CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

Fresh whole plant of *P. oleracea* was collected from Teluk Intan, Perak, Malaysia in September 2008 and January 2009. Samples were dried in the oven at 50° C for 4 days and ground into fine powder using a blender.

3.2 Extraction Techniques

3.2.1 Extraction and fractionation of extract from plant samples

Fresh whole plant of *P. oleracea* (45 kg) were washed, weighed and cut into small portions then dried in the oven at 50° C for 4 days. The dried samples (2.25 kg) were ground into powder using an electric blender. The powdered samples were stored in an air-tight container and kept at room temperature for extraction purposes. The extraction yield is shown in Table 4.1.

The dried, ground samples (2.25 kg) were soaked in methanol (1.5L) for 3 days at room temperature and allowed to shake continuously in a shaker. The extract was filtered over filter paper. The filtrate obtained was put in a beaker and anhydrous sodium sulphate was added to remove traces of water. The filtrate was then filtered and poured into a round bottom flask to be evaporated at low pressure to remove excess methanol. A dark-green, concentrated methanolic crude extract (218.00 g, 9.71 %) was obtained. A small portion of this extract was transformed to a vial using a spatula and kept in the refrigerator.

Hexane was then added to the rest of the remaining methanol extract in the round bottom flask and the resultant mixture was poured into a beaker. Anhydrous

sodium sulphate was added to the mixture to remove traces of water. The mixture was filtered over filter paper and the solvent was evaporated to give a sticky, dark-green hexane fraction (54.42 g, 24.96 %) which was then transferred to a vial and kept in the refrigerator.

The hexane-insoluble fraction was then partitioned between ethyl acetate and water. A mixture of ethyl acetate and water (1:1) was prepared and poured into a round bottom flask. The flask was shaken vigorously and the separation carried out in a separating funnel. The funnel was slightly shaken and the mixture was allowed to settle into two layers of ethyl acetate (top) and water (below). The water layer was separated from the ethyl acetate layer by opening the tap of the funnel. The water layer was collected in a beaker.

The ethyl acetate layer was then poured into the Erlenmeyer flask. The water layer was returned to the separating funnel and the extraction of the components in the water layer was repeated using fresh ethyl acetate until the ethyl acetate layer becomes colourless. Anhydrous sodium sulphate was added to the ethyl acetate to remove traces of water. The ethyl acetate was filtered over filter paper and the solvent was evaporated to give a sticky, dark-green ethyl acetate fraction (12.31 g, 5.65 %) which was then transferred to a vial and kept in the refrigerator. The water fraction was evaporated to give a hard sticky extract (94.22 g, 43.22 %) which was then stored in a vial and kept in the refrigerator.

3.2.2 Extraction and fractionation of plant samples (activated charcoal)

Fresh whole plant of *P. oleracea* (10 kg) were washed, weighed, cut into small pieces then dried in the oven at 50˚C for 4 days. The dried samples (489 g) were ground

into powder using an electric blender. The powdered plant samples were then stored in an air-tight container and kept in the refrigerator.

The dried, ground samples were soaked in methanol (1.5L) for 3 days at room temperature and allowed to shake continuously in a shaker. The methanol consisting extract was filtered over filter paper and the filtrate was then put in a beaker. Activated charcoal was added to it. The filtrate with activated charcoal was stirred with glass rod for 5 minutes and then the filtrate was filtered over glass fiber filter GC-50, 150MMD, (ADVANTEC) for a few times until a clear filtrate was obtained. The filtrate was then put in a beaker and anhydrous sodium sulphate was added to remove traces of water. After filtration, the filtrate was poured into a round bottom flask and excess solvent was evaporated at low pressure using a rotary evaporated to give a dark brown, concentrated metabolic crude extract (8.23 g, 1.68 %). A small portion of this extract was transformed to a vial using a spatula and kept in the refrigerator.

Hexane was then added to the rest of the remaining methanol extract in the round bottom flask and the resultant mixture was poured into a beaker. Anhydrous sodium sulphate was added to the mixture to remove traces of water. The mixture was filtered over filter paper and the solvent was evaporated to give a sticky, dark-brown hexane fraction (2.43 g, 25.00 %) which was then transferred to a vial and kept in the refrigerator.

