CHAPTER 3

METHODOLOGY

3.1 Plant materials

The leaves and rhizomes of *Tacca integrifolia* were collected from the Field Study Centre, Gombak, Selangor and its taxonomic identification was verified by plant taxonomist Professor Dr. Ong Hean Choi from Institute of Biological Sciences, Faculty of Science, University of Malaya.

Figure 3.00. The leaves of *Tacca integrifolia*

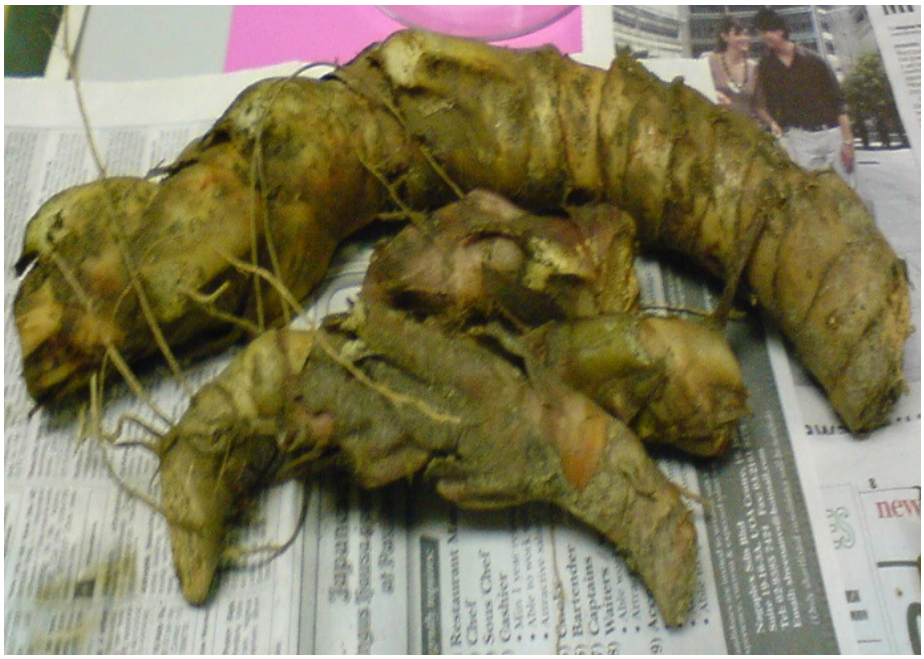

Figure 3.01. The rhizomes of *Tacca integrifolia*

3.2 Extraction of plant chemical compounds

The leaves and rhizomes of *Tacca integrifolia* were cleaned and dried in the oven at 30°C - 40°C for approximately 3 days. Dried plant materials were ground to powder form by using Continuous Chinese Herbs Grinding Machine and it was stored for further study. The extracts of hexane, petroleum ether, methanol, and chloroform were prepared by using Soxhlet apparatus (Figure 3.02). Soxhlet extractor is the most widely used leaching apparatus that extracts solid samples to a liquid phase using the technique commonly known as "solid-liquid extraction" (Castro et al., 2010). The high temperature used in this system is to provide heat to the distillation flask in order to start the extraction (Castro et al., 2010).

The ground sample was place in the thimble and extracted with 250ml of solvent in boiling flask at 40°C. When the extraction solvent was heated, the vapors condensed through the condenser and the solvent drips into the thimble contain grind sample, cause the extraction to start and the process continue until the extraction drips

into the boiling flask (Handa, 2008). The extractors were left until changes in solvent color that indicated the extraction is completed. Extractions were repeated using different solvent in order from solvent with lowest polarity followed by more polar solvent. The polar solvent will extract out polar compound and non-polar solvent extract out non-polar compound. The first solvent used in extraction was hexane, followed by petroleum ether, chloroform and methanol. The glass wool was placed in the thimble before placing the ground sample in order to prevent the sample from dripping into the solvent extractor.

The water extract was prepared using maceration method. 200g powder of ground leaves and rhizome were added separately in 1L conical flask followed by addition of 500ml distilled water and the mixture were left at room temperature approximately for 3 days (Handa, 2008). Extraction was carried out without adding any heat to avoid compound destruction (Rawani et al., 2011). All the extractions were evaporated under vacuum at 40°C by using vacuum rotary evaporator (Figure 3.03) to obtain concentrated extract. The extract were transferred into air tight amber bottles and stored at 20°C.

