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.

Table 3.1.Leaves crude extracts of *O. stamineus*

Sample	Observation			
	Methanol	**Hexane**	**Chloroform**	**Water**
Leaves of *O. stamineus*	Dark green	Yellowish brown	Dark brown	Brownish

All the solvents were heated to boiling point and the vapour condensed in the reflux condenser. The hot liquid dripped onto the plant sample (powdered dried leaves), on a porous thimble. After the liquid in the extraction chamber reached at the top of the siphon tube it flowed back into the heated flask, taking with it any dissolved plant material. After extracted with methanol, the plant sample was repeatedly extracted with chloroform and hexane solvent.

For water extraction, conventional method was used by soaking the water extract of the leaves of *O. stamineus* in water bath at 100º for 2 to 4 hours. The most important factor that influenced the solubility of material was the polarity of the solvent and the solute molecules. The solubility of solid in liquid was indicated by the affinity of molecules of the solvent. Non-polar solvent such as chloroform and hexane were used and the only force presented between molecules was dispersion, because of the production of transient charges induced in the individual molecules. Non polar solvents consisting of disordered molecules allowed the introduction of other non polar molecules easily. In this experiment, the hexane dissolved the non polar compounds such as fats and waxes, while polar solvents such as methanol and water dissolved the polar compounds such as alkaloid and sugar.

3.2 Separation of chemical compounds

A) Thin Layer Chromatography

200g of *C. stamineus* leaves in the powder form were extracted with methanol solvent and were dried by using rotary evaporator. The aluminium plates were used and TLC were developed by using 10: 90, methanol: chloroform solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and by using spray reagents including Dragendorff and Vanillin. In methanol extract, 13 labeled compounds were separated; MB1, MB2, MB3, MB4, MB5, MB6, MB7, MB8, MB9, MB10, MB11, MB12 and MB13. Table 3.2 showed 6 labeled compounds were identified as phenols, 5 labeled compounds were identified as terpenoids and 2 labeled compounds were identified as alkaloids.

Table 3.2. Thin Layer Chromatography of crude methanol extract from the leaves of *O. stamineus*

Label compound	R_f value (X100)	Observation				Comment
		Colour under visible light	Colour under UV light	Reagent		
				Dragendorff	Vanillin	
MB1	4.7	-ve	Grey (+)	Orange-brown	-ve	Alkaloid
MB2	10.6	Green (+)	Grey (+)	-ve	-ve	-
MB3	12.9	Yellow (+)	Grey(+)	-ve	Purple (+)	Terpenoid
MB4	18.8	Green (+)	Green (+)	-ve	Green (+)	Phenol
MB5	21.2	Yellow (++)	Yellow (++)	-ve	Green (+)	Phenol
MB6	26.5	Green (++)	Grey (++)	-ve	Purple (+)	Terpenoid
MB7	28.8	Yellow (++)	Yellow (++)	-ve	Purple (+)	Terpenoid
MB8	45.9	Green (++)	Grey (++)	Orange-brown(+++)	Green (+)	Phenol, Alkaloid
MB9	61.2	Green (++++)	Grey (++)	Green (+++)	Green (++++)	Phenol

MB10	65.3	Green (++)	Yellow (++)	Yellowish (+++)	Green (+++)	Phenol
MB11	74.7	-ve	Grey (+++)	Brownish (+++)	Purple (+++)	Terpenoid
MB12	88.2	Yellow(+++)	Brown (+++)	Yellowish (+++)	Green (+++)	Phenol
MB13	92.4	Green (++++)	Purple (++++)	Green (++++)	Purple (++++)	Terpenoid

Separations of phenolic compound from the methanol crude extract of *O. stamineus* and Thin Layer Chromatography (TLC) were developed by using 10:90, methanol: chloroform solvent system (Table 3.3).

Table 3.3. Thin Layer Chromatography of labeled compound (MB9) of crude methanol extract from the leaves of *O. stamineus*.

Label compound	**R_f value (X100)**	**Visible Light**	**UV Light**	**Folin-ciocalteu reagent**
M1	59.4	Yellowish green (+++)	Grey (++)	Blue (++++)
M2	78.1	Green (+++)	Yellow (+++)	Blue (++++)
M3	84.4	-ve	Grey (++++)	Blue (++++)
M4	87.5	Yellow (+++)	Brown (+++)	Green (++++)
M5	92.2	Green (++++)	Purple(++++)	Brownish(++++)

200g of *O. stamineus* leaves in the powder form were extracted with chloroform solvent and were dried using rotary evaporator. The aluminium plates were used and TLC were developed by using 10: 90, methanol: chloroform solvent system. The appearance of chemical compounds in the leaves extract were observed under visible light, UV light and by using spray reagents including Dragendorff and Vanillin. In the chloroform extract, 7 chemical compounds were isolated: CB1, CB2, CB3, CB4, CB5, CB6 and CB7. Table 3.4 showed 2 labeled compounds were identified as terpenoids and 2 labeled compounds were identified as phenols.

Table 3.4. Thin Layer Chromatography of crude chloroform extract from the leaves of *Orthosiphon stamineus*

Label compound	R_f value (X100)	Observation				Comment
		Colour under visible light	Colour under UV light	Reagent		
				Dragendorff	Vanillin	
CB1	17.3	Green (+)	Green (+)	Green (+)	-ve	Chlorophyll
CB2	19.0	Yellow (+)	Grey (+)	-ve	Purple (+)	Terpenoid
CB3	45.8	Green (++)	Green (++)	-ve	-ve	-
CB4	47.6	Yellow (++)	Grey (++)	-ve	-ve	-
CB5	64.9	Green (++)	Grey (++)	-ve	Green (++)	Phenol

CB6	88.0	Yellow (+++)	Grey (+++)	Brown (+++)	Green (+++)	Phenol, aromatic compound, alkaloids
CB7	90.0	Green (++++)	Purple (++++)	Green (++++)	Purple (++++)	Terpenoid, aromatic compound.

200g of *O. stamineus* leaves in the powder form were extracted with hexane solvent and were dried by using rotary evaporator. The aluminium plates were used and TLC were developed by using 10: 90, methanol: chloroform solvent system. The appearance of chemical compounds in the leaves extract were observed under visible light, UV light and by using spray reagents including Dragendorff and Vanillin. In hexane extract, only 4 chemical compounds were separated; HB1, HB2, HB3 and HB4. Table 3.5 showed 2 labeled compounds were identified as phenols and 2 labeled compounds were identified as terpenoids and 1 labeled compound was identified as alkaloids.

Table 3.5. Thin Layer Chromatography of crude hexane extract from the leaves of *Orthosiphon stamineus*

Label compound	R_f value (x100)	Observation				Comment
		Colour under visible light	Colour under UV light	Reagent		
				Dragendorff	Vanillin	
HB1	13.3	Green (+)	Green (+)	-ve	Green (++)	Phenol
HB2	46.6	Green (++)	Green (++)	Green (+++)	Purple (+++)	Terpenoid
HB3	84.8	Yellow (+++)	Brown (++)	Brown (++)	Purple (++++)	Terpenoid
HB4	89.7	Dark brown (+++)	Dark purple (++++)	Brownish Orange (+++)	Green (+++)	Phenol, Alkaloid

Indicator

(+)	Very light in colour
(++)	Light in colour
(+++)	Dark in colour
(++++)	very dark in colour

B) Column Chromatography

200g of *O. stamineus* leaves in the powder form were extracted with water in ratio 1:10 (ml). Column chromatography were developed by using butanol: acetic acid: water, (60:15:25) as solvent system and 5 fractions were collected. Each fraction was collected in 5 ml and was dried in the fume hood. After that, TLC was developed for each fraction and the appearance of the chemical compound in the leaves extract was observed under visible light, UV light and it was sprayed by using Vanillin reagent. The result showed only 1 labeled compound exhibited as phenols compound (Table 3.6).

Table 3.6. Column chromatography of crude water extract from the leaves of *O. stamineus*

Fraction	Labeled compound	R_f value (X100)	Observation		
			Colour under visible light	Colour under UV light	Reagent Vanillin
F1	W1	36.5	Yellow	Grey	Green
	W2	60.3	Yellow	Grey	
	W3	76.2	Yellow	Grey	
	W4	88.8	Yellow	Fluorescence	
F2	W1	31.3	Yellow	Grey	-ve
	W2	58.2	Yellow	Fluorescence	
F3	W1	22.7	Yellow	Fluorescence	-ve
F4	W1	18.0	-ve	Grey	-ve
F5	W1	44.3	-ve	Grey	-ve

C) Two dimensional Thin Layer Chromatography (2-D TLC)

The two dimensional Thin Layer Chromatography (2-D TLC) was developed to isolate and identify the phenolic compound. 2 different solvents were used as mobile phase, acetic acid: chloroform (1:9) and ethyl acetate: benzene (9:11).

Table 3.7.Thin Layer Chromatography of Standard of Phenolic Compounds

Standard	**TLC R_f (X100)**		**Colour**
	Solvent 1 **Acid acetic:$CHCl_3$**	**Solvent 2** **Etil acetate: Benzene**	**Folin reagent**
Caffeic acid	17.5	15.4	Dark blue
Anisaldehyde	80	67.7	Blue
Vanillin	-	-	-
p-coumaric acid	62.5	32.8	Blue
Ellagic acid	5.4	4.9	Dark blue

5 standard phenolic compounds (Table 3.7) were developed by thin layer chromatography (TLC). Folin reagents were sprayed onto the TLC plates to identify the phenolic compounds and R_f value of each compound was measured.

Table 3.8. Two dimensional Thin Layer Chromatography (2-D TLC) of crude methanol extract from the leaves of *Orthosiphon stamineus*

Labeled compound	**TLC R_f (X100)**		**Colour**	**Comment**
	Solvent 1 Acid acetic:$CHCl_3$	**Solvent 2 Etil acetate: Benzene**	**Folin reagent**	
P1	63.2	-ve	Blue	p-coumaric acid
P2	-ve	19.6	Dark blue	Caffeic acid

2 phenolic compounds were identified from the two dimensional Thin Layer Chromatography (2-D TLC) after they were compared with standard phenolics compound in Table 3.8.

D) High Performance Liquid Chromatography

The HPLC method applied was a modification of that reported by Akowuah *et al*., 2005. Isocratic method was used for crude extract samples of methanol, water, chloroform and hexane from the leaves of *O. stamineus*. Isocratic was a constant composition of mobile phase. 10 to 20µl of crude extract samples were separated within a total time of 30 min and their flowrate were 1ml/min. The peaks were detected at 340 nm. The results then were compared with HPLC chromatogram of the leaves extract *O. stamineus* by using different solvent (Akowuah *et al*., 2005) in order to identify the chemical compounds in *O. stamineus*. M ethanol-water-tetrahydrofuran (45: 50: 5 v/v) was using as mobile phase. Methanol was prepared in pump A and water-tetrahydrofuran was prepared in pump B.

HPLC analysis was developed to separate the presence of the chemical compounds in the leaves crude extract of *O. stamineus*.

i) Leaves methanol crude extract of *O. stamineus*

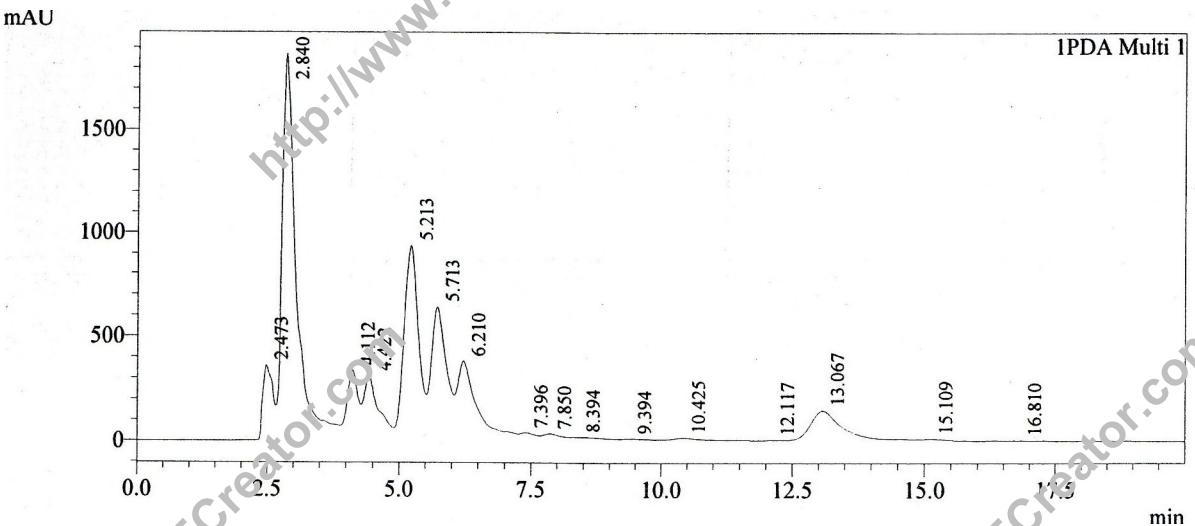

Fig. 3.1. Chromatograms of crude methanol extract from the leaves of *O. stamineus*

From Fig. 3.1, it observed that 7 high peaks were detected from the leaves methanol crude extract of *O. stamineus*.

