2.0 Materials and Methods

2.1 Plant materials

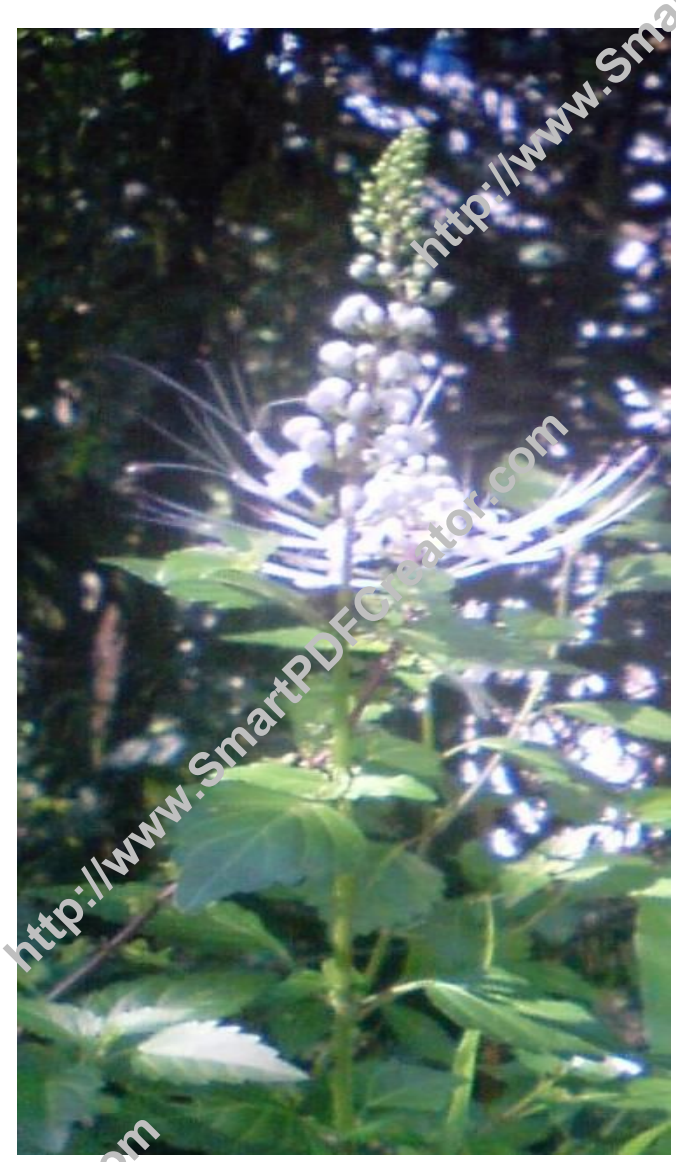

Fig.2.1. *O. stamineus*

Leaves of *O. stamineus* were collected from garden of Institute of Biological Sciences (ISB), Faculty of Science, University of Malaya (Fig.2.1). Taxonomic identification was performed and Prof. Ong Hean Chooi had confirmed it based on ethnobotanical literature.

2.2 Extraction of plant chemical compounds

The leaves of *O. stamineus* were cleaned and dried in room temperature for 48 to 72 hours. Dried plant materials which were cut into small pieces, were ground to powder form by using Continuous Chinese Herbs Grinding Machine and it was stored to further study. Crude extracts of methanol, chloroform and hexane were prepared by using soxhlet apparatus (Fig.2.2). They had to be heated in the flask to boiling point. Next, vapour was condensed in the reflux condenser. The hot liquid dripped into the plant sample. After the liquid in the extraction chamber reached at the top of the siphon tube, with any dissolved plant material, it flowed back into the heated flask. In the flask, plant materials were rapidly extracted and the soluble chemical components concentrated in the flask. Water extract was prepared by extracting the leaves powder in water bath for 4 hours at 100ºC. All the extractions were evaporated to dryness under vacuum at 40ºC by using rotary evaporator. The dried extracts which were obtained from several batches of *O. stamineus* were transferred into air-tight amber bottles and stored at 20ºC until use (Harborne, 1973).

