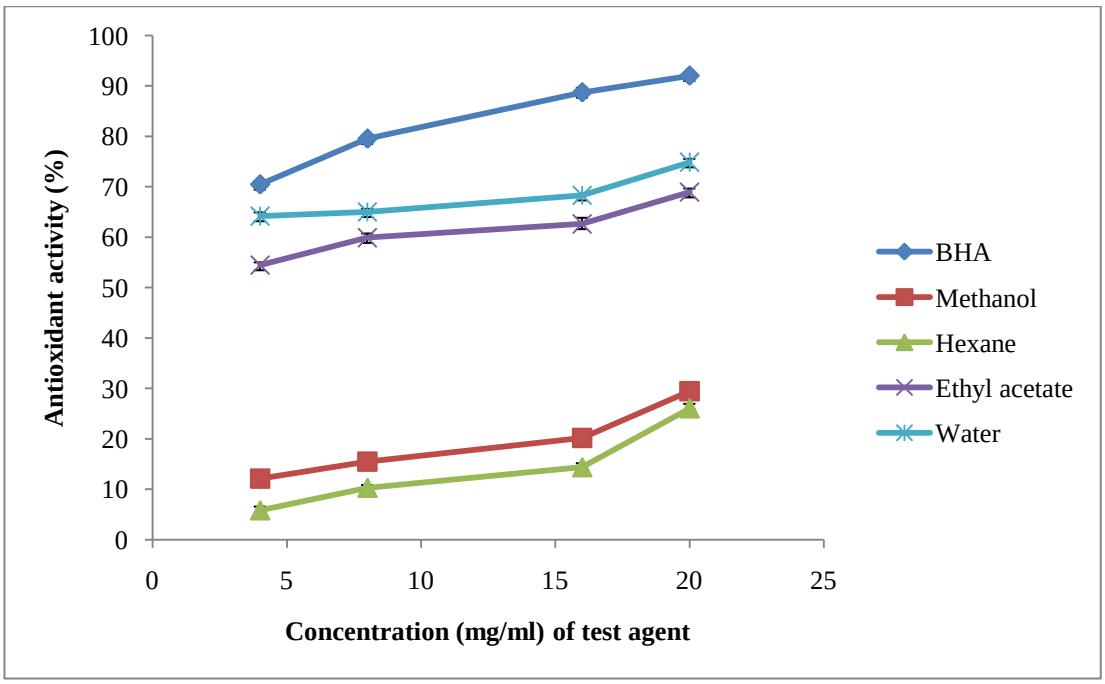


Figure 4.4: Antioxidant activity (%) of *P. oleracea* extracts and BHA measured by β-carotene bleaching method. Each value is expressed as mean ± standard deviation.

4.2.4 Comparison of antioxidant activity of *P. oleracea* extracts

The most commonly used methods for evaluating the antioxidant activity are those that involve the generation of radical species, where the disappearance of radicals determines the presence of antioxidants (Cao *et al*., 1993).

Owing to the complexity of the oxidation–antioxidation processes, different antioxidant assay can yield widely diverging results; thus Frankel and Meyer (2000) and Huang *et al*. (2005) stressed that a single antioxidant assay is not adequate for evaluating the antioxidant capacity of foods. There is no single antioxidant assay capable of providing a comprehensive picture of the antioxidant profile of a studied sample (Singh *et al*., 2007). Therefore, various methods based on different mechanisms must be used in the assessment of antioxidant activity. Rapid, sensitive, and reproducible methods, preferably requiring small sample amounts are some of the

criteria that should considered when an antioxidant activity screening is designed (Singh *et al.*, 2007).

The concentration of antioxidants, extraction medium, temperature during extraction, pH of the medium (Gazzani *et al*., 1998), chemical structures and position in the molecule (Prior *et al*., 2005) will also influence the antioxidant activity.

In this study, the ethyl acetate fraction of *P. oleracea* showed the highest antioxidant activity using DPPH radical scavenging assay. However, it was the water fraction which showed the strongest antioxidant activity in the β-carotene bleaching assay. This discrepancy is not uncommon. For example, Velioglu *et al*., (1998) reported that the lipophilic extract of coriander showed strong activity in the 15-LO assay (a method to test the activity of antioxidant against linoleic acid peroxidation), however, in the DPPH radical scavenging assay, this extract showed low antioxidant activity. Velioglu *et al*., (1998) believed that this may due to the involvement of proton donation by the active compounds may be of less importance for 15-LO inhibition than for DPPH radical scavenging. Various method used to evaluate antioxidant activity with different mechanisms will lead to different results. Kahkonen *et al.* (2001) found that in the lower density lipoproteins (LDL) oxidation method, the antioxidant activity of berry extract is mainly related to anthocyanins, but in the liposome oxidation assay, the antioxidant activity is mainly related to hydroxycinnamates. Thus, it was found that the antioxidant activities of *P. oleracea* extracts are different when different antioxidant assays are used. Metal inactivation and peroxyl radical scavenging are the major antioxidant factors in the β-carotene bleaching assay. Besides that, the physical state of the lipid system and the polarity of the compound will also affect the behavior of antioxidants.

In the reducing power assay, *P. oleracea* hexane fraction showed higher reducing power compared to the water fraction. However, in the DPPH radical

scavenging assay, the water fraction showed higher antioxidant activity compared to the hexane fraction. This might be due to the compounds present in the hexane fraction were not reactive towards DPPH. Antioxidant compounds in the hexane fraction may be more efficient to act as reducing agents for ferric iron but some may not scavenge DPPH free radicals as efficiently due to steric hindrance. Yildirim *et al*., (2000), stated that the free radical-scavenging activity in different assays can be linked to the presence of phenolic compounds in the extract / fractions. Phenolic compounds exhibited important mechanisms in antioxidative activities. The antioxidant activity of *P. oleracea* extracts are different when various antioxidant assay are used, this may due to the wide variety of antioxidant constituents in the extracts. Moreover, there are two types of antioxidants: (i) inhibitors of free radicals which initiate oxidation, and (ii) inhibitors of free radical chain propagation reactions that will affect the results of antioxidant activity. Different mechanism of action and kinetics of the inhibitory effect of antioxidants in *P. oleracea* extracts using different procedures resulted in discrepancy of these finding.

Based on the result of the antioxidant assay, the antioxidant activities of ethyl acetate fraction in *P. oleracea* were the highest in the DPPH and reducing power assay but not in the β-carotene bleaching assay. It was the water fraction that exhibited the highest antioxidant capacity in the β-carotene bleaching assay. The components in the water fraction were not investigated. However, several compounds were reported (Section 2.3.3 (iv)) in *P. oleracea* which were very polar in nature and highly likely to exist in the water extract. Some of these compounds include the phenolic glucosides, Portuloside A and B, alkaloids (oleraceins A, B, C, D and E) and many others (Youngwan *et al.*, 2003; Sakai *et al.*, 1996; Xiang *et al.*, 2005).

The synergism among the antioxidant in the mixture made the antioxidant activity not only dependent on the concentration of antioxidant but also the structure and interaction among the antioxidant. The components in the water extract was not investigated.

Thus, the *P. oleracea* extracts vary remarkably in their antioxidant activity.

4.3 *In vitro* neutral red cytotoxicity assay

In vitro cytotoxicity assay can be used for general screening of chemicals and predict human toxicity (Clemedson and Ekwall, 1999; Scheers *et al*., 2001). Cytotoxicity assay is popular for initial test because it is an *in vitro* bioassay, rapid and only small amount of sample is required. Cytotoxicity compounds are compounds that are toxic to cells in culture. These compounds may not show any selective toxicity to cancer cells as against normal cells. Cytotoxicity compounds may be cytocidal (kill cells) or cytostatic (ie. stop the cell growth, reversible or irreversibly).

The LDH leakage assay, protein assay, neutral red assay, methyl tetrazolium (MTT) assay and ATP content of treated cells are assays that employed for detection of cytotoxicity or cell viability following exposure to toxic substances. Different cytotoxicity assays might give different results. These depend on the test agent and the cytotoxicity agent that are used. Both MTT assay and neutral red assay are the most sensitive cytotoxicity assay which show statistically significant difference between the treated cells and the controls (Fotakis *et al*., 2006).

The pH of the media (Jabbar *et al*., 1989), concentration of glucose in the growth medium, and the cellular concentration of pyridine nucleotides (Vistica *et al*., 1991) will affect the results of MTT assay. Vistica *et al*., (1991) found that amount of MTT in the incubation medium can affect the reduction of MTT to MTT-formazan, and that the

concentration required to achieve maximal MTT-formazan production differed widely for various cell lines. Some of the cell lines could not be used for the MTT test, for example, L929 fibroblasts, this is probably because of their weak succinate dehydrogenase activity (Vian *et al*., 1995). In addition, free radicals generated within the mitochondria will damage the components in the mitochondria and therefore a cytotoxicity assay based on mitochondrial respiratory activity would give early sign of toxicity following exposure to a mitochondrial toxicant. Due to the false results that might be produced in the MTT assay, neutral red cytotoxicity assay is thus used to determine the cell viability in this study.

Neutral Red (NR) cytotoxicity assay is a cell survival/ viability chemosensitivity assay. It is a simple, accurate and yield producible results assay. Neutral red (2-methyl-3-amino-7-dimethylamino-phenazine) is a weak cationic supravital dye. In this assay, neutral red penetrates the cell membranes by non-ionic diffusion and accumulate intracellulary in the lysosomes of living cells. After incubation with toxic agent, the viable cells will uptake the neutral red through passive diffusion across the plasma membrane. The xenobiotic will alter the surface of the lysosomal membrane and lead to lysosomal fragility and other changes. The non-viable cells will not take up the neutral red. Thus it decreases the uptake and subsequent retention of the dye. The dye will not be retained in the dead or damaged cells after the washing and fixation steps. Thus, the viable cells can be distinguished from the damaged or dead cells.

The neutral red assay was used in this study to determine the cytotoxic effect of *P. oleracea* extracts and fractions against selected human cancer cell lines namely human hormone-dependent breast carcinoma cell line (MCF7), human cervical carcinoma cell line (Ca Ski), human colon carcinoma cell line (HT-29), human colon carcinoma cell line (HCT 116), human lung carcinoma cell line (A549), human

nasopharyngeal cell line and non-cancer human fibroblast cell line (MRC5). Doxorubicin was used as a positive control in this assay.

DMSO was used to dilute the test agents and the final concentration of DMSO in test wells and control wells was not in excess of 1 % (v/v). No effect due to the DMSO was observed. Houghton and Raman (1998) reported that at concentration below 3 % v/v, DMSO is usually not toxic to the cells.

Cytotoxicity of each test sample is expressed as IC_{50} values. IC_{50} values can be obtained by plotting the percentage of inhibition versus concentration of test agents. The IC_{50} values are established using different concentrations of each test agent tested on the cells. The IC_{50} value is the effective concentration (μg/ml) of test extracts or compound that cause 50 % inhibition of cell death (Borenfreund and Puerner, 1986, Chapuis *et al*., 1988, Liebsch and spielmann, 1995).

According to the US NCI (United States National Cancer Institute) plant screening programme, a plant extract is generally considered to have active cytotoxic effect if the IC_{50} value following incubation between 48 to 72 h, is 20 μg/ml or less, while it is 4 μg/ml or less for pure compound (Lee and Houghton, 2005; Boik, 2001; Geran *et al*., 1972, 1997).