The hexane-insoluble fraction was then partitioned between ethyl acetate and water. A mixture of ethyl acetate and water (1:1) was prepared and poured into a round bottom flask. The flask was shaken vigorously and the separation carried out in a separating funnel. The funnel was slightly shaken and the mixture was allowed to settle into two layers of ethyl acetate (top) and water (below). The water layer was separated from the ethyl acetate layer by opening the tap of the funnel. The water layer was collected in a beaker.

The ethyl acetate layer was then poured into the Erlenmeyer flask. The water layer was returned to the separating funnel and the extraction of the components in the water layer was repeated using fresh ethyl acetate until the ethyl acetate layer becomes colourless. Anhydrous sodium sulphate was added to the ethyl acetate to remove traces of water. The ethyl acetate was filtered over filter paper and the solvent was evaporated to give a sticky, dark-brown ethyl acetate fraction (0.46 g, 5.75 %) which was then transferred to a vial and kept in the refrigerator. The water fraction was evaporated to give a hard sticky extract (3.45 g, 43.13 %) which was then stored in a vial and kept in the refrigerator.

3.3 Bioassay screening

3.3.1 Antioxidant activity

3.3.1.1 DPPH (1,1-Diphenyl-2-picrylhydryl) free radical scavenging system

Antioxidant activity of *P. oleracea* was investigated through the free radical scavenging activities of the crude methanolic extract, fractionated extracts (hexane, ethyl acetate and water) via their reaction on the stable DPPH (1,1-diphenyl-2-picryhydrazyl) free radicals according to the method described by Cheung *et al*. (2003) with some modification. Ascorbic acid and BHA were used as positive reference standards in this antioxidant assay. Reaction mixtures for the positive reference standards were prepared according to Table 3.1.

Table 3.1 : Concentration mixture of ascorbic acid, DPPH and methanol for DPPH assay

Concentration of Standards (µg/ml)	Volume (µl)		
	Methanol	Standards	DPPH (8.0 mg/ml)
Control	975.00	-	25.00
1.56	971.09	3.91	25.00
3.12	967.19	7.81	25.00
6.25	959.38	15.63	25.00
12.50	943.75	31.25	25.00
25.00	912.50	62.50	25.00
50.00	850.00	125.00	25.00
100.00	725.00	250.00	25.00
200.00	475.00	500.00	25.00

The reaction mixtures were incubated at room temperature and allowed to react for 30 minutes in the dark. All measurements were done in dim light. The absorbance was measured at 520 nm with a spectrophotometer (Hitachi U2000). Methanol was used as blank. The DPPH radical without addition of ascorbic acid (as antioxidant) was used as control.

Antioxidant activity of *P. oleracea* extracts were tested at different concentrations (1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml). Reaction mixtures for the assay were prepared according to Table 3.2. All tests were run triplicates and the readings obtained were averaged.

Table 3.2 : Concentration mixtures of extracts, methanol and DPPH

Concentration of extracts (mg/ml)	Volume (µl)		
	Methanol	Extracts	DPPH (8.0 mg/ml)
Control	975.00	-	25.00
1.00	925.00	50.00	25.00
2.00	875.00	100.00	25.00
3.00	825.00	150.00	25.00
4.00	775.00	200.00	25.00
5.00	725.00	250.00	25.00

The scavenging activity (%) of each test sample was calculated according to the following formula:

$$\text{Scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extracts/ standard. Since the samples were dark green in colour, some adjustment must be made to acquire the proper reading for absorbance sample.

Abs sample = (Abs of extract+ methanol+ DPPH) – (Abs + methanol)

In the study, the scavenging activity was expressed as EC_{50} values. The EC_{50} value is the effective concentration at which DPPH radical was scavenged by 50 %. The EC_{50} value was obtained from the graph of scavenging activity (%) versus concentration of extracts. Low EC_{50} value indicates the strong ability of the extract to act as DPPH scavenger. The higher EC_{50} value indicates lower scavenging activity as more amount

of the scavengers were required to achieve 50 % scavenging reaction and thus the scavengers are less effective in scavenging the DPPH.