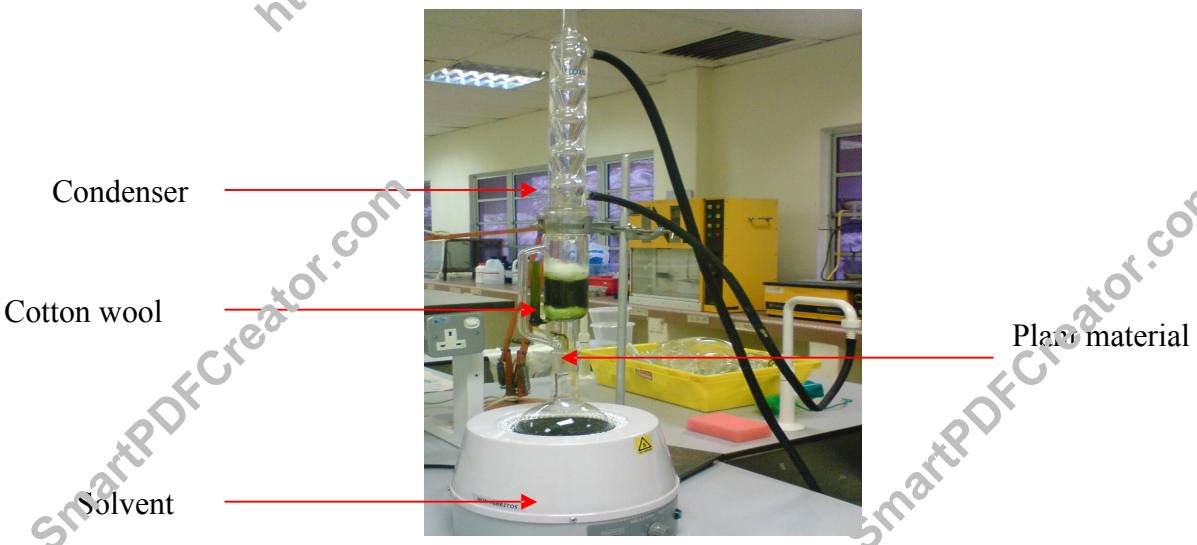

Fig.2.2.Soxhlet apparatus

2.3 Separation of chemical compound

Chromatography was a method that can be used in separation of two or more compounds or ions between two phases. Although there were many different variations of chromatography, it had the same principle.

A) Thin Layer Chromatography

In order to separate the chemical compounds from the crude extract, Thin Layer Chromatography (TLC) was used. TLC was a solid liquid form of chromatography where the stationary phase was normally polar absorbent and the mobile phase can be a single or combination of solvents. It was a quick and cheaper microscale technique. These absorbents created very polar materials and the more polar the molecule to be separated, the stronger the attractive force to the stationary phase. The polar stationary phase was more strongly attracted polar molecules (Harborne, 1973). In this study, silica gel was used as stationary phase to separate the chemical compound from each crude leaves extract of *O. stamineus*. TLC used a thin, uniform layer of silica gel (SiO_2 X H_2O), alumina (Al_2O_3 X H_2O), cellulose or polyamine that was coated onto a piece of glass, aluminium foils or polyester foil (Harborne, 1973).

Solvent was poured into the beaker (TLC chamber) to a depth of just less than 0.5cm and it was covered with watch glass. Chamber was allowed to stand and saturate while preparing for TLC plate. TLC plate (Silica gel 60 F_{254} sheet) (8cm X 2cm) and (20cm X 20cm) was prepared and a line was drawn across to mark as 'origin' of the paper. Next, sample was spotted on the 'origin' with capillary tube and the prepared TLC plate was placed in the developing beaker and it was covered with watch glass. Plate was

removed from the beaker after the solvent has risen to ¾ of the plate. Then, the solvent front was marked quickly. The plate was placed in the fume cupboard to allow the solvent to evaporate. After it sprayed with different reagent, the colored spot formed and it marked again. R_f value calculated by using this formula (Harborne, 1973):

$$R_f = \frac{\text{Distance traveled by the compound, X(cm)}}{\text{Distance traveled by the solvent, Y (cm)}}$$

The R_f value was the distance a compound moved in chromatography relative to the solvent front. It was obtained by measuring the distance from the origin to the centre of the spot produced by the substance, and this was divided by the distance between the origin and the solvent front. In identifying a plant constituent, once it had been isolated and purified, it was necessary first to determine the class of compound and then to find out which particular substance it was within that class. The class of compound was usually clear from its response to color tests, its solubility and R_f properties and its UV spectral characteristics (Harborne, 1973).

B) Two-Dimensional Thin Layer Chromatography 2-D

The best method of separating and identifying phenolics was by using Two Dimensional Thin Layer Chromatography (2-D TLC). It was normally detected after extracting by acid hydrolysis of plant sample or using concentrated aqueous alcoholic plant extract. In this study, acid hydrolysis method was used to separate the phenolic compounds using 2-D TLC. Moreover, 2D-TLC used acetic acid: chloroform and ethyl acetate-benzene. 10 g of leaves powder of *O. stamineus* was soaked in 100ml of 2M HCl for 30 minutes.

After it filtered, 100 ml of diethyl ether was added to the extract and shake well. The diethyl ether at the top layer was removed and evaporated to dryness (Harborne, 1973). The extracts were separated by using two-dimensional thin layer chromatography (2-D TLC), containing 2 different solvent systems and TLC plate was sprayed with 2 reagents, Folin and Vanillin-HCl. Solvent system that were used in this study (Harborne, 1973):

a. Acetic acid: Chloroform [1:9]

b. Ethyl acetate: Benzene [9:11]

C) Column Chromatography

Column Chromatography was a useful separation technique in identification of plant compounds. The principle of this method was the same with TLC, but it can separate in larger quantities. It allowed separating and collecting the compounds individually. In this study, column chromatography technique was used to separate the chemical compounds in the crude water extract of *O. stamineus* (Fig.2.3) (Harborne, 1973).