4.3.1 Cytotoxic activity of *P. oleracea* extracts

i. Human hormone-dependent breast carcinoma cell line (MCF7)

The *in vitro* growth inhibition of MCF7 cells by *P. oleracea* extracts (without treatment with activated charcoal) is shown in Figure 4.5. The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against MCF7cell lines is shown in Table 4.8. The

methanol extract, hexane and water fractions did not exhibit cytotoxic activity (IC_{50} >100.00 μg/ml). The percentage of growth inhibition of MCF7 cells increases with increase of ethyl acetate fraction concentration. The ethyl acetate fraction did not exhibit inhibitory activity (IC_{50} 64.00 ± 1.53 μg ml^{-1}) against MCF7 cell lines. Upon treatment with activated charcoal, all extracts exhibited no activity against all the tested cancer. Figure 4.6 shows the *in vitro* growth inhibitions of MCF7 cells by *P. oleracea* extracts (upon treatment with activated charcoal).

Table 4.8: The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against MCF7 cell line

Extract / Fractions	Treatment	MCF7
Methanol	Without treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		64.00 ± 1.53 μg/ml
Water		>100.00 μg/ml
Methanol	Upon treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml

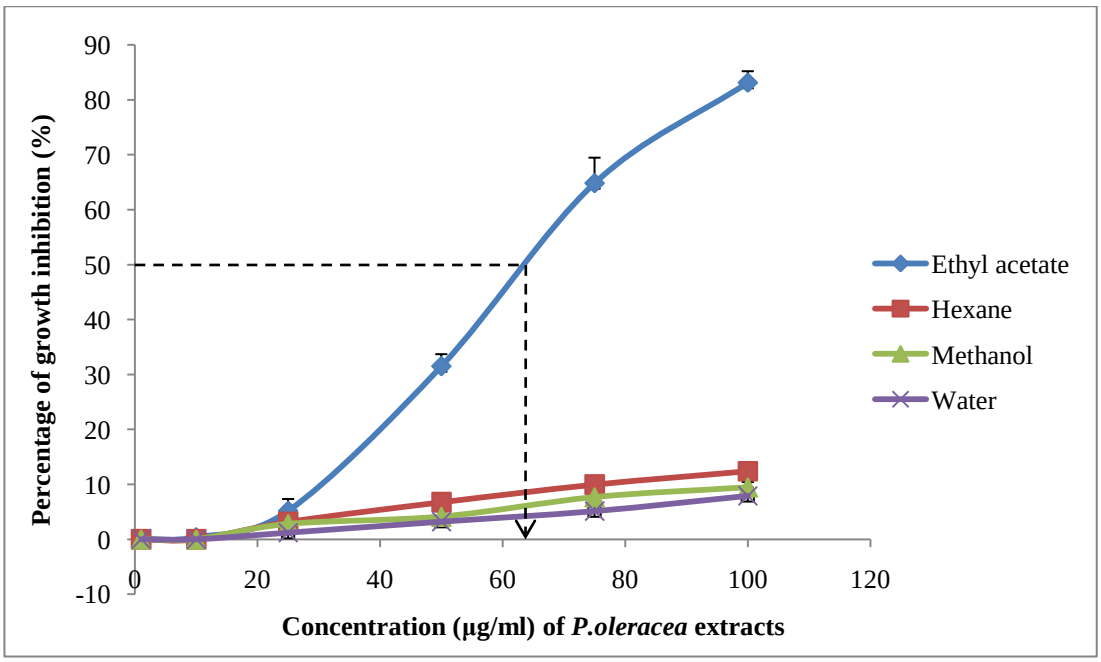


Figure 4.5: The *in vitro* growth inhibitions of MCF7 cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay

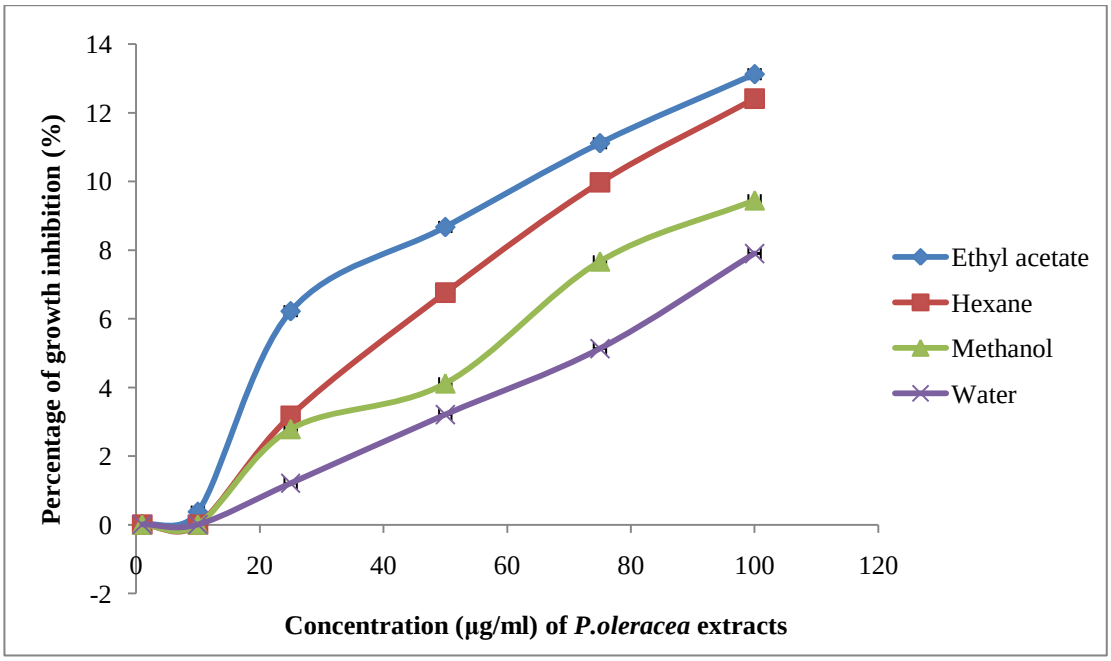


Figure 4.6: The *in vitro* growth inhibitions of MCF7 cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

ii. Human cervical carcinoma cell line (Ca Ski)

The methanol, hexane and water fractions did not exhibit cytotoxic activity (IC_{50} >100.00 μg/ml) against all tested cell lines. Table 4.9 shows the cytotoxic effect (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against human cervical carcinoma cell lines (Ca Ski). Figure 4.7 shows the *in vitro* growth inhibitions of Ca Ski cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay. Whilst, Figure 4.8 shows the *in vitro* growth inhibitions of Ca Ski cells by *P. oleracea* extracts (upon treatment with activated charcoal). The percentage of growth inhibition of Ca Ski cells increases with increase of ethyl acetate fraction concentration. The ethyl acetate fraction did not exhibit cytotoxic activities (IC_{50} 60.00 ± 1.00 μg ml^{-1}) on Ca Ski cell lines based on the guideline proposed by the US NCI plant screening program.

Table 4.9 : The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against Ca Ski cell line

Extract / Fractions	Treatment	Ca Ski
Methanol	Without treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		60.00 ± 1.00 μg/ml
Water		>100.00 μg/ml
Methanol	Upon treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml

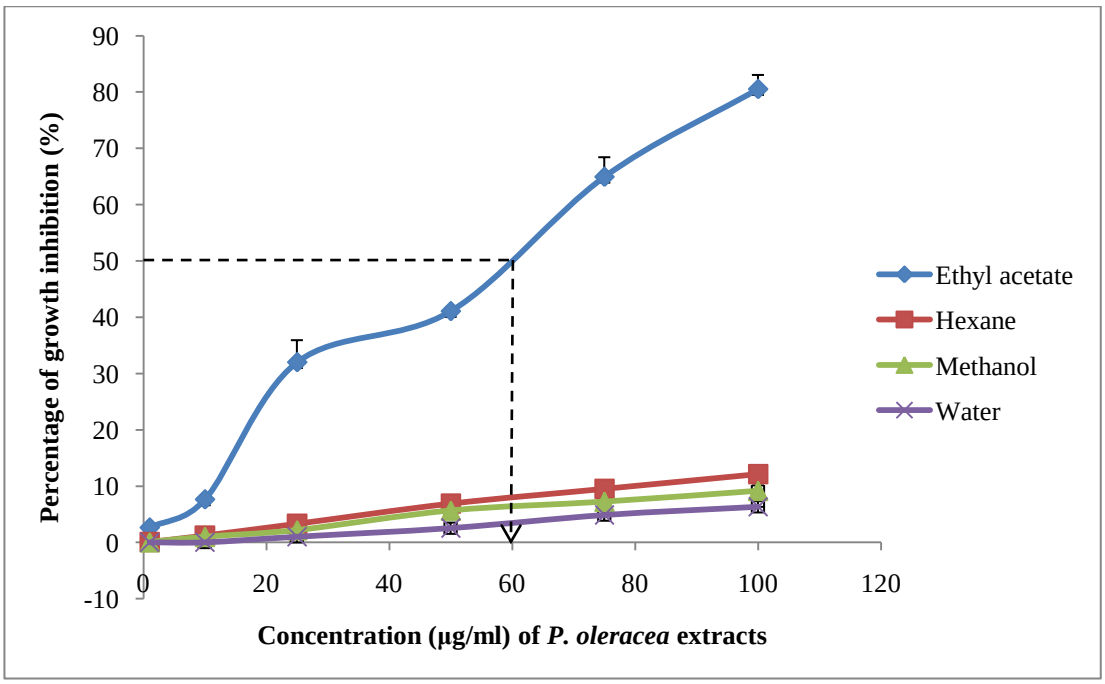


Figure 4.7 : The *in vitro* growth inhibitions of Ca Ski cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay

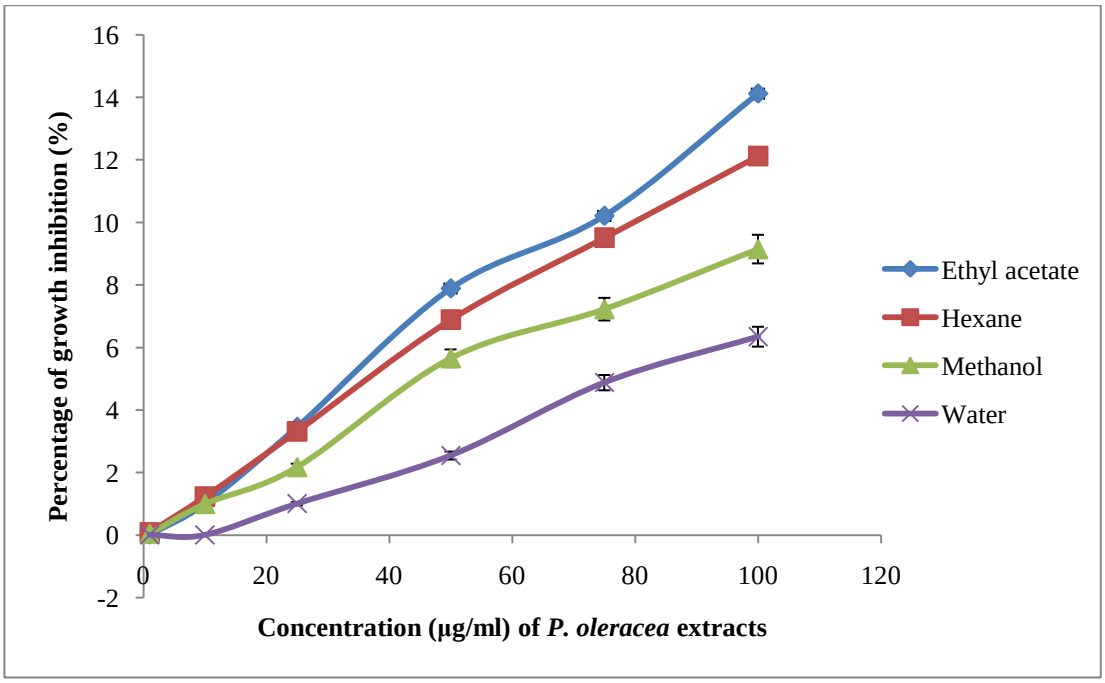


Figure 4.8 : The *in vitro* growth inhibitions of Ca Ski cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

iii. Human lung carcinoma cell line (A549)

