Chapter – 6

STUDIES ON ANTIOXIDANT ACTIVITY OF THE LEAVES OF *BISCHOFIA JAVANICA* AND *FRAXINUS FLORIBUNDA*

6.1 Introduction:

Oxygen free radical induced cellular damage has been implicated in many pathological conditions, like malignancy, ageing process, inflammation and degenerating diseases. [1-2] Reactive oxygen species (ROS) have been known to cause tissue injury through covalent bonding and lipid peroxidation.[3] Anti-inflammatory drugs could conceivably affect oxidant damage at the sites of inflammation in several ways. They might directly scavenge such reactive oxidants as hydroxyl radical ($OH^{\cdot}$) and hypochlorous acid (OHCl). [4] It has been shown in most cases, if not all, anti-inflammatory drugs are capable of reacting quickly with OH radical. Hence, any anti-inflammatory drug present at a site of inflammation would remove OH radical. Antioxidants act as major defense against radical-mediated toxicity by protecting the damages caused by free radicals. Plant-derived antioxidants may function as reducing agents, scavengers of free radicals and metal ion chelators, among others. [5] The antioxidant activity in plasma has been shown to increase after consumption of foods high in antioxidants. [6] Thus, phytochemicals may combat oxidative stress in human body by maintaining a balance between oxidants and antioxidants

6.1.1 DPPH Radical Scavenging Assay:

The 1, 1-diphenyl- 2picryl hydrazyl (DPPH) radical was widely used a reagent to evaluate free radical scavenging activity of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen atom to become a stable diamagnetic molecule. [7-8] The reduction capability of DPPH radical is determined by the decrease in absorbance induced by antioxidants.

6.1.2 Lipid Peroxidation Assay:

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves formation of lipid radicals leading to membrane damage. Free radicals induce lipid peroxidation in poly unsaturated lipid rich areas like brain and liver. Malondialdehyde (MDA), the secondary product of lipid peroxidation, is estimated in the plasma samples utilizing the colorimetric reaction with thiobarbituric acid (TBA). [9] It

gives the index of the extent of lipid peroxidation. Since the assay estimates the amount of TBA reactive substance e.g. MDA, it is also known as TBARS (thiobarbituric acid reactive substance) test.

6.1.3 Hydroxyl Radical Scavenging Assay:

The hydroxyl radical scavenging activity is measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture, by studying the competition between deoxy ribose and the test drug for hydrogen radicals generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 systems. The hydroxyl radicals attack deoxy ribose which eventually results in TBARS formation. [10]

6.2 Instruments, Chemicals and Drugs:

UV-Visible double beam spectrophotometer (Shimadzu Corporation, Japan), cold centrifuge are available in the laboratory of Himalayan Pharmacy Institute. DPPH (1,1-diphenyl-2-picryl hydrazyl), Tris-Hcl, Ferrous sulphate, Ascorbic acid, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Potassium dihydrogen phosphate-pottasium hydroxide buffer, Deoxy ribose, α-tocopherol, EDTA, Ferric chloride and hydrogen peroxide were purchased from Sigma and other chemicals were procured from local firms and were of analytical grade.

6.3 Animals:

Wistar female albino rats (150-250g) were used in this study. They were housed in large propylene cages and kept at 22±2°C in 12 h dark-light cycle. The animals were fed with pellet food and water *ad libitum*. All animals were acclimatized to the laboratory environment for at least one week before the experimental session. The animal experiment was approved by Institutional Animal Ethical Committee (IAEC) *No: HPI/07/60/IAEC/0006*.

6.4 Antioxidant Studies of the leaves of *Bischofia javanica*

6.4.1 DPPH Radical Scavenging Assay:

To different concentrations (20, 40, 80,160 and 320μg/ml) of methanol extract of the leaves of *B.javanica* and its isolated compound, Friedeline-3α-acetate in methanol, 1ml of a methanolic solution of 0.2Mm DPPH [11] was added. After mixing thoroughly, the mixture was allowed to stand in dark for 30min and the absorbance at 523 nm was measured using methanol for the baseline correction. The results were then compared with that of the control prepared in the same manner but without the sample. Radical scavenging activity was calculated and expressed in percentage, α-tocopherol at the concentration range of 20, 40, 80, 160, 320 μg/ml was used as a positive control for each concentration. Readings were taken in quadruplicate. The antioxidant activity was expressed as IC_{50} value, which is defined as the concentration (μg/ml) of extract required to inhibit the formation of DPPH radicals by 50%.

6.4.2 Lipid Peroxidation Assay:

The brains of normal female albino rats of the Wistar strain (age 3months) were dissected and homogenized in chilled 20mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 20,000×g for 15 min at 4°C. A 0.5-ml aliquot of the supernatant was then taken to incubate with 0.3 ml of the test sample of methanol extract of leaves of *B.javanica* (BJ) and Friedelin-3α-acetate at the concentrations of 20, 40, 80,160 and 320μg/ml), 0.1 ml of 10 mM FeSO4, and 0.1 ml of 0.1mM ascorbic acid at 37°C for 1h. The reaction was then stopped by addition of 0.75 ml of 10% solution of trichloroacetic acid (TCA) and 0.5 ml of 1% (w/v) solution of thiobarbituric acid (TBA). The mixture was then heated at 100°C for 45 min. After centrifugation, the precipitated proteins were removed and the color of the malondialdehyde (MDA)-TBA complex in the supernatant was measured at 532 nm [12] in Uv-Vis spectrophotometer. α-tocopherol at the concentrations of 20,40,80,160 and 320 μg/ml was used as a positive control. For each concentration readings were taken in quadruplicate.

6.4.3 Hydroxyl Radical Scavenging Assay:

The hydroxyl radical scavenging activity of methanol extract of *B.javanica*, its pure compound, Friedelin-3α-acetate and the reference compound α-tocopherol was determined by the method of Aruoma et al.[13] The assay mixture, in a total volume of 1.2 ml, contained deoxyribose (2.8mM), $FeCl_3$ (25mM), EDTA (100mM), H_2O_2 (2.8mM), KH_2PO_4/KOH buffer, pH 7.4 (10mM), various concentrations of test drugs and standard (20-320μg/ml) and the pH was then readjusted to 7.4. The ascorbate (100mM) was then added to start the reaction. After incubation at 37 °C for 1 h, 1 ml of 1% (w/v) thiobarbituric acid (TBA) in 50mM NaOH and 1ml of 2.8% (w/v) trichloroacetic acid (TCA) was added to the reaction mixture and placed in a hot water bath maintained at 80°C for up to 20 min. The contents were cooled, centrifuged and absorbance of the supernatant was read at 532 nm against the control. Readings were taken in quadruplicate.

6.5 Antioxidant Studies of the Leaves of *Fraxinus floribunda*

6.5.1 DPPH Radical Scavenging Assay:

To different concentrations (20, 40, 80,160 and 320μg/ml) of methanol extract of the leaves of *F.floribunda* and its isolated compounds β-amyrin in methanol, 1ml of a methanolic solution of 0.2Mm DPPH [11] was added. After mixing thoroughly, the mixture was allowed to stand in dark for 30min and the absorbance at 523 nm was measured using methanol for the baseline correction. The results were then compared with that of the control prepared in the same manner but without the sample. Radical scavenging activity was calculated and expressed in percentage, α-tocopherol at the concentration range of 20, 40, 80, 160, 320 μg/ml was used as a positive control for each concentration. Readings were taken in quadruplicate. The antioxidant activity was expressed as IC_{50} value, which is defined as the concentration (μg/ml) of extract required to inhibit the formation of DPPH radicals by 50%.