The silica gel was packed into the column and air bubbles were avoided. Butanol, Acetic Acid and Water, BAW (60:15:25) were used as mobile phase. 100µl of water extract was loaded at the top of the packed column and it was eluted with solvent system at flow rate 1ml per minute. 5 ml of each fraction were collected in specimen tubes until the final fraction and it were dried in the fume hood (Harborne, 1973).

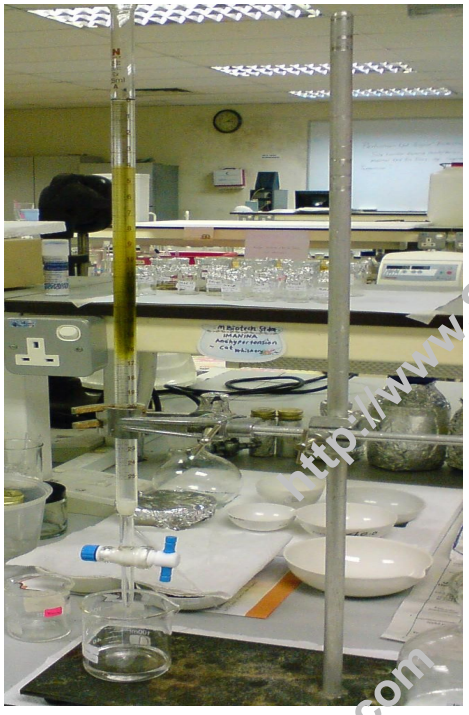

Fig.2.3.Column Chromatography

D) High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was a highly improved form of column chromatography. It was forced under high pressure and that makes it much faster. Smaller particle size was used for the column packing material that gave much higher surface area for interactions between the stationary phase and molecules passing through it. Usually, detection of the eluted compounds was one of the most difficult problems, but in HPLC the detection method was highly automated and very sensitive (Loon, *et al*., 2005).

The major parts of HPLC were solvent reservoir, pump or solvent delivery system, sample injector, column, detector, data analysis and waste bottle. HPLC grade solvent was used because of its purity. Solvent, mobile phase and test samples had to be filtered with 0.45µm micro filter for separation of an impurity which can damage column and system.

Pump was used to force the eluent through the column and detector. If a non-polar stationary phase [C-18] used with polar mobile phase (methanol: water), non-polar solute was retained more and polar solute was eluted. The retention time was varied depending on the interactions between the stationary phases and molecules that have been analyzed and the solvents that used (Loon, *et al*., 2005).

i. Preparation of High Performance Liquid Chromatography (HPLC) for crude extract of *O. stamineus.*

In this project, an isocratic method was used for crude extract sample of methanol, water, chloroform and hexane from leaves of *O. stamineus*. HPLC analysis was performed using a Shimadzu chromatograph equipped with an automatic injector, column oven and UV detector. Isocratic was a constant composition of mobile phase. The HPLC method applied was a modification of that reported by Akowuah *et al*., 2005. 10 to 20µl of crude extract sample were separated within a total time of 30 min and flow rate of 1ml per min.

The peaks were detected at 340 nm. The result was then compared with HPLC chromatogram of the *O. stamineus* leaf extract by using different solvents (Akowuah *et al*., 2005) in order to identify the chemical compound in *O. stamineus*. Methanol-water-tetrahydrofuran (45: 50: 5 v/v) was used as mobile phase. Methanol was in pump A and water-tetrahydrofuran was in pump B. The distilled water was filtered by using 0.45µm membrane filter for 3 times before it mixed with tetrahydrofuran (Akowuah, *et al*., 2005).

Degasser or sonicator was used to get rid of air bubble of the solvent before it pumped into the HPLC machine. pH measurement was also important in HPLC solvent system because it can change the hydrophobicity of the analyte. The pH of the mobile phase must be >2 and <10. For this reason, we must use a buffering agent such as sodium phosphate to control the pH. The buffers can control the pH and neutralized the charge to improve the chromatography. The setting of the Shimadzu HPLC machine was carefully done because all the HPLC equipments were very sensitive and easily broken (Akowuah, *et al*., 2005).

After the samples were filtered with 0.45µm microfilter, their solubility was tested by using mobile phase, and only test sample that was dissolved in the mobile phase proceed in the next step. We have to wait for about 20 to 30 minutes to make sure that the pressure was stable before the injection started. Injector was a syringe that was used to inject the sample into the eluent stream through a rubber septum. The syringe must be rinsed with deionized water before being used to prevent contamination. Chromatogram can be observed through computer that was connected with HPLC system (Akowuah, *et al*., 2005).