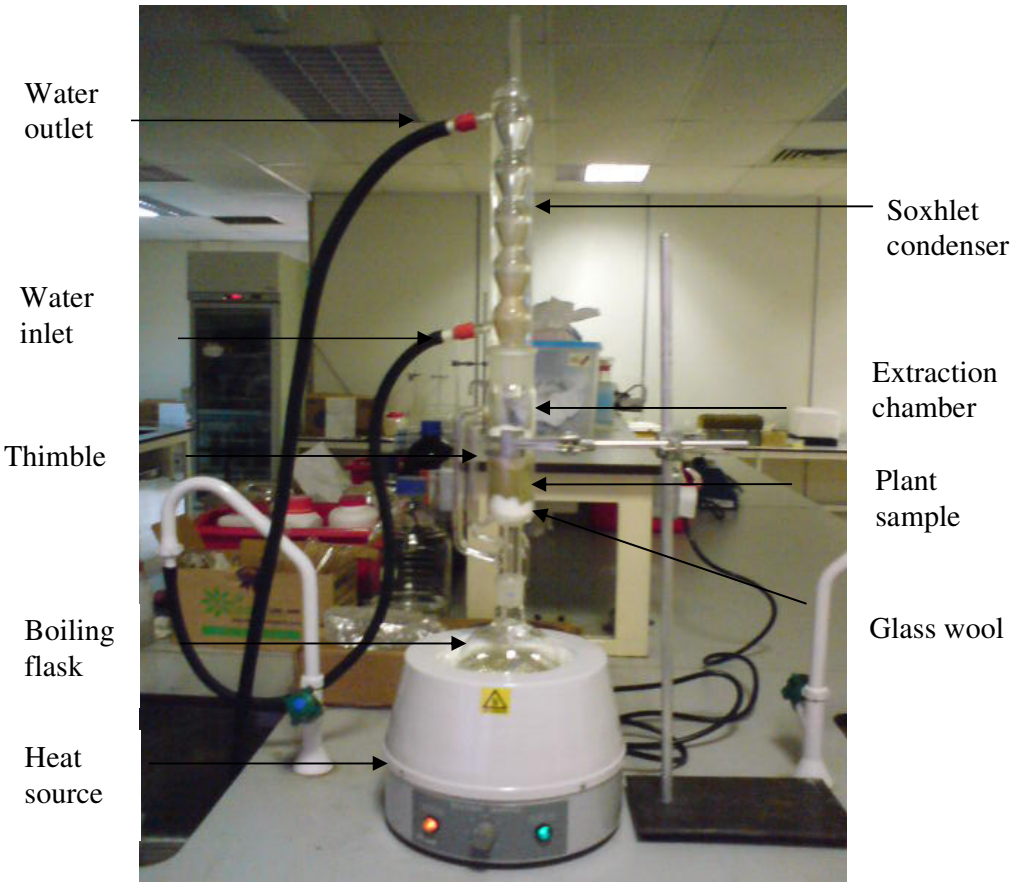

Figure 3.02. The soxhlet apparatus

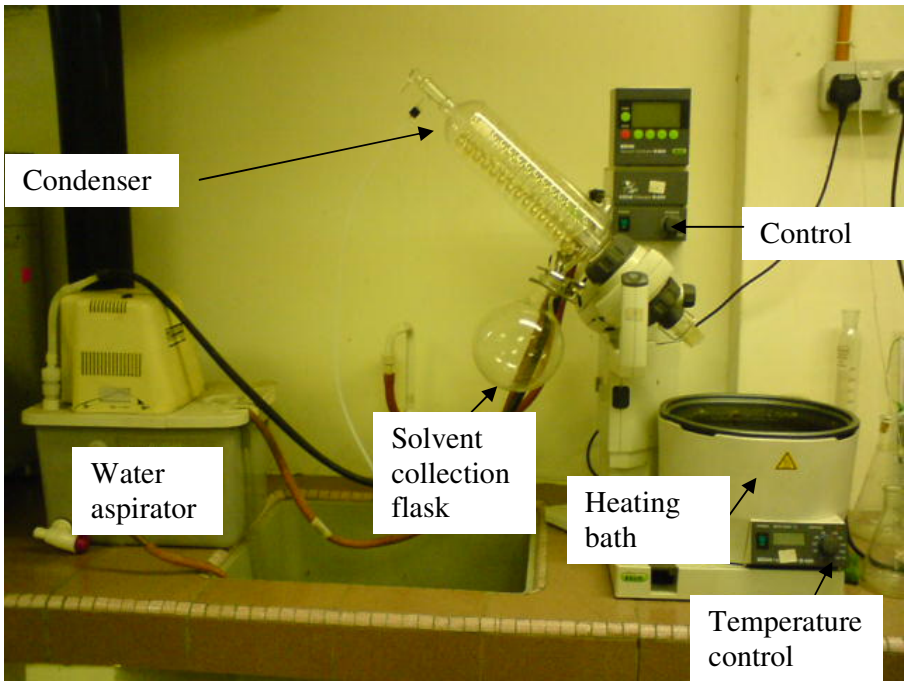

Figure 3.03. The vacuum rotary evaporator

3.3 Separation and isolation of chemical compounds

Chromatography is used widely as a separative technique and analytical technique (Hamilton et al., 1977). It consist of various methods to separate, isolate, identify and quantify the components in a test sample (Fried, 1999). Difference in the equilibrium distribution (*K*) of the components between mobile phase and stationary phase provide separation of components in a mixture (Hamilton et al., 1977). Differences in migration rates in the sample during distribution process is the factor that contribute in the separation of chemical compounds (Fried, 1999).

3.3.1 Thin Layer Chromatography (TLC)

In the late 1950s, the term 'thin layer chromatography' was created by Egon Stahl in Germany (Fried, 1999). Thin layer chromatography is a liquid chromatographic separation process in which started by application of a small spot of test sample at the origin of a thin sorbent layer usually made of silica gel, aluminum oxide, or cellulose, supported on a glass, plastic, or metal plate (Hahn-Deinstrop, 2007; Fried, 1999). This layer of adsorbent is known as the stationary phase while the mobile phase will pass through it by capillary action (Fried, 1999). The stationary phase is a polar phase and contained 10-50 µm particle while the mobile phase is a non- polar phase (Fuchs et al., 2011).

The mobile phase use can be a single solvent or a mixture of solvents that can be organic and/or aqueous solvents as long as the solvents chosen are match and suitable to the nature of the analytes and sorbent layer being used. The selection of mobile phase is generally done by trial and error based on the analyst's experience and literature review. There are variety of sorbents have been used, including silica gel, cellulose, alumina, polyamides, ion exchangers, and chemical bonded polar and nonpolar phases, coated onto a suitable support. The mobile phase contends with the chromatographed substance for sorbent sites. A polar substance will require a polar solvent to migrate on a silica gel or alumina adsorbent layer while a stronger solvent would increase retention factor (R_f) value. TLC is a favorite choice to run analytical technique and preparative technique (Fried, 1999).

In this study, TLC plate (Silica gel 60 F_{254} sheet) of size 20cm x 20cm and 10cm x 2cm was used. A line was drawn with a pencil, across at the bottom and at the top of TLC plate to mark the origin (bottom) and the end line (top) of the plate (Figure 3.05). A small amount of respective solvent was poured in to a TLC chamber (Figure

3.04) approximately less than 1 centimeter in depth. Mobile phase used were chloroform, chloroform-ethanol (9.7:0.3) (Sherma, 2000), and buthanol-acetic acid-water (60:15:25) (Beug et al., 1981).

A spot of each leaves and rhizomes extract were applied to a TLC aluminum plate at the origin line with a capillary tube. TLC plates were place inside the TLC chamber contain solvent system and covered with watch glass. Different analytes ascend the TLC plate at different rates to achieve separation of compound. TLC plates were removed from the chamber after solvents have been risen up to ¾ of the plate (Fried, 1999).

The appearances of colour substances were viewed in daylight and detection of colourless substances was observed under short-wave and long-wave UV light (Fried, 1999). Each colour substances were marked with a pencil. TLC plates were place in the fume cupboard to let the solvents evaporated before sprayed with chemical reagents. Different chemical reagent were used to determined different chemical compound contain in leaves and rhizome extract. Reagents used were Dragendorff reagent, Vanillin-sulphuric acid, Anesaldehyde-sulphuric acid and iodine vapor. Dragendorff reagent was used to detected alkaloid by the observation of orange spots (Jia et al., 2009), Vanillin-sulphuric acid used to detected phenol by arising of pink and red colour (Sharma et al., 1998) and blue or purple colour that represent terpenoid, anesaldehyde-sulphuric acid was used to detected saponin that presence as black spot, essential oil as blue spot and flavonoids as red and other colour (Zschocke et al., 2000) while iodine vapor was used to detected unsaturated compound with double bond chain by observing the brown spot presence (Zschocke et al., 2000).

Distance travel by the compounds divided by the distance travel by the solvent in TLC plate was valued as the retention factor, or R_f, and were calculated as the formula below;

$$R_f = \frac{\text{Distance of spot center from start point (x) cm}}{\text{Distance of solvent front from start point (y) cm}}$$

The migration distances of substances on thin layer chromatograms are generally fixed as R_f values (Stahl, 1969). The larger the distance of the compound took on the TLC plate give larger R_f value is a compound with less polarity compared to compound with smaller R_f value and smaller distance its travel on the TLC plate because it interacts less strongly with the polar adsorbent on the TLC plate. The R_f values can provide distinct evidence to the identification of unknown compound.

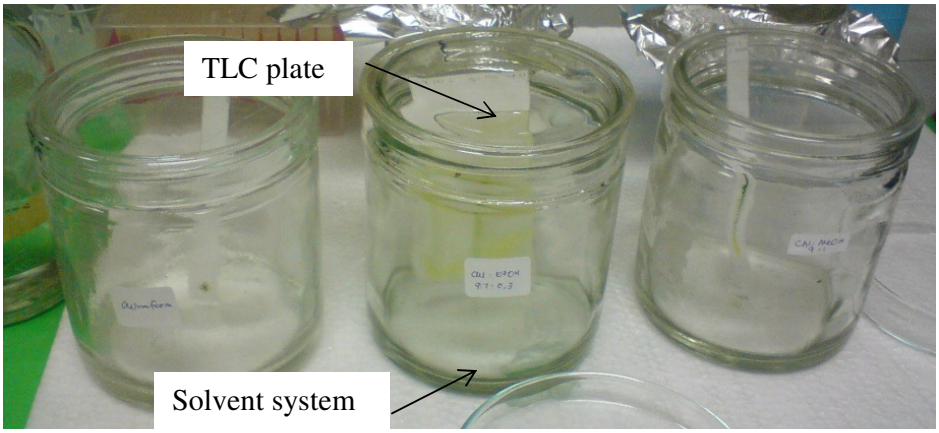

Figure 3.04. Thin Layer Chromatography (TLC) systems.