3.3.1.2 Reducing power assay

The reducing power of the prepared extract was determined according to method of Oyaizu (1986). Each extract in varying amounts of 5 mg, 10 mg, 15 mg and 20 mg was dissolved in 1.0 ml of methanol (for methanol extract, hexane and ethyl acetate fractions) and distilled water (for water fraction) to which, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % (w/v) solution of potassium ferricyanide was added. The mixture was incubated in a water bath at 50 ˚C for 20 minutes. After that, 2.5 ml of 10 % (w/v) trichloroacetic acid was added and the mixture was centrifuged at 1000 rpm for 10 minutes. A 2.5 ml aliquot of the supernatant was combined with 2.5 ml of distilled water and 0.5 ml of 0.1 % (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read spectrophotometrically (Hitachi U2000) at 700 nm. Methanol was used as blank. Increased absorbance of the reaction mixture indicates greater reducing power. The tests were run triplicates and the reading obtained was averaged for all samples. Ascorbic acid and BHA were used as positive reference standards. The higher absorbance of the reaction mixture indicated greater reducing power; the extracts that showed comparable absorbance readings with the positive standards (ascorbic acid and BHA) are considered having high reducing power.

3.3.1.3 β - carotene bleaching assay

Antioxidant activity was determined by measuring the coupled oxidation of carotene and linoleic acid, as described by Cheung *et al*. (2003). 1 ml of β-carotene solution in chloroform (0.2 mg/ml) was pipetted into a round-bottomed flask, which contained 0.02 ml linoleic acid and 0.2 ml Tween 80. After the removals of the chloroform by using rotary evaporator, 50 ml of oxygenate distilled water (which was bubbled by an air pump for overnight) was added to the flask followed by vigorous stirring to form a liposome solution. 5 ml aliquots of the liposome solution were transferred to test tubes which contained 0.2 ml of extracts at different concentrations (4 to 20 mg/ml). Methanol or water (instead of extract) was used as control while the blank contained all the earlier chemicals (0.02 ml of linoleic acid and 0.2 ml of Tween 80 in 50 ml of oxygenated distilled water) except β-carotene solution. The absorbance of each extracts was measured immediately (t = 0) at 470 nm using spectrophotometer (Hitachi U2000). The absorbance was measured again at time intervals of 20 minutes for 120 minutes. The test systems are placed in oven at 50 ˚C during the 2 hours the test was conducted. All samples were assayed in triplicate. BHA was used as positive reference standard in this assay.

The rate of β-carotene bleaching (R) was calculated according to the following formula:

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

a: initial absorbance reading at time = 0 min

b: final absorbance reading at time = 120 min

T: 120 minutes

The antioxidant activity (%) was calculated using the equation:

$$\text{Antioxidant activity (\%)} = \frac{R_{control} - R_{sample}}{R_{control}} \times 100\%$$

The tests were run in triplicates and the reading obtained was averaged. Graph of concentration of extracts versus antioxidant activity were plotted.

3.3.1.4 Statistical analysis

Data were recorded as means ± standard deviation of triplicate measurements. Statistical analysis was carried out with Microsoft excel.

3.3.2 Cytotoxicity screening

3.3.2.1 Neutral red cytotoxicity activity assay

Cytotoxic investigation of *P. oleracea* extracts were conducted using the Neutral Red Assay (NR assay). The cytotoxicity activities of the methanolic crude extracts, fractionated extracts (hexane, ethyl acetate and water fractions) of *P. oleracea* were screened using the neutral red cytotoxicity assay on the following selected cell lines:

a) MRC5 (non-cancer human fibroblast cell line)
b) MCF7 (hormone-dependent breast carcinoma cell line)
c) Ca Ski (human cervical carcinoma cell line)
d) HT-29 (human colon adenocarcinoma cell line)
e) HCT 116 (human colon carcinoma cell line)
f) A549 (human lung adenocarcinoma cell line)
g) KB (human nasopharyngeal epidermoid carcinoma cell line)

The viability of the cells was checked before and after treatment by the tryphan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (-196 ˚C) prior to use.

KB cells were maintained in Medium 199 (Sigma); Ca Ski, A549, HT-29 and MCF7 cells in RPMI 1640 (Sigma); HCT 116 in McCOY'S 5A Medium (Sigma) and MRC5 cells in EMEM (Eagle Minimum Essential Medium) (Sigma), supplemented with 10 % foetal (FBS, PAA Lab, Austria), 100.0 µg/ml penicillin/streptomycin (PAA Lab, Austria) and 50.0 µg/ml of amphostericin B (PAA Lab, Austria). The cells were cultured in 5 % CO_2 incubator kept at 37 ℃ in a humidified atmosphere. The culture was subcultured every two to three days as needed and routinely checked under an inverted microscope (IMT- 2 Olympus, Japan) for any contamination.