ii) Leaves chloroform crude extract of *O. stamineus*

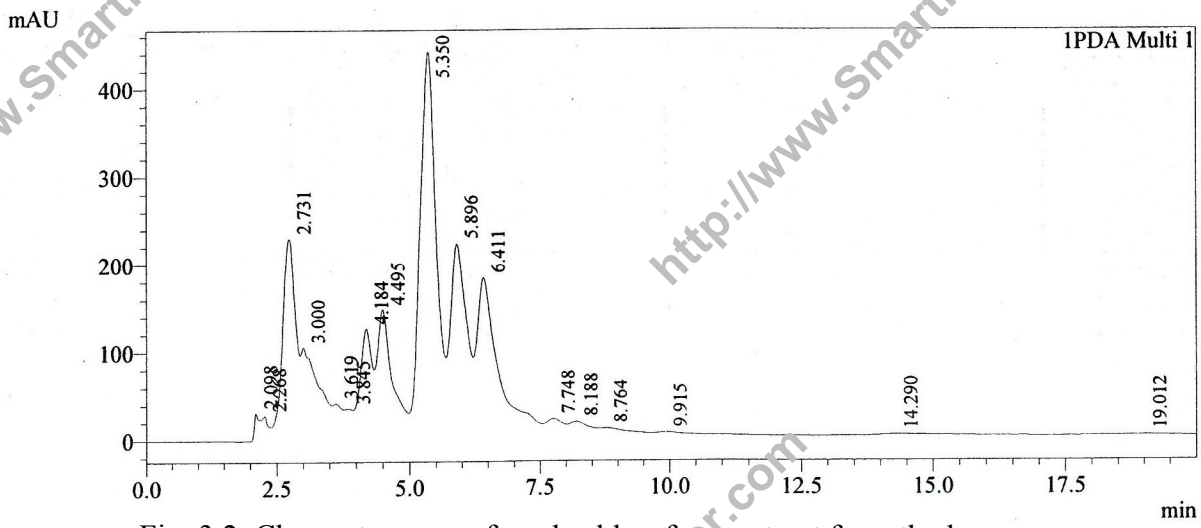

Fig. 3.2. Chromatograms of crude chloroform extract from the leaves of *O. stamineus*

From Fig. 3.2, it observed that 6 high peaks were detected from the leaves chloroform crude extract of *O. stamineus.*

iii) Leaves hexane crude extract of *O. stamineus*

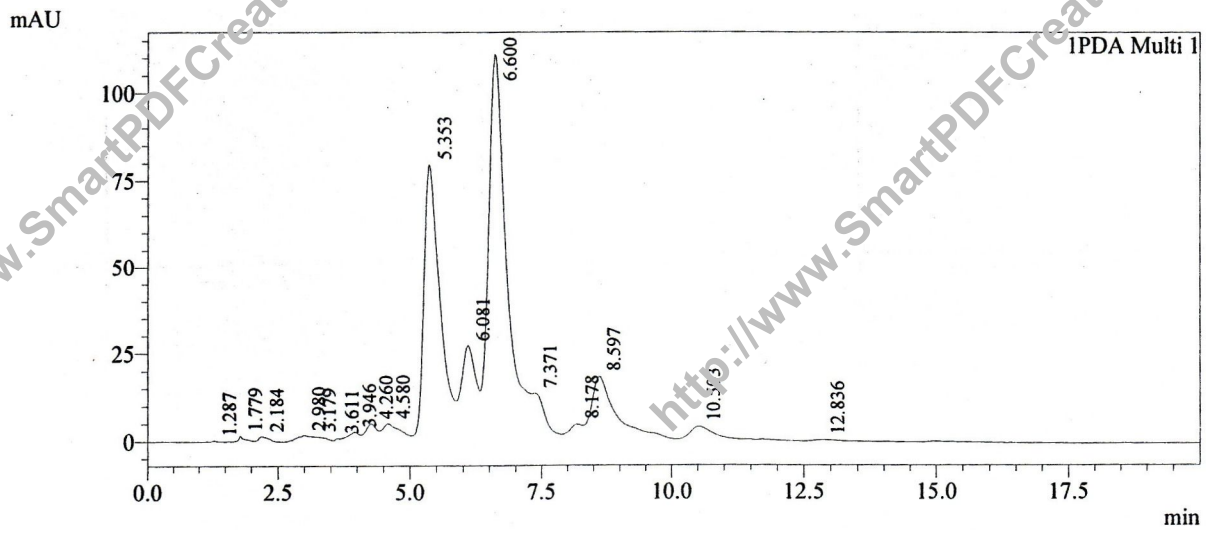

Fig. 3.3. Chromatograms of crude hexane extract from the leaves of *O. stamineus*

From Fig. 3.3, it observed that 4 high peaks detected from the leaves hexane crude extract of *O. stamineus.* From Fig. 3.4, it observed that 4 high peaks were detected from the leaves water extract of *O. stamineus.*

iv) Leaves water extract of *Orthosiphon stamineus*

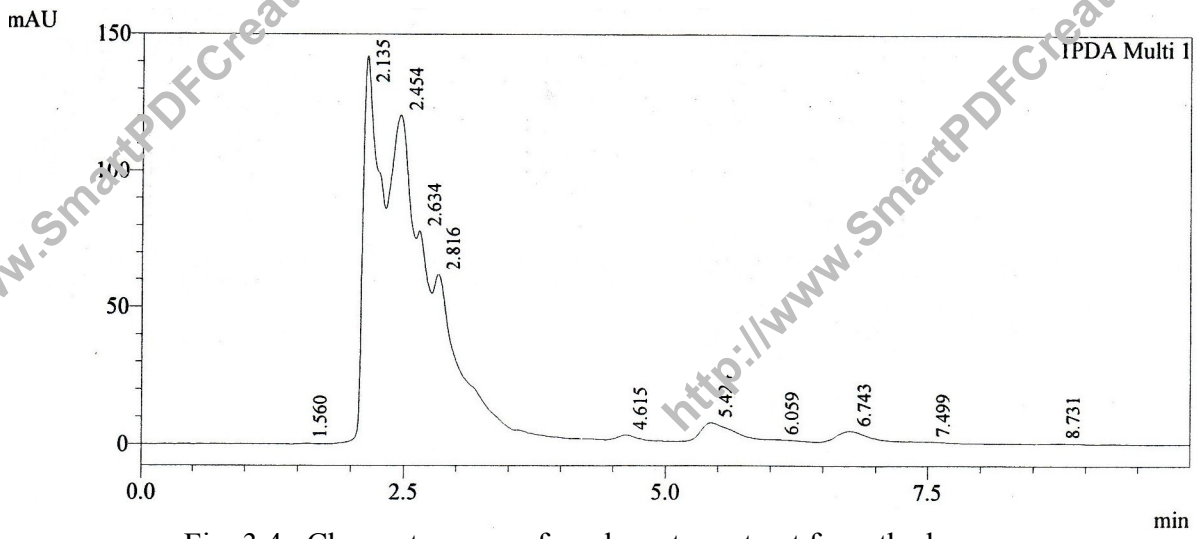

Fig. 3.4. Chromatograms of crude water extract from the leaves of *Orthosiphon stamineus*

v) Standard Hippuric acid (HA)

10µl of HA at concentration of 12.5 µg/ml was injected into HPLC system with flow rate of 1ml/min. The peaks were detected at 228 nm and identified by standard substances (Wu *et al*., 2002). HPLC developed to separate the HA compound. Test sample was separated with two solvent systems:

A) 0.05% TFA in water.

B) 0.05% TFA in acetonitrile.

Standard of Hippuric acid [62.5mg/ml]

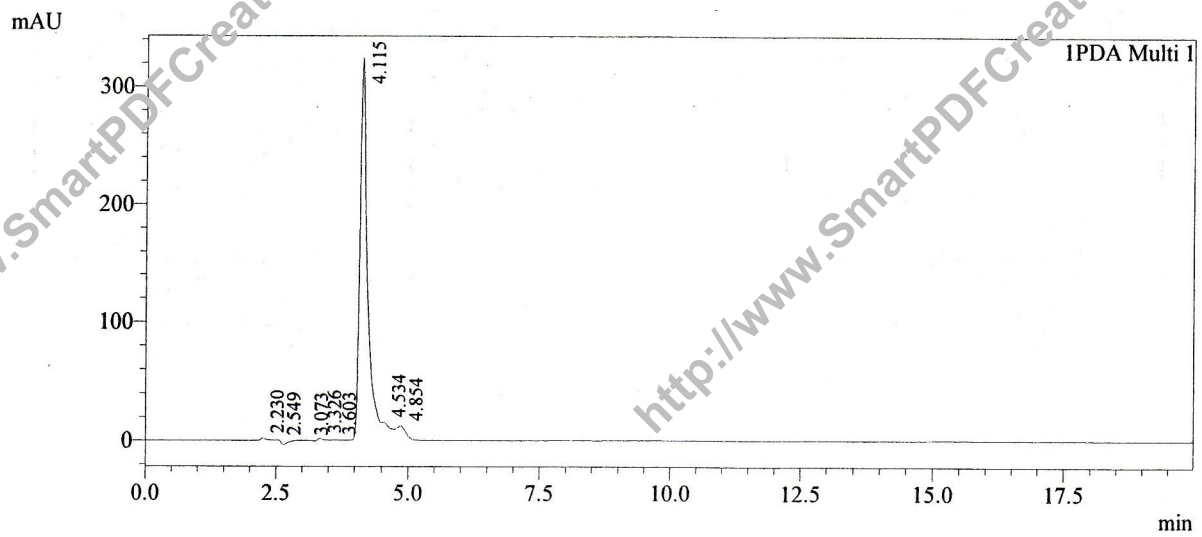

Fig. 3.5. Chromatograms of standard of Hippuric acid [62.5mg/ml]

From Fig. 3.5, it observed that a standard HA compound showed a highest peak at the concentration of 62.5mg/ml.

3.3 Determination of antioxidant activity

A) DPPH Radical Scavenging Assay

The ability of methanol, chloroform, hexane and water crude extracts from the leaves of *O. stamineus* to scavenge the free radical were determined using DPPH radical scavenging assay. The absorbance at 517 nm was used to determine the percentage inhibition of DPPH radical. IC_{50} value can be observed from the graph, where 50% inhibition to the DPPH radical. IC_{50} was the concentration which the extract inhibited 50% of DPPH free radical.

i) Standard Ascorbic acid

Ascorbic acid was used as the reference standard in the DPPH radical scavenging assay. All the reaction mixtures including ascorbic acid, DPPH and methanol were incubated at room temperature and absorbance reading taken at 517 nm. Methanol was used as blank. Table 3.9 showed the scavenging ability of the ascorbic acid on DPPH radicals. At 200 µg/ml, the percentage inhibition of Ascorbic acid against DPPH radicals was 91.7% while at 1.56 µg/ml, percentage inhibition of Ascorbic acid against DPPH radicals was 4.4%. Figure 3.6 shows the curve of inhibition of Ascorbic acid against DPPH radicals and IC_{50} value was determined as 12.83µg/ml.

Table 3.9. DPPH radicals scavenging activity of Ascorbic acid

Concentration of Ascorbic acid [µg/ml]	Absorbance at 517nm			Percentage Inhibition (%)
	1	2	Mean±S.D	
200	0.263	0.240	0.252±0.016	91.7
100	0.332	0.324	0.328±0.006	89.2
50	0.351	0.349	0.350±0.001	88.5
25	0.659	0.624	0.642±0.025	78.9
12.5	1.492	1.505	1.499±0.009	50.7
6.25	2.131	2.042	2.087±0.063	31.3
3.12	2.335	2.449	2.392±0.081	21.3
1.56	2.754	2.695	2.725±0.042	4.4
Control	3.068	3.010	3.039±0.041	-

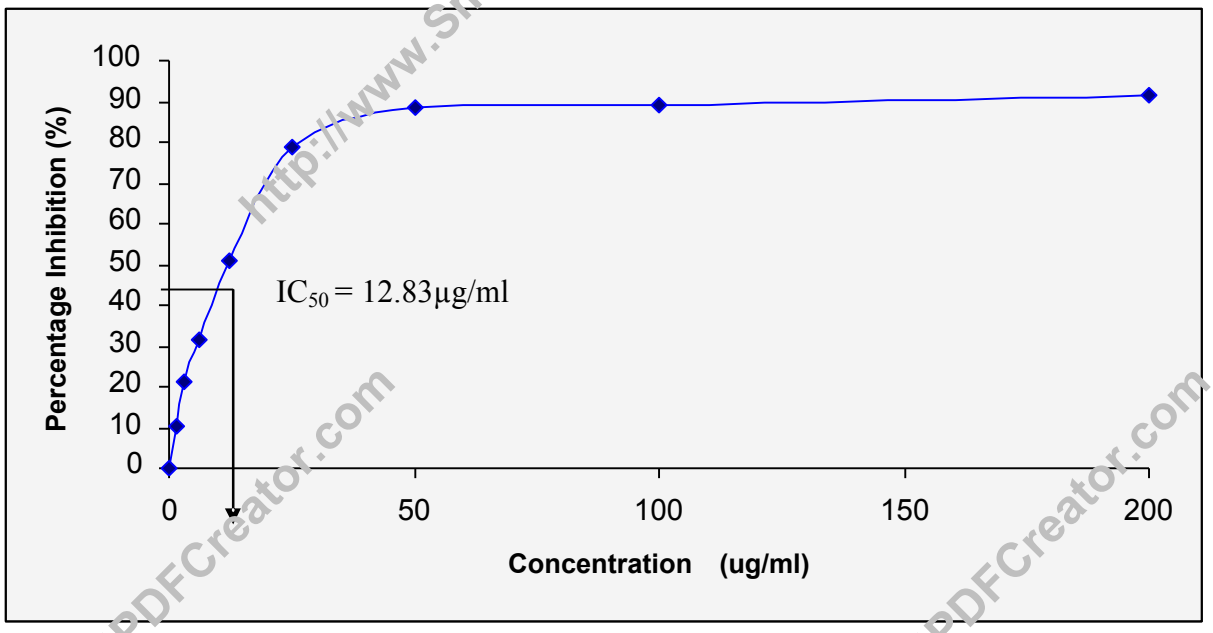

Fig. 3.6. Percentage inhibition of DPPH by Ascorbic acid

ii) DPPH radical scavenging activity of leaves crude extract of *O. stamineus*

Table 3.10 to Table 3.13 showed the scavenging effect of methanol, chloroform, hexane and water crude extract from the leaves of *O. stamineus* on DPPH radicals. The radical scavenging activity was tested at 5 different concentrations and the results shown in the tables below. Figure 3.7 illustrated the inhibition of crude extracts from leaves of *O. stamineus* and Ascorbic acid against DPPH radicals. IC_{50} value of methanol extract was 1.83mg/ml, chloroform extract was 2.59mg/ml, hexane extract was 1.98mg/ml and water extract was 1.44mg/ml.