6.5.2 Lipid Peroxidation Assay:

The brains of normal female albino rats of the Wistar strain (age 3months) were dissected and homogenized in chilled 20mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 20,000×g for 15 min at 4°C. A 0.5-ml aliquot of the supernatant was then taken to incubate with 0.3 ml of the test sample of methanol extract of leaves of *Fraxinus floribunda* and β-amyrin at the concentrations of 20, 40, 80,160 and 320μg/ml), 0.1 ml of 10 mM FeSO4, and 0.1 ml of 0.1mM ascorbic acid at 37°C for 1h. The reaction was then stopped by addition of 0.75 ml of 10% solution of trichloroacetic acid (TCA) and 0.5 ml of 1% (w/v) solution of thiobarbituric acid (TBA). The mixture was then heated at 100°C for 45 min. After centrifugation, the precipitated proteins were removed and the color of the malondialdehyde (MDA)-TBA complex in the supernatant was measured at 532 nm [12] in UV-Vis spectrophotometer. α-tocopherol at the concentrations of 20,40,80,160 and 320 μg/ml was used as a positive control. For each concentration readings were taken in quadruplicate.

6.5.3 Hydroxyl Radical Scavenging Assay:

The hydroxyl radical scavenging activity of methanol extract of *F.floribunda,* its isolated pure compound, β-amyrin and the reference compound α-tocopherol was determined by the method of Aruoma et al. [13] The assay mixture, in a total volume of 1.2 ml, contained deoxyribose (2.8mM), $FeCl_3$ (25mM), EDTA (100mM), H_2O_2 (2.8mM), KH_2PO_4/KOH buffer pH 7.4 (10mM), various concentrations of test drugs and standard (20–320μg/ml) and the pH was then readjusted to 7.4. The ascorbate (100mM) was then added to start the reaction. After incubation at 37 °C for 1 h, 1 ml of 1% (w/v) thiobarbituric acid (TBA) in 50mM NaOH and 1ml of 2.8% (w/v) trichloroacetic acid (TCA) was added to the reaction mixture and placed in a hot water bath maintained at 80°C for up to 20 min. The contents were cooled, centrifuged and absorbance of the supernatant was read at 532 nm against the control. Readings were taken in quadruplicate.

6.6 Results of the Antioxidant Studies of the Leaves of *Bischofia javanica*:

6.6.1 DPPH Radical Scavenging Assay:

The percentage of DPPH radical scavenging activity was calculated by using the formula ----

$$\text{Scavenging (\%)} = \frac{\text{Absorbance of Control - Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

The test drugs, methanol extract of *Bischofia javanica* (BJ) and Friedelin-3α-acetate shown to have concentration dependent antioxidant activities. The IC_{50} value of BJ was found to be 118.29 µg/ml, for the isolated compound, Friedelin-3α-acetate it was188.08 µg/ml **(Table 6.1** and **Figure 6.1, 6.2, 6.3).**The scavenging activity of BJ and Friedelin-3α-acetate at 320µg/ml was found to be 84.26 and 68.21% respectively. The slope of the curve showed the precision of the linearity of concentration dependent radical scavenging activity in the **Figures 6.1 to 6.3.**

6.6.2 Lipid Peroxidation Assay:

The percentage of scavenging activity of the drugs was calculated using the formula given in **6.6.1.** The results of the study revealed that the antioxidant activities of the test drugs are more in the concentration range of 160-320 µg/ml. The coloration produced by the Thio Barbituric Acid Reactive Substance (TBARS) was reduced by the test drug in between these concentrations in remarkable manner. The IC_{50} values of the drugs BJ, Friedelin-3α-acetate and α-tocopherol were 142,191.10 and 135.31 µg/ml **(Tables 6.2)** respectively. Linear regression curve depicted the R^2 of Friedelin-3α-acetate is 0.9713 which is much more than that of BJ (0.9184) and α-tocopherol (0.9126) as shown in the **Figures 6.4, 6.5 and 6.6.**

6.6.3 Hydroxyl Radical Scavenging Assay:

The percentage of hydroxyl radical scavenging activity was determined using the formula given in **6.6.1**. The IC_{50} value of hydroxyl radical scavenging activity of the test drugs are found to be higher compared to DPPH and Lipid peroxidation assay (Table 6.3).The free radical (OH) scavenging activity for the test drugs are found to be concentration dependent as shown in the **Figure 6.7, 6.8 and 6.9**. In the concentration range of 20-320μg/ml of test drugs, the IC_{50} for the test drugs BJ and friedelin-3α-acetate was found to be 185.23 and 163.37μg/ml respectively and for the standard drug α-tocopherol it was 158.95μg/ml.

6.7 Results of Antioxidant Studies of the Leaves of *Fraxinus floribunda:*

6.7.1 DPPH Radical Scavenging Assay:

The percentage of DDPH radical scavenging activity was calculated by using the formula---

$$\text{Scavenging (\%)} = \frac{\text{Absorbance of Control-Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

In this study the DPPH radical scavenging activity test drugs was found to be concentration dependent in the concentration range of 20-320μg/ml. β-amyrin, the pure compound isolated from the leaves of *Fraxinus floribunda* (FF) shown to have antioxidant activity (72.14%) better than the methanol extract (61.42%) of the leaves **(Table 6.4)**. The IC_{50} values of FF, β-amyrin and α-tocopherol were determined by Linear regression analysis as shown in the **Figures 6.10, 6.11 and 6.12.**

6.7.2 Lipid Peroxidation Assay:

The percentage of scavenging activity of the drugs was calculated using the formula given in **6.7.1**. The results of the Lipid peroxidation assay revealed that the antioxidant activity of the test drugs increase on increasing the concentration of the drugs. The isolated phytoconstituent β-amyrin showed better antioxidant activity (IC_{50}137.92μg/ml)

compared to FF (IC_{50} 172.52 µg/ml and α-tocopherol (IC_{50} 149.13µg/ml) **(Table 6.5)**. The linear regression curve of FF, β-amyrin and α-tocopherol are represented in the **Figures 6.13, 6.14 and 6.15.**

6.7.3 Hydroxyl Radical Scavenging Assay:

The percentage of hydroxyl radical scavenging activity was determined using the formula given in **6.7.1**. The results of the hydroxyl radical scavenging activity assay are presented in the **Table 6.6** and **Figures 6.16, 6.17 and 6.18.** The IC_{50} value of methanol extract of *F.Floribunda* was found to be more (166.63µg/ml) than that of standard drug α-tocopherol (149.15µg/ml) while the isolated compound, β-amyrin showed better activity (129.75µg/ml) than both FF and α-tocopherol.

Table 6.1 Antioxidant activity of leaves of *Bischofia javanica* by DPPH radical scavenging assay

Drug	Concentration (µg/ml)	Scavenging activity (%)	IC_{50} (µg/ml)
BJ	20	23.42±1.64	118.29
	40	32.56±1.56	
	80	46.15±2.84	
	160	69.23±3.32	
	320	84.26±2.11	
Friedeline-3α-acetate	20	18.45±3.41	188.08
	40	29.24±2.98	
	80	36.86±3.42	
	160	48.24±2.59	
	320	68.21±4.36	
α-tocopherol	20	20.22±3.12	146.28
	40	37.51±3.23	
	80	41.19±1.14	
	160	53.20±4.56	
	320	78.67±1.64	