TLC system consists of solvent system as mobile phase and TLC plate as stationary phase. TLC for each extracts were developed to separate chemical compounds in *Tacca integrifolia* extracts.

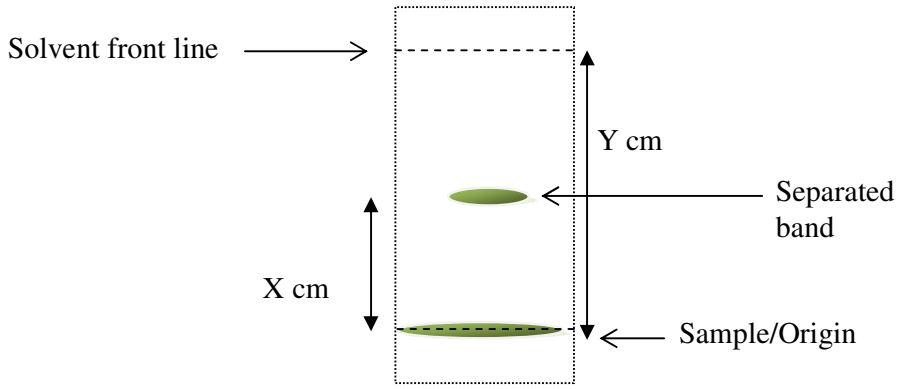

Figure 3.05. Diagram of TLC plate

3.3.2 Column Chromatography (CC)

Mixture of compounds was purified using Column chromatography to obtain individual chemical compounds. An ordinary preparative column chromatography is a glass tube diameter from 5 mm to 50 mm, height between 50 cm to 1 m and a tap at the bottom.

A Silica gel 60 was used as a stationary phase. On its powder form, it was mixed with solvent. The mixture was poured into the column and air bubbles were avoided. Glass wool was place inside the column to prevent stationary phase being washed out of the column. Buthanol, acetic acid and water (60:15:25) were used as mobile phase (Gocan et al., 1996). 100 µl of water extract from leaves and rhizome of *Tacca integrifolia* were loaded separately at the top of the packed column. Samples were eluted with solvent system at flow rate of 1ml per minute (Figure 3.06). 20 fractions were collected in specimen tubes with 2ml for each fraction and place in fume cupboard for evaporation. Dry weight were measured and Thin layer chromatography (TLC) for each fraction were done to select fraction that contain chemical compound thus determine the compound presence.

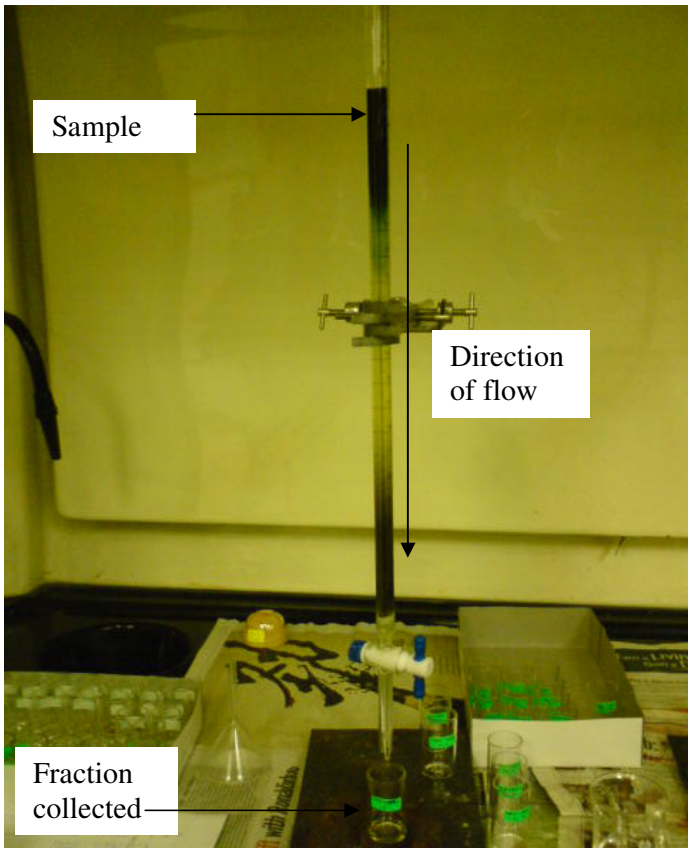

Figure 3.06. The column chromatography

3.3.3 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is an advanced chromatographic technique that provide separation of a mixture of compounds and is commonly used to identify, quantify and purify the individual components of the mixture in biochemistry and analytical chemistry . Chromatographers and pharmaceutical industry used HPLC for the accurate and precise chemical analysis of various drug from nature (Gupta et al., 2008). The HPLC column are reusable without regeneration and the resolution achieved are far exceeds compared to the older methods are the advantages of HPLC. The analysis times of a sample are generally shorter and preparative liquid chromatography are possible on a larger scale

(Hamilton et al., 1977). Other than that, the technique is less dependent on the operator's skill and its reproducibility is greatly improved while the HPLC instrumentation lends itself to automation and quantization. Process-scale HPLC as a purification tool has been increased as the demand for phytomolecules has increased. HPLC has features such as high resolving power, fast separation, continuous monitoring of column effluent, qualitative and quantitative measurements and isolation and automation of analytical procedures and data handling (Gupta et al., 2008).

Chemical compounds were separated by passing through the mobile phase that contains a mixture of the components through the stationary phase that contain a column packed of solid particles. Physical and chemical forces between the solute and the two phases cause a retention on the chromatographic column as there is differences in the magnitude forces, resulting the resolution and separation of the individual solute. Distribution of solutes between the two phases causes the separation to occur (Gupta et al., 2008).

Chromatography were categorized according to the mechanism of separation which are adsorption chromatography, partion chromatography, ion exchange chromatography, size exclusion chromatography and affinity chromatography where HPLC has mainly focused on adsorption and partition chromatography (Gupta et al., 2008). There are two modes of analysis that depends on the operation techniques known as isocratic and binary gradient (Gupta et al., 2008). In isocratic analysis, mobile phase composition remains constant during the elution process while the composition of mobile phase continuously change in binary gradient analysis (Gupta et al., 2008). HPLC with stationary phase is more polar than the mobile phase is named normal phase HPLC while reversed phase HPLC (RP-HPLC) has a polar mobile phase compare to stationary phase, thus most of the biomedical substances were separated by reversed

phase HPLC using mobile phase such as methanol, acetonitrile and mixture of buffer (Gupta et al., 2008).