Table 4.10 summarizes the cytotoxic activity of *P. oleracea* extracts against human lung carcinoma cell lines (A549). Figure 4.9 shows the *in vitro* growth inhibitions of A549 cells by *P. oleracea* extracts (without treatment with activated charcoal). The percentage of growth inhibition of A549 cells increases with increase of ethyl acetate fraction concentration. The IC_{50} value for the methanol, hexane and water fractions are >100.00 μg/ml, thus all these fractions did not exhibit cytotoxic activity. The extracts treated with activated charcoal also did not exhibit cytotoxic activity. Figure 4.10 shows the *in vitro* growth inhibitions of A549 cells by *P. oleracea* extracts (upon treatment with activated charcoal). Only the ethyl acetate fraction of *P. oleracea* showed some cytotoxic effect (IC_{50} 54.00 ± 4.00 μg ml^{-1}) towards A549 cell lines based on the guideline proposed by the US NCI plant screening programme.

Table 4.10 : The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against A549 cell line

Extract / Fractions	Treatment	A549
Methanol	Without treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		54.00 ± 4.00 μg/ml
Water		>100.00 μg/ml
Methanol	Upon treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml

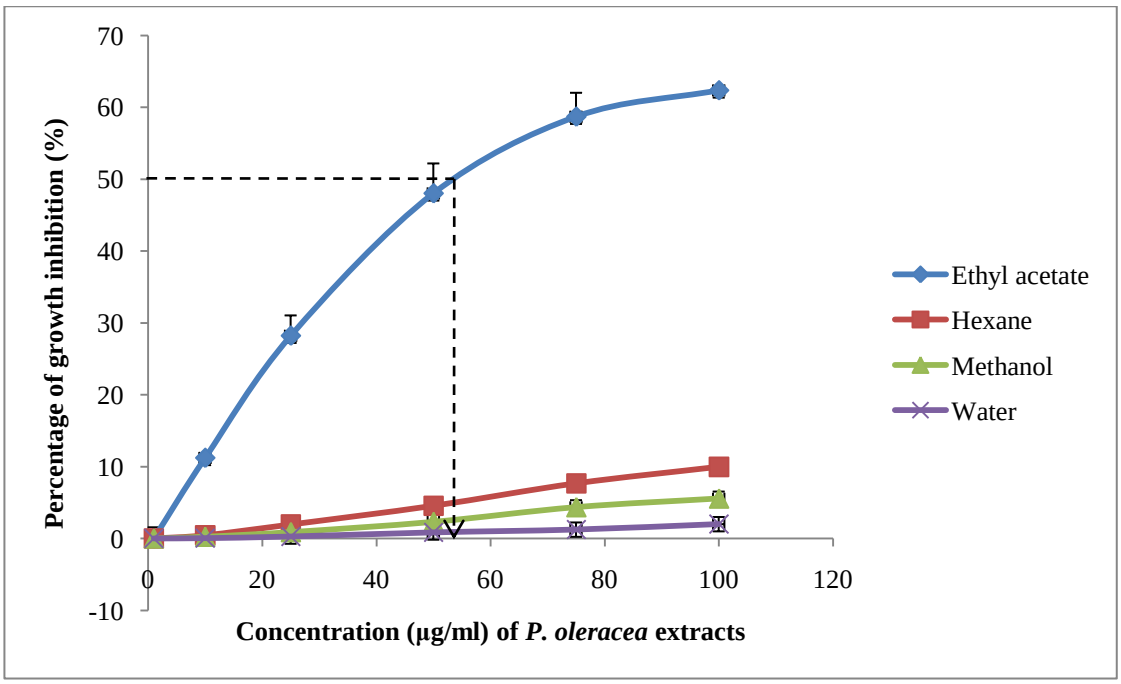


Figure 4.9: The *in vitro* growth inhibitions of A549 cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay

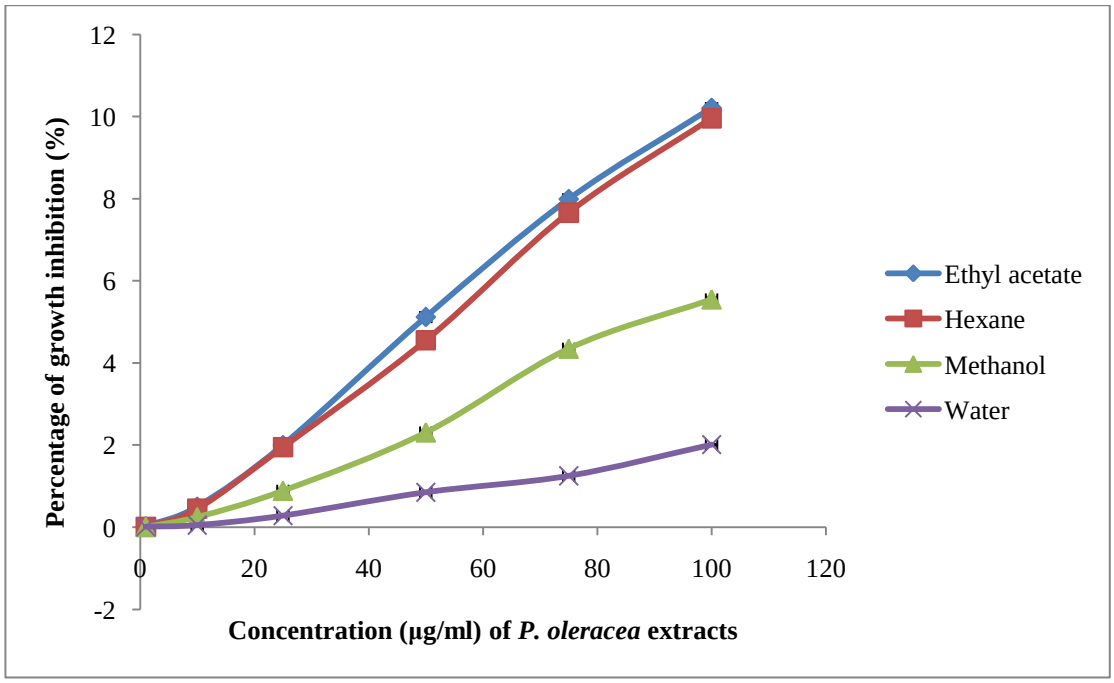


Figure 4.10 : The *in vitro* growth inhibitions of A549 cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

iv. Human colon carcinoma cell line (HT-29)

The cytotoxic activity of *P. oleracea* extracts against HT-29 cell lines is shown in Table 4.11 and Figure 4.11. The percentage of inhibition of HT-29 cells increases with increase concentration of hexane and ethyl acetate fractions. The IC_{50} for the methanol and water fractions are >100.00 µg/ml. The ethyl acetate fraction possessed the strongest cytotoxicity with an IC_{50} value of 8.00 ± 0.58 µg ml^{-1}. Whilst, the hexane fraction showed moderate cytotoxic effect (IC_{50} 39.00 ± 3.06 µg ml^{-1}) against HT-29 cells. All the extracts of *P. oleracea* did not exhibit cytotoxic activity after treated with activated charcoal as shown in Figure 4.12

Table 4.11 : The cytotoxic activity (IC_{50} µg ml^{-1}) of *P. oleracea* extracts against HT-29 cell line

Extract / Fractions	Treatment	HT-29
Methanol	Without treatment with activated charcoal	>100.00 µg/ml
Hexane		39.00 ± 3.06 µg/ml
Ethyl acetate		8.00 ± 0.58µg/ml
Water		>100.00 µg/ml
Methanol	Upon treatment with activated charcoal	>100.00 µg/ml
Hexane		>100.00 µg/ml
Ethyl acetate		>100.00 µg/ml
Water		>100.00 µg/ml

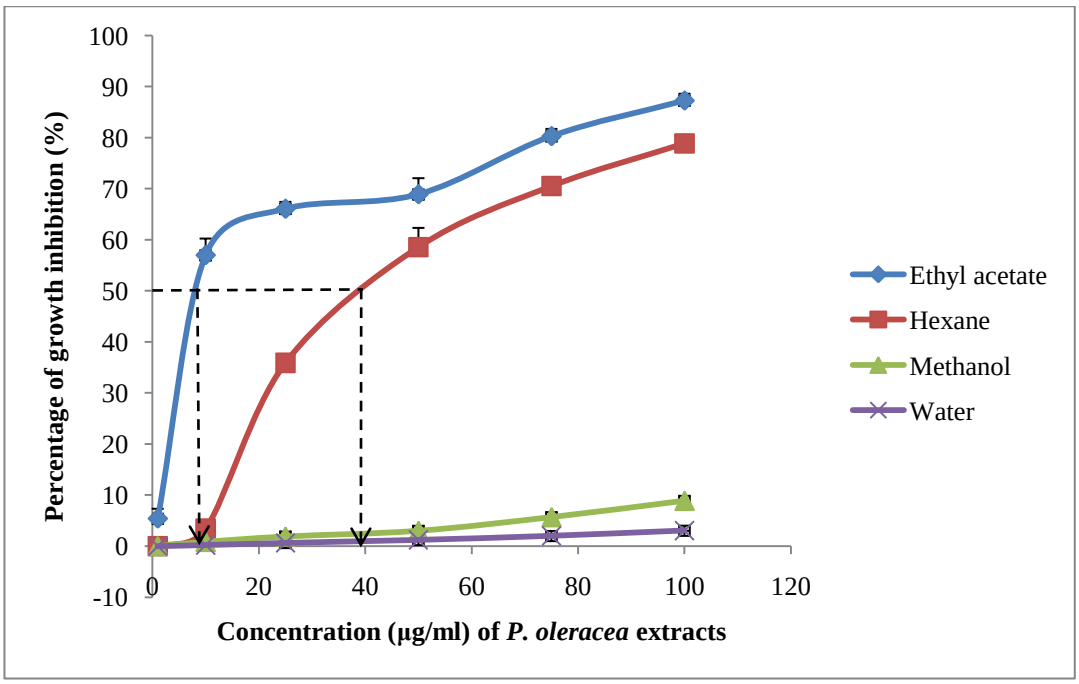


Figure 4.11: The *in vitro* growth inhibitions of HT-29 cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay.