When we observed the HPLC chromatogram, retention time played an important role. Retention time was the time that taken for a particular compound to travel through the column of the detector. Time was measured from time at which the sample was injected to the point at which the display showed a maximum peak height for that compound. After the HPLC was run about 30 minutes, the mobile phase for washing had to prepare. Usually if the buffer was used as a mobile phase, 100% of deionized water was used for 30 minutes (for both pumps) and continued with 70:30, methanol or acetonitrile: deionized water. If no

buffer was used, only 70:30, methanol or acetonitrile: deionized water was used for washing (Akowuah *et al*., 2005).

ii. Preparation of High Performance Liquid Chromatography (HPLC) for Hippuric acid (HA) standard.

All the test samples were filtered with 0.45µm micro filter. Solubility test was done to the filtered samples and only dissolved samples were injected into HPLC machine. Hippuric acid at different concentrations: 500µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml were prepared freshly on a daily basis. 10µl of injection volume were taken from each test sample and its flow rate was 1ml/min. All the samples were separated with two solvent systems: 0.05% TFA in water and 0.05% TFA in acetonitrile. The peaks were detected at 228 nm and were identified by standard substances (Wu *et al*., 2002).

2.4 Detection of chemical compound

After the compound has been isolated and purified, we have to determine the class of compound to take a further test. The detection of chemical compound of TLC plates was carried out by observing under visible light, under UV light and spraying with Dragendorff and Vanillin reagent. The identification of the isolated compound within the class depends on measuring other properties when compared to the literature (Harborne, 1973).

A) Visible Light

Under visible light, the coloured of the chemical compound can be seen clearly. The chemical compound that appeared was a compound that contains pigments that absorb lights. TLC chromatograms of each sample were observed under visible light. Available bands on the plate were mark and R_f value were calculated. (Green colour band indicate the presence of chlorophyll in the sample while carotene gives red, orange or yellow bands) (Harborne, 1973).

B) Ultra Violet Light

Under Ultra violet (UV) light, the aromatic compounds can be identified. Aromatic compounds represent phenolic, quinine and flavonoid compounds. TLC chromatograms were observed under ultra violet light in the dark room by using UV lamp to spot for fluorescent band and R_f value of each band was calculated. (Fluorescent green band indicated presence of aromatic compound in the sample) (Harborne, 1973).

C) Dragendorff Reagent

Apart from using UV light, Dragendorff reagent can be used to detect the chemical compounds that may present. If the chemical compounds showed orange colour, it indicated the presence of alkaloids. Dragendorf Reagent was prepared by mixing 5ml of Solution A [1.7g basic bismuth nitrate in 100ml H_2O-HOAc (80:20)] with 5ml Solution B [40g KI in 100ml distilled water], 20g acetic acid and 70ml distilled water were added. Dragendorf Reagent was used to dye the TLC chromatograms to determine the presence of alkaloid compound in the samples. TLC chromatograms dyed with Dragendorff reagent.

Then the sample was aired dry. (Orange spot on the plate indicated the presence of alkaloid and choline in the sample, koumarin gave an orange spot). R_f value for the alkaloid compounds was calculated and recorded (Harborne, 1973).

D) Vanillin Reagent

Another reagent that can be used to detect the chemical compound was Vanillin reagent. It was sprayed onto the TLC plate and it heated at about 100ºC. If the purple colour appeared, the compound was indicated as terpenoid but if the green colour appeared, it showed the presence of phenol compound. Vanillin reagent was prepared by mixing 5% ethanol sulfuric acid with 1% ethanol vanillin. TLC chromatograms were dyed with Vanillin Reagent. Then they were heated in a hot plate at about 100ºC. (Terpenoid gave purple band while phenolic compound gave red or other colour spot). R_f value of each coloured spot was calculated and recorded. All the chemical compounds available in each sample were isolated from TLC plate and they were transferred into sample tubes. 1ml of distilled water was added to dissolve the powder form of the chemical compound samples for bioassay test (Harborne, 1973).

2.5 Determination of antioxidant activity

Antioxidant was a molecule that presents in small concentrations compared to that of an oxidizable substrate. It delayed or prevent the oxidation of the substrate (Yu, 2008). Antioxidants can function in vivo to prevent the oxidation of biological targets such as:

i) Chelate metal ions to prevent the formation of reactive oxygen (ROS) or nitrogen species (RNS).

ii) Inhibit oxidative enzyme including cyclooxygenases

iii) Induce antioxidant enzyme activity.

iv) Directly react with and scavenge the ROS or RNS species.