HPLC instrument used in this study was a reverse phase HPLC system (RP-HPLC) from Shimadzu with C-18 column (Figure 3.07). It consist of 2 mobile phase; mobile phase A that connected to Pump A, and mobile phase B that connected to Pump B, Diode Array Detector (DAD) (Shimadzu SPD-MIDA VP) 250nm and System controller that link to a computer. HPLC with RP C-18 column separate difference classes of bioactive compound including phenolic compounds (Dai et al., 2010).

HPLC is a commonly used method in natural products separation thus its development in chemical analysis has combine the chromatographic separation system on-line with a spectroscopic detector, thus information of analytes present in sample such as its chemical structure are easily obtain leading us to identify the target or unknown bioactive compounds (Tierney et al., 2010).

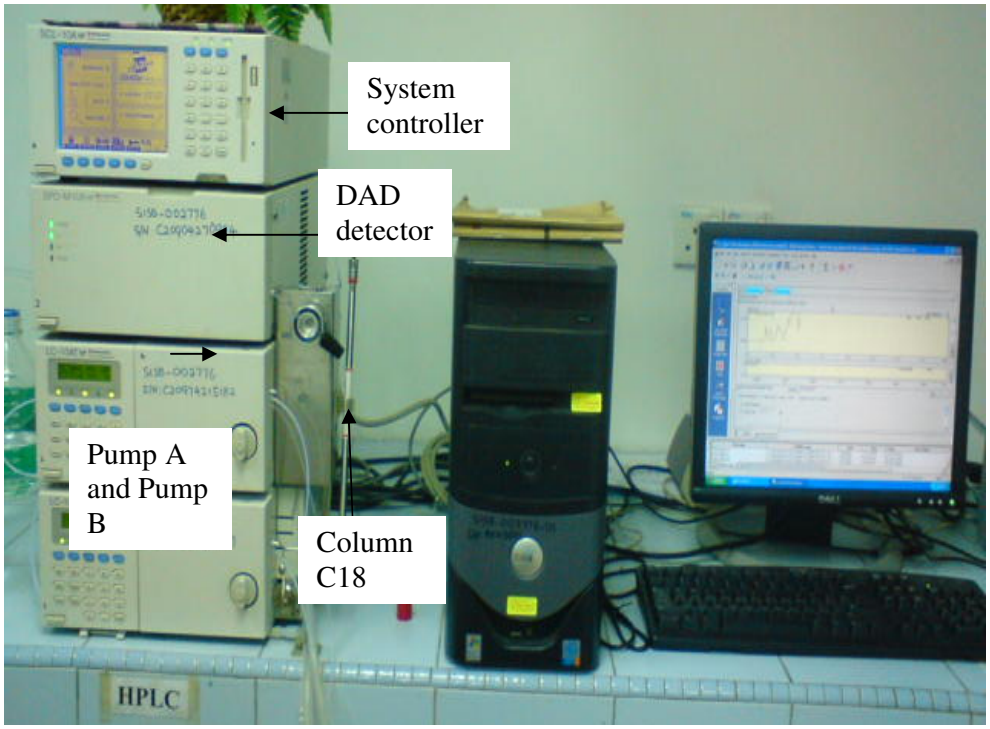

Figure 3.07. High Performance Liquid Chromatography (HPLC)

3.3.3.1 Detection of standard phenol and flavonoid

10mg/ml standard of gallic acid and tannic acid were dissolved in HPLC pure water and 10mg/ml standard of quercetin was dissolved in methanol HPLC grade. All standard and mobile phase were filtered through 0.45µm regenerated cellulose membrane filter and mobile phase were sonicated before use. Mobile phase used were water-acetic acid (97:3) as mobile phase A and methanol HPLC grade as mobile phase B. Standard compound were detected at 280 nm with isocratic analysis (Chen et al., 2001). Retention time detected from RP-HPLC analysis from each standard compound was further used to compare with RP-HPLC analysis of sample.

3.3.3.2 Separation of chemical compounds in extract of *Tacca integrifolia*

10mg/ml of extract were prepared and filtered using regenerated cellulose (RC) membrane filter 0.45µm before they were tested with solubility test using 0.1% acetic acid in pure water as mobile phase A and methanol HPLC grade as mobile phase B (Chen et al., 2001). Solubility test are compulsory before sample were injected into the RP-HPLC systems. Samples that are not soluble in mobile phase will not be injected as this is to prevent the damage to the C-18 column and damage to HPLC equipment. Only chloroform extract, methanol extract and water extract from both leaves and rhizome of *Tacca integrifolia* were soluble in mobile phase and injected to the column. Mobile phase A and B were filtered with membrane filter paper 0.45µm using solvent filtration kit (Figure 3.08), followed by degassing in sonicator (Figure 3.09) for one hour to remove air bubbles.

HPLC were switch on starting with Pump A, Pump B, Diode Array Detector (DAD), system controller and computer. After the LabSolutions for High Performance Liquid Chromatography (LC) software were connected to the system, mobile phase were place in solvent reservoir and purging for 2 minutes to detect the presence of air bubble if any. HPLC analysis only can be started after the mobile phase is free from air bubbles. Instrument parameters were set up, save and download to system controller. Instrument was switch on and left to warm up for 30 minutes to stabilize the pressure of the system. 10µl from 10mg/ml of extracts from leaves and rhizome of *Tacca integrifolia* were injected through Octadecylsilane (ODS) Column C18 separately, with flow rate at 0.8 ml/min. Compound were detected at 280nm in isocratic analysis (Chen et al., 2001).