Each value represents means of quadruplicate determinations ± SD,

BJ - Methanol extract of *Bischofia javanica*

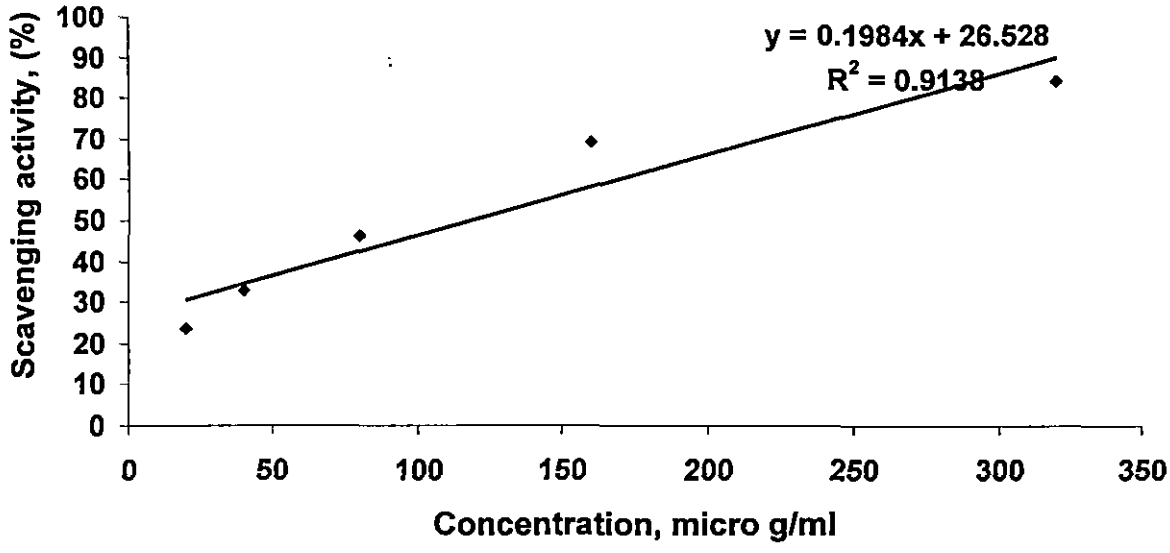

Figure 6.1 Linear regression analysis of antioxidant activity of methanol extract of *Bischofia javanica* by DPPH assay

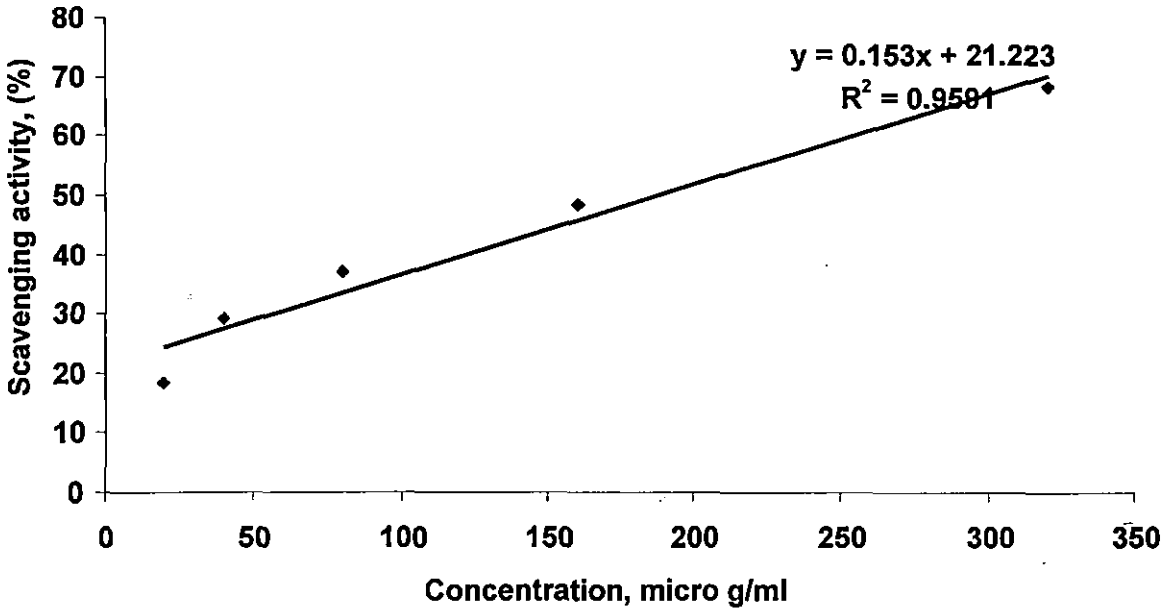

Figure 6.2 Linear regression analysis of antioxidant activity of Friedelin-3alpha-acetate by DPPH assay

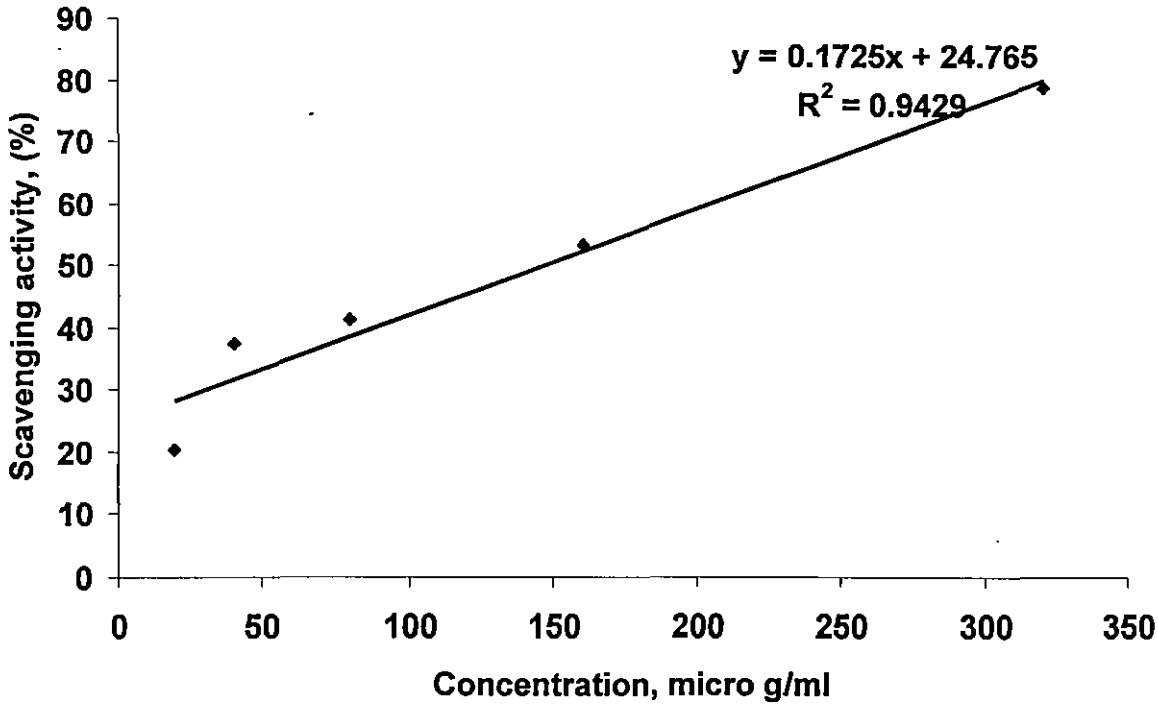

Figure 6.3 Linear Regression analysis of antioxidant activity of alpha-tocopherol by DPPH assay

Table 6.2 Antioxidant activity of leaves of *Bischofia javanica* by Lipid peroxidation assay

Drug	Concentration (µg/ml)	Scavenging activity (%)	IC_{50} (µg/ml)
BJ	20	16.11±2.43	
	40	37.06±2.76	
	80	43.14±3.42	142.00
	160	55.23±1.32	
	320	81.43±2.34	
Friedeline-3α-acetate	20	12.10±1.45	
	40	20.26±2.23	191.10
	80	32.57±1.65	
	160	48.19±3.78	
	320	72.13±1.96	
α-tocopherol	20	10.18±2.45	135.31
	40	27.43±1.43	
	80	42.67±2.13	
	160	67.20±4.21	
	320	88.66±3.79	