Table 3.10. DPPH radical scavenging activity of crude methanol extract from the leaves of *O. stamineus.*

Concentration [µg/ml]	**Absorbance at 517nm**			**Percentage Inhibition (%)**
	1	**2**	**Mean±S.D**	
200	0.438	0.465	0.452±0.019	84.5
100	0.554	0.618	0.586±0.045	79.9
50	0.895	0.973	0.934±0.055	67.9
25	1.451	1.435	1.443±0.011	50.4
0	1.995	2.101	2.048±0.0750	29.7

Table 3.11. DPPH radical scavenging activity of crude chloroform extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 517nm			Percentage Inhibition (%)
	1	2	Mean±S.D	
200	0.614	0.644	0.629±0.021	78.4
100	0.736	0.753	0.745±0.012	74.5
50	0.872	0.914	0.893± 0.030	69.4
25	1.367	1.334	1.351±0.023	53.7
0	2.203	2.246	2.225±0.030	23.7

Table 3.12. DPPH radical scavenging activity of crude hexane extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 517nm			Percentage Inhibition (%)
	1	2	Mean±S.D	
200	0.759	0.436	0.5975±0.228	79.5
100	0.789	0.664	0.7265±0.088	75.1
50	1.238	1.517	1.3775±0.197	52.7
25	1.649	1.964	1.807± 0.223	38.0
0	2.395	2.142	2.2685±0.179	22.2

Table 3.13. DPPH radical scavenging activity of crude water extract

from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 517nm			Percentage Inhibition (%)
	1	2	Mean±S.D	
200	0.389	0.406	0.398±0.012	86.4
100	0.536	0.614	0.575±0.055	80.3
50	0.753	0.769	0.761±0.011	73.9
25	1.113	1.224	1.169±0.078	59.9
0	1.695	1.742	1.719±0.033	41

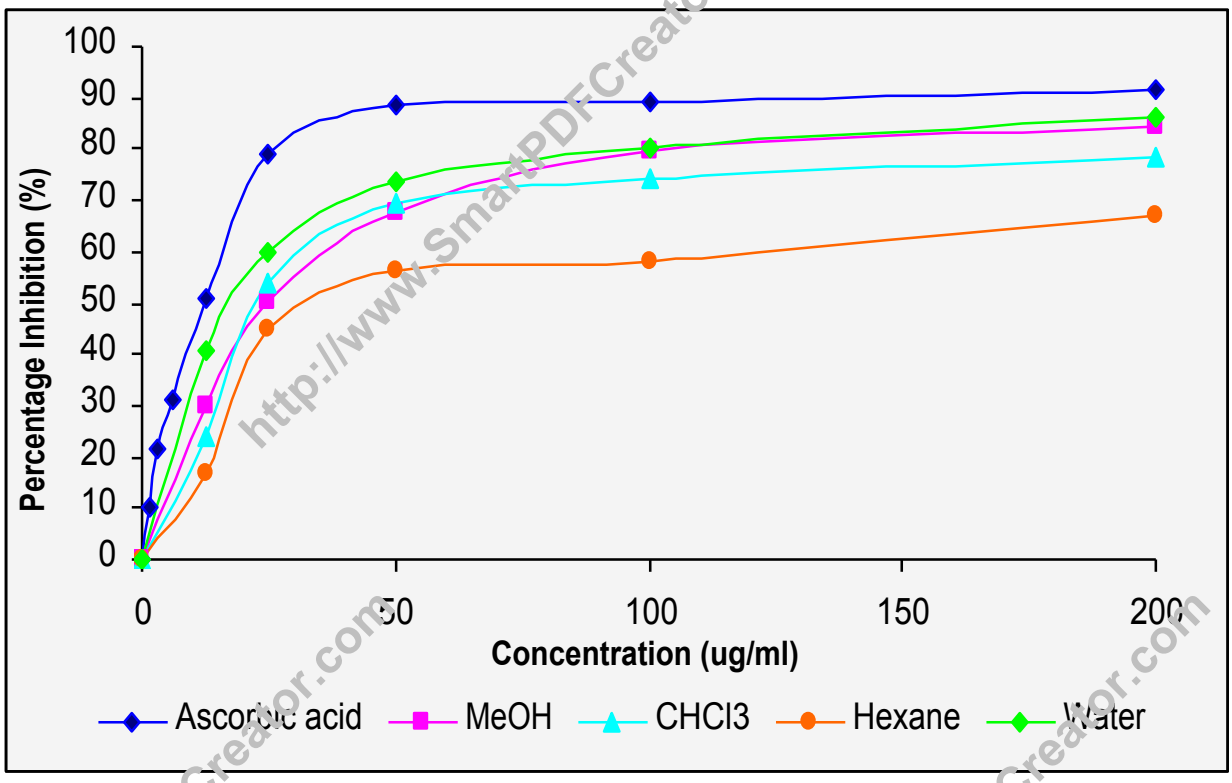

Fig. 3.7. DPPH radical scavenging activity of crude extracts

from the leaves of *O. stamineus*

B) Reducing Power Assay

The antioxidant activities of methanol, chloroform, hexane and water crude extracts from the leaves of *O. stamineus* were determined by using reducing power assay. The reactions mixtures were turned from yellow to Perl's Prussian blue complex and it depending on the reducing power of each extract used. The absorbance reading at 562 nm was taken. This assay was carried out in triplicates and the average reading was recorded.

i) Standard Butylated hydroxyanisole (BHA)

Butylated hydroxyanisole (BHA) was used as the standard in the reducing power assay. Table 3.14 showed the absorbance of reducing power of BHA at 562 nm. The results were shown in the tables below. Figure 3.8 showed the curve of BHA in the reducing power assay. The reducing power increased slightly from 400 to 500µg/ml.

Table 3.14. Reducing power of Butylated Hydroxyanisole (BHA)

Concentration [µg/ml]	**Absorbance at 562nm**			**Mean±S.D**
	1	**2**	**3**	
500	0.205	0.199	0.211	0.205±0.006
375	0.191	0.196	0.189	0.192±0.004
250	0.189	0.178	0.187	0.185±0.006
125	0.157	0.156	0.159	0.157±0.002
0	0.064	0.061	0.069	0.065±0.004

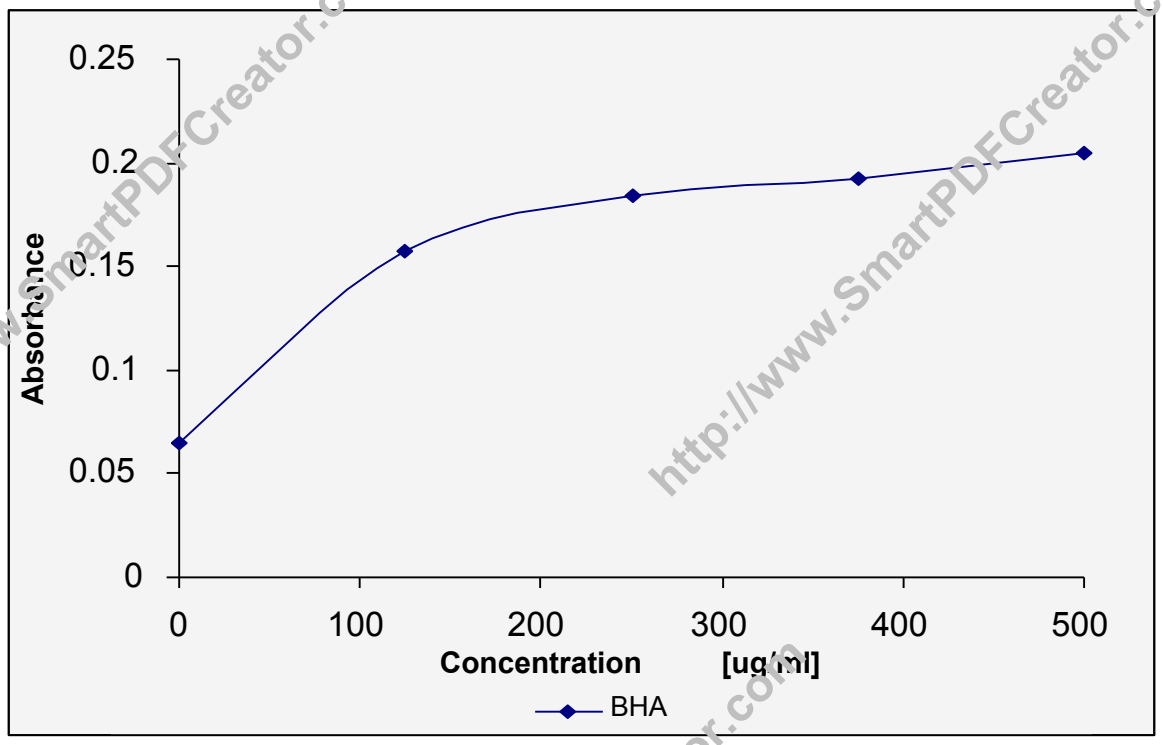

Fig. 3.8. Standard curve of Butylated hydroxyanisole (BHA)

ii) Reducing power of leaves crude extract of *O. stamineus*.

The methanol, chloroform, hexane and water crude extracts from the leaves of *O. stamineus* were evaluated for its reducing power at various different concentrations 125, 250, 375 and 500 µg/ml. The results were shown in the Table 3.15 to Table 3.18. Figure 3.9 illustrated the curve of reducing power of crude extract from the leaves of *O. stamineus*.

Table 3.15. Reducing power of crude methanol extract from the leaves of *O. stamineus*.

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D
	1	2	3	
500	0.193	0.183	0.185	0.187±0.005
375	0.181	0.178	0.186	0.182±0.004
250	0.183	0.177	0.174	0.178±0.005
125	0.163	0.165	0.152	0.160±0.007
0	0.056	0.057	0.059	0.0573±0.002

Table 3.16. Reducing power of crude chloroform extract from the leaves of *O. stamineus*.

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D
	1	2	3	
500	0.155	0.152	0.175	0.161±0.013
375	0.142	0.147	0.149	0.146±0.004
250	0.123	0.142	0.132	0.132±0.010
125	0.09	0.095	0.091	0.092±0.003
0	0.053	0.055	0.060	0.056±0.004

Table 3.17. Reducing power of crude hexane extract from the leaves of *O. stamineus*.

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D
	1	2	3	
500	0.140	0.153	0.110	0.134±0.022
375	0.106	0.131	0.117	0.118±0.013
250	0.098	0.108	0.104	0.103±0.005
125	0.083	0.084	0.087	0.085±0.002
0	0.049	0.051	0.05	0.050±0.001

Table 3.18. Reducing power of crude water extract from the leaves of *O. stamineus*.