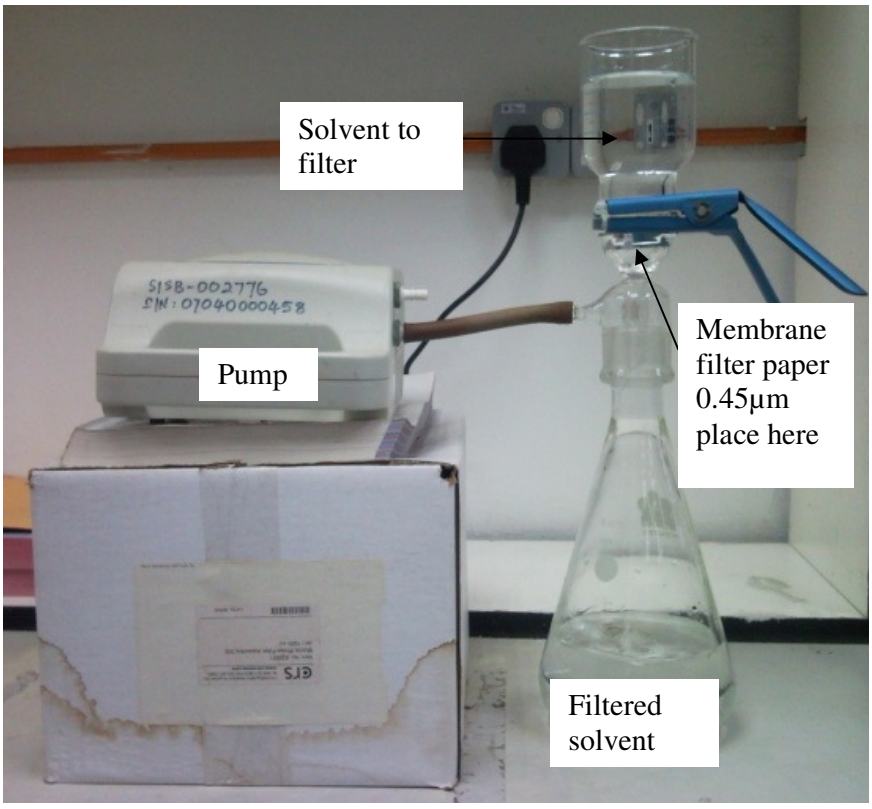

Figure 3.08. The Solvent Filtration Kit

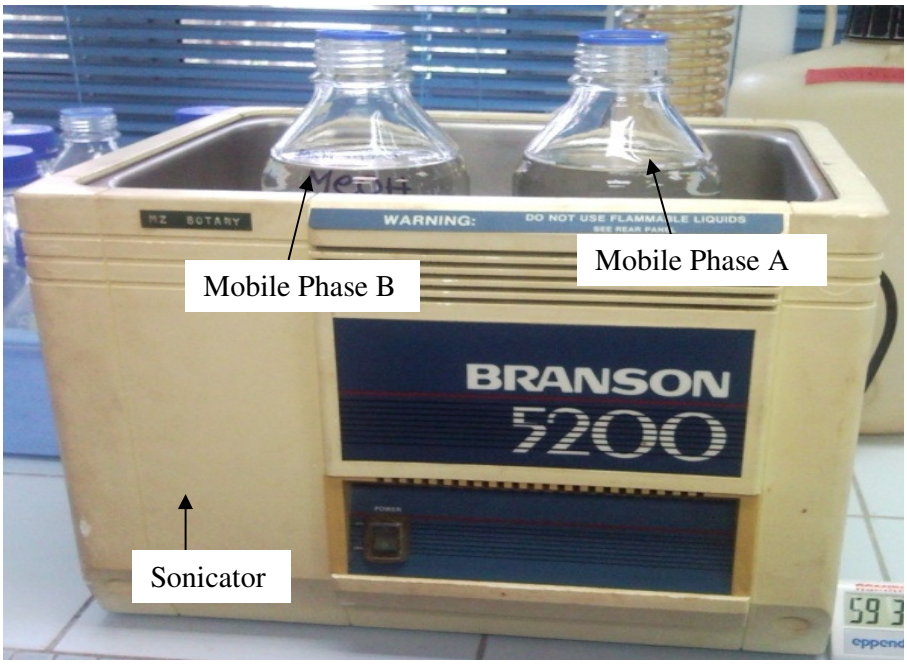

Figure 3.09. Degassing of mobile phases A and B using sonicator

3.3.3.3 Determination of standard Hippuric acid

Hippuric acid was freshly prepared in five difference concentration at 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and 31.25µg/ml. All samples were filtered with 0.45µm regenerated cellulose (RC) membrane filter paper followed by solubility test with a mixture of 0.5% trifluoroacetic acid (TFA) in water as mobile phase A and 0.5% trifluoroacetic acid (TFA) in acetonitrile as mobile phase B. 10µl of each sample were injected to HPLC through reversed-phase C18 column with flow rate set at 1ml/min in isocratic analysis (Meng et al., 1995).

3.3.4 Determination of chemical compounds in extracts of *Tacca integrifolia* using Liquid Chromatography Mass Spectrometry combined with Mass Spectrometry (LCMS/MS)

Liquid Chromatography has become an important separation tools in many research area including biomedical applications and natural products (Bocxlaer et al., 2000). Mass Spectrometry is a technique that used to identify unknown compound including identifying quantification of pesticides in water samples, steroids in athletes and many various important area (Bramer, 1997). Liquid Chromatography- Mass Spectrometry (LCMS) is a combination instruments that are upgraded in speed of development, its acceptance and high spread (Niessen, 1999). While LCMS/MS has been a preferred method in drug metabolism studies as it's offered a very high selectivity and sensitivity (Little et al., 2006). This technique that has established in many research areas are advanced in ionization approaches especially in atmospheric pressure (Bocxlaer et al., 2000). This upgraded instrument is an important tool to support analytical analysis for drug development in pharmaceutical industry, thus it is also a favorite choice of instrument in environmental analysis, biochemical and biotechnological applications and other fields of application (Niessen, 1999).

In pharmaceutical industry, LCMS used virtually in every stage of drug development as its sensitivity, selectivity and speed has been an important factor (Niessen, 1999). Compared to Photodiode Array detector (PDA), LCMS has enhanced its confirmation of identity, selectivity, ease of operation and has achieved its level of automation (Niessen, 1999).

In determination of chemical compounds of *Tacca integrifolia*, extracts has been fully screen with AB Sciex 3200QTrap LCMS/MS and fully scan with MS/MS data collection with negative ionization mode. Phenomenex Aqua C18 (50mm x 2.0mm x 5µM) was used as a column in a rapid screening at 15 minutes run time. Water with 0.1% formic acid and 5mM ammonium formate were used as buffer A and a mixture of

acetonitrile with 0.1% formic acid and 5mM ammonium formatted as buffer B. Sample were run with gradient mode; 10% A to 90% B from 0.01 minute to 8.0 minute and were held for 3 minutes and back to 10% A in 0.1 minute and re-equilibrated for 4 minutes. Pre-run equilibration time was 1.0 minute. 1 ml of water extract and methanol extracts were diluted with 5ml methanol separately, while 1ml of chloroform extract were diluted with 1ml acetonitrile and 4ml methanol. 1 ml hexane extract and 1 ml petroleum extract were diluted with 1ml dichloromethane, 1ml acetonitrile and 3 ml methanol separately. All samples were appropriately diluted and filtered with 0.22µM nylon filter. Injection volume for all samples was 20µL.

3.4 Detection of chemical compounds

Chemical compounds isolated from column chromatography and thin layer chromatography (TLC) were observed in visible light and in UV light for any colour presence and TLC plate were dried in fume cupboard before were sprayed with different chemical reagents to detect different chemical compound present.

3.4.1 Visible light

After TLC plate has been removed from TLC chamber, TLC plates were observed for any colour presence. Each band marked with pencil and R_f value were calculated and recorded. Colour that usually detected was green and yellow that shows the presence of chemical compounds.

3.4.2 UV light

TLC plates were observed under a short wavelength ultraviolet (UV) lamp to detect UV absorbing and aromatic compound. The appearance of green fluorescence spot indicates the presence of aromatic compound such as phenolic, quinine and flavonoid.

3.4.3 Dragendorff reagent

Dragendorff reagent was used widely to detect the presence of alkaloid in Thin Layer Chromatography (TLC) of plant extracts (Jia et al., 2009). The mixture of 0.8 g of Bismuth nitrate ($BiONO_3$) in 10 ml of glacial acetic acid (HAc) was diluted with distilled water to obtain solution A. Solution B was prepared by dissolving 20g Potassium Iodide (KI) powder to 50 ml distilled water in brown volumetric flask. To prepare 100 ml of Dragendorff reagent, 5 ml of solution A and 5 ml of solution B was added in 100 ml brown volumetric flask and mixture was diluted until 100 ml solution (Jia et al., 2009). Dragendorff reagent was stored in covered brown bottle (Urban-Morlan et al., 2008).

TLC plates that have been removed from TLC chamber were placed in the fume cupboard to allow the solvent to evaporate before they were sprayed with fresh preparation of Dragendorff reagents. The reagent reacts with alkaloid to produce an orange spots. Orange spots were mark with pencil and R_f value were calculated. To maintain its freshness, Dragendorff reagent discarded after 48 hours (Urban-Morlan et al., 2008).