Each value represents means of quadruplicate determinations ± SD,

BJ - Methanol extract of *Bischofia javanica*

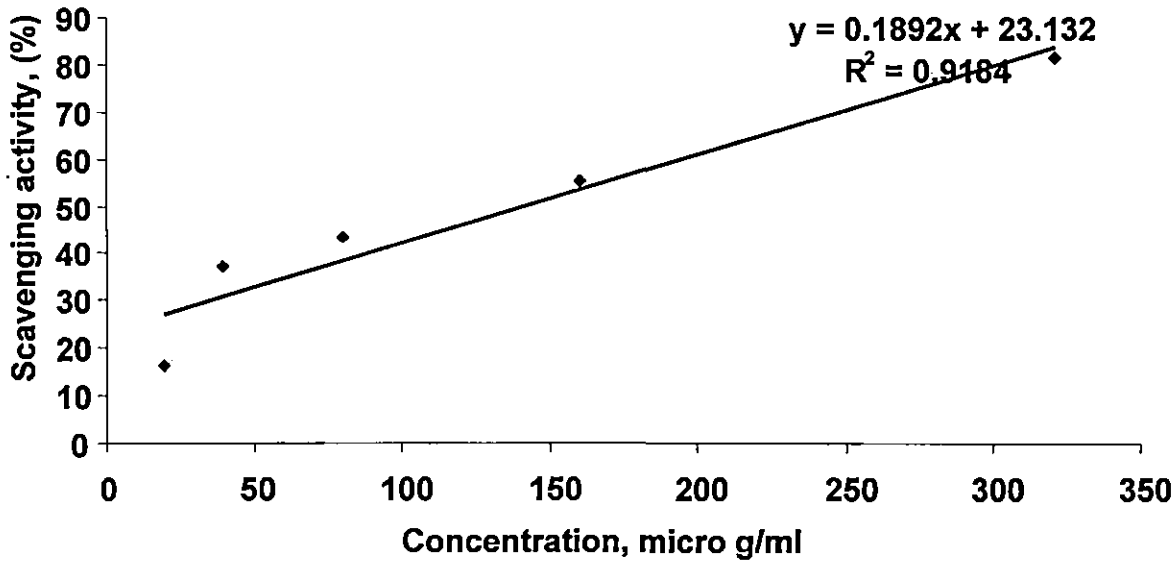

Figure 6.4 Linear regression analysis of antioxidant activity of methanol extract of leaves of *Bischofia javanica* by Lipid peroxidation assay

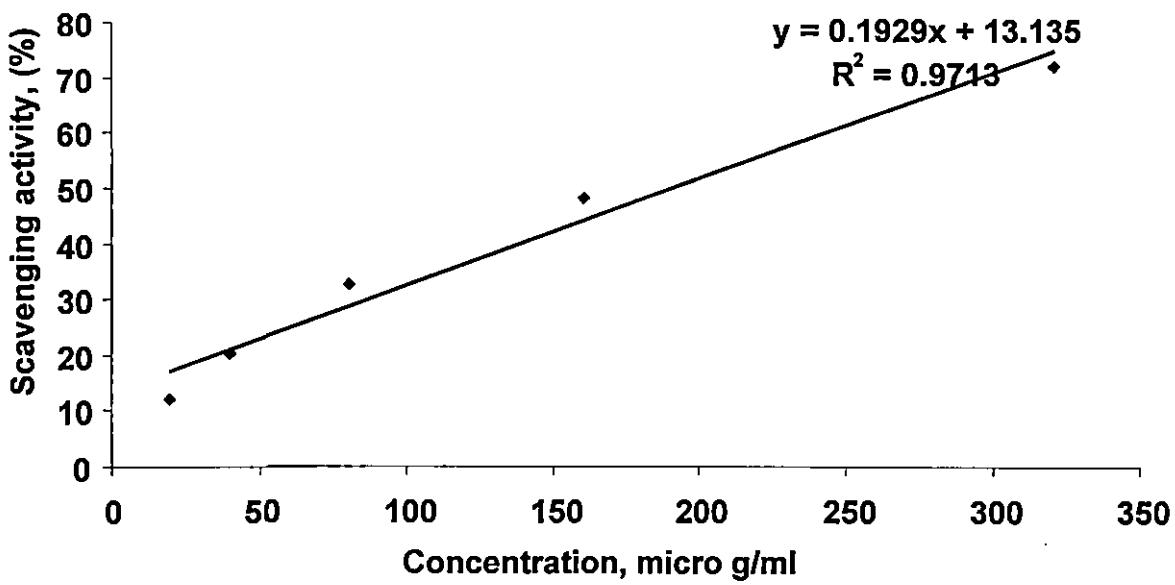

Figure 6.5 Linear regression analysis of antioxidant activity of Friedelin-3alpha-acetate by Lipid peroxidation assay

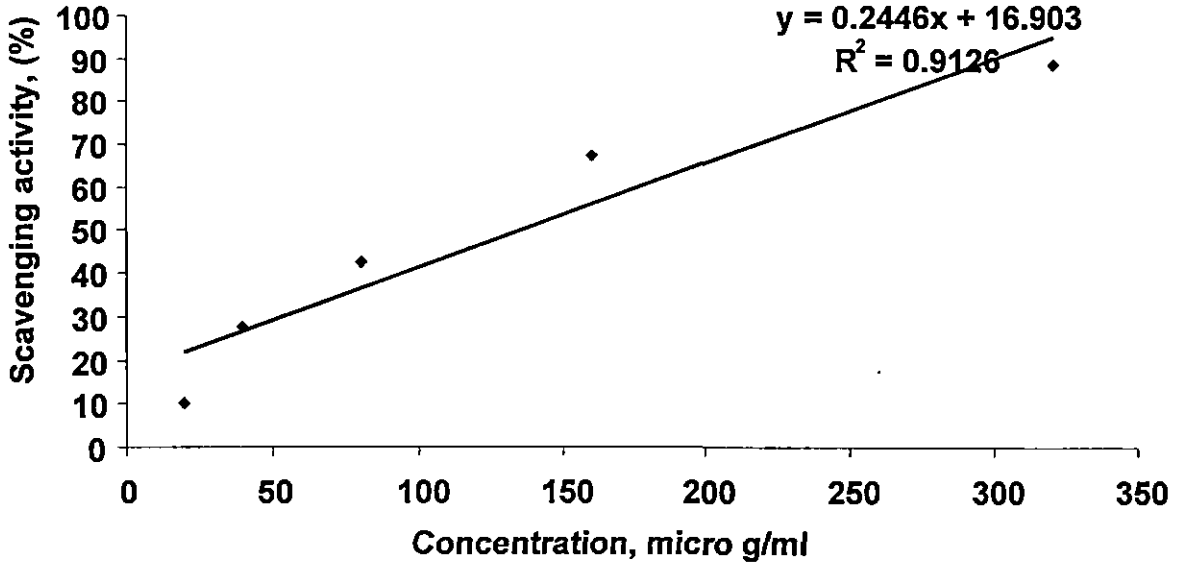

Figure 6.6 Linear regression analysis of antioxidant activity of alpha-tocopherol by Lipid peroxidation assay

Table 6.3 Antioxidant activity of leaves of *Bischofia javanica* by Hydroxyl radical scavenging assay

Drug	Concentration (µg/ml)	Percentage of scavenging	IC_{50} (µg/ml)
BJ	20	10.01±2.21	185.23
	40	28.19±3.42	
	80	35.25±1.23	
	160	48.26±1.43	
	320	72.14±5.14	
Friedeline-3α-acetate	20	12.17±4.67	163.37
	40	23.32±1.23	
	80	38.67±3.45	
	160	55.19±2.31	
	320	79.11±1.97	
α-tocopherol	20	17.18±2.87	158.95
	40	23.46±3.56	
	80	43.57±4.57	
	160	69.20±1.23	
	320	84.79±2.54	