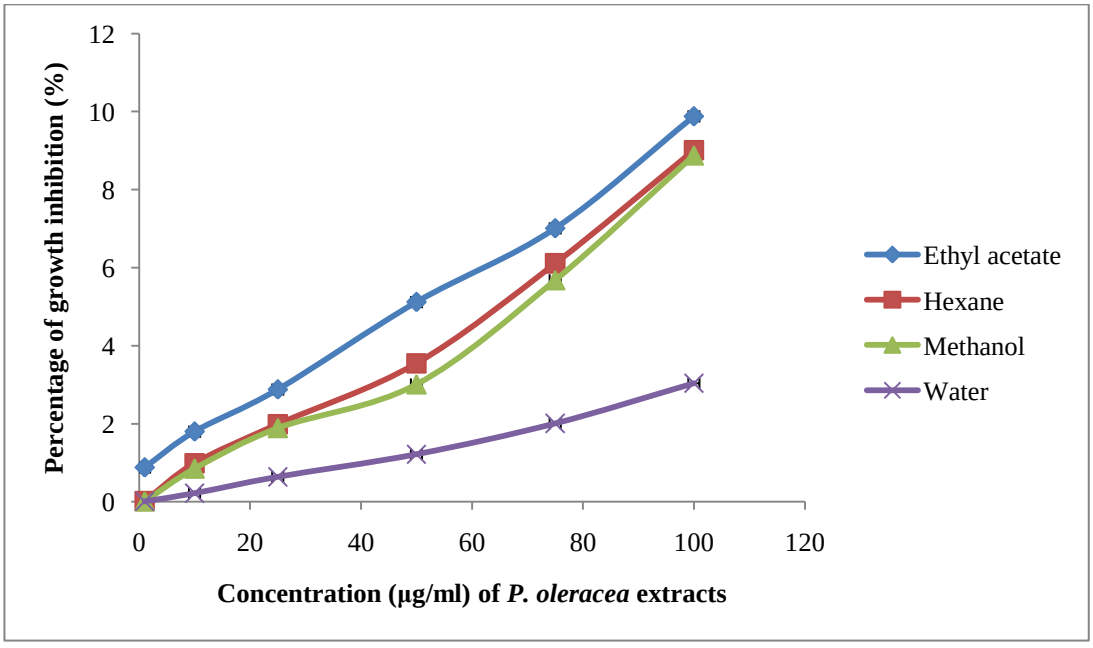


Figure 4.12 : The *in vitro* growth inhibitions of HT-29 cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

v. Human colon carcinoma cell line (HCT 116)

The cytotoxic activity of *P. oleracea* extracts against HCT 116 cell lines is shown in Table 4.12 and Figure 4.13. Percentage of inhibition of HCT 116 cells increases with increase of ethyl acetate fraction concentration. The methanol, hexane and water fraction did not exhibit cytotoxic activity (IC_{50} > 100 μg/ml). Upon treatment with activated charcoal, all extracts exhibited no activities against the tested cancer cell lines as shown in Figure 4.14. The ethyl acetate fraction of *P. oleracea* showed moderate cytotoxic effect against HCT 116 cell lines (IC_{50} 32.00 ± 3.06 μg ml^{-1}).

Table 4.12 : The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against HCT 116 cell line

Extract/ Fractions	Treatment	HCT-116
Methanol	Without treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		32.00 ± 3.06 μg/ml
Water		>100.00 μg/ml
Methanol	Upon treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml

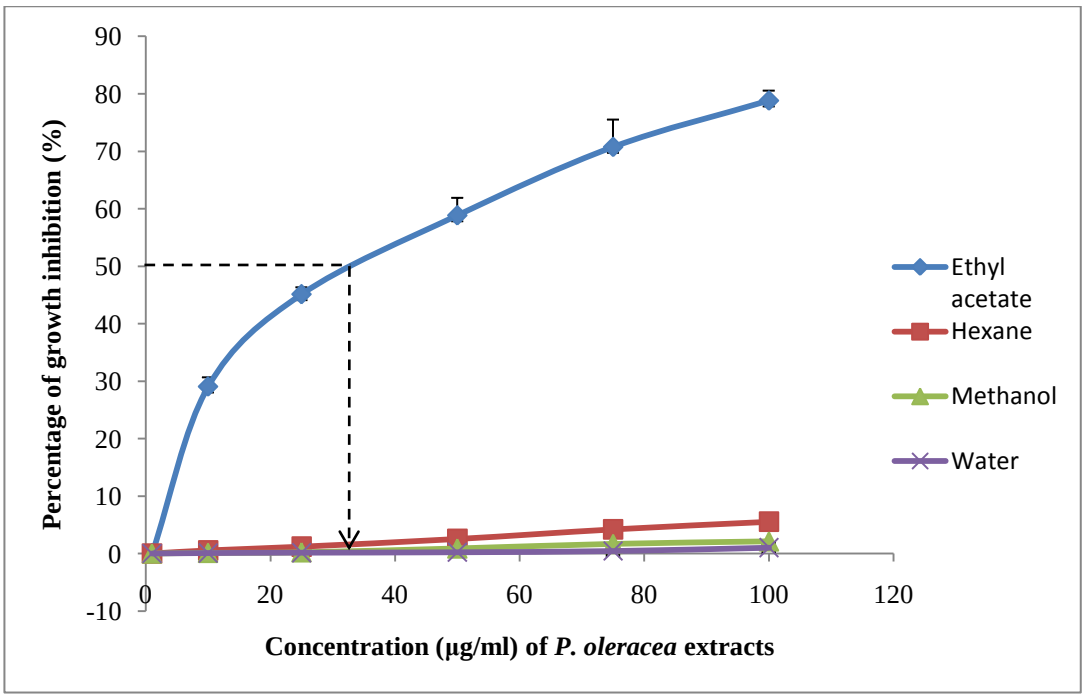


Figure 4.13 : The *in vitro* growth inhibitions of HCT 116 cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay

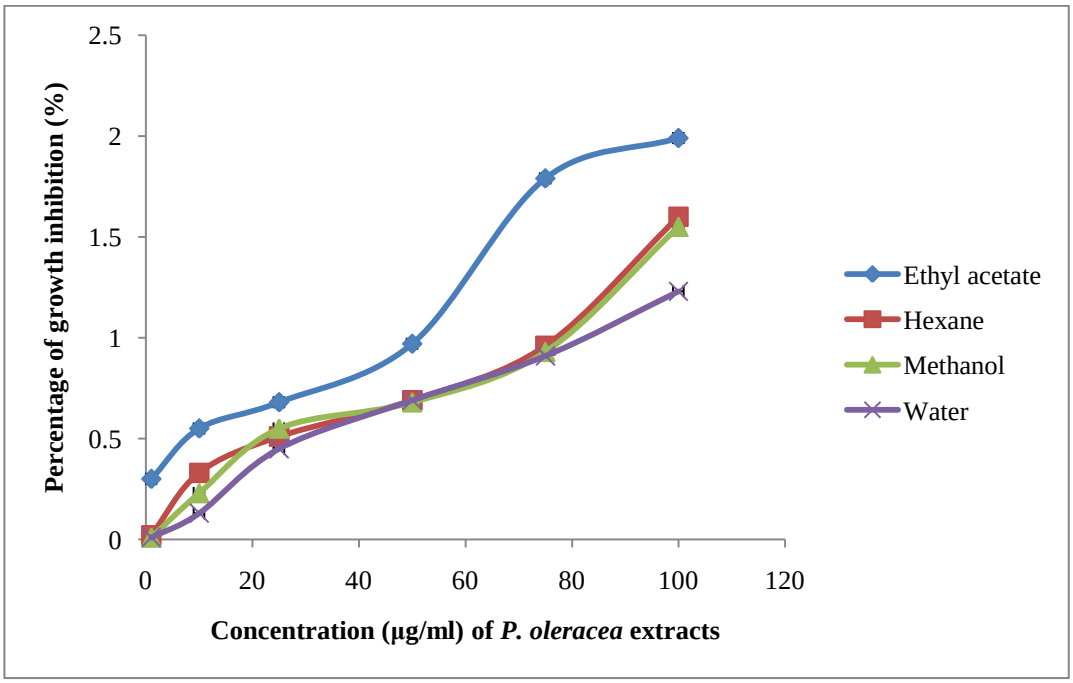


Figure 4.14: The *in vitro* growth inhibitions of HCT 116 cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

vi. Human nasopharyngeal cell line (KB)

The cytotoxic activity of *P. oleracea* extracts against KB cell lines is shown in Table 4.13 and Figure 4.15. The percentage of inhibition of KB cells increases with increase of ethyl acetate fraction concentration. The IC_{50} for the methanol, hexane and water fractions are >100.00 μg/ml. The ethyl acetate fraction possessed the strongest cytotoxicity with an IC_{50} value of 69.00 ± 0.58 μg ml^{-1}. Figure 4.16 shows the *in vitro* growth inhibitions of KB cells by *P. oleracea* extracts (upon treatment with activated charcoal). All the extracts of *P. oleracea* did not exhibit cytotoxic activity after treated with activated charcoal.

Table 4.13 : The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against KB cell line

Extracts / Fraction	Treatment	KB
Methanol	Without treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		69.00 ± 0.58 μg/ml
Water		>100.00 μg/ml
Methanol	Upon treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml

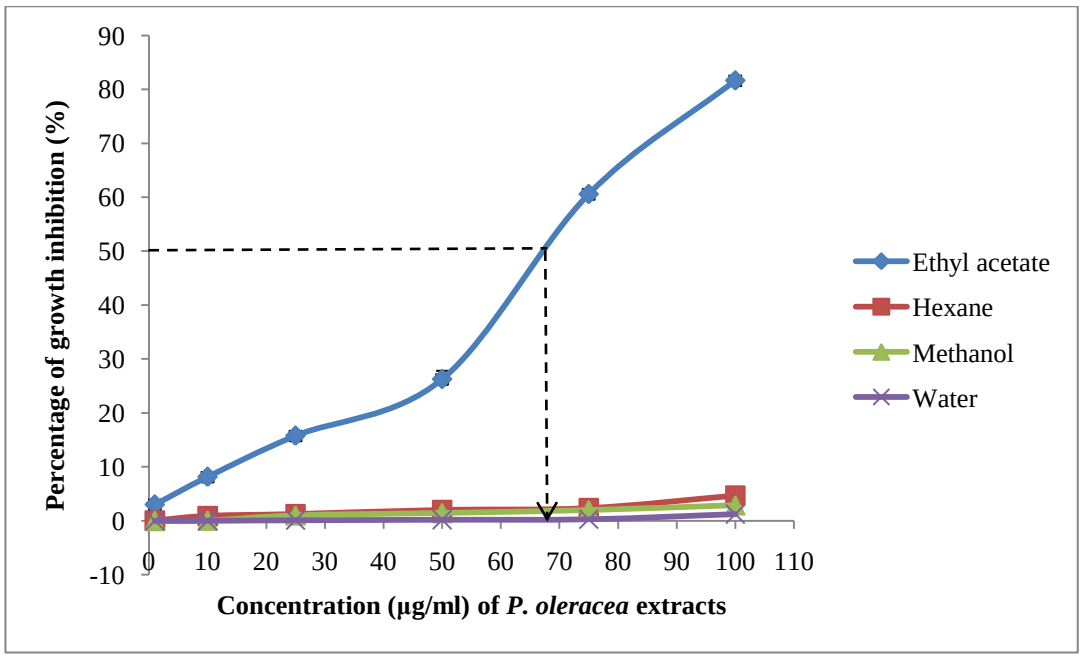


Figure 4.15: The *in vitro* growth inhibitions of KB cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay

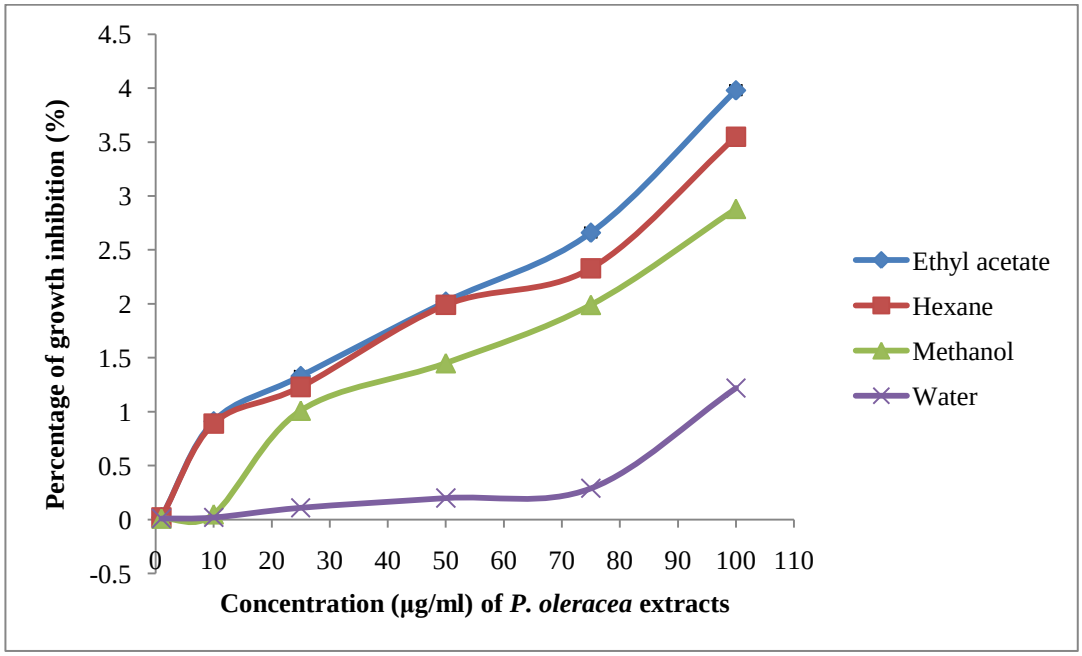


Figure 4.16: The *in vitro* growth inhibitions of KB cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

vii. Non-cancer human fibroblast cell line (MRC5)

The cytotoxic activity of *P. oleracea* extracts against MRC5cell lines is shown in Table 4.14. All extracts of *P. oleracea* (methanol, hexane, ethyl acetate and water fraction did not exhibit cytotoxic activity (IC_{50} >100 μg/ml). Upon treatment with activated charcoal, all extracts exhibited no activities against the tested cancer cell lines. Figure 4.17 shows the *in vitro* growth inhibitions of MRC5cells by *P. oleracea* extracts (without treatment with activated charcoal). Whilst, Figure 4.18 shows the *in vitro* growth inhibitions of MRC5 cells by *P. oleracea* extracts (upon treatment with activated charcoal).

Table 4.14: The cytotoxic activity (IC_{50} μg ml^{-1}) of *P. oleracea* extracts against MRC5 cell lines

Extract/ Fractions	Treatment	MRC-5
Methanol	Without treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml
Methanol	Upon treatment with activated charcoal	>100.00 μg/ml
Hexane		>100.00 μg/ml
Ethyl acetate		>100.00 μg/ml
Water		>100.00 μg/ml

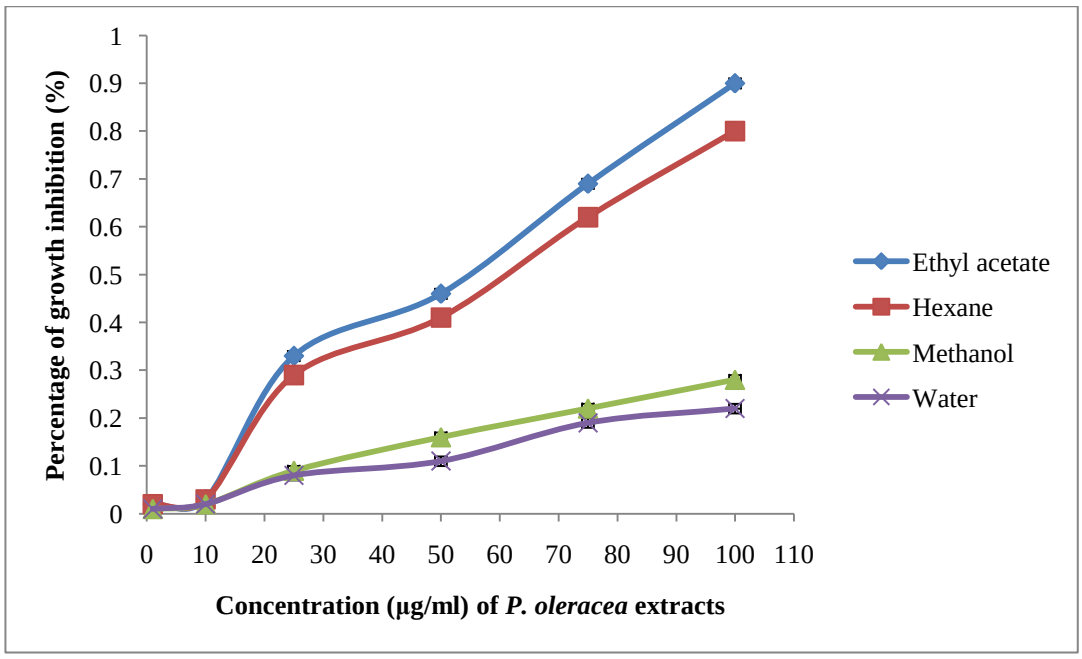


Figure 4.17 : The *in vitro* growth inhibitions of MRC5 cells by *P. oleracea* extracts (without treatment with activated charcoal) determined by neutral red cytotoxicity assay

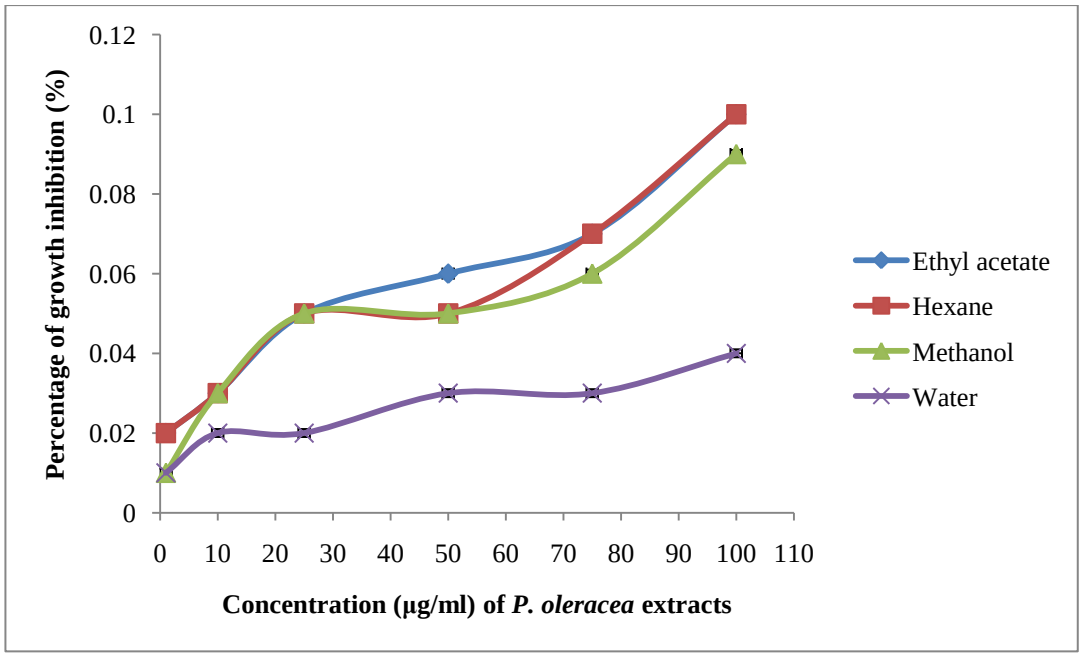


Figure 4.18 : The *in vitro* growth inhibitions of MRC5 cells by *P. oleracea* extracts (upon treatment with activated charcoal) determined by neutral red cytotoxicity assay

4.3.2 Cytotoxic activity of Doxorubicin

Table 4.15 shows the cytotoxic activity of doxorubicin against several cell lines. A plant extract is generally considered to have active cytotoxic effect if the IC_{50} value following incubation between 48 to 72 h, is 20 μg/ml or less, while it is 4 μg/ml or less for pure compound. In the present study, doxorubicin was used as the positive control for the cytotoxicity assay. Doxorubicin was screened for its cytotoxic activity on the selected human cell lines namely MCF7, Ca Ski, A549, KB, HT-29, HCT 116 and MRC5. Doxorubicin possessed very strong cytotoxicity with the IC_{50} values of 0.05 ± 0.01 μg/ml, 0.58 ± 0.01 μg/ml, 0.18 ± 0.06 μg/ml, 0.24 ± 0.04 μg/ml, 0.33 ± 0.03 μg/ml, 0.27 ± 0.01 μg/ml and 0.40 ± 0.03 μg/ml against MCF7, A549, Ca Ski, HCT 116, HT-29, KB and a non-cancer MRC5 cell lines respectively.

Doxorubicin is an anti-cancer chemotherapy drug and classified as an "anthracycline antibiotic." Doxorubicin is isolated from the bacterium *Streptomyces peucetius* var. Caesius. Doxorubicin prevents DNA replication by intercalates between the base pairs in the DNA helix. It ultimately inhibits protein synthesis. Moreover, doxorubicin inhibits topoisomerase II resulting in an increased and stabilized cleavable enzyme-DNA linked complex during DNA replication and subsequently prevents the ligation of the nucleotide strand after double-strand breakage. The IUPAC name for doxorubicin is (8*S*,10*S*)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene -5,12-dione. The molecular formula is $C_{27}H_{29}NO_{11}$ with molecular weight 543.52 g/mol. Doxorubicin is administered intravenously, in the form of hydrochloride salt. Cancers treated with Doxorubicin include: pancreas, prostate, sarcomas, bladder, breast, liver, lung, lymphomas, mesothelioma, multiple myeloma, thyroid, neuroblastoma, head and neck, leukemia (some types), ovary, stomach, testis (germ cell), uterus. Although

doxorubicin is able to inhibit the growth of cancerous cell line, at the same time it also inhibits the growth of non-cancer human fibroblast cell line (MRC5). Based on the result, doxorubicin is cytotoxic against human cancer cell lines and non-cancer human MRC5 cell line. Thus, doxorubicin is known as potent cytostatic drug.

Table 4.15: The cytotoxic activity of doxorubicin against several cell lines

Cell lines	IC_{50} (in μg/ml)	IC_{50} (in μM)
MCF7	0.05 ± 0.01	0.09 ± 0.01
A549	0.58 ± 0.01	1.01 ± 0.02
Ca Ski	0.18 ± 0.06	0.31 ± 0.10
HCT 116	0.24 ± 0.04	0.41 ± 0.07
HT-29	0.33 ± 0.03	0.33 ± 0.03
KB	0.27 ± 0.01	0.46 ± 0.02
MRC5	0.40 ± 0.03	0.69 ± 0.05

4.3.3 Comparison of cytotoxic activity of *P. oleracea*

The results of preliminary cytotoxicity screening of the crude methanol and fractionated extracts of *P. oleracea* against various cancer and non-cancer cell lines are summarizes in Table 4.16.