(Yu, 2008)

The antioxidant activity estimation was highly affected by the ROS or RNS in the assay and while an antioxidant may be effective in scavenging one type of free radicals. It may ineffective towards another type of free radicals or chelating a metal ion. The antioxidants activity of each crude extracts from *O. stamineus* were evaluated by using three different methods:

i) 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

ii) Reducing Power Assay

iii) Metal Chelating Assay

A) DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity assay was a decolorization assay that can determine the activity of antioxidants to directly react with DPPH stable free radical by observing its absorbance at 517 nm with a spectrophotometer (Yu, 2008). The DPPH radical was stable organic nitrogen centered free radical, dark purple in color that become colorless when it was reduced to its non-radical form by antioxidants (Yu, 2008). A purple colored 1,1-Diphenyl-2-picryl hydrazyl (DPPH), a stable free radical which was reduced to α, α-diphenyl-β-picryl hydrazine. DPPH was observed yellow in color if reacts with antioxidant. The decolorization of purple colour indicated the potential of antioxidants of the samples. When the more decolorization of purple colour occurred, the antioxidant concentration of the sample was higher (Adedapo, *et al*., 2008).

The DPPH radical scavenging activity was found commonly use in the screening of the antioxidant properties of pure compounds and plant extracts. This method was technically simple and can be determined by using single read spectrophotometer. However, the disadvantage of the DPPH activity scavenging assay was the use of non-physiologically relevant free radicals which resembled to free radicals that involved in the oxidation process in biological system (Adedapo, *et al*., 2008).

i) Preparation of extracts

5 different concentrations of crude extract of *O. stamineus* were prepared and dissolved in 1ml methanol.

ii) Preparation of Ascorbic acid

Ascorbic acid was used as the positive reference standard in DPPH radical scavenging assay. Reaction mixtures of ascorbic acid, DPPH, and methanol were prepared as shown in Table 2.1. A stock of Ascorbic acid was prepared at the concentration of 400 µg/ml. 0.02 g of Ascorbic acid was dissolved in 50 ml methanol. The stock solution was kept in flask which was wrapped in aluminium foil (Adedapo, *et al*., 2008).

iii) Preparations of 1,1-Diphenyl-2-picryl hydrazyl (DPPH)

A stock of DPPH in methanol was prepared at the concentration of 8 mg/ml. 0.04 g of DPPH stock was dissolved in 5 ml methanol. The stock solution was kept in flask which was wrapped in aluminium foil (Adedapo, *et al*., 2008).

Table 2.1 Reaction of mixtures containing ascorbic acid, DPPH, and methanol as positive standard references.

Concentration of ascorbic acid (µg/ml)	Volume of methanol(µl)	Volume of ascorbic acid (µl)	Volume of DPPH solution (µl)
200.00	475.00	500.00	25.00
100.00	725.00	250.00	25.00
50.00	850.00	125.00	25.00
25.00	912.00	62.50	25.00
12.50	943.75	31.25	25.00
6.25	959.38	15.63	25.00
3.12	967.19	7.81	25.00
1.56	971.09	3.91	25.00
Control	975.00	-	25.00
Blank	1000.00	-	25.00

The reaction mixtures were incubated at room temperature to react for 30 minutes. Methanol was used as blank. The DPPH radical was used alone without any ascorbic acid as negative control. The quenching of free radicals by ascorbic acid was measured spectrophotometrically at 517 nm. The degree of discoloration indicated the free radical scavenging efficiency of ascorbic acid (Adedapo, *et al*., 2008).

v) Determination of percentage of inhibition of DPPH

Radical scavenging activity was expressed as percentage of inhibition which was calculated by using this formula:

Percentage of Inhibition (%)= [($OD_{control} - OD_{sample}$) / OD control] X 100

Where;

$OD_{control}$ = absorbance value of control

OD_{sample} = absorbance value of sample or crude extract

Percentage of inhibition for each concentration for the active extract was calculated by using the above formula. A graph of percentage of inhibitions against concentration was plotted so that IC_{50} value can be determined. The IC_{50} value was the concentration at which 50% of DPPH radicals inhibited (Table 2.2) (Adedapo *et al*., 2008).

Table 2.2. Reaction mixtures of crude extract, DPPH and methanol for DPPH radical scavenging assay to determine IC_{50} value.

Concentration of crude extract (mg/ml)	**Volume of methanol (µl)**	**Volume of crude extract (µl)**	**Volume of DPPH solution (µl)**
5	725.00	250.00	25.00
4	775.00	200.00	25.00
3	825.00	150.00	25.00
2	875.00	100.00	25.00
1	925.00	50.00	25.00
Control	975.00	-	25.00
Blank	1000.00	-	-

B) Reducing Power Assay

The reducing power assay was one of the mechanism actions of antioxidants and it can be used to measure the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. The ferric ion or ferricyanide complex was reduced by the antioxidants to the ferrous form and Perl's Prussian blue complex was observed. Fe (III) reduction was often used as an indicator of electron-donating activity and it can be strongly correlated with other antioxidant properties (Ozsoy, *et al*., 2008).