Each value represents means of quadruplicate determinations ± SD,

BJ – Methanol extract of *Bischofia javanica*

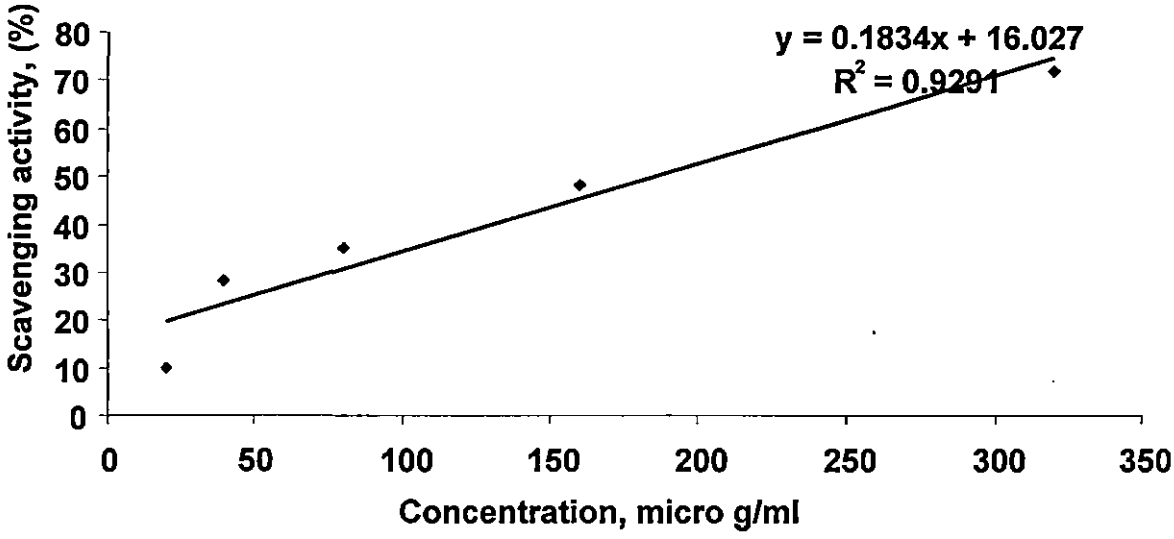

Figure 6.7 Linear regression analysis of antioxidant activity of methanol extract of leaves of *Bischofia javanica* by Hydroxy radical scavenging assay

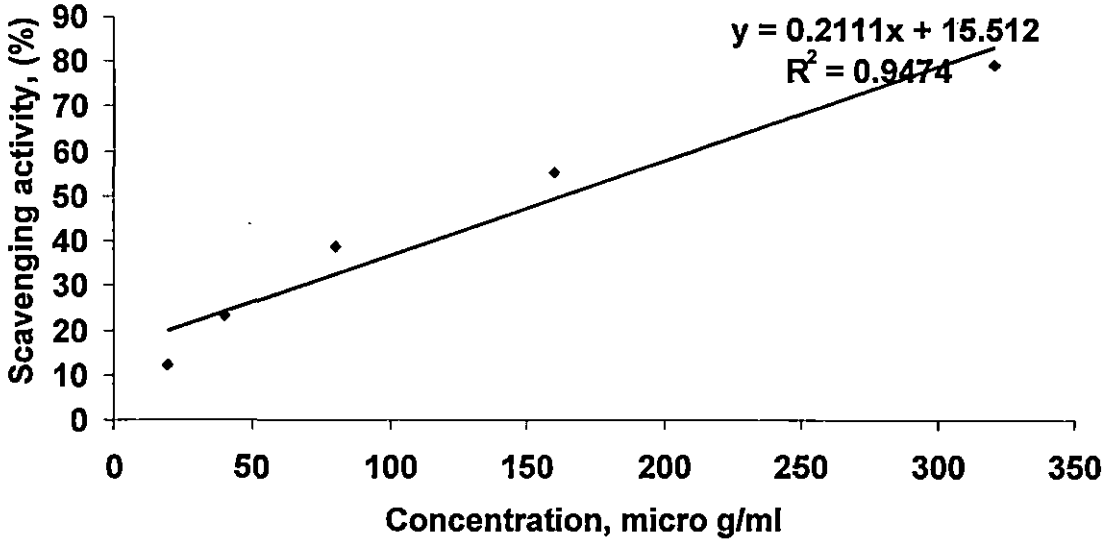

Figure 6.8 Linear regression analysis of antioxidant activity of Friedelin-3alpha-acetate by hydroxyl radical scavenging assay

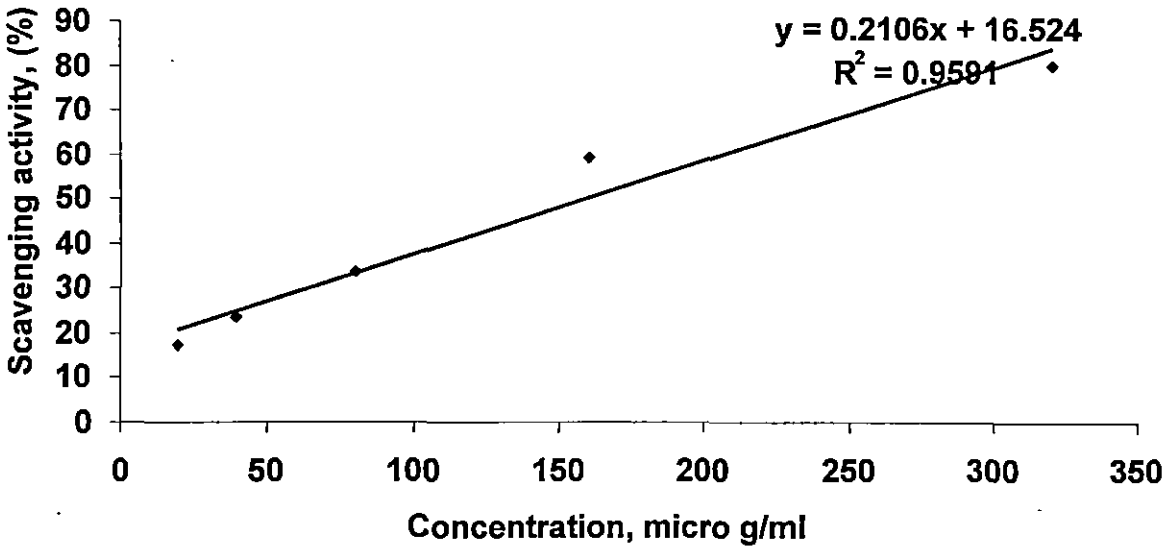

Figure 6.9 Linear regression analysis of antioxidant activity of alpha-tocopherol by hydroxyl radical scavenging assay

Table 6.4 Antioxidant activity of leaves of *Fraxinus floribunda* by DPPH reduction assay

Drug	Concentration (µg/ml)	Scavenging activity(%)	IC_{50} (µg/ml)
FF	20	12.43±2.31	218.77
	40	26.98±3.21	
	80	38.32±1.56	
	160	44.15±2.98	
	320	61.42±1.44	
β-amyrin	20	14.54±0.98	168.47
	40	29.67±1.23	
	80	40.97±2.87	
	160	54.26±1.29	
	320	72.14±6.14	
α-tocopherol	20	18.25±2.78	154.68
	40	34.57±2.44	
	80	39.11±1.67	
	160	56.01±2.90	
	320	75.54±1.43	