3.4.4 Vanillin-Sulphuric acid reagents

Vanillin reagent was used to detect phenolic compound (Sharma et al., 1998). Fresh vanillin-sulphuric acid reagent was prepared by dissolving 0.5 g vanillin in 100 ml sulphuric acid-ethanol (40:10) (Sharma et al., 1998).

TLC plates were removed from TLC chamber and dried in fume cupboard before sprayed with vanillin-sulphuric acid reagent. Then the plates were heated on hot plate at 100°C. The formation of pink and red colour showed the presence of phenol while purple colour presence terpenoid.

Vanillin-sulphuric acid reagent used to detect phenolic compound such as pyrogallol, phloroglucinol, resorcinol, catechin and epicatechin, and cinnamic acid after heating in hot plate 120°C for 20 minutes (Sharma et al., 1998).

3.4.5 Anisaldehyde-sulphuric acid

A mixture of 0.5 ml anisaldehyde, 10 ml glacial acetic acid (HAc), 85 ml methanol (MeOH) and 5 ml concentrated sulphuric acid (H_2SO_4) were prepared freshly prior to use (Zschocke et al., 2000)

TLC plate were sprayed with anisaldehyde-sulphuric acid in the fume cupboard and heated to 110°C until colour presence. Black spot colour indicates saponin, blue colour indicates the presence of essential oil and red and other colour indicate the presences of flavonoid.

3.4.6 Iodine vapor

TLC plates that have been removed from TLC chamber were place in a chamber that contain crystal iodine and was left for a few minutes. The brown spot detect the presence of double bond chain chemical compound and unsaturated compound (Zschocke et al., 2000).

3.4.7 Saponin froth test

The ground dried leaves and rhizome of plant sample was extracted with methanol, respectively and were left overnight. Extractions were filtered and 1 ml of the filtered sample was transferred into a small test tube. 5 ml of distilled water was added and the mixture were shaken vigorously for 30 seconds and allowed to stand at room temperature. A formation of a stable froth over a period of 30 minutes indicates the presence of saponin.

3.4.8 Tannin and phenolic compounds

The ground dried leaves and rhizome were extracted separately with methanol analytical grade for overnight. Extractions were filtered and 2 ml of the filtrate was transferred into test tube. 6 drops of 1% Ferric chloride ($FeCl_3$) solution was added and changes in colors were observed. Methanol leaves extract that was in green colour changes to dark green while methanol rhizome extract that was brown colour was changes to dark brown. Color changes indicated the presence of tannin and phenolic compounds.

3.5 Determination of the total phenolic contents

Determination of total phenol content in extracts of *Tacca integrifolia* was initially started by adding 500 µl samples to the mixture of 5ml Folin Ciocalteu reagent; water (1:10) and 4ml 1M sodium carbonate solution. Mixtures were incubated at 45°C for 15 minutes in water bath. Absorbance was read at 765nm. Gallic acid was used as an internal standard for the calibration curve (Hsouna et al., 2011). The total phenolic content was expressed as mg of gallic acid equivalents (mgGAE/g) of dry sample using the linear equation based on the calibration curve (Hsouna et al., 2011).

3.6 Determination of total flavonoid contents

Total Flavonoid content was determined using Quercetin as standard. 1 ml diluted extracts were mix with 0.3ml 5% sodium nitrate and was incubated for 5 minutes in water bath at 37°C. 0.3ml 10% aluminum chloride was added to the mixture and was left incubated for 6 minutes in water bath at 37°C. 2ml of 1M sodium hydroxide and 10 ml distilled water were added to the mixture before absorbance reading was taken at 510 nm.

3.7 Angiotensin Converting Enzyme (ACE) Inhibition Activity

ACE inhibition assay is *in vitro* method that was used to determine the activity of ACE enzyme in the experiment thus to calculate the percentage of inhibition of ACE enzyme by the extract from leaves and rhizome of *Tacca integrifolia*. In the search for new antihypertensive agents from medicinal plants, inhibition of ACE will be one of the effective screening methods besides using animal model.

The plant extracts were screened for ACE-inhibitory activities using purified ACE extracted from rat lung and Hippuryl-L-Histidyl-L-Leucine (HHL) as substrate. The *in vitro* inhibitory activities of the ACE were determined by spectrophotometric method. It is based on the hydrolysis of HHL in the presence of ACE to produce Hippuric acid (HA) and histidyl-L-leucine (HL) as byproducts (Figure 3.10) (Wu J. et al, 2002). The absorbances of hippuric acid extracted into ethyl acetate were measured using spectrophotometer at 228 nm (Wu J. et al, 2002). The extent of hippuric acid release is directly related to the ACE activity (Meng et al., 1995).

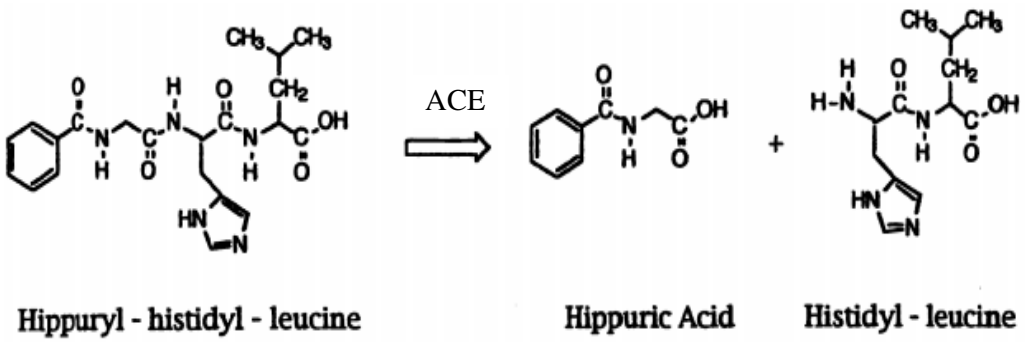

Figure 3.10. Hydrolysis of the substrate Hippuryl- Histidyl- Leucine by angiotensin converting enzyme (ACE) (Mehanna et al., 1999).

3.7.1 Preparation of Angiotensin Converting Enzyme (ACE) enzyme extract

10 ICR-mice were collected from Animal House, University of Malaya. They were killed using dislocation of the neck, and were immediately dissected to collect the lungs. Lungs collected were immediately rinsed using 0.05 M phosphate buffer pH8.3 before homogenized using homogenizer, followed by centrifugation at 400rpm for 10 minutes to obtain ACE enzyme.

3.7.2 Angiotensin Converting Enzyme (ACE) assay

Extract of leaves and rhizome of *Tacca integrifolia* were prepared in five difference concentration which are 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml. ACE inhibition activity was measured using spectrophotometric assay of the reaction of hippuric acid, a product of HHL hydrolysis according to the Cushman and Cheung (1971) with slight of modification (Choi et al., 2001). HHL is a synthetic substrates used as substitute of ANG I as it hydrolyzed 20 times more rapid compare to ANG I with the same amount (Meng et al., 1996).