The cytotoxicity study was evaluated against MCF7, KB, HCT 116, HT–29, Ca Ski, A549 and MRC5 cells according to previously described protocols (Borenfreund and Puerner, 1985). The cell lines used in the present study were purchased from the American Type Culture Collection (ATCC, USA).

Briefly, the cells were detached from the tissue culture flask with 0.25 % trypsin-EDTA solution and PBS solution and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 minutes. The density of the viable cells was counted by 0.4 % of tryphan blues exclusion in a haemacytometer. Cells at a density of 3 x 10^4 /ml were plated into a 96-well microtitre plate (Nunc) and were incubated in a CO_2 incubator at 37 ℃ for 24 hours to allow the cells to adhere and achieve 60 - 70 % confluence at the time of the addition of crude extracts. After 24 hours of incubation, the cells were treated with different concentrations (1, 10, 25, 50, 75 and 100 µg/ml) of each test agents. The cells were incubated at 37 ℃ for an additional 72 hours. DMSO was used to dilute the test agents and the final concentration

of DMSO in test wells and control wells used was not in excess of 1 % (v/v). No effect due to the DMSO was observed. Doxorubicin was used as the positive control. The well with untreated cells is the negative control.

After the incubation period (72 hours), the cells were stained with neutral red (50.0 µg/ml) and were incubated for another 3 hours to allow maximum uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the media were removed and the cells were rapidly washed with washing solution (mixture of 1.0 % v/v formaldehyde and 1.0 % w/v calcium chloride). The neutral red dye was eluted from the cells by adding 200 µl of neutral red resorb solution and further incubated for 30 minutes at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). Dye absorbance (optical density, OD) was measured at 540 nm using the ELISA reader (Titertek Multiskan MCC/340).

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

The percentage of inhibition (%) of each of the test samples was calculated according to the following formula**:**

$$\text{Percentage of inhibition (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100\%$$

Cytotoxicity of each test extract is expressed as IC_{50} value. The IC_{50} is the concentration of test extracts that causes 50 % inhibition or cell death, averaged from the three experiments and was obtained through plotting the graph percentage of

inhibition (%) versus concentration of test extracts. According to the US NCI (United States National Cancer Institute) plant screening program, 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).

3.3.2.2 Statistical analysis

All data were recorded as means ± standard deviation of three experiments. Statistical analysis was carried out with Microsoft excel.

3.4 Analysis of thin layer chromatography (TLC)

TLC was performed to determine the suitable solvent system for the isolation of chemical compounds from the ethyl acetate fraction of *P. oleracea*. Silica gel 60 F_{254} TLC plates from Merck were used for TLC analysis. By using a capillary tube, a drop of sample was spotted about 1.0 cm from the bottom of the TLC plate. The TLC plate was placed into a TLC developing tank filled with premixed solvent system when the sample had dried. When it was fully developed, the TLC plate was removed from the developing tank. Then it was dried by using hair blower. The TLC plate was viewed under UV light (254 and/ or 343 mm), followed by placing into an iodine vapor chamber.

3.5 Preparative - Thin Layer Chromatography (PTLC)

Preparative-Thin Layer Chromatography (PTLC) was used in the purification of compounds. The technique was performed on 10 cm x 10 cm normal phase thin layer chromatography plates (glass coated silica gel 60 F_{254} plate from Merck). The samples were introduced onto the plates as a continuous streak 1 cm above the base of the plates using capillary tubes. The plates were developed in a chromatographic tank saturated with the developing solvent at room temperature. The plates were then air-dried, and the desired band was scrapped out and extracted with a suitable solvent.

3.6 Isolation of chemical compounds from the ethyl acetate fraction of *P. oleracea*

Based on the results of preliminary cytotoxicity screening (Section 4.), the ethyl acetate fraction of *P. oleracea* was found to have strong inhibitory activity on HT-29 cell lines. Thus further chemical investigation was directed to this fraction. The high performance liquid chromatography (HPLC) profile for ethyl acetate fraction is shown is Appendix A30.