Each value represents means of quadruplicate determinations ± SD,

FF – Methanol extract of *Fraxinus floribunda*

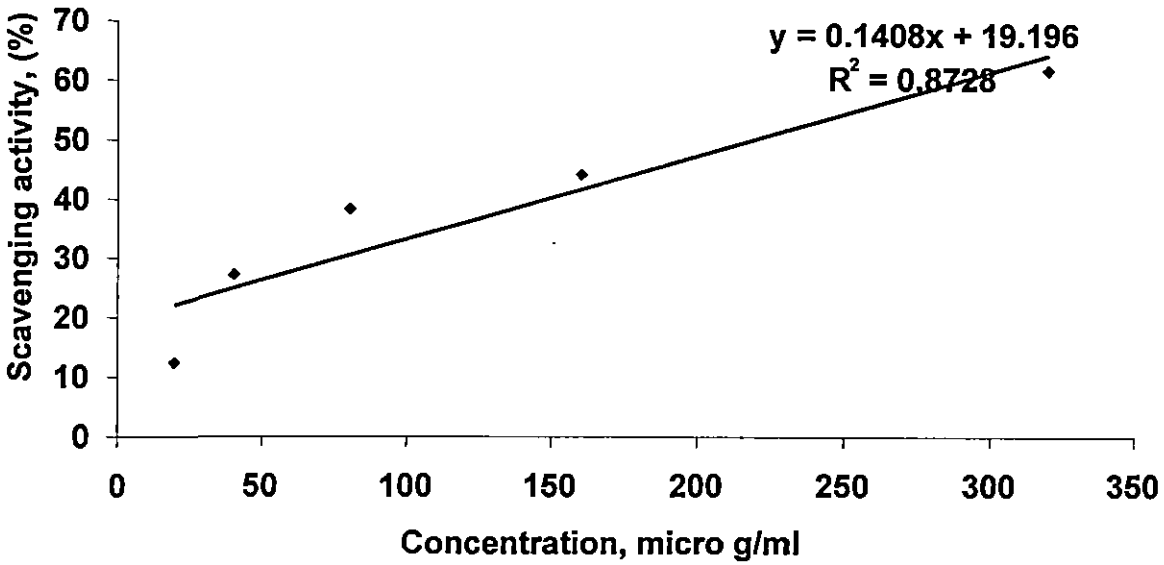

Figure 6.10 Linear regression analysis of antioxidant activity of methanol extract of leaves of *Fraxinus floribunda* by DPPH assay

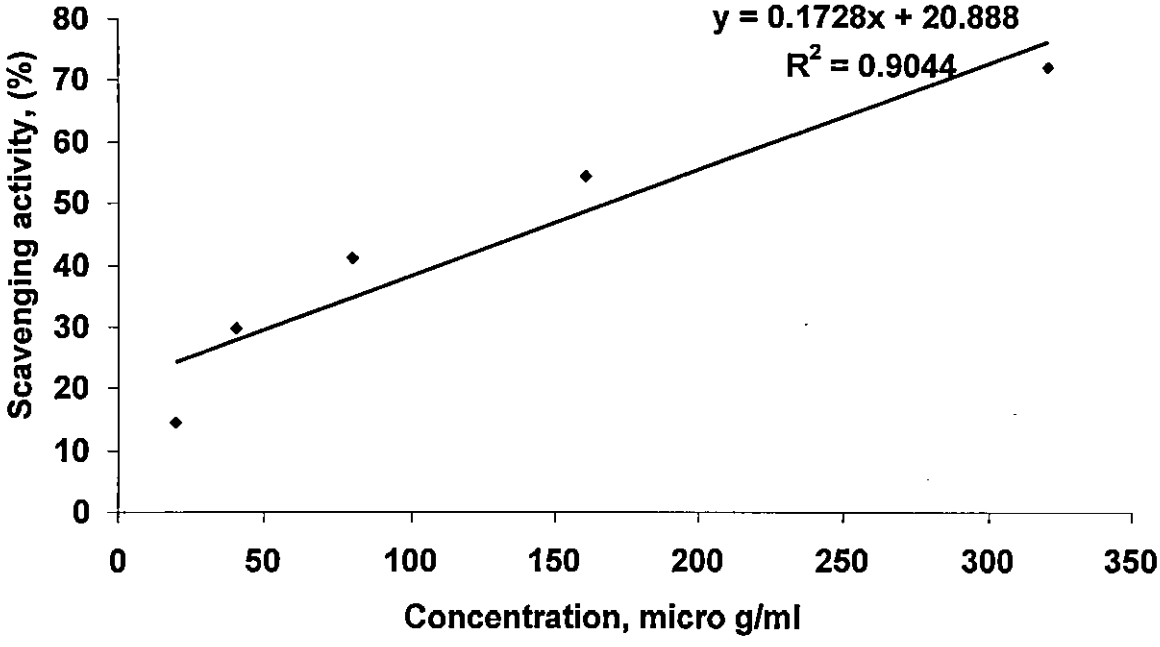

Figure 6.11 Linear regression analysis of antioxidant activity of Beta amyrin by DPPH assay

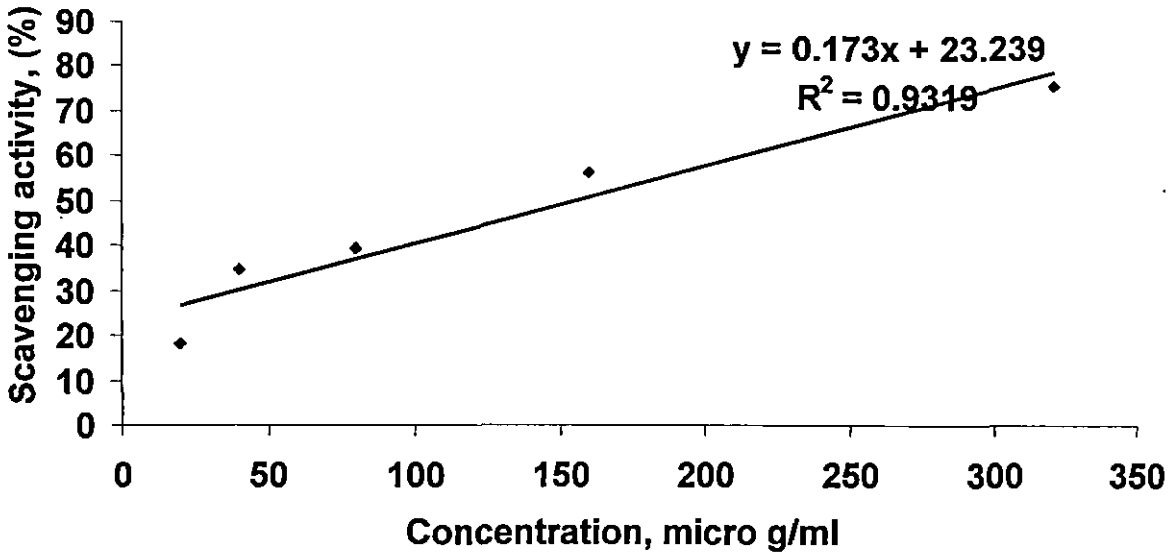

Figure 6.12 Linear regression analysis of antioxidant activity of alpha-tocopherol by DPPH assay

Table 6.5 Antioxidant activity of leaves of *Fraxinus floribunda* by Lipid peroxidation assay

Drug	Concentration (µg/ml)	Scavenging activity (%)	IC_{50} (µg/ml)
FF	20	18.14±3.78	
	40	26.45±2.88	
	80	39.16±1.43	172.54
	160	54.27±2.14	
	320	71.00±2.72	
β-amyrin	20	14.45±1.98	137.92
	40	28.29±1.78	
	80	46.27±3.11	
	160	65.32±2.41	
	320	81.23±1.10	
α-tocopherol	20	12.32±1.08	149.13
	40	24.43±3.18	
	80	46.67±2.46	
	160	62.20±2.67	
	320	78.66±2.34	