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D
	1	2	3	
500	0.19	0.182	0.181	0.184±0.005
375	0.172	0.181	0.172	0.175±0.005
250	0.169	0.168	0.166	0.168±0.002
125	0.142	0.145	0.143	0.143±0.002
0	0.072	0.069	0.061	0.067±0.006

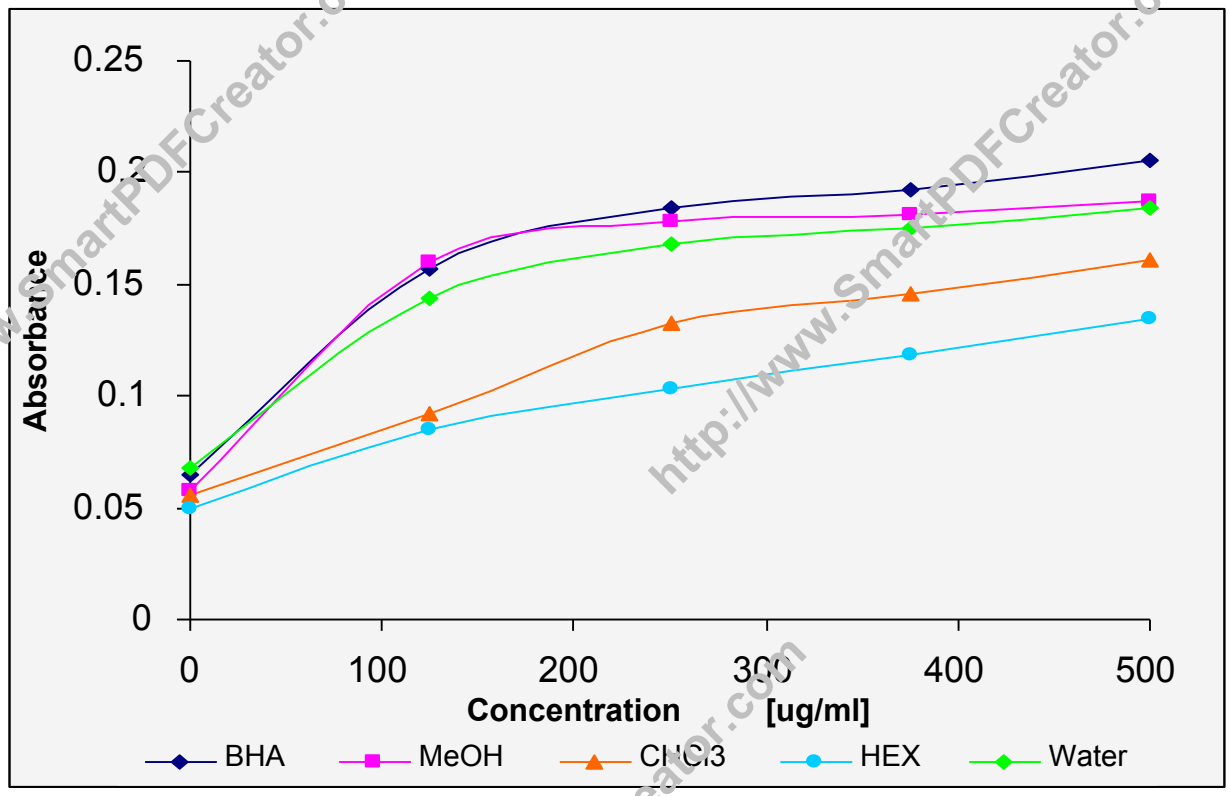

Fig. 3.9. Reducing power of leaves crude extract of *O. stamineus*.

C) Metal Chelating Assay

The antioxidant activities of methanol, chloroform, hexane and water extract from the leaves of *O. stamineus* were determined by using metal chelating assay. It was based on the chelating effect of Fe^{2+} ions by ferrozine reagent. Antioxidants from the test samples disturbed the formation of ferrozine- Fe^{2+} complexes. The colour intensity of the reaction mixtures reduced. As the concentration of the samples and standard increased, the metal chelating activity was increased. This assay was also carried out in triplicates and the average reading was recorded.

i) Standard Ethylene-diamine-tetraacetic acid (EDTA)

Ethylene-diamine-tetraacetic acid (EDTA) was used as the standard in the metal chelating assay. It was tested at various concentrations of 20, 40, 60, 80 and 100 µg/ml. The results were shown in the Table 3.19. Figure 3.10 illustrated the standard curve of EDTA in metal chelating assay. The metal chelating activity increased slightly at concentration of 60 to 100 µg/ml.

Table 3.19. Metal chelating activity of Ethylene-diamine-tetraacetic acid (EDTA)

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D	Percentage Inhibition (%)
	1	2	3		
80	0.019	0.023	0.022	0.021±0.002	99
60	0.029	0.026	0.029	0.028±0.002	98.7
40	0.198	0.194	0.199	0.197±0.003	91.5
20	1.101	1.108	1.098	1.102±0.005	52.3
0	1.835	1.889	1.859	1.861±0.027	19.5

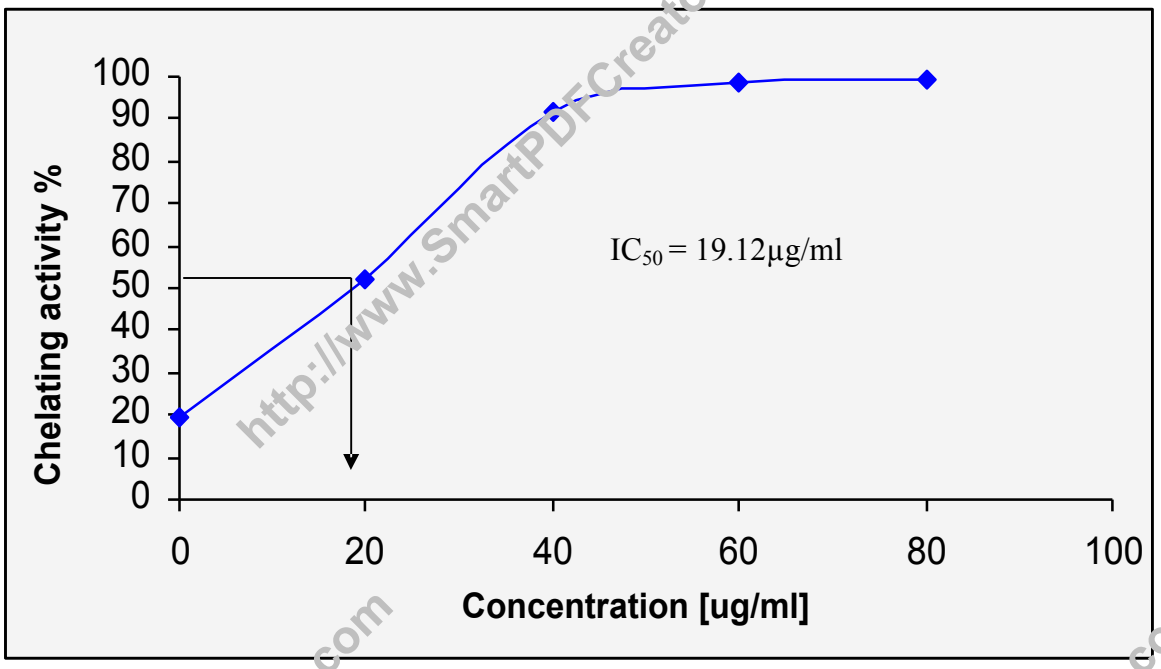

Fig. 3.10. Standard curve of Ethylene-diamine-tetraacetic acid (EDTA)

ii) Metal chelating activity of leaves crude extract of *Orthosiphon stamineus*

The metal chelating activity of methanol, chloroform, hexane and water extracts from the leaves of *O. stamineus* were determined by using metal chelating assay at various concentration; 20, 40, 60, 80 and 100 µg/ml. The results were shown in the Table 3.20 to Table 3.23.

Figure 3.11 showed the curve of the crude extract from the leaves of *O. stamineus* and standard in metal chelating assay. The metal chelating activity of crude extracts were lower than standard (EDTA).

Table 3.20. Metal chelating activity of crude methanol extract from the leaves of *O. stamineus*

Concentration [µg/ml]	**Absorbance at 562nm**			**Mean±S.D**	**Percentage Inhibition (%)**
	1	**2**	**3**		
80	0.998	1.111	1.013	1.041±0.061	55
60	1.116	1.181	1.167	1.155±0.034	50
40	1.246	1.194	1.233	1.224±0.027	47
20	1.609	1.613	1.617	1.613±0.004	30.2
0	1.947	1.959	1.955	1.954±0.006	15.5

Table 3.21. Metal chelating activity of crude chloroform extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D	Percentage Inhibition (%)
	1	2	3		
80	1.215	1.199	1.292	1.235±0.050	46.5
60	1.312	1.313	1.299	1.308±0.008	43.4
40	1.361	1.356	1.299	1.339±0.034	42.1
20	1.652	1.645	1.718	1.672±0.040	27.7
0	2.025	2.114	2.122	2.087±0.054	9.69

Table 3.22. Metal chelating activity of crude hexane extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D	Percentage Inhibition (%)
	1	2	3		
80	1.369	1.412	1.411	1.397±0.025	39.5
60	1.412	1.413	1.409	1.411±0.002	38.9
40	1.561	1.556	1.499	1.539±0.034	33.4
20	1.957	1.949	1.955	1.954±0.004	15.4
0	2.225	2.234	2.232	2.230±0.005	3.5

Table 3.23. Metal chelating activity of crude water extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 562nm			Mean±S.D	Percentage Inhibition (%)
	1	2	3		
80	1.116	1.181	1.167	1.155±0.034	50
60	1.213	1.223	1.222	1.219±0.006	47.3
40	1.317	1.322	1.309	1.316±0.007	43.1
20	1.755	1.783	1.755	1.764±0.016	23.7
0	1.967	1.996	1.983	1.982±0.015	14.2

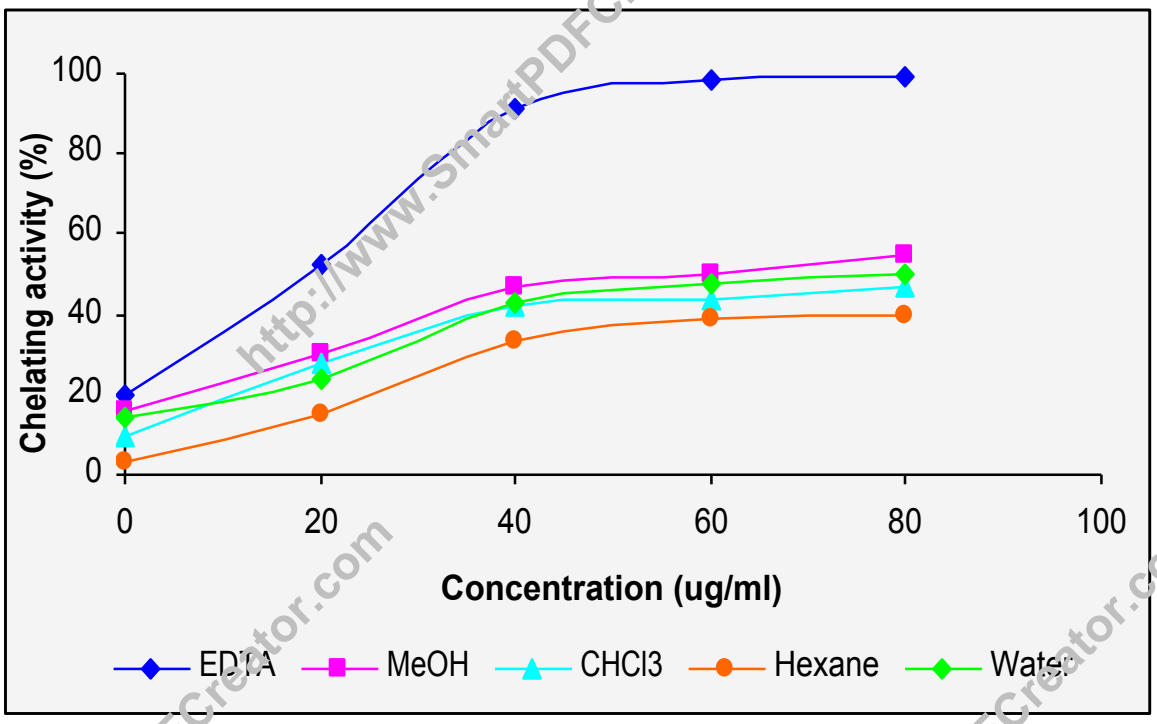

Fig. 3.11 Metal chelating activity of crude extract from the leaves of *O. stamineus*

3.4 Angiotensin Converting Enzyme (ACE) Bioassay

A) Angiotensin Converting Enzyme Inhibitory Assay

ACE inhibitory assay was done base on the hydrolysis of Hippuryl-L-Histidyl-L-Leucine (HHL) by ACE to Hippuric Acid and Histidyl-L-Leucine (HL). The extent of the Hippuric acid form was directly related to the ACE activity and it was determined spectrophotometrically by reading the absorbance at 228 nm.

i) Determination of ACE inhibition of standard captopril

Captopril was used as the standard in the ACE inhibitory assay. Table 31 showed the ability of the Captopril acted as ACE inhibitor to inhibit the process of HHL to form Hippuric Acid and Histidyl-L-Leucine (HL). Various concentrations of Captopril were tested; 250, 200, 150, 100 and 50 µg/ml and at 250 µg/ml (Table 3.24). At the concentration of 250 µg/ml, the percentage of ACE inhibition was 88%. Figure 3.12 showed the IC_{50} value of Captopril, 46.1 µg/ml.