The yellow colour of the test solution can change to various blue and green colors depending on the reducing power potential of each extract. The reductants that present in the crude extracts can caused the Fe^{3+}/Ferricyanide complex to be reduced to ferrous ion, Fe^{2+}. When the Perl's Prussian blue was formed in the reaction mixture, it indicated the presence of Fe^{2+}. The more intense of blue color showed high reducing power capability of the crude extract and the increase of OD reading (700 nm) indicated greater reducing power of the extract. Reducing power can evaluate the ability of extracts to reduce from Fe^{3+} to Fe^{2+} (Chang, *et al*., 2007).

i) Preparations of solutions

a) Potassium Ferricyanide 1%

A stock of potassium ferricyanide was prepared at the concentration of 10 mg/ml. 0.6 g of potassium ferricyanide stock was dissolved in 60 ml distilled water (Chang, *et al*., 2007).

b) Trichloroacetic acid (TCA) 10%

A stock of trichloroacetic acid was prepared at the concentrations of 100 mg/ml. 6 g of trichloroacetic acid stock was dissolved in 60 ml distilled water (Chang, *et al*., 2007).

c) Ferric chloride solution 0.1%

A stock of ferric chloride was prepared at the concentrations of 1 mg/ml. 0.01 g of ferric chloride stock was dissolved in 10 ml distilled water (Chang, *et al*., 2007).

d) 0.4 M Monobasic stock

13.9 g of sodium phosphate monobasic Na_2HPO_4 (anhydrous) was dissolved in 500 ml of distilled water (Chang, *et al*., 2007).

e) 0.4 M Dibasic stock

28.4 g of sodium phosphate dibasic (heptahydrate) NaH_2PO_4 (anhydrous) was dissolved in 500 ml of distilled water (Chang, *et al*., 2007).

f) 0.2 M Phosphate Buffer

187.5 ml of monobasic solution was mixed with 112.5 ml dibasic solution, followed by 600 ml distilled water. The solution was mixed well and adjusted to pH 6.6 (Chang, *et al*., 2007).

ii) Determination of reducing power for crude extracts samples

Generally, varied amount of 5mg, 10mg, 15mg, and 20mg of each crude extract were dissolved in 1ml methanol and it were vortexed until it mixed completely. 1ml of crude extracts prepared was then added with 2.5ml of 0.2M Phosphate Buffer (pH 6.6) and later with 2.5ml of 1% (w/v) potassium ferricyanide. The mixtures were incubated in water bath at 50º C for 20 minutes (Gulcin, *et al*., 2007).

Following incubation, 2.5ml of 10% trichloroacetic acid (TCA) solution was added to each mixture and then centrifuged at 1000 rpm for approximately 10 minutes. 2.5 ml of aliquot of the upper layer was transferred in test tubes and added with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The colour changes were observed. The mixtures were then transferred in cuvetts. Optical density (OD) or absorbance of the reaction mixtures was taken using spectrophotometer at 700nm. Increased absorbance of the reaction mixtures indicated greater reducing power (Gulcin, *et al*., 2007).

Butylated hydroxyanisole (BHA) was used as positive reference standard using the same methods mentioned above. All tests were carried out in triplicates and the readings were averaged out. Mean values for three independent samples were calculated for each extract. Methanol was used as blank (Gulcin, *et al*., 2007).

C) Metal Chelating Assay

The metal or iron (Fe^{2+}) chelating assay can be used to measure the capacity test of antioxidant samples to compete with a synthetic chelator, to form chelating complexes with iron (II) under controlled pH. Iron chelation may produce important antioxidative effects by preventing metal-catalyzed oxidation. The effective iron chelators gave protection against oxidative damage by removing the iron (II) which participates in hydroxyl radical-generating Fenton-type reactions. By minimizing the iron (II), the

oxidative damage was protected by inhibiting production of ROS and lipid peroxidation. The main objective of this assay was to evaluate the ability of each extract to chelate ferrous ion and preventing the formation of ferrozine Fe^{2+} complex (Yu, 2008).

i) Preparation of stock solution

a) Ferrozine 5mM

0.0246 g ferrozine stock was dissolved in 10 ml deionized water (Yu, 2008).

b) Ferum chloride ($FeCl_2$) 2mM

0.0397 g of $FeCl_2$ stock was dissolved in 10ml deionized water (Yu, 2008).

ii) Preparation of standard (ethylenediaminetetraacetic acid) EDTA

Ethylenediaminetetraacetic acid (EDTA) was used as positive reference standard in this assay. EDTA stock of 0.1 g/ml was prepared by dissolving 1g of EDTA in 4 ml deionized water. The pH was adjusted while stirring with NaOH solution until most of EDTA was dissolved. Once the EDTA was dissolved, total volume of EDTA stocks was mixed with deionized water, followed by ferric ($FeCl_2$) and ferrozine respectively in centrifuge tubes according to Table 2.3 (Yu, 2008).