The extraction and fractionation procedures leading to the isolation of mixtures is shown in Figure 3.1. For isolation purposes, the plant fraction was not pre-treated with activated charcoal to remove chlorophyll. The fraction was found to lose its cytotoxic activity after treatment with activated charcoal (Section 4).

The ethyl acetate fraction of *P. oleracea* (10.0 g) was subjected to vacuum liquid chromatography (VLC) using a column (30.0 cm in length and 7.0 cm id) packed with 300.0 g of silica gel (0.063 – 0.200 mm) as the stationary phase. The ratio of the fraction to silica gel is 1:30. Initially, elution of fractions commenced with hexane

followed by hexane enriched with increasing percentage of acetone and finally with methanol. A total of 11 fractions were collected from this separation and was labelled from A to K (Table 3.3).

Table 3.3: Fractions obtained from VLC of ethyl acetate of *P. oleracea*

Fraction	Weight (mg)
A	0.02
B	1.12
C	0.28
D	0.48
E	0.66
F	0.52
G	1.36
H	0.56
I	0.08
J	0.28
K	3.92
Total	9.28

3.6.1 Isolation of mixtures from the ethyl acetate fraction of *P. oleracea*

The isolation of mixtures was directed to subfraction C, subfraction G and subfraction H because only these three subfractions showed strong inhibitory activity against HT-29 cell line.

Subfraction C (0.28 g) was subjected to preparative-TLC (PTLC) to give Mixture (I) (6.80 mg), using ethyl acetate: hexane (1:9) as developing solvent; Rf = 0.5.

Subfraction G (1.36 g) was subjected to PTLC to give Mixture (II) (12.00 mg); Rf = 0.7 and Mixture (III) (20.00 mg) Rf = 0.4 using ethyl acetate: hexane (6:4) as developing solvent.

Subfraction H (0.56 g) was subjected to PTLC to give Mixture (IV) (8.00 mg) using ethyl acetate: hexane (6:4) as developing solvent; Rf = 0.8.

The isolation of mixtures I-IV from the active ethyl acetate fraction of *P .oleracea* was shown in Figure 3.2.

3.7 Spectrophotometry and spectroscopy analysis

3.7.1 Gas Chromatography-Mass Spectrophotometry (GC-MS)

GC-MS analysis was performed using Agilent Technologies 6980N gas chromatography equipped with a 5979 Mass Selective Detector (70 eV direct inlet). On a fused silica capillary column HP–5ms (equivalent to SE 54, 30 m, ID 0.25 mm, 0.25 μm film thickness) with helium as carrier gas.

GC-MS is used to determine the molecular weight of the components separated from a sample of mixture. A HP-5ms (5 % phenylmethylsiloxane) capillary column (30.0 m x 250 μm x 0.25 μm) is used. The oven temperature is programmed at an initial temperature of 100 ℃, then programmed to 300 ℃ at 5 ℃ min^{-1} and held for 20 minutes at 300 ℃ using helium as the carrier gas at a flow rate of 1 $mlmin^{-1}$. The mass spectrometry mode being used is the electron ionization (EI) mode with a current of 70eV. The injection mode is programmed with the sample injection volume of 1 μl with a split mode of ratio 1 : 20. The injection port temperature is set into 230 ℃ and the detector/ interface temperature is set to 250 ℃. The results of the sample can be collected after 40 minutes of run. The total ion chromatogram obtained was auto integrated by ChemStation and the components were identified by comparison with an accompanying mass spectral database (NIST 05 MS Library, USA).

3.7.2 Liquid Chromatography- Mass Spectrophotometry (LC-MS/MS)

LC-MS is used to determine the molecular weight of polar components in a sample mixture. The column used is Phenomenex Aqua C18- 50mm x 2.0mm x 5 uM. Water and acetonitrile were the eluting solvents. 0.2 % formic acid and 2mM ammonium formate used as buffer.

3.7.3 Nuclear Magnetic Resonance (NMR)

NMR (Nuclear Magnetic Resonance) spectra were recorded on a JOEL 400 MHz FT NMR spectrometer at 400 MHz for ^{1}H NMR (^{1}H Nuclear Magnetic Resonance) and at 100.40 MHz for ^{13}C NMR (^{13}C Nulcear Magnetic Resonance). Internal standards used in ^{1}H NMR spectra was TMS (δ: 0.00) for $CDCl_3$; in ^{13}C NMR was $CDCl_3$ (δ: 77.0).