The ethyl acetate fraction of *P. oleracea* in general gave higher inhibition and stimulation values against all the cancer cell lines compared to other extracts. The ethyl acetate fraction of *P. oleracea* was selectively toxic towards HT-29 cells with IC_{50} value 8.00 ± 0.58 μg/ml. The ethyl acetate fraction showed very weak inhibition against other

cell lines such as MCF7, Ca Ski, A549, KB and MRC5 cells. The active ingredients in the ethyl acetate fraction may lead to valuable compounds that may have the ability to kill HT-29 cancer cells but do not cause damage to normal cells (IC_{50} >100.00 μg/ml against MRC5). The stronger inhibitory effect of the ethyl acetate fraction in comparison to the crude methanol extract was probably due to the partial purification process. Other extracts of *P. oleracea* did not exhibit any cytotoxic effect on all tested cancer cell lines.

All the extracts of *P. oleracea* did not show cytotoxic activity against all the selected cell lines after treatment with activated charcoal. Initially, activated charcoal was used to remove the chlorophyll present in the extracts. However, it was observed that the ethyl acetate extracts (upon treatment with activated charcoal) lost its activity towards all MCF7, Ca Ski, A549, HT-29, HCT 116 and KB cells. Besides chlorophyll, the activated charcoal was able to bind to compounds with double bonds (http://www.chemvironcarbon.com/en/activated-carbon/adsorption) and phenolic compounds. The adsorption of complex phenolic compounds on the activated charcoal was demonstrated by Dominique R. *et al.* (2010). The compounds such as squalene, phytol, methyl linolenate and methyl linoleate in the extract may be adsorb by the activated charcoal. The binding of activated charcoal to the compounds were through ionic interactions, hydrogen bonding, and also Van der Waals forces. The lost of cytotoxic activity may be due to the adsorption of active compounds with double bond in the extracts by the activated charcoal.

The cytotoxic effects of doxorubicin (positive reference standards) were relatively more pronounced than the tested extracts. The extracts were not as effective as doxorubicin in the cytotoxic activity.

In the present study, the water fraction of *P. oleracea* did not exhibit cytotoxic activity, while the patent (patent number 5869060) by Yoon *et al*. (1999) showed that the water extract of *P. oleracea* showed cytotoxic activity against KATO III (human gastric carcinoma cell line). The dissimilarity of the cytotoxic activity result may be due to the use of different cytotoxicity assay. Different cytotoxicity assays might give different results. For example, Fotakis and Timbrel (2006) exposed HTC cells (rat hepatoma cell line) to $CdCl_2$ for 24 hours, and the cytotoxicity assay was determined with the neutral red assay and the MTT assay. Finally, the EC_{50} values obtained by neutral red assay and MTT assay were 20.00 μM ± 3.31 and 100.00 μM ± 14.47 respectively. In this study, neutral red assay was used, while Yoon *et al*. (1999), used tryphan blue incorporation assay to determine the cytotoxic effect of *P. oleracea* water extract. Thus this explains the difference in the outcome of the experiment. Moreover, the difference in cell line employed also produced different cytotoxic effect. The results indicated that various gastric cell lines differed in their susceptibility to *P. oleracea* water extract. The compounds in *P. oleracea* water extract act selectively on the type of cancer cell used. The compounds in water extract only showed strong cytotoxic activity to KATO III cell line.

In the neutral red cytotoxicity assay, the methanol extract, hexane and water fractions did not show cytotoxic activity against MCF7, Ca Ski, A549, HT-29, HCT 116 and MRC5 cell lines. Only the ethyl acetate fraction of *P. oleracea* exhibited strong cytotoxic activity against HT-29 cells (IC_{50} 8.00 ± 0.58 μg/ml). Thus, the ethyl acetate fraction was selectively toxic to HT-29 cells. The compounds in the ethyl acetate fraction have the ability to kill cancer cells but not toxic against MRC5 cells.

The ethyl acetate fraction of *P. oleracea* showed very strong cytotoxic activity on HT-29 cells with IC_{50} 8.00 ± 0.58 μg/ml compared to HCT 116 cells with IC_{50} 32.00 ± 3.06 μg/ml. Although both HT-29 and HCT 116 are human colon carcinoma cell lines,

however, only HT-29 expresses COX-2 protein and *p*53 gene. The levels of colonic cyclooxygenase (COX) enzymes are important in colon cancer carcinogenesis and chemoprevention. COX-1 and COX-2 are two isoforms of COX enzyme. COX-1 is expresses in most mammalian cell while COX-2 is inducible at the sites of inflammation and tissue repair. HT-29 cells express COX-2 protein while HCT 116 cells do not express COX-2 protein. The levels of COX-2 expression may modulate apoptosis. Tsuji and Dubois (1995) indicated that COX-2 protein play a significant role in apoptosis. A study done by Hsi *et al*. (1999) showed that transfection of APC gene in HT-29 will decrease the COX-2 protein level and increase susceptibility to apoptosis. The over-expression of COX-2 protein will inhibit apoptosis in colonic epithelial cells. In contrast, the inhibition of COX-2 protein will induce apoptosis in colonic epithelial cells. Agarwal *et al*. (2003) reported that SC236 (a selective COX-2 inhibitor) at the concentration > 75 μM will reduce the COX-2 protein expression in HT-29 and induce greater levels of apoptosis in HT-29 rather than in HCT 116 cells. The ethyl acetate fraction of *P. oleracea* may selectively target cells that express COX-2 protein. The difference in percentage of growth inhibition showed by HT-29 and HCT 116 may be due to the difference in COX-2 expression in these cell lines and COX-2 inhibition by the compounds in the ethyl acetate fraction. The compounds in the ethyl acetate fraction may cause transfection of APC gene in HT-29 and decrease the COX-2 protein level.

Besides COX-2 protein, the presence of different type of *p*53 genes also distinguishes the HT-29 cell line from HCT 116 cell line. HT-29 cell line carries a mutant form of the *p53* gene, while HCT 116 cell line carries wild-type *p53* gene (Raffaella *et al*., 2004). The compounds in the ethyl acetate fraction may selectively act on the mutant form of the *p53* gene causing HT-29 cell death by apoptosis.

However, the actual mechanism of apoptosis or cell death elicited by *P. oleracea* ethyl acetate extract requires further investigation.

In summary, the findings from the cytotoxic activity of *P. oleracea* support the common belief that ethnopharmacological selection of *P. oleracea* is a useful criterion in drug discovery. The extracts of *P. oler*acea showed much less cytotoxicity against the normal cell line. The results from this study thus provide some scientific support on the use of *P. oleracea* in folk medicine for cancer treatment. Further investigation was thus directed to investigation on the hexane and ethyl acetate extracts to identify the components present which may lead to the discovery of valuable lead compounds.

Table 4.16 : Comparison between IC_{50} values of *P. oleracea* extracts/ fractions against various cancer and non-cancer cell lines

Plant / standard	Extracts / fractions	IC_{50} (µg/ml)						
		MCF7	**CasKi**	**A549**	**HT29**	**HCT116**	**KB**	**MRC-5**
P. oleracea	Methanol	>100	>100	>100	>100	>100	>100	>100
	Hexane	>100	>100	>100	39.00 ± 3.06	>100	>100	>100
	Ethyl acetate	64.00 ± 1.53	60.00 ± 1.00	54.00 ± 4.00	**8.00 ± 0.58**	32.00 ± 3.06	69.00 ± 0.58	>100
	Water	>100	>100	>100	>100	>100	>100	>100
***P.oleracea* (treated with activated charcoal)**	Methanol	>100	>100	>100	>100	>100	>100	>100
	Hexane	>100	>100	>100	>100	>100	>100	>100
	Ethyl acetate	>100	>100	>100	>100	>100	>100	>100
	Water	>100	>100	>100	>100	>100	>100	>100
Doxorubcin (Positive reference standard)		0.05 ± 0.01	0.18 ± 0.06	0.58 ± 0.01	0.24 ± 0.04	0.33 ± 0.03	0.27 ± 0.01	0.40 ± 0.03

4.4 Chemical investigation on the hexane fraction of *P. oleracea*

Analysis of the hexane fraction of *P. oleracea* by GC-MS showed that it contains methyl palmitate (**1**, 11.10 %), methyl oleate (**2**, 2.88 %), methyl linoleate (**3**, 4.07 %), methyl linolenate (**4**, 8.70 %), phytol (**5**, 41.55 %), palmitic acid (**6**, 7.86 %) and squalene (**7**, 19.81%). All the chemical constituents in the hexane extract of *P. oleracea* were identified by comparison of the mass spectral data with that of the NIST mass-spectral Library. The structures of compounds **1-7** are illustrated in Figure 4.19.

According to Sarkar *et al*. (2006), methyl palmitate (1) has the ability to inhibit lipopolysaccharide-stimulated phagocytic activity of rat peritoneal macrophages. Takeara *et al.* (2008) also reported that methyl palmitate exerted cytotoxic effect on T-cell leukemia cell line (Molt-4) with IC_{50} value of 2.28 μg ml^{-1} while methyl stearate was cytotoxic to acute promyeloblastic leukemia cell line (HL-60) and Molt-4 cell line with IC_{50} values of 3.08 and 4.65 μg ml^{-1} respectively. Methyl oleate (2) was reported to be able to promote the induction of malignant lymphomas (Arffmann and Glavind, 1974). Methyl linoleate (3) is essential for the maintenance of growth and shown to be potent cycloxygenase-2 (COX-2) catalyzed prostaglandin biosynthesis inhibitors (Ringbom *et al*., 2001). Phytol (5) is part of chlorophyll which is important for plant biosynthesis. Phytol showed significant anti-tumor activity against molt 4B lymphoid leukemia cells (Hibasami *et al.*, 2002), P388 mouse lymphocytic leukemia cells (Phutdhawong *et al.*, 2004), HT-29 human colon cancer cells, MG-63 osteosarcoma cells and AZ-521 gastric cancer cells (Lee *et al.*, 1999). The cytotoxic activity of phytol was due to an iduction of apoptosis (Komiya *et al*., 1999). Squalene (7) has been reported to possess antilipidemic, antioxidant and membrane-stabilizing properties (Qureshi *et al*., 1996; Ko *et al*., 2002; Ivashkevich *et al*., 1981). Squalene acts as antioxidant besides playing an important role in enhancing health through its part in the building blocks of hormones and cholesterol. Squalene was reported to have protective

activity against several carcinogens, including azoxymethane- induced colon cancer (Rao *et al*., 1998) and nicotine-derived nitrosaminoketone-(NMK) induced lung carcinogenesis (Smith *et al*., 1998).