Each value represents means of quadruplicate determinations ± SD, FF- Methanol extract of *Fraxinus floribunda*

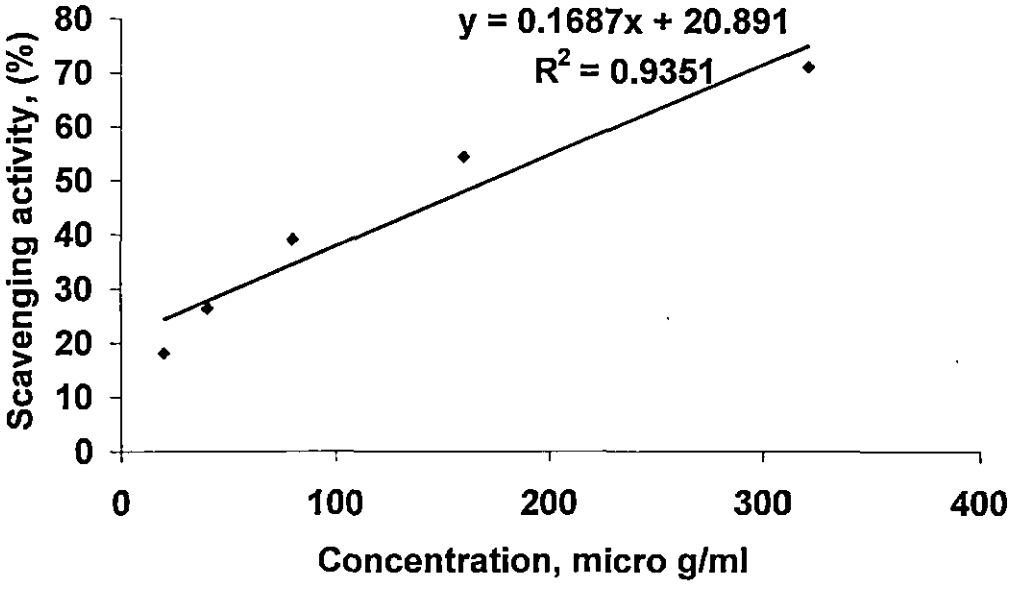

Figure 6.13 Linear regression analysis of antioxidant activity of methanol extract of leaves of *Fraxinus floribunda* by Lipid peroxidation assay

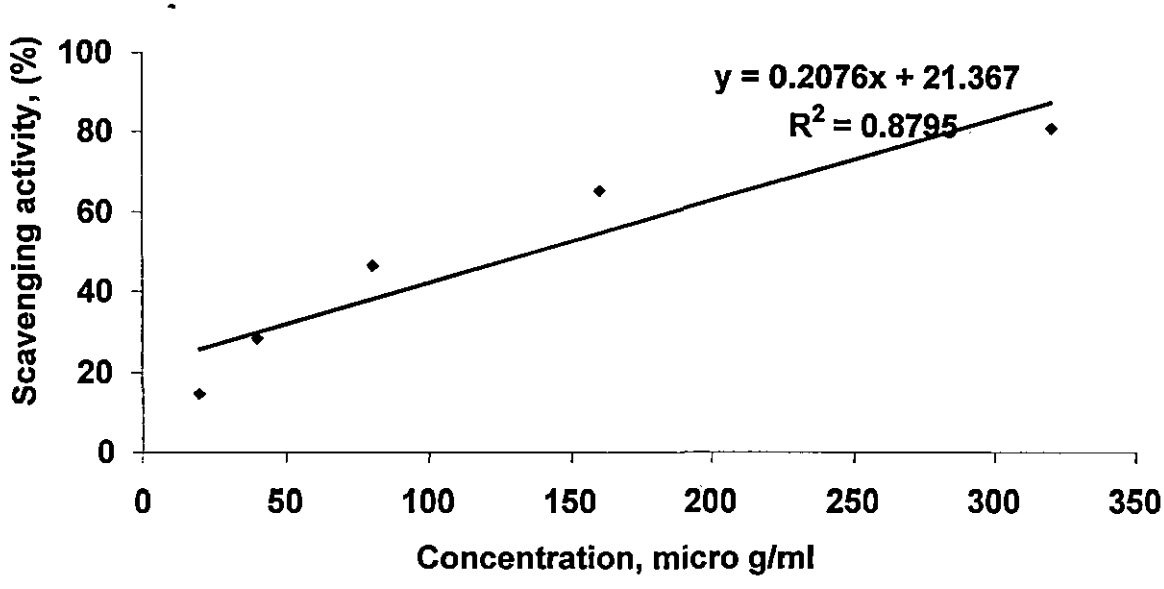

Figure 6.14 Linear regression analysis of antioxidant activity of Beta-amyrin by Lipid peroxidation assay

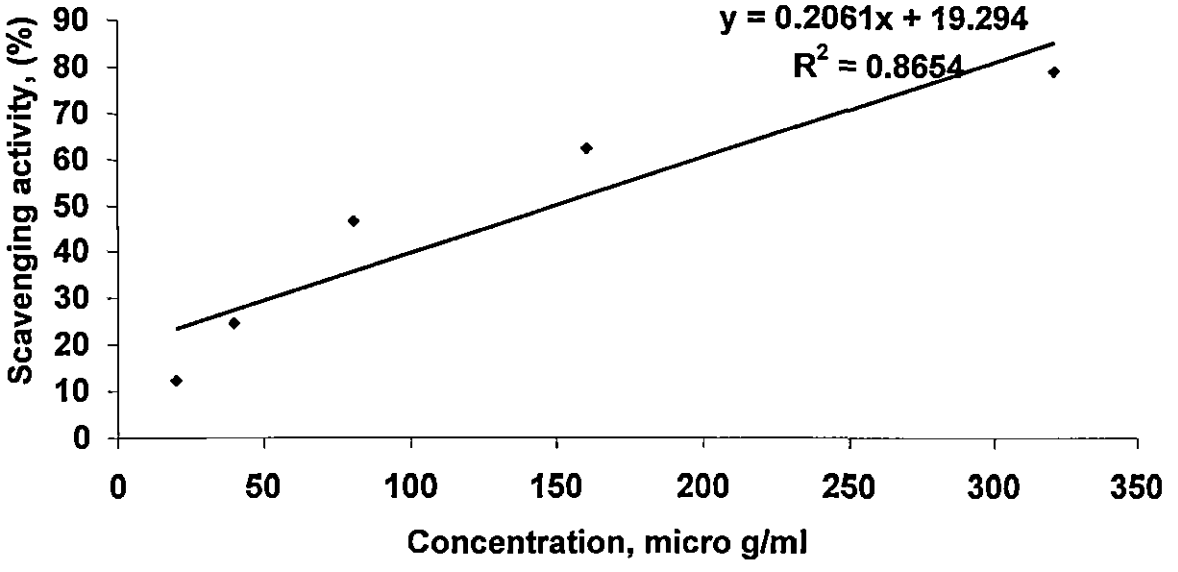

Figure 6.15 Linear regression analysis of antioxidant activity of Alpha-tocopherol by Lipid peroxidation assay

Table 6.6 Antioxidant activity of leaves of *Fraxinus floribunda* by Hydroxyl radical scavenging assay