3.7.4 Microplate reader

The neutral red dye absorbance (optical density, OD) was measured at 540 nm using a ELISA reader (Titertek Multiskan MCC/340).

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3.7.5 UV-visible spectrophotometer

The spectrometric absorbance reading for scavenging effect on DPPH free radicals assay, reducing power assay and β-carotene bleaching assay were performed on a Hitachi U2000 Spectrophotometer.

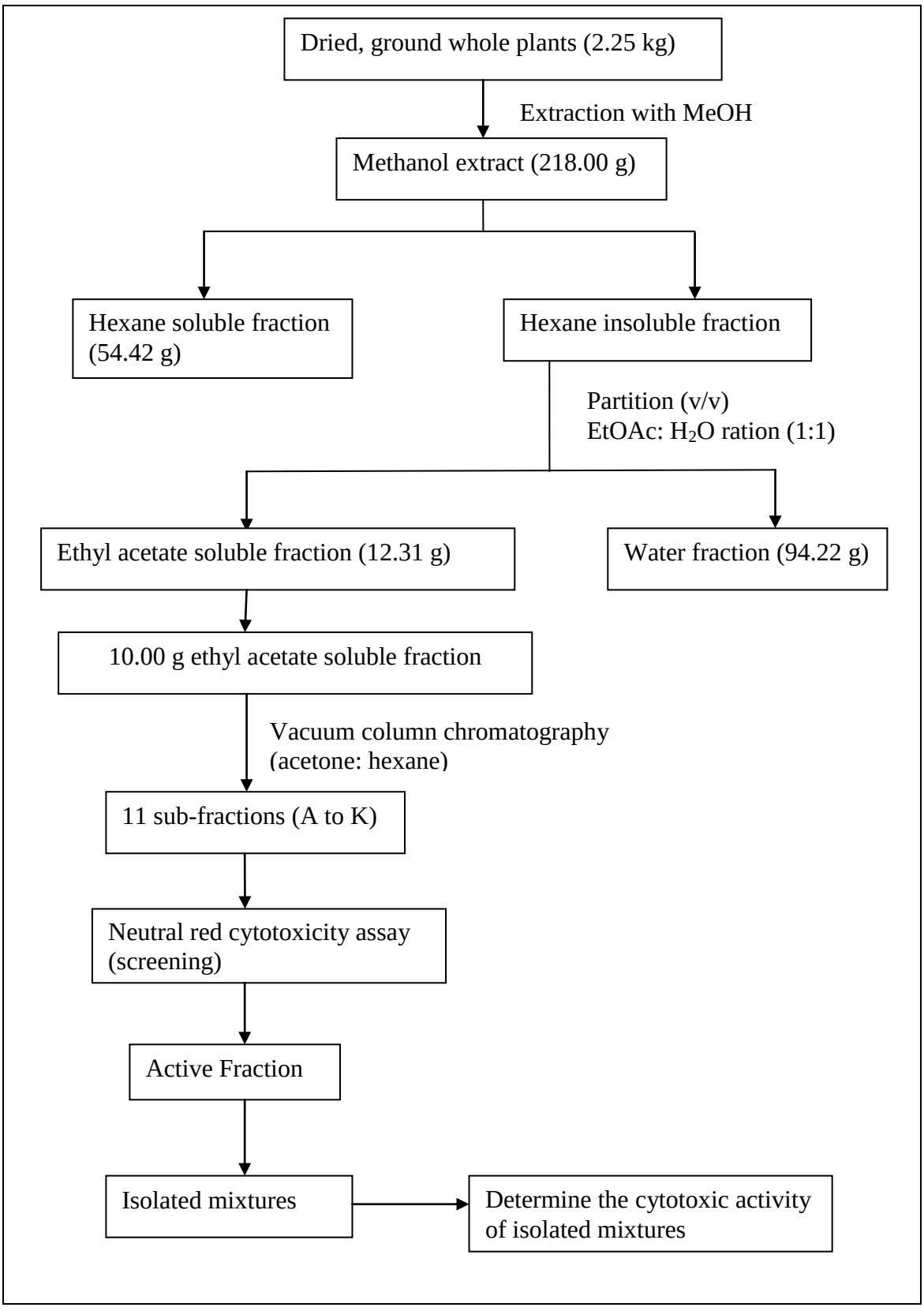

Figure 3.1: The extraction and fractionation procedures leading to the isolation of mixtures from the active ethyl acetate fraction of *P. oleracea*