A mixture of 0.25 ml 100 mM phosphate buffer pH8.3, 0.25 ml 300 mM sodium chloride (NaCl), 0.25 ml 5 mM HHL and 0.25 ml sample were added in test tube. 0.25 ml crude enzymes were added into the mixture and were incubated in water bath at 37°C for 30 minutes. The reaction then was terminated by adding 0.25 ml 1 N hydrochloric acid (Lahogue et al., 2010). 2 ml ethyl acetate was added to each test tube and respective test tubes were vortex for 5 seconds. The top layer was removed using Pasteur pipette and were placed into the crucible and left in fume cupboard to dryness. 3 ml of distilled water was added and mixture was transferred into quartz cuvette. The absorbance was measured at 228 nm using spectrophotometer and the percentage of ACE inhibition of the standard and samples were determined using the formula below:

$$\text{Percentage of ACE inhibition (\%)} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

Percentage of ACE inhibition and ACE activity of the plant extract were compared with standard of captopril.

3.7.3 Preparation of Hippuric acid (HA) as standard curve

Standard solution of Hippuric acid was prepared in five difference concentration which are 1.9 µg/ml, 3.8 µg/ml, 7.5 µg/ml, 15 µg/ml and 30µg/ml. Absorbance were read in triplicate at 228nm to obtain mean absorbance and standard curve were plotted in a graph before the equation was used in the calculation of ACE activity.

3.8 Animal Study

3.8.1 Sub-Acute Toxicity test of water extracts from leaves and rhizome of *Tacca integrifolia* on Spontaneously Hypertensive Rats (SHR)

The oral toxicity test of water extract from leaves and rhizome of *Tacca integrifolia* was studied according to Organization for Economic Cooperation and Development (OECD) Test Guideline 423 with Animal Care and Use Committee (ACUC) ethic number ISB/10/11/2008/JM(R) approved by Animal House, University of Malaya. This study has been conducted to characterize the potential toxic effects of water extract from *Tacca integrifolia*.

21 female spontaneously hypertensive rats (SHR) with body weight between 100-200g supplied by Animal House, University of Malaya, were acclimatized for 2 weeks before experiment. The SHR rats were divided into 7 groups which are (i) control SHR group; (ii) low dose (50mg/kg); (iii) medium dose (100mg/kg); (iv) high dose (500mg/kg) for water leaves extract of *Tacca integrifolia* and (v) low dose (50mg/kg); (vi) medium dose (100mg/kg) and (vii) high dose (500mg/kg) for water rhizomes extract of *Tacca integrifolia*. Water extract from leaves and rhizomes of *Tacca*

integrifolia were given orally on a daily basis using feeding needle and fed between 9.00 am to 12.00 noon. All SHR rats were caged individually.

Clinical signs of the SHR were observed at interval of 30 minutes, one hour, and three hours on the first day and once a day until 28 days. Body weights were measured before dosing, on days 7, 14, 21 and day 28. SHR were fast approximately 12 hours before sacrificed using diethyl ether. Blood serums were taken to perform liver function test and renal function test.

3.8.2 Spontaneously Hypertensive Rats (SHR) Treatment of hypertension using water leaves extract and water rhizome extract of *Tacca integrifolia* compared with Captopril as positive reference standard

Animal used in this study were Spontaneously Hypertensive Rats (SHR) supplied from Animal House, University of Malaya with an Animal Care and Use Committee (ACUC) ethic number ISB/10/11/2008/JM(R). This study has used 8 male Sprague Dawley rats as control normal and 56 male SHR with body weight between 180-190g that were acclimatized for 2 weeks before experiments. SHR were divided into 7 groups which are (i) control group of SHR given normal saline (Ctrl); (ii) standard group given low dose of captopril as positive reference standard (50mg/kg); (iii) high dose of captopril (100mg/kg); (iv) low dose of water leaves extract (50mg/kg); (v) high dose of water leaves extracts (100mg/kg); (vi) low dose of water rhizomes extract (50mg/kg) and (vii) high dose of water rhizome extracts (100mg/kg). Daily administration were given orally at dose of 5ml/kg/day (Wang et al., 2008). Treatment took duration for 28 days and SHR body weights were measured from day 0 and every week until before they were sacrificed. Captopril, water leaves extract and water rhizomes extract were given orally using feeding needles between 9.00 am to 12 noon. Systolic blood pressures were measured every week via tail cuff method using PowerLab instrument in conscious, lightly restrained rat (Ibrahim et al., 2005). SHR rats

were fast approximately 12 hours before sacrificed. SHR were sacrificed using diethyl ether and blood serums were taken for liver function test and renal function test purposes.

3.9 Antioxidant activity

3.9.1 DPPH Radical Scavenging Activity

Singlet electron in purple-coloured free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) paired with a hydrogen atom coming from a potential antioxidant will form a yellow-coloured diphenylpicrylhydrazine (Atmani et al., 2009, Kirby et al., 1997). Figure 3.11 showed the structure of free stable radical DPPH. Radical-scavenging activity of different extracts were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as reagent according to the method of Hsouna and Trigui (2011) with some modifications as in Table 3.00. The stock solution of DPPH was freshly prepared prior to use by weighing 3.94mg of DPPH stock dissolved in 10 ml of methanol to obtain 0.001M of stock solution of DPPH. The stock solution was kept in flask wrapped with aluminum foil.

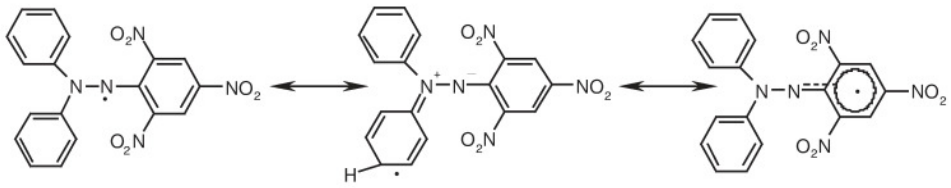

Figure 3.11. The structure of the free stable radical DPPH (Ionita, 2005).

5mg of stock ascorbic acid was dissolved in 5 ml methanol to obtain 1 mg/ml stock solutions. Ascorbic acid stock solution was kept in flask wrapped with aluminum foil. A stock of sample extracts from leaves and rhizomes of *Tacca integrifolia* were prepared in 1mg/ml.

3.9.1.1 DPPH radical scavenging activity of standard ascorbic acid

Stock solution of DPPH and ascorbic acid were mixed according to the Table 3.00. Reaction mixtures were incubated at room temperature and were left for 30 minutes to allow reaction. All mixtures were covered with a box wrapped with aluminum foil and lights were switch off to avoid oxidation by direct sunlight and light. Absorbances reading were taken in triplicate after 30 minutes using ELISA at 517nm wavelength. Discoloration of purple colour to yellow colour was observed and the presence of free radical compound was suggested. The lower absorbance reading indicates higher free radical scavenging activity. A graph of standard curve of ascorbic acid was plotted.

Table 3.00. DPPH Radical Scavenging activity of standard ascorbic acid

Concentration of Ascorbic acid (µg/ml)	Volume of MeOH (µl)	Volume of Ascorbic acid (µl)	Volume of DPPH (µl)
Control	4000	0	500
2.5	3990	10	500
5.0	3980	20	500
10.0	3960	40	500
12.5	3950	50	500
37.5	3850	150	500
125	3500	500	500
250	3000	1000	500
500	2000	2000	500

3.9.1.2 DPPH radical scavenging activity of extracts from leaves and rhizome of *Tacca integrifolia*

The aim of DPPH radical scavenging activity of extracts was to determine the active extract that has a potential in scavenging the DPPH radical activity. The DPPH radical scavenging activity of leaves and rhizome extract were carried out as in Table 3.01.