Drug	Concentration (µg/ml)	Percentage of scavenging	IC_{50} (µg/ml)
FF	20	11.23±1.98	166.63
	40	23.87±2.64	
	80	41.76±3.21	
	160	58.91±1.76	
	320	73.21±1.33	
B-amyrin	20	19.24±1.27	129.85
	40	29.36±2.24	
	80	48.57±2.95	
	160	65.49±1.31	
	320	81.57±3.07	
α-tocopherol	20	15.16±2.87	149.13
	40	28.76±1.66	
	80	47.37±2.59	
	160	68.20±2.23	
	320	81.99±1.55	

Each value represents means of quadruplicate determinations ± SD, FF - Methanol extract of *Fraxinus floribunda*

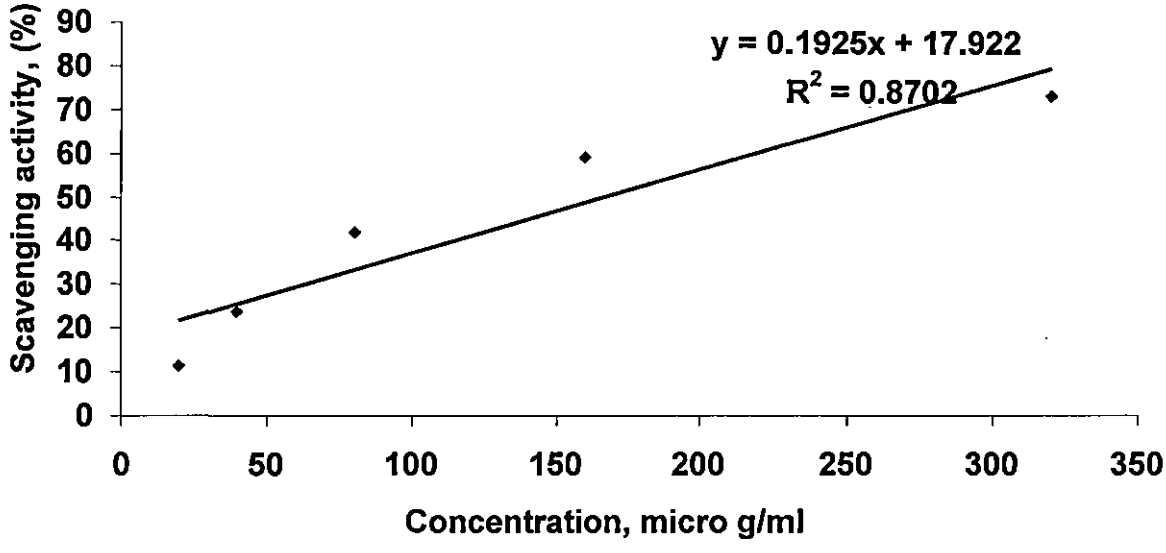

Figure 6.16 Linear regression analysis of antioxidant activity of methanol extract of leaves of *Fraxinus floribunda* by hydroxyl radical scavenging assay

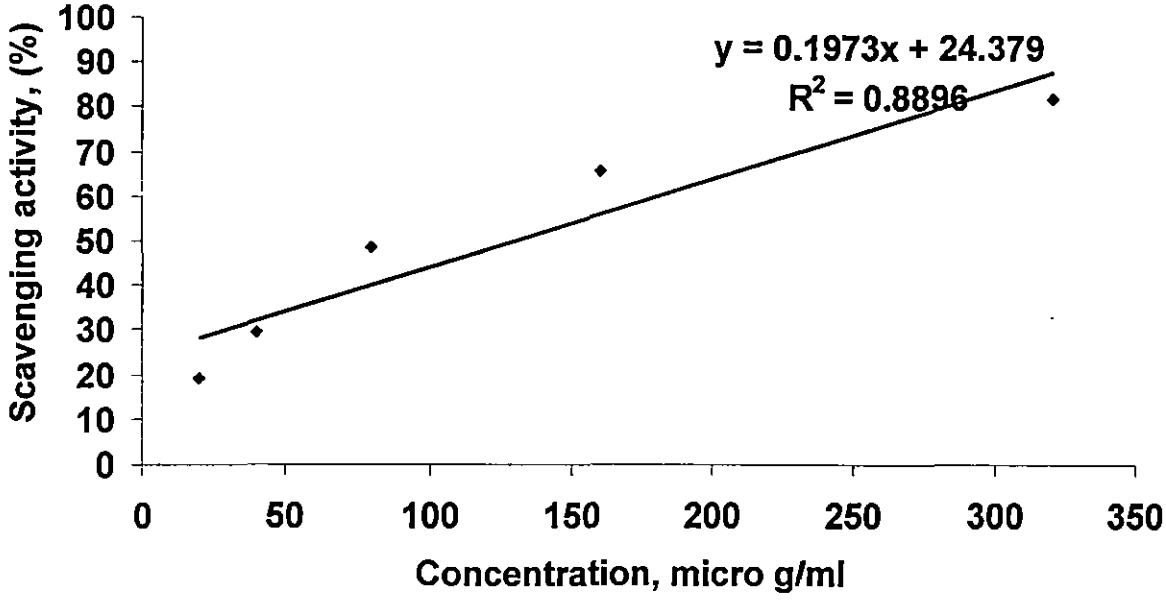

Figure 6.17 Linear regression analysis of antioxidant activity of Beta-amyrin by hydroxyl radical scavenging assay

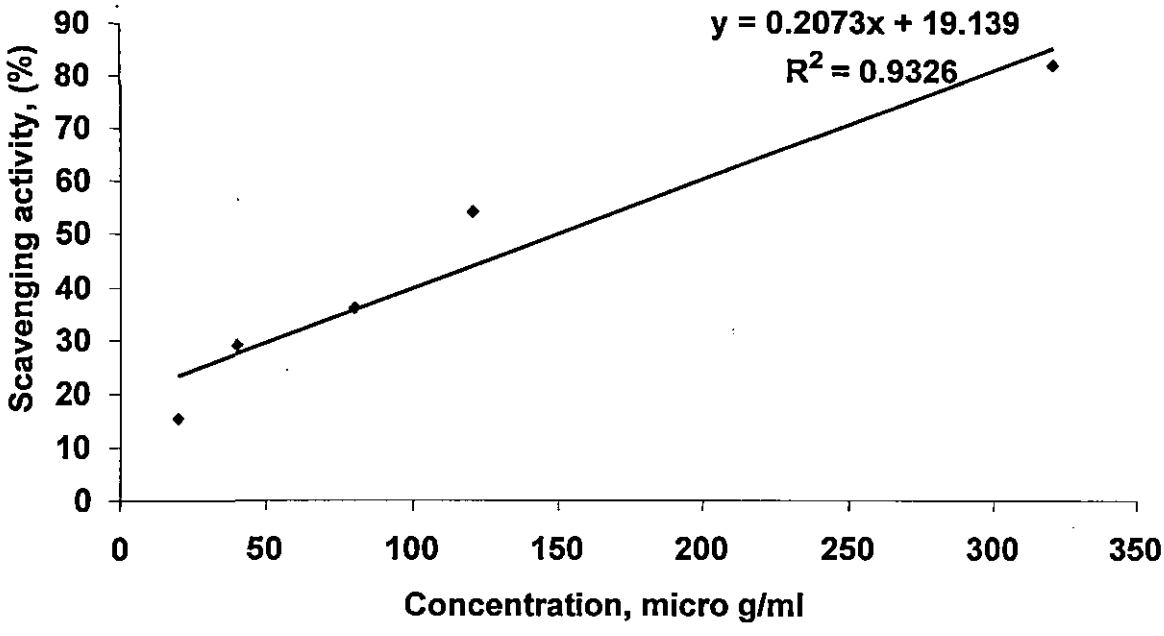

Figure 6.18 Linear regression analysis of antioxidant activity of Alpha-tocopherol by hydroxyl radical scavenging assay

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