Table 3.24. Standard of Captopril

Concentration of Captopril [µg/ml]	Absorbance at 228nm			Percentage Inhibition (%)	ACE Activity [M/min] ($X 10^{-5}$)
	1	2	Mean±S.D		
250	0.172	0.164	0.168±0.006	88.0	7.5
200	0.177	0.175	0.176±0.001	87.3	7.8
150	0.199	0.205	0.202±0.004	85.5	9.0
100	0.274	0.263	0.269±0.008	80.6	12.0
50	0.648	0.625	0.637±0.016	54.2	28.4

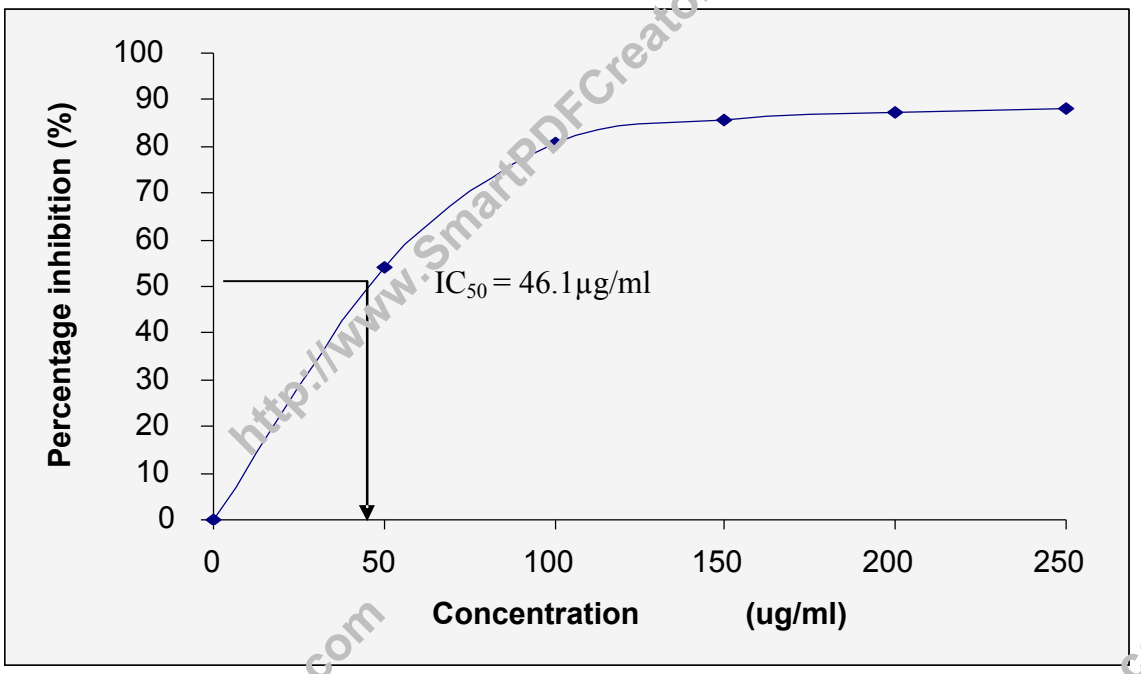

Fig. 3.12. Percentage of ACE inhibition of Captopril

ii) Determination of ACE inhibition of crude extracts of *O. stamineus*

The ACE inhibitory activities of methanol, chloroform, hexane and water extracts from the leaves of *O. stamineus* were determined (Table 3.25 to Table 3.28). All the crude extracts with 5 different concentrations were also tested by using ACE inhibitory activity to measure the percentage inhibition of ACE, its IC_{50} value and ACE activity. The percentage of ACE inhibition increased when the concentration of test samples increased.

The ACE activity decreased in the high concentration of test samples indicated that the ACE inhibitor inhibited the Angiotensin converting enzyme (ACE) from the reaction mixtures. Methanol extract showed IC_{50} value of 128 µg/ml, chloroform showed 262.5 µg/ml and water extract showed 121.8 µg/ml. Figure 3.13 showed the ACE inhibition of leaves extract of *O. stamineus* and standard (Captopril) and Figure 3.14 showed the ACE activity of the test samples.

Table 3.25. Percentage of ACE inhibition and ACE activity of crude methanol extract from the leaves *O. stamineus*

Concentration [µg/ml]	Absorbance at 228nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D		
250	0.222	0.234	0.228±0.008	83.6	10.2
200	0.236	0.235	0.236±0.001	83.1	10.5
150	0.374	0.369	0.372±0.004	73.2	16.6
100	0.658	0.645	0.652±0.009	53.1	29.1
50	0.936	0.942	0.939±0.004	32.4	41.8

Table 3.26. Percentage of ACE inhibition and ACE activity of crude chloroform extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 228nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D		
250	0.609	0.614	0.612±0.003536	56	27.3
200	0.682	0.674	0.678±0.005657	51.2	30.2
150	0.717	0.726	0.723±0.006364	48.1	32.2
100	0.825	0.838	0.833±0.009192	40.2	37.1
50	0.995	0.989	0.992±0.004243	28.7	44.2

Table 3.27. Percentage of ACE inhibition and ACE activity of crude hexane extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 228nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D		
250	0.709	0.711	0.710±0.001	48.9	31.6
200	0.826	0.834	0.830±0.006	40.3	37.0
150	0.901	0.909	0.905±0.006	34.9	40.3
100	1.058	1.053	1.056±0.004	23.7	47.1
50	1.215	1.209	1.212±0.004	12.8	54.0

Table 3.28. Percentage of ACE inhibition and ACE activity of crude water extract from the leaves of *O. stamineus*

Concentration [µg/ml]	Absorbance at 228nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D		
250	0.245	0.238	0.242±0.00495	82.6	10.8
200	0.255	0.259	0.257±0.002828	81.5	11.5
150	0.394	0.391	0.393±0.002121	71.8	17.5
100	0.711	0.699	0.705±0.008485	49.3	31.4
50	0.981	0.974	0.978±0.00495	29.7	43.5

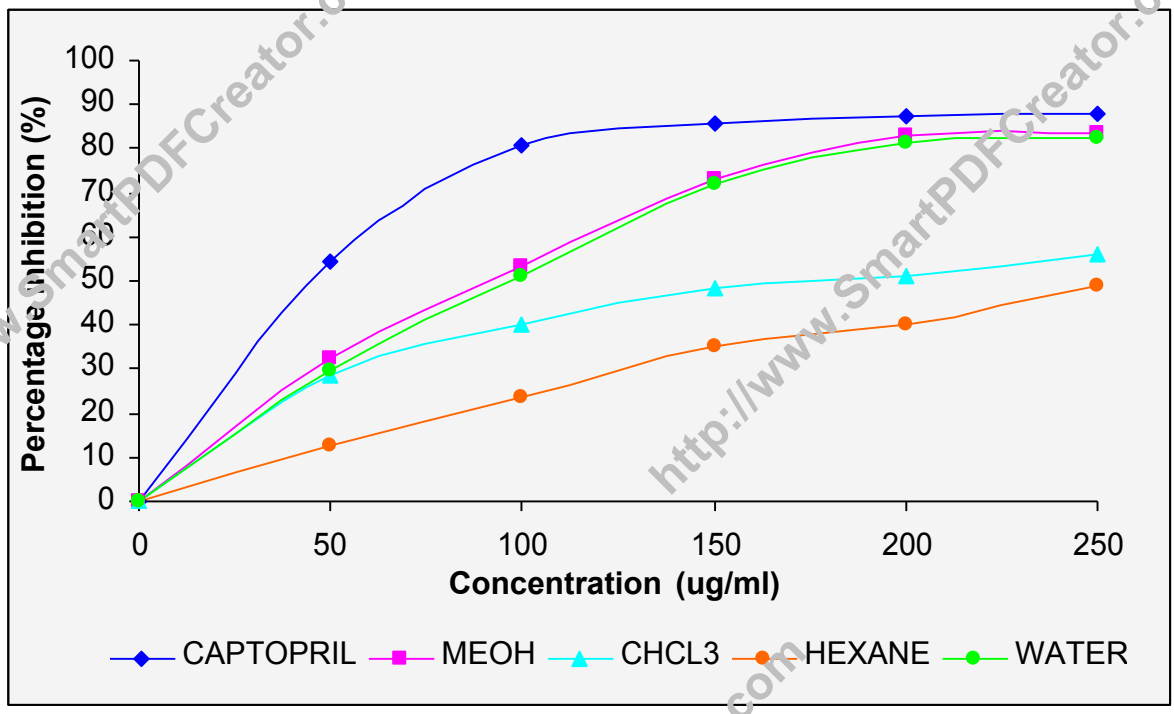

Fig. 3.13. Percentage of ACE inhibition of leaves crude extract of *O. stamineus*

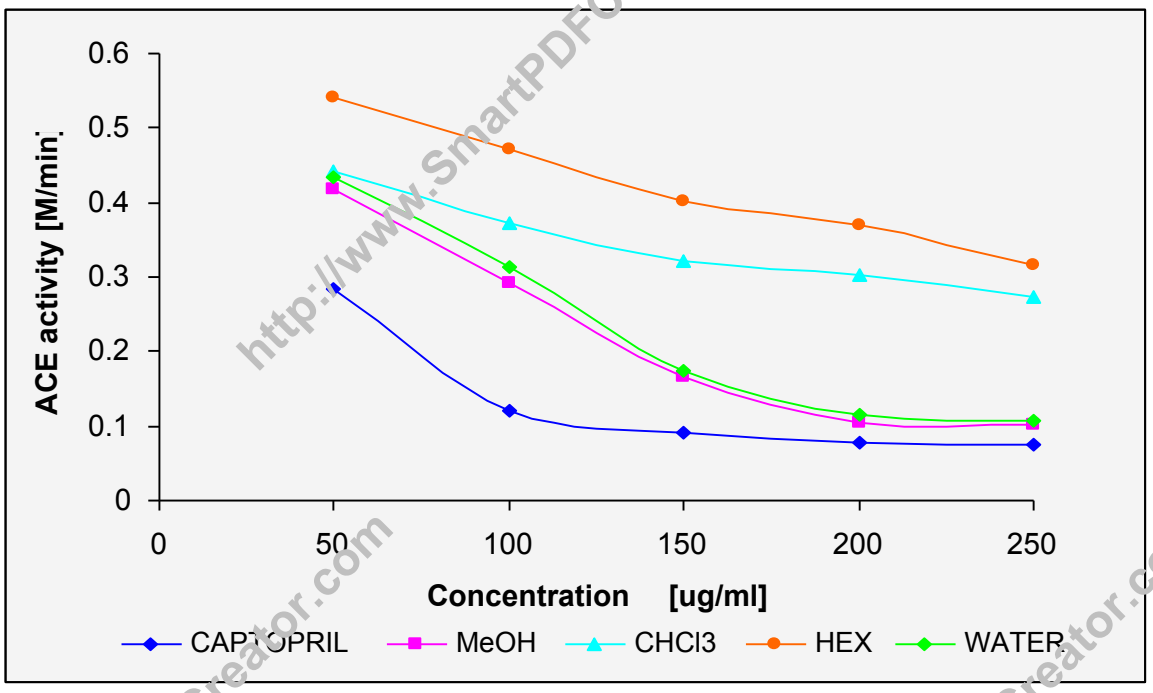

Fig. 3.14. ACE activity of leaves crude extract of *O. stamineus*

iii) Determination of ACE inhibitory activity of the chemical compounds isolated from leaves extract of *O. stamineus* by using TLC and CC

After the chemical compounds (in powdery form) isolated from the TLC plates, 1ug of each compound was mixed with 1ml of distilled water. It was centrifuged before continued with ACE inhibitory activity assay. Table 3.29 to Table 3.32 showed the ACE inhibition of the isolated chemical compounds from crude extracts of *O. stamineus*. Figure 3.15 to Figure 3.18 illustrated the histogram of the ACE inhibition of the isolated chemical compounds from crude extracts of *O. stamineus.*

Table 3.29. Percentage of ACE inhibition of the chemical compounds isolated crude methanol extract of *O. stamineus* by using TLC

Chemical compound	Absorbance at 228 nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D.		
MB1	0.234	0.213	0.224±0.015	88.9	10.0
MB2	0.232	0.178	0.205±0.038	89.7	9.1
MB3	0.148	0.320	0.234±0.121	88.4	10.4
MB4	0.400	0.308	0.354±0.065	82.4	15.8
MB5	0.288	0.246	0.267±0.029	86.8	11.9
MB6	0.504	0.289	0.397±0.152	80.2	17.7
MB7	0.221	0.190	0.206±0.022	89.7	9.1
MB8	0.308	0.222	0.265±0.061	86.8	11.8
MB9	0.172	0.223	0.198±0.036	90.1	8.8
MB10	0.262	0.210	0.236±0.037	88.3	10.5
MB11	0.203	0.274	0.239±0.050	88.1	10.7
MB12	0.313	0.347	0.330±0.024	83.6	14.7
MB13	0.638	0.466	0.552±0.122	72.5	24.6

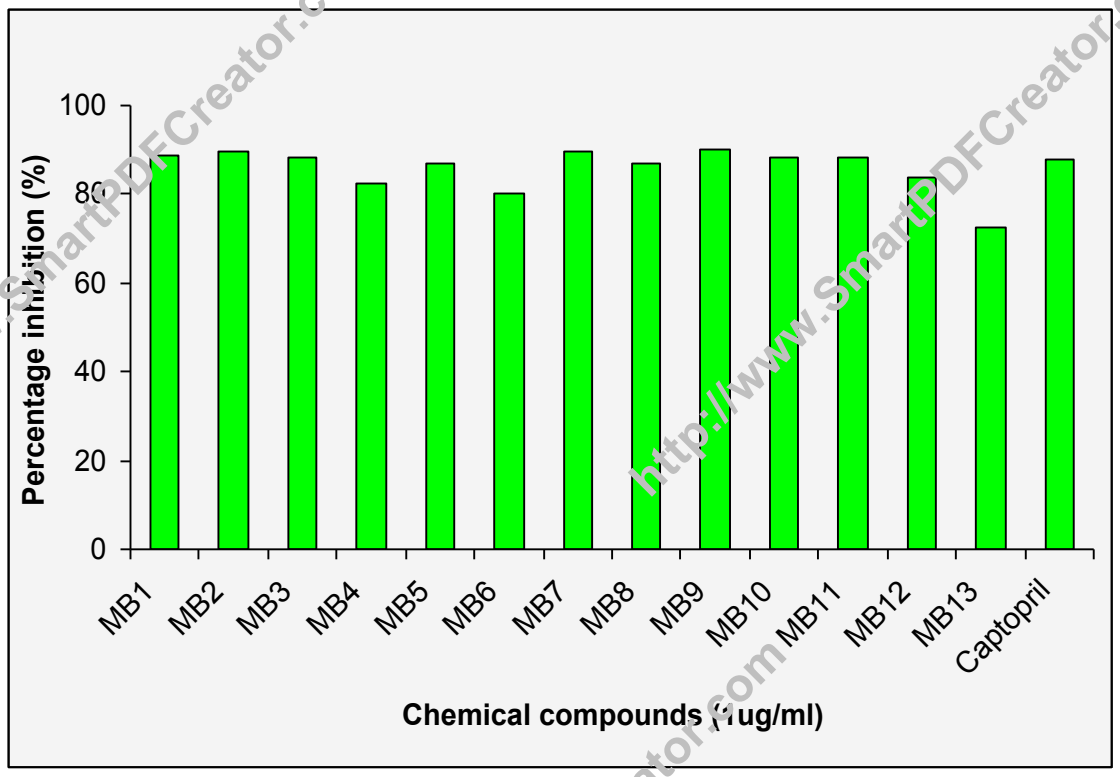

Fig. 3.15. Percentage of ACE inhibition of the chemical compounds isolated from crude methanol extract by using TLC