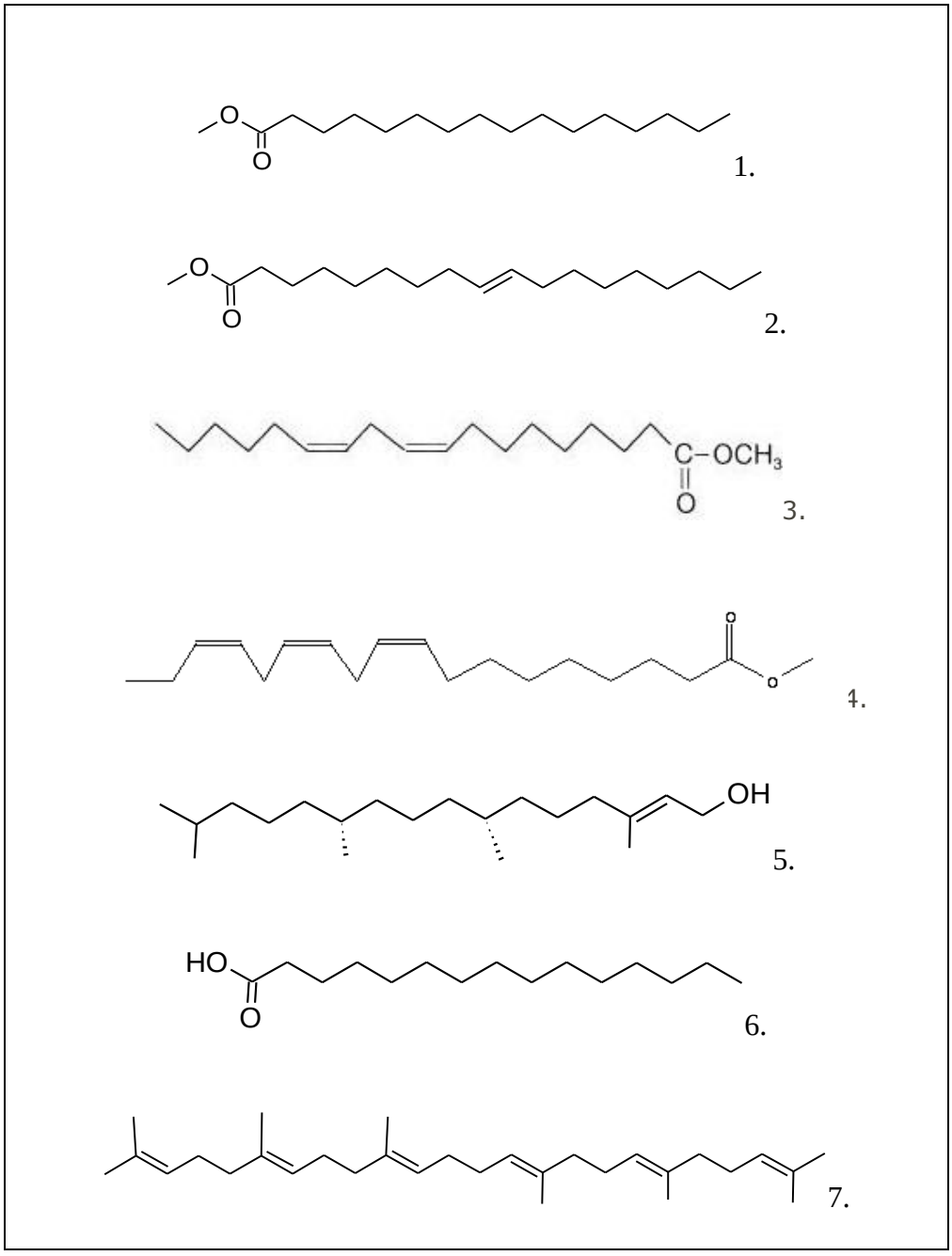


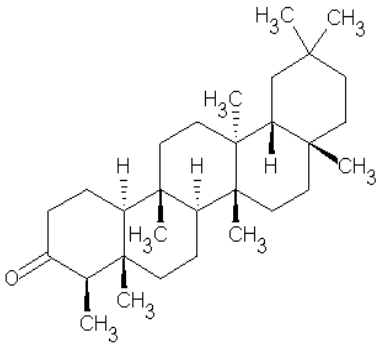
Figure 4.19 : Structures of compounds 1-7 identified in the hexane fraction of *P. oleracea*

4.5 Chemical constituents from the ethyl acetate fraction of *P. oleracea*

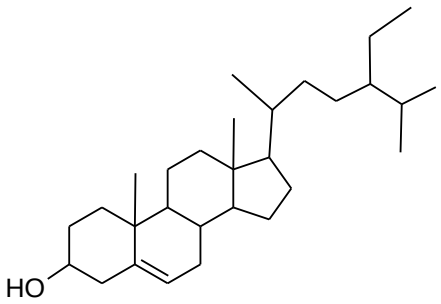
In this investigation, several chemical components were identified present in the ethyl acetate fraction, namely friedelin, β – sitosterol, campesterol, 3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0] hept-1-yl) and 4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one. There were other components in the ethyl acetate fraction that were not identified.

(i) Chemical constituents in mixture (I)

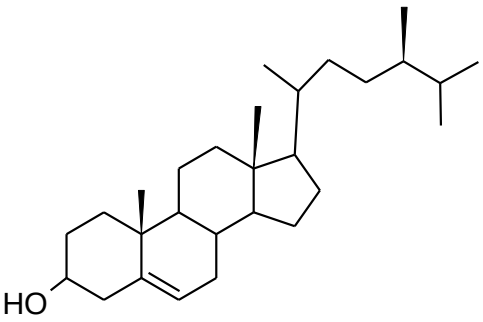
Mixture (I) (6.8 mg) was obtained as a white amorphous substance from subfraction C through PTLC. Mixture (I) contained 1 major and 2 minor components with molecular weights 426, 414 and 410 respectively identified as friedelin (8), β-sitosterol (9) and campesterol (10) respectively by GC-MS. Friedelin (8) was isolated from the mixture by PTLC and was positively identified by NMR analysis. Figure 4.20 shows the structures of chemical compounds found in mixture (I). The total ion chromatogram of mixture (I) is shown in Appendix A13.



Friedelin (8)



β-sitosterol (9)



Campesterol (10)

Figure 4.20 : The structures of chemical compounds found in mixture (I)

(ii) Chemical constituents in mixture (II)

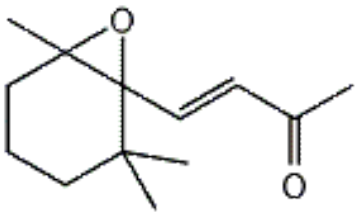
Mixture (II) (12 mg) was obtained as a green coloured substance from subfraction G through PTLC. Mixture (II) contained 1 major and 4 minor components with molecular weights 565, 339,572, 624 and 570 respectively as shown in the LC-MS/MS analysis. The components could not be identified. Purification was not attempted as the amount of the sample was insufficient for further isolation procedures. Plant samples were also not available. The chromatogram of mixture (II) and the mass spectra of the compounds in the chromatogram are shown in Appendix A22. The proton NMR spectrum is shown in Appendix 23 but it was not able to identify the components as they are present as a mixture of 5 components.

(iii) Chemical constituents in mixture (III)

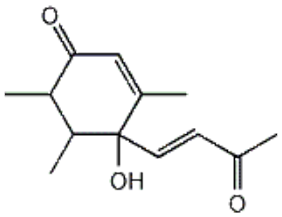
Mixture (III) (20 mg) was obtained as a yellow coloured substance from subfraction G through PTLC. Mixture (III) contained 1 major and 5 minor components with molecular weights 678, 565, 142, 666, 583 and 552 respectively through LC-MS/MS analysis. The components could not be identified. Purification was not attempted as the amount of the sample was insufficient for further isolation procedures. The chromatogram of mixture (III) and the mass spectra of the compounds in the chromatogram are shown in Appendix A24. The proton NMR spectrum is shown in Appendix 25 but it was not able to identify the components as they are present as a mixture of 6 components.

(iv) Chemical constituents in mixture (IV)

Mixture (IV) (8 mg) is a green coloured compound obtained from subfraction H through PTLC. Mixture (IV) contained 1 major and 2 minor components with molecular weights 196, 208 and 222 respectively as shown in the GC-MS. The 2 minor components were identified as 3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0] hept-1-yl) (11) and 4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one (12). Whilst the component with the molecular weight 196 could not be identified. A close look at the mass spectral and NMR data suggested that this component could be a sesquiterpenoid. Figure 4.21 shows the structures of chemical compound found in mixture (IV). The total ion chromatogram of mixture (IV) is shown in Appendix A26.



3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0] hept-1-yl) (11.)



4-Hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one (12.)

Figure 4.21: The structures of chemical compound found in mixture (IV)

4.6 Cytotoxic effect of mixtures isolated from the ethyl acetate fraction of *P. oleracea*

4.6.1 Cytotoxic effect of mixture (I)

Table 4.17 shows the cytotoxic investigation result of mixture (I) against HT-29 and MRC5 cell lines. Figure 4.22 shows the *in vitro* growth inhibitions of HT-29 cells by mixture (I) determined by neutral red cytotoxicity assay. The percentage of growth inhibition of HT-29 cells increases with increase of mixture (I) concentration. The IC_{50} of mixture (I) tested against HT-29 cell line and MRC5 cell line was >100.00 μg ml^{-1}. Mixture (I) did not exhibit cytotoxic activity against HT-29 and MRC5 cell lines.

Table 4.17 : The cytotoxic activity (IC_{50} μg ml^{-1}) of mixture (I) tested against HT-29 and MRC5 cell lines

Cell line	IC_{50}
HT-29	>100.00 μg/ml
MRC5	>100.00 μg/ml

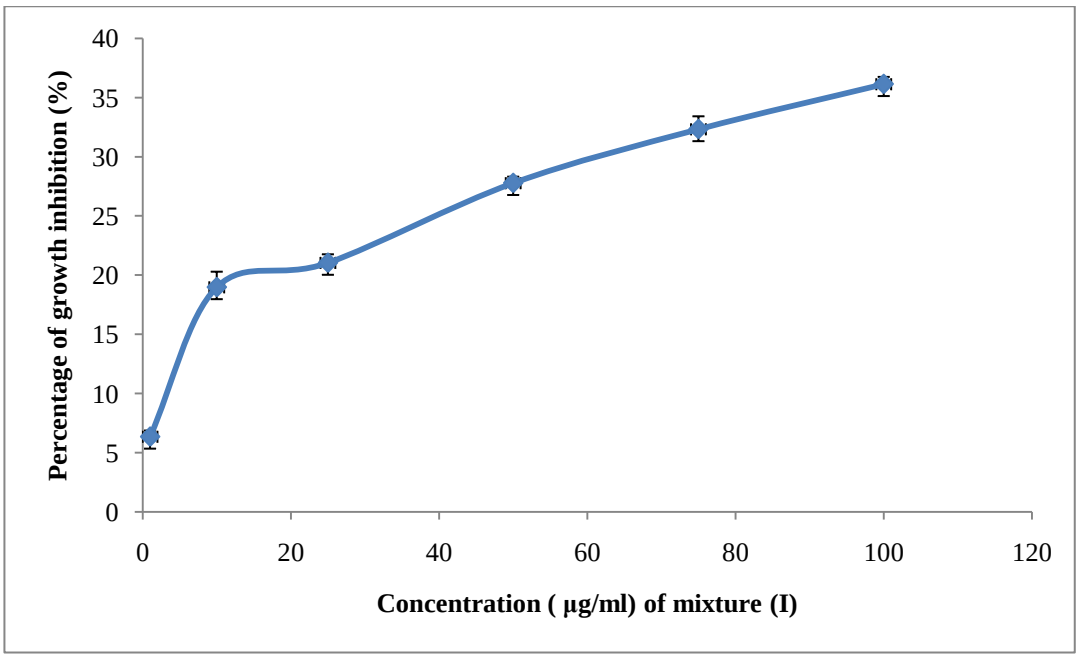


Figure 4.22 : The *in vitro* growth inhibitions of HT-29 cells of mixture (I) determined by neutral red cytotoxicity assay

4.6.2 Cytotoxic effect of mixture (II)

The results of cytotoxic effect of mixture (II) on HT-29 and MRC5 cell lines are shown in Table 4.18. Mixture (II) did not exhibit cytotoxic effect on the investigated cell lines as shown by the IC_{50} values >100.00 µg ml^{-1}.