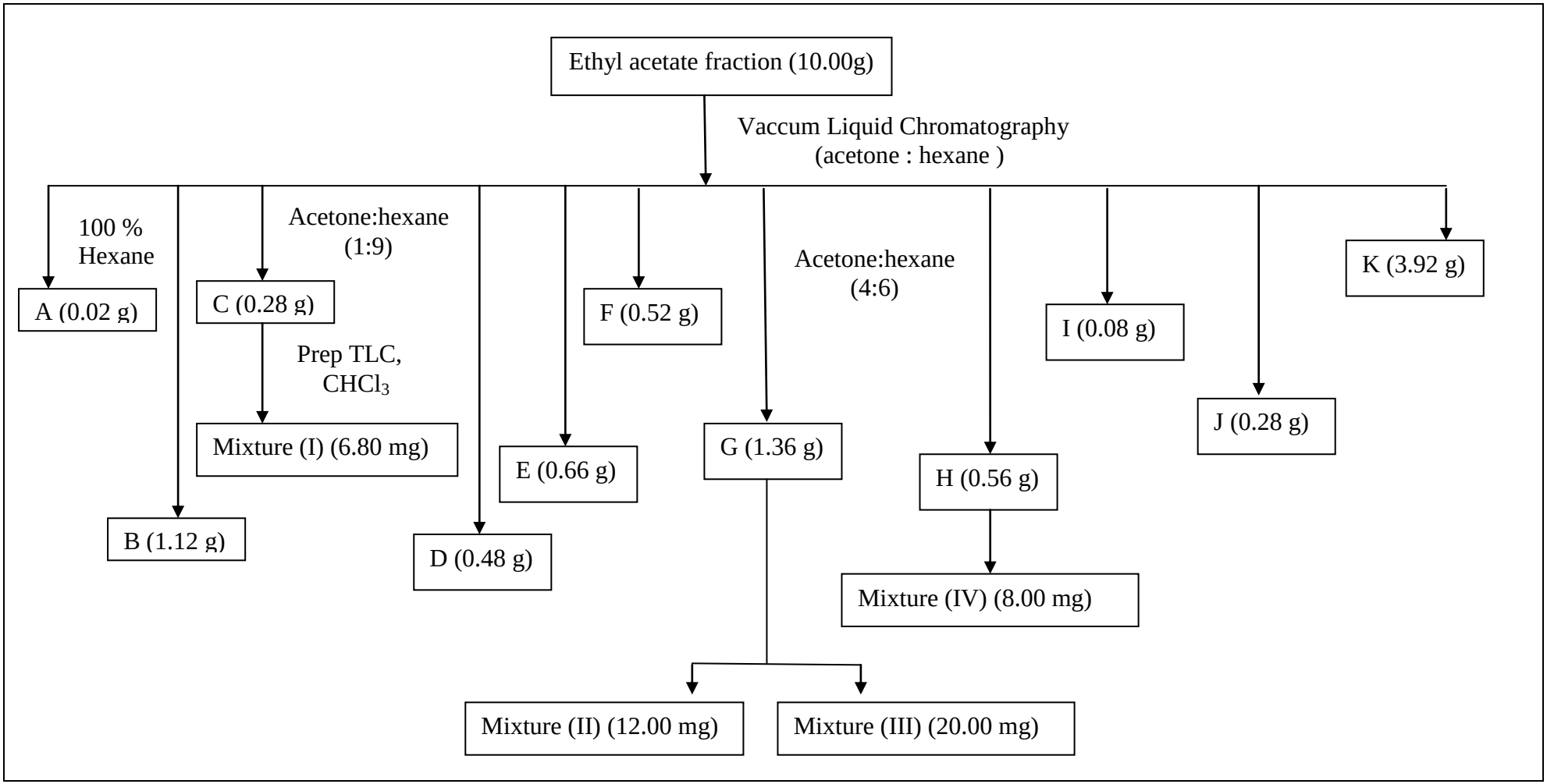

Figure 3.2 : Isolation of mixtures I – IV from the active ethyl acetate fraction of *P. oleracea*