Table 3.30. Percentage of ACE inhibition of the chemical compounds isolated crude chloroform extract of *O. stamineus* by using TLC

Chemical compound	Absorbance at 228 nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D.		
CB1	0.446	0.308	0.377±0.098	81.2	16.8
CB2	0.348	0.502	0.425±0.109	78.8	18.9
CB3	0.545	0.547	0.546±0.001	72.8	24.3
CB4	0.573	0.33	0.452±0.172	77.5	20.1
CB5	0.594	0.403	0.499±0.135	75.2	22.2
CB6	0.395	0.309	0.352±0.061	82.4	15.7
CB7	0.585	0.600	0.593±0.011	70.5	26.4

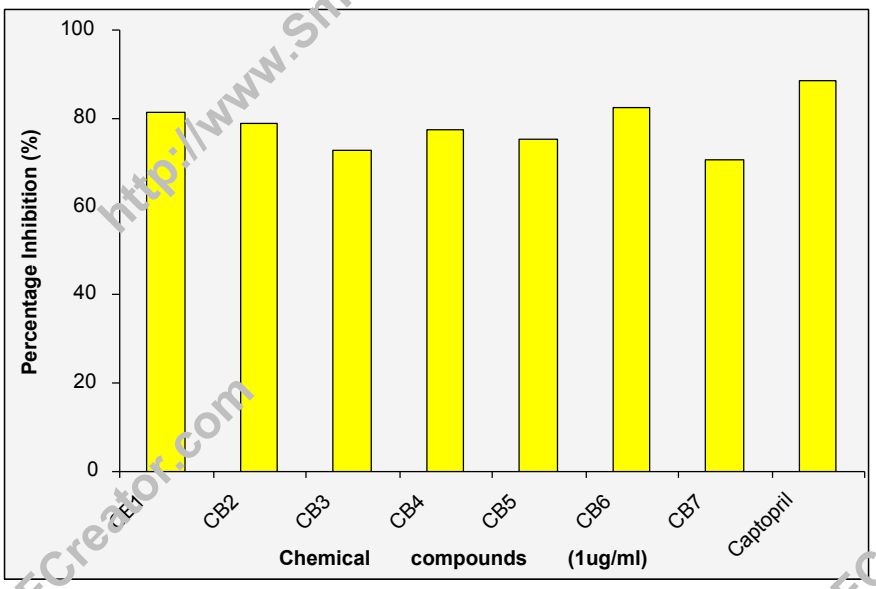

Fig. 3.16. Percentage of ACE inhibition of the chemical compounds isolated from crude chloroform extract by using TLC

Table 3.31. Percentage of ACE inhibition of the chemical compounds isolated crude hexane extract of *O. stamineus* by using TLC

Chemical compound	Absorbance at 228 nm			Percentage Inhibition (%)	ACE activity [M/min] (X 10^{-5})
	1	2	Mean±S.D		
HB1	0.334	0.198	0.266±0.096	86.8	11.9
HB2	0.387	0.605	0.496±0.154	75.3	22.1
HB3	0.295	0.317	0.306±0.016	84.8	13.6
HB4	0.156	0.239	0.198±0.059	90.1	8.8

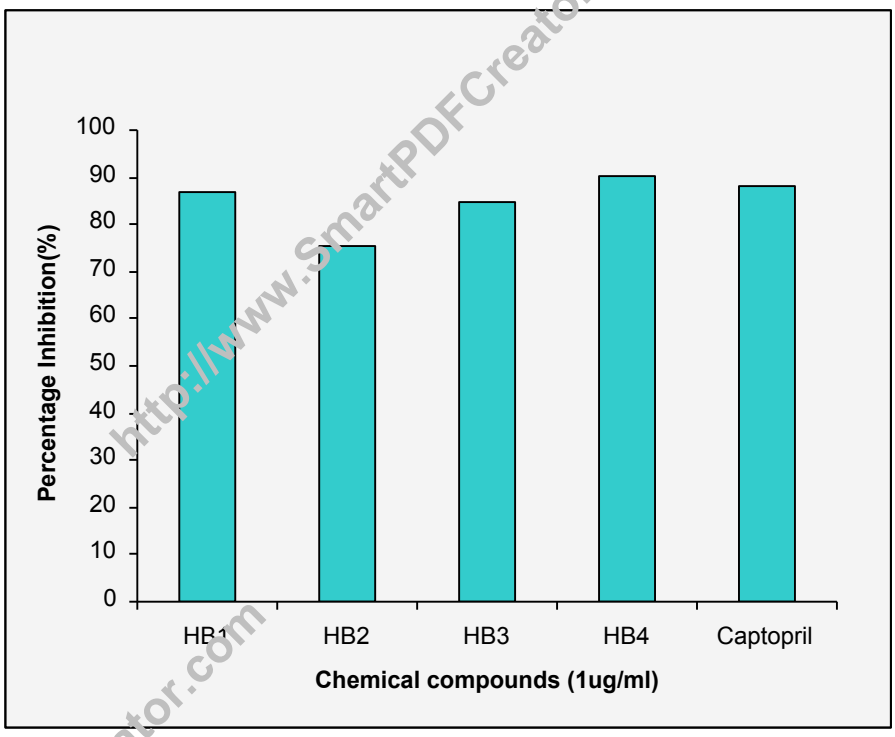

Fig. 3.17. Percentage of ACE inhibition of the chemical compounds isolated from crude hexane extract by using TLC

Table 3.32. Percentage of ACE Inhibition of the chemical compounds isolated from crude water extract of *O. stamineus* by using column chromatography

Fraction	Absorbance at 228nm			Percentage Inhibition (%)	ACE Activity [M/min] (X 10^{-5})
	1	2	Mean±S.D.		
1	0.226	0.224	0.225±0.001	88.8	10.0
2	0.225	0.216	0.221±0.006	89	9.8
3	0.422	0.428	0.425±0.004	78.8	18.9
4	0.623	0.636	0.630±0.009	68.7	28.1
5	0.896	0.823	0.860±0.052	57.2	38.3

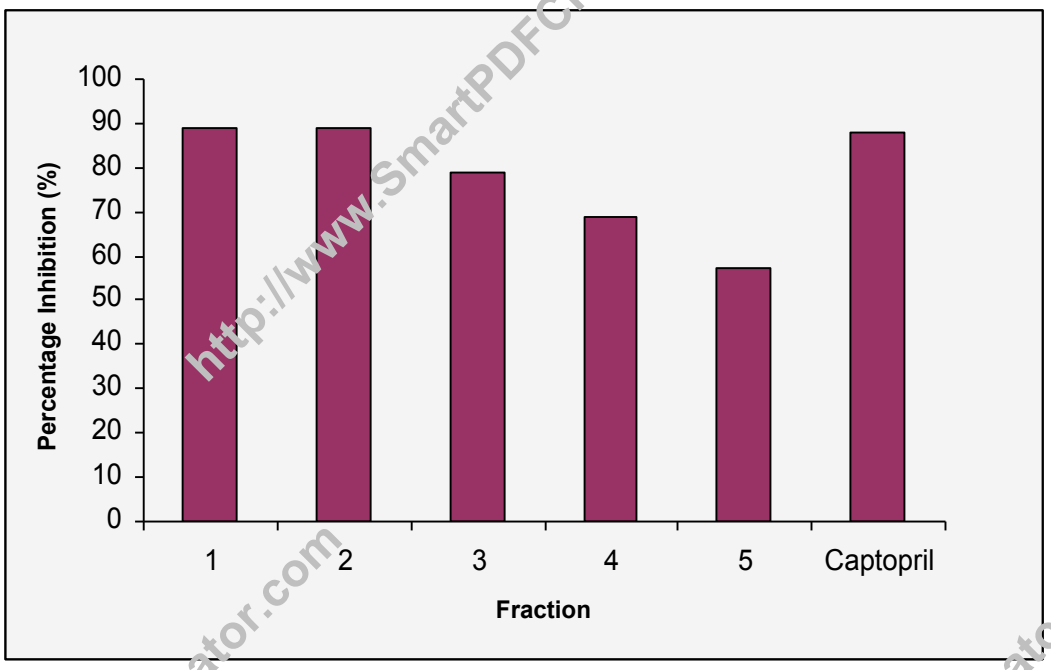

Fig. 3.18. Percentage of ACE inhibition of the chemical compounds isolated from crude water extract by using column chromatography

B) Hippuric Acid Standard Curve

Standard curve of Hippuric acid (HA) was done to measure the ACE activity of the test samples. Low ACE activity indicated that the ACE inhibitor was effective in this reaction (Table 3.33). Figure 3.19 illustrated the standard curve of HA.

Table 3.33. Standard curve of Hippuric acid (HA)

Concentration HA[µg/ml]	Absorbance at 228nm		Mean±S.D
	1	2	
20	1.465	1.471	1.468±0.004
4	0.401	0.411	0.406±0.007
0.8	0.189	0.197	0.193±0.006
0.16	0.085	0.095	0.090±0.007
0.032	0.033	0.039	0.036±0.004

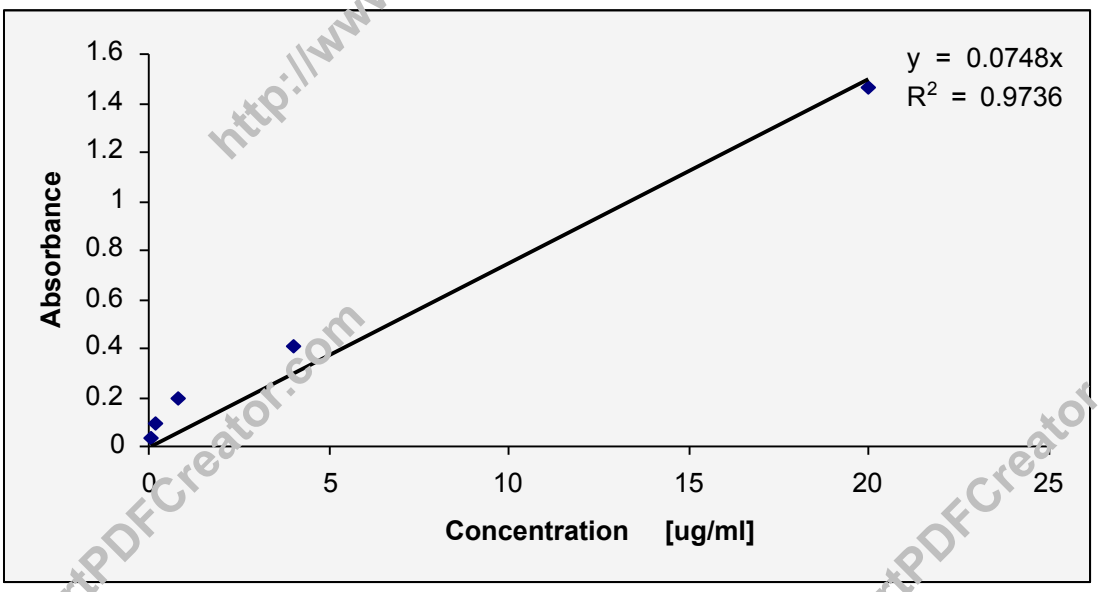

Fig. 3.19 Standard Curve of Hippuric Acid (HA)

From the standard curve (Fig.3.19), y=0.0748x, (y=absorbance, x=ACE activity), so the ACE activity of each sample can be calculated.