Table 4.18: The cytotoxic activity (IC_{50} µg ml^{-1}) of mixture (II) tested against HT-29 and MRC5 cell lines

Cell line	IC_{50}
HT-29	>100.00 µg/ml
MRC5	>100.00 µg/ml

4.6.3 Cytotoxic effect of mixture (III)

Table 4.19 shows the cytotoxic activity of mixture (III) on HT-29 and MRC5 cell lines. Figure 4.23 shows the *in vitro* growth inhibitions of HT-29 cells of mixture (III) determined by neutral red cytotoxicity assay. The percentage of growth inhibition of HT-29 cells increases with increase of mixture (III) concentration in a dose dependent manner. The IC_{50} of the mixture (III) was 15.00 ± 0.87 μg ml^{-1}. Mixture (III) exhibited weak cytotoxic activity against HT-29 cell line but no activity on MRC5 cell line (IC_{50} >100.00 μg/ml).

Table 4.19: The cytotoxic activity of mixture (III) tested against HT-29 and MRC5 cell lines

Cell line	IC_{50}
HT-29	15.00 ± 0.87 μg/ml
MRC5	>100.00 μg/ml

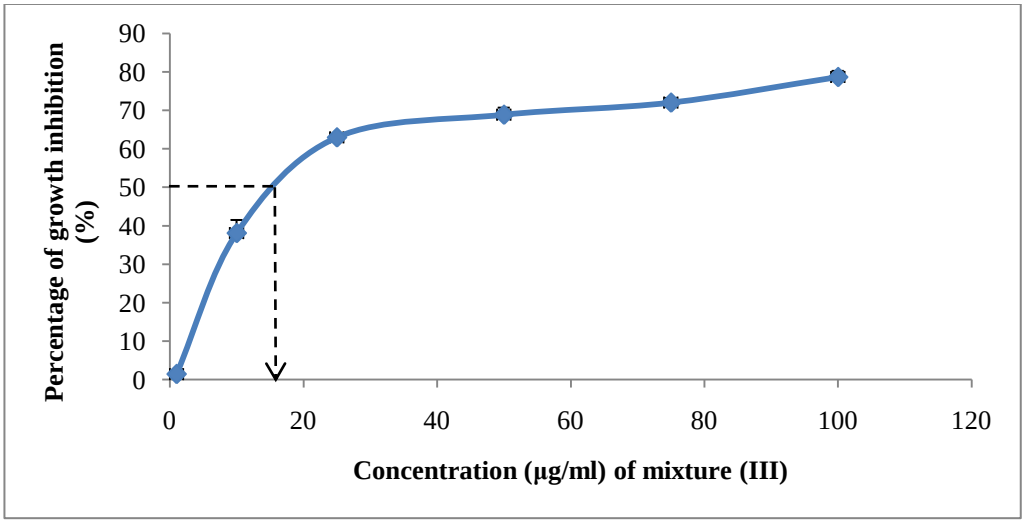


Figure 4.23 : The *in vitro* growth inhibitions of HT-29 cells of mixture (III) determined by neutral red cytotoxicity assay

4.6.4 Cytotoxic effect of mixture (IV)

Table 4.20 shows the cytotoxic activity of mixture (IV) on HT-29 and MRC5 cell lines. Figure 4.24 shows the *in vitro* growth inhibitions of HT-29 cells of mixture (IV) determined by neutral red cytotoxicity assay. The percentage of growth inhibition of HT-29 cells increases with increase of mixture (IV) concentration. Mixture (IV) exhibited weak cytotoxic activity against HT-29 cells (IC_{50} 22.00 ± 0.58 µg ml^{-1}) but no activity against MRC5 cells (IC_{50} >100.00 µg ml^{-1}).

Table 4.20 : The cytotoxic activity (IC_{50} µg ml^{-1}) of mixture (IV) tested against HT-29 and MRC5 cell lines

Cell line	IC_{50}
HT-29	22.00 ± 0.58µg/ml
MRC5	> 100.00 µg/ml

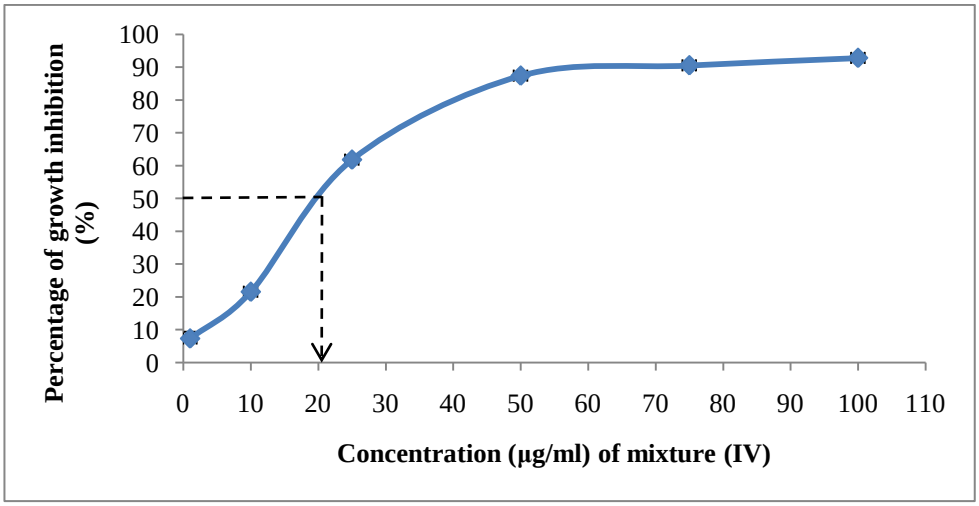


Figure 4.24: The *in vitro* growth inhibitions of HT-29 cells of mixture (IV) determined by neutral red cytotoxicity assay

4.6.5 Comparison of cytotoxic effect of mixtures isolated from the ethyl acetate fraction of *P. oleracea*

Chemical investigation on the ethyl acetate fraction showed that it contained mixtures I, II, III and IV. Mixture (I) which was composed of friedelin (8), β-sitostreol and campesterol exhibited no cytotoxic effect on HT-29 cells. β-Sitosterol was not expected to have cytotoxic effect and the results obtained were in agreement with Awad *et al*. (2000), Block *et al*. (2004), Chaturvedula *et al*. (2002), Jackson *et al*. (2000) and Moghadasian (2000). Mixture (II) also did not exhibit any cytotoxic effect on HT-29 cells. However, Mixture (III) and (IV) exhibited weak cytotoxic effect with IC_{50} 15.00 ± 0.87 μg/ml and 22.00 ± 0.58μg/ml respectively. Cytotoxic activities of the isolated mixtures were lower than that shown by the ethyl acetate fraction from which the mixtures were obtained. It appears therefore that the cytotoxic activity were lost upon purification. Together the components in the ethyl acetate fraction displayed good cytotoxic effect. The interactions between different biological active components were probably responsible for their total effects. The different compounds can modulate unrelated signaling pathways and therefore can exert synergistic effects. The ethyl acetate fraction of *P. oleracea* contained a combination of active constituents which interacted within themselves and enhanced (synergized) the therapeutic effect. The purification of fraction ends up in loss of biological activity indicating that synergistic activities occurred between the components. Synergism among the components in the mixture contributed to the cytotoxic activity which not only depended on the structure and interaction among the components, but also the concentration of certain components. The differences in the cytotoxic effect between the crude fraction and isolated mixtures against the same cell lines can thus be explained by synergistic effect.

Wills *et al*. (2000) stated that synergy is an important concept in medicinal plant. In the context of chemical complexity it applies if the pharmacological action of a

chemical mixture is greater than the arithmetic sum of the effects of individual components.

Some reports have shown that crude plant extracts are more active pharmacologically than their isolated active principles. The dichloromethane and methanolic extracts of *Andrographis paniculata* were reported to be more potent than the individual pure compounds in the cytotoxicity assay. The cytotoxic effect of the methanolic extract on HT-29 cells was 10 μg/ml. However, the isolated compounds 14-deoxyandrographolide and 14-deoxy-11,12-didehydroandrographolide did not exhibit cytotoxic activity against the cancer cell lines **(**Ajaya Kumar *et al*., 2004). Puri *et al*. (1993) reported that the whole extract of *Andrographis paniculata* extract was more active in stimulating both antigen specific and non-specific immune system in mice compared to the individual compounds.

Another report by Woldemichael and Wink (2001) showed that the crude saponin mixture of *Chenopodium quinoa* inhibited the growth of *Canalida albicans* at 50 μg/ml, but the pure compounds showed little or no activity. Thus, they suggested a possibility of synergistic effect occuring between the crude saponin mixtures.

Luna-Herrer *et al.* (2007) evaluated the synergistic effect of methanol extract of Laurus spp. on antimycobacterial activity. The methanolic extract of Laurus *spp.* showed very strong antimycobacterial activity against *M. tuberculosis H37Rv*, with an MIC of 3.25 mg/l. However, the isolated compounds, costunolide and dehydrocostuslactone have MIC value of 12.5 and 12.5 respectively. Thus they concluded that further steps of purification of extracts and fractions end up in loss of biological activity. This indicated that synergistic activities had occurred between the components.

The isolated compounds may lose their biological activity or may not behave the same way as the compounds in whole foods. Blot *et al*. (1993) showed the intake of vitamin C alone did not reduce the cancer incidence. Whilst, Hennekens *et al*. (1996) showed that the intake of β-carotene supplement did not reduced cancer incidence. Whereas Omenn *et al.* (1996) and The Alpha-tocopherol and β-carotene Cancer Prevention Study Group (1994) reported that smokers receiving β-carotene supplement have increase the lung cancer incidence. However, the intake of vitamin C and carotenoid in diets high in fruits, green and yellow vegetables are generally associated with cancer prevention. This showed the synergistic effects between vitamin C and β-carotene in the mixture of whole foods for cancer prevention.

Khafif *et al.* (1998) reported that chemopreventive synergism was observed between epigallocatechin-3-gallate (EGCG) and curcurmin from *Curcuma longa*. The combination of EGCG and curcurmin showed synergistic interactions in growth inhibition of malignant and premalignant human oral epithelial cells and increased sigmoidicity of the dose-response curves.

Zhou *et al*. (2004) reported that in an immune deficient mouse model, with implanted MCF7 human breast cancer cells, soy (the soy isoflavone genistein as well as soy phytochemical concentrate) combined with green tea showed synergistic inhibition of tumor cell growth. This inhibition was associated with inhibited tumor angiogenesis and reduced estrogen receptor (ER)-alpha expression and serum levels of insulin-like growth factor (IGF)-I, both crucial factors in breast cancer development. Modulation of these two different mechanisms of action may explain the synergistic effects of the combined phytochemicals.

Horie *et al.* (2005) found synergistic effect of green tea catechins on cell growth and induction of apoptosis in gastric carcinoma cells. Epicatechin (EC) had almost no

effect on cell growth or induction of apoptosis but when EC combined with other catechins, significant synergistic effect on the induction apoptosis was observed.

The findings demonstrated by the above researchers support the theory of synergistic effect. Thus, it can be concluded that synergistic effect of the compounds in the ethyl acetate fraction is responsible for the cytotoxic activity shown by the extract.