Table 3.01. DPPH radical scavenging activity of extract of *Tacca integrifolia*

Concentration of sample µg/ml)	Volume of MeOH (µl)	Volume of sample (µl)	Volume of DPPH (µl)
Control	4000	0	500
2.5	3990	10	500
5.0	3980	20	500
10.0	3960	40	500
12.5	3950	50	500
37.5	3850	150	500
125	3500	500	500
250	3000	1000	500
500	2000	2000	500

Mixture of sample, methanol and DPPH were added as in Table 3.01 and were left incubated for 30 minutes at room temperature with the absence of direct sunlight. The absorbance reading was taken using ELISA at 517 nm and discoloration of purple colour to yellow colour was observed.

DPPH scavenging effect was calculated using equation as below:

$$\text{DPPH scavenging effect (\%)} = \frac{(OD_{control} - OD_{sample})}{OD_{control}} \times 100$$

Where $OD_{control}$ is the absorbance of the control reaction, OD_{sample} is the absorbance of the sample reaction (Adedapo et al., 2008).

Tests were carried out in triplicate.

A graph of percentage of DPPH inhibitions against concentration was plotted to determine IC_{50} value.

3.9.2 Ferric Reducing Power Assay (FRAP)

The Ferric Reducing Power Assay (FRAP) of extracts from leaves and rhizome of *Tacca integrifolia* was determined spectrophotometrically according to the Atmani et al. (2009). Experiment was started initially by adding 1 ml of sample plant extracts to the mixture of 2.5 ml of 2.0 M phosphate buffer pH6.6 and 2.5 ml of 1% (w/v) potassium ferricyanide. The mixtures were then incubated in water bath at 50°C for 20 minutes. 2.5 ml of 10% trichloroacetic acid (TCA) were added to the mixture before centrifuged at 1000rpm for 10 minutes. 2.5 ml of aliquot of upper layer was transferred into another test tube before added with 2.5 ml distilled water followed by addition of 0.5 ml 0.1% ferric chloride. The observation of colour changes was made and absorbance was read at 700 nm using methanol as a blank. Experiment was done in triplicate and result was compared with Butylated Hydroxyanisole (BHA) as reference standard.

3.9.3 Metal Chelating Assay

The aim of the assay is to evaluate the ability of each extracts to chelate ferrous ion and prevent the formation of ferrozine-Fe^{2+} complex. 0.0246 g of ferrozine stock was dissolved in 10ml deionized water in centrifuge tube and wrapped in aluminum foil prior to assay. Stock solution of $FeCl_2$ was prepared by dissolving 0.0397g of $FeCl_2$ stock in 10 ml deionized water and wrapped with aluminum foil prior to use.

Ethylenediaminetetraacetic acid (EDTA) was used as positive reference standard by preparing 0.1g/ml EDTA in deionized water. The pH was adjusted using sodium hydroxide (NaOH) until all EDTA was completely dissolved. Deionized water

was added until total volume of the solution reach 10 ml. Solution were mixed according to Table 3.02.

Table 3.02. Metal Chelating Assay of EDTA

Concentration of EDTA (mg/ml)	Volume of deionized water (ml)	Volume of EDTA (ml)	Volume of $FeCl_2$ 2mM (ml)	Volume of ferrozine 5 mM (ml)
Control	3.7	-	0.100	0.200
1	3.7	0.040	0.100	0.200
2	3.7	0.080	0.100	0.200
3	3.7	0.120	0.100	0.200
4	3.7	0.160	0.100	0.200
5	3.7	0.200	0.100	0.200

The mixtures were shaken vigorously and were left in the room temperature for 10 minutes. 30 µl of mixture was transferred into ELISA plate and absorbances reading were read at 562 nm. All samples were in triplicate and the absorbance reading were average out. Percentage of ferrozine-Fe^{2+} complex inhibition was calculated using formula below;

$$\% \text{ inhibition} = [(\text{Abs control} - \text{Abs sample})/\text{Abs control}] \times 100$$

Where, Abs control=absorbance of control

Abs sample= absorbance of sample

Extracts then were tested with difference concentration of 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml that were prepared in methanol. The solution and sample were mixed according to Table 3.03.

Table 3.03. Metal Chelating Assay of extracts of *Tacca integrifolia*

Concentration of sample (mg/ml)	Volume of deionized water (ml)	Volume of sample (ml)	Volume of $FeCl_2$ 2mM (ml)	Volume of ferrozine 5 mM (ml)
Control	3.7	-	0.100	0.200
1	3.7	0.200	0.100	0.200
2	3.7	0.400	0.100	0.200
3	3.7	0.600	0.100	0.200
4	3.7	0.800	0.100	0.200
5	3.7	1.000	0.100	0.200

The mixtures were shaken vigorously and left in room temperature for 10 minutes before transferred into ELISA plate. The absorbance reading was measured at 562 nm in triplicates using ELISA. The reading were average out and percentage of inhibition of ferrozine-Fe^{2+} complex each reading were calculated.

3.10 Brine Shrimp Lethality Assay (BSLA)

High doses of bioactive compounds are usually toxic. To investigate the cytotoxicity of plant extracts, Brine Shrimp Lethality Assay (BSLA) widely known as a convenient method to determine the lethal dose concentration of sample as preliminary analysis. Brine Shrimp is a small organism that was used as convenience monitor for screening in the discovery of new bioactive natural products. The brine shrimp assay is a very useful technique for the isolation of biogenic compounds from plant extracts (Manilal et al., 2009).

Preparation of BSLA analysis was started by dissolving 38g of sea salt in 1L of distilled water. The mixture was then filtered with Whatman Filter paper No1. 15cm. Prepared seawater were transferred into a small tank that were divided into two side, with one side covered with aluminum foil and another side was not covered.

1 g of *Artemia salina* (Linnaeus) cysts was aerated in the cover side of small tank contains sea water. Lamp was placed and switched on above the uncovered side of small tank to attract hatched shrimp through perforations in the dam. After 48 hours, the freshly hatched free-swimming nauplii were used for the bioassay. The brine shrimp assay has advantages of being simple, inexpensive and rapid. It is easily utilizes a large number of organisms for statistical validations, small amount of sample and with no special equipment.

Vials for testing were prepared with 3 vials for each concentration and test initially were at 1000, 100 and 10µg. Sample of extract from leaves and rhizome were weighed 20mg respectively before added with 2 ml of water to prepared stock of 20mg/2ml of each sample. 500,50,5 µl of the mixture were transferred into vials corresponding to 1000, 100, or 10 µg/ml, respectively. Sea water was added to each vial, followed by adding 10 shrimp per vial and the volume was adjusted with sea water until 5 ml/vial. 24 hours later, the number of survived shrimp was counted and recorded. The data was analyzed using Finney computer program to determined LC_{50} values and 95% confidence intervals for each sample.

3.11 Statistical analysis

Data collected are in triplicate and presented as average ± standard deviation (SD). The data were analyzed using analysis of variance (ANOVA). One-way ANOVA was used to evaluate the value of blood serum test taken from *in vivo* test and two-way ANOVA was used in analysis of body weight and systolic blood pressure measurement of spontaneously hypertensive rats, and in Ferric reducing power assay analysis. Post hoc test used was Tukey test to compare between means and determined significance at $p<0.05$. All statistical analysis was by using SPPS 14.0.