3.5 Determination of Phenol Content

Standard curve of Gallic acid was developed to determine the total phenolic contents of methanol, chloroform, hexane and water extract from the leaves of *O. stamineus* (Table 3.34). Figure 3.20 showed the standard curve of Gallic acid. The phenol content of each extract was measured by using equation from the standard curve. Table 3.35 showed the total phenolic contents of crude methanol, chloroform, hexane and water extracts from the leaves of *O. stamineus*.

i) Standard phenol (Gallic acid)

Table 3.34. Standard curve of Gallic acid

Concentration gallic acid [ug/ml]	Absorbance at 765nm		Mean±S.D.
	1	2	
250	4	4	4±0
200	4	4	4±0
150	1.963	1.952	1.9575±0.008
100	0.934	0.942	0.938±0.006
50	0.819	0.825	0.822±0.004
0	0.055	0.052	0.0535±0.002

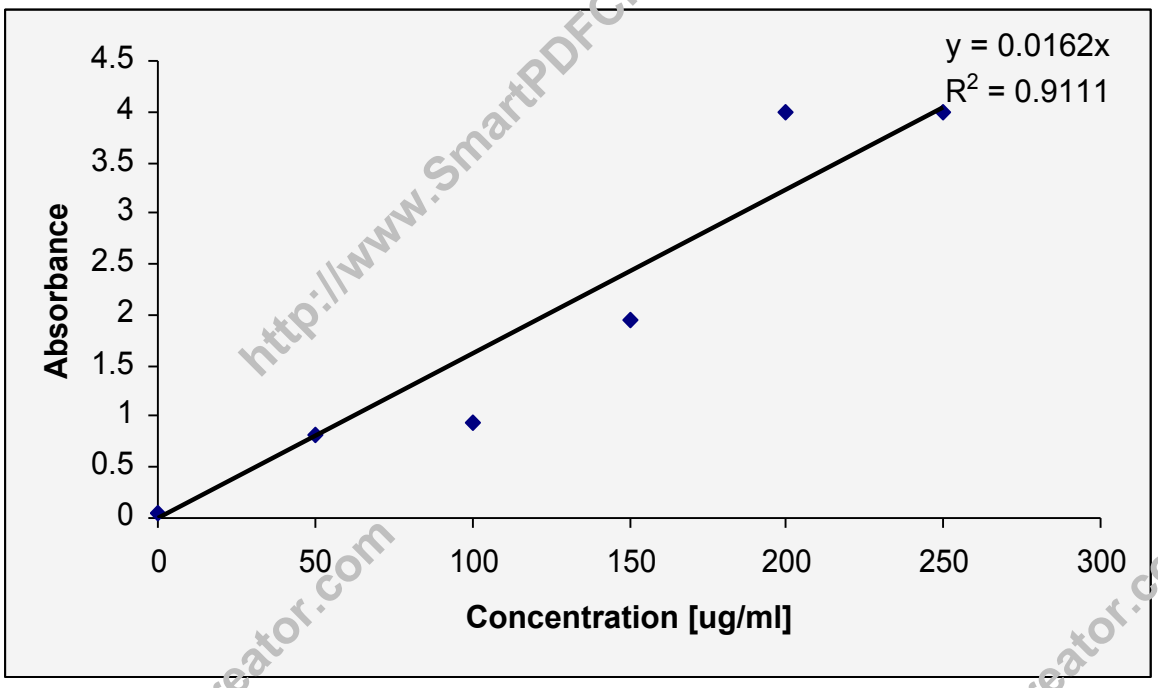

Fig. 3.20. Standard curve of Gallic acid

ii) Total phenolic of leaves crude extracts of *O. stamineus*

Table 3.35. Total phenolic of leaves crude extracts of *O. stamineus*

Crude extract	Absorbance at 765nm			Total Phenolic Content [mg/g dry mass]
	1	2	Mean±S.D.	
Control	0.052	0.049	0.051±0.002	-
Hexane	0.094	0.097	0.096±0.002	23.70
Chloroform	0.155	0.147	0.151±0.006	37.28
Methanol	0. 731	0. 734	0.734±0.02	181.23
Water	0.752	0.745	0.749±0.005	184.94

Table 3.36 showed the IC_{50} value of 3 different assays from this study and total phenolic contents of crude methanol, chloroform, hexane and water from the leaves of *O. stamineus.*

Table 3.36. IC_{50} value and total phenol content of leaves crude extract of *O. stamineus*

Sample	IC_{50} (DPPH) [µg/ml]	IC_{50} (Metal chelating) [µg/ml]	IC_{50} (ACE) [µg/ml]	Total Phenol Content [mg/g dry mass]
Methanol	24. 80	60	94.16	181.23
Chloroform	23.28	-	195.31	37.28
Hexane	47.44	-	-	23.70
Water	20. 87	80	101.42	184.94

3.6 Brine Shrimp Lethality Assay (BSLA)

From these tables, the LC_{50} value for different crude extracts of *O. stamineus*. When the LC_{50} value was higher it meant that the toxicity of the crude extract was lower. The highest LC_{50} value was the water extract from leaves of *O. stamineus*, which was 532459.30µg/ml, while the lowest LC_{50} value was the hexane extract with 47.54µg/ml. This meant that 47.54µg/ml was needed to inhibit the 50% population of the brine shrimp.Table 3.37. Number of dead shrimp in leaves crude extract of *O. stamineus*

Concentration sample [µg/ml]	**Total number of shrimp**				**Number of dead**			
	MeOH	**CHCl3**	**HEX**	**H20**	**MeOH**	**CHCl3**	**HEX**	**H20**
1000	10	10	10	10	8	7	10	4
100	10	10	10	10	3	6	6	2
1	10	10	10	10	2	3	2	3

Table 3.38. Probit analysis table of crude methanol extract from the leaves of *O. stamineus*

Concentration sample [µg/ml]	**Log_{10} [Concentration sample]**	**Total no. of shrimp**	**Number of dead**	**Percentage mortality (%)**	**LC_{50} [µg/ml]**	**95 percent confidence**
1000	3	10	8	80	171.49	33.53-2165. 71
100	2	10	3	30		
10	1	10	2	20		

Table 3.39. Probit analysis table of leaves chloroform crude extract of *O. stamineus*

Concentration sample [µg/ml]	**Log_{10} [Concentration sample]**	**Total no. of shrimp**	**Number of dead**	**Percentage mortality (%)**	**LC_{50} [µg/ml]**	**95 percent confidence**
1000	3	10	7	70	67.59	0-infinity
100	2	10	6	60		
10	1	10	3	30		

Table 3.40. Probit analysis table of crude hexane extract from the leaves of *O. stamineus*

Concentration sample [µg/ml]	**Log_{10} [Concentration sample]**	**Total no. of shrimp**	**Number of dead**	**Percentage mortality (%)**	**LC_{50} [µg/ml]**	**95 percent confidence**
1000	3	10	10	100	47.54	13.92-134.94
100	2	10	6	60		
10	1	10	2	20		

Table 3.41. Probit analysis table of crude water extract from the leaves of *O. stamineus*

Concentration sample [µg/ml]	**Log_{10} [Concentration sample]**	**Total no. of shrimp**	**Number of dead**	**Percentage mortality (%)**	**LC_{50} [µg/ml]**	**95 percent confidence**
1000	3	10	4	40	532459.30	21.44-185.22
100	2	10	2	20		
10	1	10	3	30		

The extraction process from the leaves of *O. stamineus* was started by using Soxhlet apparatus. The dried-powdered leaves of *O. stamineus* were extracted by using 4 different types of solvents such as methanol, chloroform, hexane and water. From (Table 3.1) it observed that the methanol extraction showed dark green extract, while hexane showed yellowish brown extract, chloroform showed dark brown extract and water extract exhibited brownish extract. According to Ross and Brain, 1977, the polar solvent will extracted out the polar compound and the non-polar compound will be extracted by the non-polar solvent. Therefore, the high polarity solvent such as methanol may extract high polarity chemical compound such as amino acid, sugar and glycosides while chloroform solvent may extract alkaloids and volatile oil. Hexane, as non polar solvent may extract fats, waxes and fixed oil.

After extraction, separation of chemical compound from the leaves crude extract of *O. stamineus* was done by using Thin Layer Chromatography (TLC), Column Chromatography (CC) and High Performance Liquid Chromatograhy (HPLC). TLC was one separation technique that was inexpensive and quick. In the methanol extract, 13 labeled compounds were separated from the silica gel TLC plate; MB1, MB2, M B3, MB4, MB5, MB6, MB7, MB8, MB9, MB10, MB11, MB12 and MB13. In the chloroform extract, 7 compounds were separated: CB1, CB2, CB3, CB4, CB5, CB6 and CB7 while only 4 labeled compounds were separated from the hexane crude extract of *O. stamineus*; HB1, HB2, HB3 and HB4.

The TLC plates were sprayed with Vanillin reagent and heated, if purple band or spot appeared, it indicated the presence of terpenoid. Terpenoids was separated as labeled compound; MB3, MB6, MB7, MB11, MB13 from the methanol crude extract (Table 3.2). In addition, from the hexane extract, terpenoids was found in HB2 and HB3 (Table 3.5). CB2 and CB7 represented terpenoids in chloroform extract (Table 3.4). Study had shown the presence of terpenoids compound (diterpenes) in *O. stamineus* (Tezuka, 2000).

After spraying the TLC plate with Vanillin and green labeled compound was appeared, it showed the presence of phenols. In the methanol extract, labeled compound MB4, MB5, MB8, MB9, MB10 and MB12 contain phenols (Table 3.2). Whereas, in the chloroform and hexane extract, labeled compounds: CB5, CB6, HB1 and HB4 represented as phenols. From the previous studies, twenty phenolic compounds including flavonol glycosides, lipophilic flavones and caffeic acid derivatives were isolated from *O. stamineus* (Khamsah, *et al*., 2006). In addition, a flavonoid (Methylripariochromene A) that was isolated from the leaves extract of *O. stamineus* was found to reduce blood pressure in spontaneously hypertensive rats (Wiart, 2002). To confirm the detection of phenolic compounds, the two dimensional Thin Layer Chromatography (2-D TLC) was developed. (Harborne, *et al*., 1973). The TLC plates were then sprayed by using Folin reagent and 2 phenolic compounds detected; p-coumaric acid and caffeic acid (Table 3.8).

TLC plates were sprayed with Dragendorff reagent and orange compound appeared indicated the presence of alkaloids. MB1 and MB8 labeled compound from methanol crude extract were observed as alkaloids. In addition, HB3, HB4 and CB6 labeled compounds from the hexane and chloroform extract also showed positive result of alkaloids. Alkaloids had been reported to inhibit the percentage of Angiotensin converting enzyme (ACE) in ACE bioassay (Nyman, *et al*., 1998) but no research have been reported about the importance of alkaloids which was isolated from *O. stamineus* in antihypertension activity.

Purification test had been developed from labeled compound (MB9) of methanol extract. From (Table 3.3), it observed that 5 compounds were separated on the TLC plate and after it were sprayed with Folin-ciocalteu reagent, blue colour appeared, indicated the presence of phenolic compounds.

Column chromatography was one of the chromatography techniques to separate the chemical compound using glass column. It worked on much larger scales by packing the same materials into the glass column. This technique was applied to water extraction and 5 fractions were collected. After TLC plates were sprayed with vanillin reagent, the green coloured compound appeared; it indicated the presence of phenolic compound (Table 3.6).

High performance liquid chromatography (HPLC) was a highly improved form of column chromatography. It was performed under high pressure and the detection method in HPLC was highly automated and very sensitive. From the previous study, it was reported that polyphenols compound, polymethoxylated flavonoids and caffeic acid

derivatives were identified and quantitatively determined by HPLC from *O. stamineus* (Olah, *et al*., 2003). Moreover, other flavonoids compounds such as sinensetin, eupatorin and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) were also detected from *O. stamineus* by using HPLC method with ultraviolet detection (Leon, *et al.,* 2005). In this study, we used HPLC to separate the chemical compounds that present in the leaves crude extract of *O. stamineus.*

From Fig.3.1, chromatograms were observed and 7 compounds were separated from methanol crude extract of *O. stamineus* within 30 minutes. It had been reported that methanol extracts from the leaves of *O. stamineus* showed the presence of rosmarinic acid, TMF, sinensitin, and eupatorin (Akowuah, *et al*., 2005).

Fig.3.2 and Fig.3.3 showed HPLC detected 6 chemical compounds from chloroform and 4 compounds were separated from hexane crude extract of *O. stamineus* within 30 minutes. From the previous study, it was reported that chloroform extract of *O. stamineus* gave the highest amount of sinensetin and eupatorin (Akowuah, *et al*., 2005).

After 30 minutes run in the HPLC system, only 4 compounds were detected from the water extract of *O. stamineus* (Fig.3.4). The separation of the chemical compounds from water extract was not good because the mobile phase may not be suitable for water extract.

HPLC analysis showed less chemical compounds compared with thin layer chromatography and column chromatography analysis. The peaks of the chemical compounds were confirmed by comparing of their retention time with reference standards (Akowuah, *et al*., 2005). After comparing their retention time, compound at peak 5

(methanol) that separated at the retention time of 5.213 was identified as rosmarinic acid (RA). Standard of RA represents at retention time 5.563 (Akowuah, *et al*., 2005).

Standard of Hippuryl-histidyl-leucine (HHL) and Hippuric acid (HA) were also separated by using HPLC. The previous study had been reported to separate the standard compound of HHL and HA by using HPLC on C18 column with gradient elution (Wu, *et. al*., 2002). From Fig. 3.5, the HA sample showed one of the highest peaks at retention time of 4.115, and it indicated that the sample was pure and not contaminated with other compounds. For separation of 5mM of HHL compound, the highest peak was detected in the retention time of 2.184. The separation of HA and HHL can be achieved only in 8 to 10 minutes.

In this study, the antioxidant activity of the methanol, hexane, chloroform and water extracts from leaves of *O. stamineus* were examined using DPPH radical scavenging activity. Referring to the method described earlier and the results of the screening were shown in (Fig. 3.7) as compared with known antioxidant Ascorbic acid. At the concentration of 200µg/ml, the leaves water extract of *O. stamineus* showed the highest percentage of antioxidant activity, which was 86.4% and it was followed by methanol extract with 84.5% . Chloroform and hexane extract showed the lowest antioxidant activity with 78.4% and 79.5% respectively. The overall activity of the crude extracts was lower than ascorbic acid, which was 91.7%.