Table 2.3.Reaction mixtures of deionized water, EDTA, $FeCl_2$ 2mM and ferrozine 5mM

Concentration of EDTA (mM/ml)	Volume of deionized water (ml)	Volume of EDTA (ml)	Volume of $FeCl_2$ 2mM (ml)	Volume of ferrozine 5mM (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 reaction mixture was shaken vigorously and left incubated in the room temperature for 10 minutes. 1ml of mixture was transferred into 3 cuvettes each. The absorbance reading was measured at 562nm. Deionized water was used as blank. All of 6 samples were tested in triplicate and the readings were averaged out. Percentage of inhibition of ferrozine Fe^{2+} complex in each reading was calculated (Yu, 2008).

iii) Determination of percentage of inhibition of ferrozine Fe^{2+} complex

The percentage of inhibition of ferrozine Fe^{2+} complex formation was calculated using the formula below (Yu, 2008).:

Percentage of Inhibition (%)= [(Abs control – Abs sample) / Abs control] X 100

Where;

Abs control = absorbance reading of control

Abs sample = absorbance reading of sample

iv) Determination of metal chelating activity of crude extracts samples

Crude extracts were tested in different concentration of 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml and were prepared by dissolving 20mg/ml of crude extract in 1ml methanol. The crude extracts were then added with deionized water, followed by ferric chloride ($FeCl_2$) and ferrozine respectively in centrifuge tubes according to Table 2.4 (Yu, 2008).

Table 2.4. Reaction mixtures of crude extracts, deionized water, $FeCl_2$ and ferrozine.

Concentration of crude extract (mg/ml)	**Volume of deionized water (ml)**	**Volume of crude extract (ml)**	**Volume of $FeCl_2$ 2mM (ml)**	**Volume of ferrozine 5mM (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 reaction mixture was shaken vigorously and left incubated in the room temperature for 10 minutes. 1ml of mixture was transferred into 3 cuvettes each. The absorbance reading was measured at 562nm. All 6 samples were tested in triplicate and the readings were averaged out. Percentage of inhibition of ferrozine Fe^{2+} complex of each reading was calculated (Yu, 2008).

2.6 Angiotensin Converting Enzyme (ACE) Bioassay

The renin angiotensin system was involved in the regulation of blood pressure and electrolyte metabolism. Angiotensin II was the primary hormone that made from angiotensinogen. Angiotensinogen, an α_2-globulin made in liver, was the substrate for renin. Renin acted at the substrate angiotensinogen to produce the decapeptide angiotensin I. The synthesis of angiotensinogen in liver was enhanced by hormones such as glucocorticoids and estrogen. These hormones can cause hypertension because of the increased plasma levels of angiotensinogen (Murray, *et al.,* 1998). The active angiotensin II also increased blood pressure by causing vasoconstriction. Therefore, ACE bioassay was done in this study to see whether the leaves of *O. stamineus* contain chemical compounds that may act as ACE inhibitor to reduce high blood pressure in human (Goh, *et al*., 1995).

A) Preparation of ACE crude extract

Angiotensin converting enzyme (ACE) was a glycoprotein that mainly found in lungs, endothelial cells and plasma. It removed two carboxyl terminal amino acids from the decapeptide Angiotensin I to form Angiotensin II. Angiotensin II increased blood pressure by causing vasoconstriction of the arteriole (Murray, *et al*., 1998). In this study, we have extracted ACE from rat lungs. About 20 ICR rat was dissected and their lungs

were soaked in the 50mM phosphate buffer, pH8.3. The lungs were homogenized and centrifuged at 4000rpm for 15min. The clear supernatant which contains ACE was stored in the fridge for future bioassay study (Braga, *et al*., 2007).

B) Angiotensin Converting Enzyme (ACE) Inhibitory Assay

Angiotensin Converting Enzyme (ACE) inhibitory assay was a method that was developed by Cushman and Cheung (1971) with modified based on the hydrolysis of hippuryl-histidyl-leucine (HHL) by ACE to form hippuric acid (HA) and histidyl-leucine as products. The HA was extracted into ethyl acetate and it was quantified by measuring the absorbance in a spectrophotometer at 228nm (Wu *et al.,* 2002). If the reading of the absorbance was lower, it means that the ACE activity in the test reaction was reduced and it indicated that the plant samples presented high concentration of Angiotensin converting enzyme inhibitor (ACEI) agent (Duncan *et al*., 1999).

i) Preparation of samples

5 different concentrations of *O. stamineus* were prepared and dissolved in 1 ml of methanol. The concentration of each crude extract were calculated using $M_1V_1=M_2V_2$. The concentrations for all TLC samples were same (1µg/ml). The powdered TLC samples were dissolved into 1ml of distilled water before proceed it to ACE inhibitory assay (Braga, *et al*., 2007).