From Thin Layer Chromatography (TLC) and Column Chromatography (CC) separation techniques, 3 chemical compounds were separated including terpenoids, alkaloids and phenolics. Water and methanol extracts of *O. stamineus* showed highest

activity of DPPH and it known as the effective scavenger of DPPH free radical. The main chemical compounds that presence in the water and methanol extracts were phenolic compounds. Phenolic compounds played an important role in scavenging DPPH radical. In the previous study, it was reported that the leaves extracts of *O. stamineus* with different solvent system shows significant radical scavenging activity of DPPH in vitro model system (Akowuah, *et al*., 2005). In addition, the methanol extract of *O. stamineus* from different localities showed the effective activity towards DPPH radical scavenging assay (Khamsah, *et al*., 2006).

Ascorbic acid was used as a standard in the DPPH radical scavenging assay. Half maximal inhibitory concentration (IC_{50}) was a measure of the effectiveness of a compound in inhibiting biological or biochemical functions. According to Fig.3.6, the percentage of DPPH radical scavenging activity of ascorbic acid at 200µg/ml was 91.7% and the IC_{50} value of ascorbic acid was 12.83 µg/ml. The IC_{50} value of leaves water and methanol crude extract from *O. stamineus* were 20.87 µg/ml and 24.8 µg/ml respectively. Besides, IC_{50} values of hexane and chloroform extract were 47.44 µg/ml and 23.28 µg/ml respectively (Fig.3.7).

The reducing power assay was one of the mechanism actions of antioxidants. Antioxidants will inhibit or reduce the ferricyanide complex to the ferrous form and Perl's Prussian blue complex appeared if the reaction occurred. Fe (III) reduction was often used as an electron-donating activity and it can be strongly correlated with other antioxidant properties. The extract which showed a reducing power could function as

electron donor and could also reduce the oxidized intermediates which generate from the lipid peroxidation reaction (Thitilertdecha, *et al*., 2008).

Fig.3.9 showed the dose response curves of the reducing power activity from leaves crude extracts of *O. stamineus*. The reducing power of methanol and water extract increased from 0.160±0.007 and 0.143±0.002 at 125µg/ml respectively to 0.187±0.005 and 0.184±0.005 at 500µg/ml. The reducing power of chloroform and hexane extract increased from 0.092±0.003 and 0.085±0.002 at 125µg/ml respectively to 0.161±0.013 and 0.134±0.022. The leaves methanol extract of *O. stamineus* showed the highest activity at all concentrations.

Matkowski, 2008 reported that the ethyl acetate extract of *O. stamineus* had a capability to reduce transition metal ions in the phosphomolybdenum assay, but there was no research been reported using reducing power assay to reduce the formation of Fe^{3+} to Fe^{2+} from the extract of *O. stamineus*. The metal chelating assay was basically to determine the ability of each extract to chelate ferrous ion and prevent the formation of ferrozine Fe^{2+} complex. In this study, various concentration of crude *O. stamineus* extract were determined their ability to chelate ferrous ion and reduce the formation of this complex.

Fig.3.11 summarized the results obtained for chelating effects of crude extract on iron (II). It was noted that the chelating activity of the leaves crude extract of *O. stamineus* increased when the concentration increased, but it only showed a little Fe^{2+} chelating activity. Only methanol and water extract exhibited IC_{50} value. IC_{50} value of EDTA was 38.24 µg/ml and the IC_{50} values of methanol and water extract of *O.*

stamineus were 30 µg/ml and 100 µg/ml respectively. None of the leaves crude extract of the *O. stamineus* appeared to be better chelator of iron (II) ions than the standard EDTA in this assay. No study had been reported about the metal chelating activity of extract from *O. stamineus*.

Angiotensin converting enzyme (ACE) inhibitory assay was used to measure the inhibition of the ACE activity in the test sample. A method that was modified from Cushman and Cheung, 1971 was based on the hydrolysis of Hippuryl-histidyl-leucine (HHL) by ACE to form Hippuric acid (HA) and Histidyl-leucine as products (Wu, *et al*., 2002). In this assay, HHL was used as a substrate.

ACE was prepared from the fresh rat's lungs in the same day of bioassay test. Previous study showed that, other than rats lungs, ACE can also extracted from rabbit lungs (Braga *et al*., 2007). Captopril was used as positive control in this ACE inhibitory assay. It was a synthetic ACE inhibitor which was widely used as antihypertension drugs in medical treatment of hypertension in humans (Watanabe, *et al.,* 2005). Captopril exhibited high ACE inhibition, 88%±0.002 at concentration of 250 µg/ml. The IC_{50} value of captopril was determined by 46.13µg/ml (Fig.3.12).

From Table 3.25, the leaves methanol crude extract of *O. stamineus* showed the highest ACE inhibition, 81.7%±0.004 and hexane extract represented the lowest ACE inhibition, 39.6%±0.013 at the concentration of 250 µg/ml. The main active compound that presented in the methanol crude extracts of *O. stamineus* was phenolics compound and they may act as ACE inhibitor in this ACE inhibitory assay.

The polyphenol compound from *O. stamineus* had been reported to be very effective in reducing oxidative stress (Akowuah, *et al.,* 2005), which can develop hypertension. Various concentrations of the leaves crude extract of *O. stamineus* were screened for their ACE inhibitory activity. Fig.3.13 illustrated Captopril which showed the highest ACE inhibitory activity 88.0%±0.006 at the concentration of 250µg/ml followed by leaves methanol, water, chloroform and hexane extract of *O. stamineus*. The higher concentration of crude extracts showed higher ACE inhibition and low ACE activity. From the results, the leaves methanol extract of *O. stamineus* showed the highest ACE inhibition at 83.6%±0.008 with IC_{50} value of 94.16 µg/ml followed by water extract, 82.6%±0.005 with IC_{50} value of 101.42 µg/ml and chloroform extract, 56%±0.004 with IC_{50} of 262.45 µg/ml. Hexane crude extract showed the lowest ACE inhibition (48.9%±0.001).

Isolated labeled compounds from TLC plates were screened for their inhibitory effect on ACE. In methanol crude extract (Table 3.29) all labeled compounds showed more than 80% of ACE inhibition except for MB13 compound that exhibited only 72.5%±0.122. Labeled compound MB9 showed the highest ACE inhibition (90.1%±0.036). MB9 contained phenolic compound while MB13 contained terpenoid and aromatic compounds showed the lowest ACE inhibition (72.5% ±6.1). MB1 containing alkaloid compound also showed high ACE inhibiton (88.9%±0.015). Therefore it was proven that alkaloids posses important role in ACE inhibitory activity.

From the two dimensional Thin Layer Chromatography (TLC-2D) result (Table 3.8), phenolic compounds were presence in the leaves methanol crude extract, so it showed that phenolic compounds were ACE inhibitor agents from *O. stamineus*. Moreover, flavonoids compound has also been isolated from leaves of *O. stamineus* and it was reported to exhibit antihypertensive effect (Lee, *et al.,* 2004).

CB6 labeled compound from chloroform extract (Table 3.30) showed the highest percentage of ACE inhibition, 82.4%±0.061 and the lowest percentage was observed from CB7, 70.5%±0.011. CB6 consisting of phenol, aromatic compound and alkaloid, while CB7 represented terpenoid and aromatic compound. Alkaloids in the leaves of *O. stamineus* may be the new ACE inhibitors found.

In addition, HB4 labeled compound from TLC plate of hexane extract showed the highest ACE inhibition (90.1%±0.059), while HB2 showed the lowest percentage of ACE inhibition, 75.3%±0.154 (Table 3.31). HB4 contained phenol, aromatic compound and alkaloids while HB2 contained terpenoid and aromatic compound. All of the potentials ACE inhibitors that mentioned above contained aromatic compounds.

Terpenoids was also an active compound that present in the *O. stamineus* and it plays an important role in ACE inhibitory activity. Labeled compound, MB7 that contained terpenoids and aromatic compounds showed the second highest ACE inhibitory activity (89.7%±0.022). HB3 showed 84.8%±0.016 of ACE inhibition and it also contained terpenoids. No research has been reported about the terpenoids compound as ACE inhibitor was extracted *O. stamineus*.

Table 3.32 showed that fraction 1 and 2 from column chromatography of leaves water extract of *O. stamineus* exhibited the highest percentage of ACE inhibition, 88.8%±0.001 and 89%±0.0006 respectively. Fraction 1 showed the presence of phenolic compound. The percentage of ACE inhibitory activity of crude extract was lower than inhibitory activity of isolated labeled compound from TLC plate. The chemical compounds in the crude extract were not separated well and the compounds become more complex in the test sample.

The crude extract was a good antihypertension agent in when it was in the higher concentration. The percentage of ACE activity decreased and the percentage of ACE inhibition increased. In order to measure the ACE activity, standard curve of Hippuric acid (HA) was developed (Table 3.33). Fig. 3.19, showed standard curve of HA and the equation was used to determine the ACE activity of each test sample. Low ACE activity indicated the ACE inhibitors from the test samples were effectively inhibiting the ACE from the mixed reactions.

Standard curve of Gallic acid was developed to determine the total phenolic contents of each crude extract from the leaves of *O. stamineus*. Fig. 3.20 showed the standard curve of gallic acid and the equation was used to determine the total phenol content of each extract. From (Table 3.35), the leaves water extract of *O. stamineus* showed the highest phenolic content, 184.94 mg/g dry mass and hexane crude extract exhibited the lowest phenolic content, 23.70 mg/g dry mass.

Table 3.36 showed the IC_{50} value of 3 different assays, DPPH free radical scavenging assay, metal chelating assay and ACE inhibitory assay of crude methanol, chloroform, hexane and water extracts from the leaves of *O. stamineus*. Crude methanol extract exhibited IC_{50} value of 24.8 µg/ml in DPPH free radical scavenging assay, 60 µg/ml in metal chelating assay and 181.23 µg/ml in ACE inhibitory assay. While crude water extract exhibited IC_{50} value of 20.87 µg/ml in DPPH free radical scavenging assay, 80 µg/ml in metal chelating assay and 184.94 µg/ml in ACE inhibitory assay.

BSLA test was used to determine the toxicity of the plant extract in different concentrations. The LC_{50} value of the brine shrimp assay was obtained from the crude extract, methanol, chloroform, hexane and water. BSLA results presented by (Table 3.41) showed that the leaves water extract of *O. stamineus* was non-toxic to the brine shrimp. They exhibited very low toxicity, gave LC_{50} values of 532459.30. The leaves hexane extract of *O. stamineus* was the most toxic (Table 3.40), with LC_{50} value of 47.5µg/ml. Leaves chloroform and methanol extract of *O. stamineus* exhibited LC_{50} value of 67.6 µg/ml and 171.49 µg/ml. The methanol and water extracts of *O. stamineus* were the most active in antioxidant and ACE inhibitory activity. They exhibited low toxicity on brine shrimp, so it is suggested that the leaves methanol and water extract of *O. stamineus* were more suitable to be used in medical treatment. No research had been reported to determine the toxicity values of extract from *O. stamineus* by using brine shrimp lethality assay (BSLA).

In the previous study, it was reported that reactive oxygen species (ROS) played an important role in the development of hypertension. The ROS was not critical in the early stages of hypertension, but it can be important in severe hypertension (Touyz, 2004). When the antioxidant defense mechanism in human body was reduced, the oxidative stress occurred and it induced hypertension disease so, antioxidant should be consumed by human not only to inhibit or prevent the oxidative stress diseases but can also reduce high blood pressure. Synthetic antioxidant and ACEI that were widely used by people can cause many harmful effects, therefore natural sources such as medicinal plant that contain antioxidant and antihypertensive properties such as *O. stamineus* can be consumed safely to treat these diseases.

Leaves extract of *O. stamineus* were not only can be used to treat oxidative stress but can also treat high blood pressure. The treatment of hypertension by consuming antioxidant from dietary intake of plant such as *O. stamineus* will reduce or slow down the process of free radicals damage.

From the antioxidant activity results that were observed before, the leaves extract of *O. stamineus* showed high percentage of antioxidant activity and it can be useful in the treatment of various types of diseases that related to oxidative stress such as cardiovascular disease, cancer, ageing and hypertension. From the ACE inhibitory activity, leaves extract of *O. stamineus* showed high potential as ACE inhibitor and it can be useful in medical treatment of hypertension.

The combination of potential as antioxidant and antihypertension agent made *O. stamineus* can be used for traditional treatment safely. Nowadays, there were a few products from *O. stamineus* or 'Misai Kucing' that have been produced in Malaysia, such as in the forms of tea; 'HERBagus-Teh Misai Kucing', Reelear Tea, Polens Herbal Tea, 'The Tropika-Misai Kucing', NusaHerba Misai Kuching Organic Herbal Tea and '3 in 1 coffee' to treat hypertension and antioxidant related diseases.

CONCLUSION

From this study, it can be concluded that methanol leaves extract of *O. stamineus* had the highest ACE inhibitor and antioxidant activity if compared with chloroform, hexane and water extract. The phenolic compounds that were extracted from *O. stamineus* played important role in ACE inhibitory activity. Crude methanol extract from the leaves of *O. stamineus* also exhibited lower toxicity compared to other extracts. The chemical compounds such as phenolics, terpenoid and alkaloids from the leaves extract of *O. stamineus* were separated through Thin Layer Chromatography (TLC), Column Chromatography (CC) and High Performance Liquid Chromatography (HPLC) and this compounds were found to possess high antioxidant activity and acted as natural Angiotensin Converting Enzyme (ACE) Inhibitor, therefore it can be useful to treat not only hypertension but can also be used to treat cardiovascular disease, cancer and ageing.