ii) Determination of ACE inhibitory activity

250 µl of sample was transferred to the test tube. 250 µl of 100mM Phosphate buffer (pH8.3) was added to the tubes. 250 µl of 300mM NaCl, 250 µl of 5mM HHL was added. Finally 250µl of crude ACE was added. All the test tubes were incubated in water bath (37ºC) for 30 minutes. 250 µl of 1N HCl was added to terminate the reaction. 200µl. Ethyl acetate was added to the mixture and it vortexed for 5 sec. The upper layer was taken

out by using glass pipette and it was transferred to crucible and evaporated to dry. Absorbance reading was taken by using spectrophotometer at 228nm with cuvette quartz. 1 cuvette quartz filled with distilled water (dH_20) as blank. All reading had to be duplicated. The percentage of inhibition of the standard and samples were measured according to the formula below (Loizzo, *et al.,* 2008):

$$\text{Percentage of ACE Inhibitory Activity (\%)} = \frac{\text{Abs Control-Abs Sample}}{\text{Abs Control}} \times 100\%$$

C) Preparation of Hippuric Acid standard curve

Hippuric acid (HA) was a water soluble glycine conjugates that was formed from food additives benzoate. The standard curve of Hippuric acid has been done to measure the ACE activity of the mixture reaction. The ACE activity indicated the effectiveness of ACE inhibitor. If the ACE activity was high, it means that the ACE inhibitor did not inhibit the formation of angiotensin II. If the ACE inhibitors were effective, they will inhibit the convertion of inactive angiotensin I to the active angiotensin II. 0.0020g of Hippuric acid diluted with 4ml dH_2O. Then, they were diluted into 5 different concentrations. After that, the absorbance reading at 228nm was taken and standard curve was plotted (Braga *et al*., 2007).

From standard graph y=mX, (y=absorbance reading)

(ACE activity) X = y/ 30 minutes

2.7 Determination of Total Phenols

Phenolic compounds have been found to be strong antioxidants against free radicals and they were also reported to be an ACE inhibitor agent to reduce high blood pressure in human body (Loizzo, *et al*., 2008). Total phenolic content has been expressed in the terms of gallic acid equivalents, catechin equivalents, tannic acid equivalents and caffeic acid equivalents. Measurement of total phenolic content indicated the amount of phenolic compounds presented in the plant samples. In this study we used gallic acid as a standard to measure the phenolic compound from the leaves extract of *O. stamineus* (Adedapo, *et al.,* 2008).

500µl samples/standard solution was added with 5ml Folin Ciocalteu reagent (1:10 is diluted with distilled water) and 4ml of 1M Na_2CO_3 solution. The reaction mixture incubated in water bath at 45ºC for 15 min. The absorbance was measured at 765nm with spectrophotometer. Standard curve was prepared by diluting 0, 50, 100, 150, 200, 250mg/ml gallic solution in methanol: water (50:50,v/v). The total phenol was calculated as gallic acid equivalents mg/g dry mass (Adedapo, *et al.,* 2008).

2. 8 Brine Shrimp Lethality Bioassay (BSLA)

Brine shrimp lethality bioassay was carried out to investigate the cytoxicity of plant extracts. The method has also been applied to plant extracts in order to facilitate the isolation of biologically active compound. The technique was easily done and the cost was cheaper, and it utilized small amount of test material. This in vivo lethality test has been successfully employed for bioassay-guide fractionation of active cytotoxic and antitumor agents from medicinal plants. In this study, we used BSLA to measure the toxicity of the leaves crude extract of *O. stamineus* (Olaleye, 2007).

38 g sea salt per liter of water was prepared to make 'sea water'. The sea water was prepared in small tank and shrimp eggs were added to one side of the divided tank and were covered with aluminium foil. The lamp above the uncovered side will attract hatched shrimp through perforations in the dam. The shrimps were allowed 2 day to hatch and mature as nauplii. Vials for testing were prepared; test initially at 1000, 100 and 10µg/ml; 3 vials were prepared at each concentration for a total of 9 vials; 20mg of sample weigh and 2ml of water (20mg/2ml) were added; from this solution, 500, 50 or 5µl was transferred to vials corresponding to 1000, 100 or 10µg/ml respectively (Olaleye, 2007).

After 2 days when the shrimp larvae were ready, the sea water was added to each vial, 10 shrimps per vial (30 shrimp per dilution) were counted and the volume was adjusted with sea water to 5ml/vial (shrimp can be used 48-72 hours after the initiation of hatching). After 72 hours, they should be discarded. 24 hours later, they were counted and the number of survivors was recorded. The data was analyzed with Finney computer program to determine LC_{50} values and 95% confidence (Olaleye, 2007).

3.1 Extraction of plant chemical compounds

The leaves of *O. stamineus* were extracted by using Soxhlet apparatus. Methanol, chloroform and hexane were used as solvent (Table 3.1). Extraction process was started with low polarity of solvent: hexane, followed by medium polarity of solvent, chloroform and finally with high polarity of solvent, methanol. The polar solvent extracted out the polar compound and the non-polar compound extracted by the non-polar solvent.