# CHAPTER 7

# ANTIOXIDANT ACTIVITY

## 7.1. Introduction

Free radicals are chemical species possessing an unpaired electron that can be considered as fragment of molecules and which are generally extremely reactive and short lived. They are produced continuously in cells either as accidental byproducts of metabolism or deliberately during different pathological disorders and phagocytosis. Free radicals can be formed in mechanisms.

- By the homolytic cleavage of a covalent bond of a molecule, with each fragment retaining one unpaired electron.
- By the loss of a single electron from a normal molecule.
- By the addition of a single electron to a normal molecule.

The electron transfer is a far more common process in biological systems, than that of first mechanism. The most important free radicals in biological systems are the radicals derived from oxygen. With increasing acceptance of free radicals as common and important biochemical intermediates, they have been implicated in a large number of human diseases.

Free radical reaction is an important pathway in wide range of unrelated biological systems. It has been implicated in causing diabetes, liver cirrhosis, nephrotoxicity etc [1]. Reactive oxygen species (ROS) such as superoxide anions ($O_2^-$), hydroxyl radical ($OH^\bullet$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent bonding and lipid peroxidation [2], and thus have been shown to augment collagen synthesis and fibrosis. The increased production of toxic oxygen derivatives is considered to be a universal feature in stress conditions. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human body against infection and degenerative diseases. Presently, there is an increasing realization that herbs can influence the oxidative stress related diseases by maintaining equilibrium between rates of generation of ROS and their neutralization by endogenous antioxidant enzymes [3]. Use of herbs as a source of antioxidant has opened a novel lead for the development of new modalities of treatments.

The aim of the present study is to determine the antioxidant and free radical scavenging activities of the methanol extract of *C.oppositifolia* leaf and *H.nepalense* root and their isolated compounds. For this purpose, plant extracts and their isolated compounds were tested for different free radical scavenging activities and their capacity to reduce lipid peroxidation.

## 7.2. Materials and Methods

### 7.2.1. Plant materials

Methanol extracts of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

### 7.2.2. Chemicals

Thiobarbituric acid was obtained from Loba Chemie, India. 1,1-Diphynyl-2-picryl hydrazyl (DPPH), NADH and nitroblue tetrazolium (NBT) were obtained from Sigma chemicals, St. Louis, USA. Deoxy ribose was obtained from Merck India. Dimethyl sulphoxide, ethylene diamine tetra acetic acid (EDTA), ferrous sulphate, trichloroacetic acid, hydrogen peroxide, ascorbic acid, mannitol, potassium dihydrogen phosphate, potassium hydroxide, deoxy ribose, phenazine methosulphate were of analytical grade and were obtained from Ranbaxy fine chemicals.

### 7.2.3. Antioxidant activity of *C.oppositifolia* leaf

#### 7.2.3.1. Lipid peroxidation model

The extent of lipid peroxidation in goat liver homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method [4] with minor modifications [5]. Goat liver was purchased from local slutter house. Its lobes were dried between blotting papers (to remove excess blood) and were cut into small pieces with a heavy-duty blade. They were then homogenized in glass-teflon homogenizer tube in cold phosphate buffer saline (pH 7.4). The content was centrifuged at 2000 rpm for 10 min and supernatant was diluted with phosphate buffer

saline up to final concentration of protein 0.8-1.5 mg/0.1 ml. Protein concentration was measured by using standard method of Lowery et.al [6]. To study the comparative response, the experiment was performed in nine glass petri dishes (35 mm ID). Liver homogenate (3ml) was aliquoted to each of the petri dishes. The dish number one and two were treated as control and standard where buffer and vitamin E were added respectively. To the third to seventh dishes, different concentrations of methanol extract (200-1000 µg/ml) and the eighth & ninth dishes compound I (25, 50 µg/ml) were added. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate in nine dishes [7]. After 30 min, 100 µl of each reaction mixture was taken into tubes containing 1.5 ml of 10% Trichloro acetic acid. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% thiobarbituric acid. The mixture was heated in a water bath at 85°C for 30 min and in a boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 535nm in a spectrophotometer (Shimadzu model 1601). The TBARS concentration was calculated by using Equation 1 (molar extinction coefficient of TBARS) and expressed as nanomoles (nM)/mg of tissue [8, 9]. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the Equation 2.

$$\text{nM of TBARS/mg of tissue} = \frac{\text{OD} \times \text{volume of homogenate} \times 100 \times 10^3}{(1.56 \times 10^5) \times \text{volume of extract taken}} \quad \text{---------- Eqn 1}$$

$$\% \text{ Inhibition} = \frac{[(\text{OD of control} - \text{OD of test}) \times 100]}{\text{OD of control}} \quad \text{---------- Eqn 2.}$$

**7.2.3.2. DPPH radical scavenging activity**

DPPH scavenging activity of *C.oppositifolia* was measured by spectrophotometric method [7]. To each of the nine glass tubes methanolic solution of DPPH (100 µM, 2.95ml) was taken. Tube No. one was treated as control without test compound where 0.05 ml of methanol was added. The second tube contained the standard compound vitamin E. To the tubes numbered from three to seven methanol extract of *C.oppositifolia*

(0.05 ml) was added at concentration ranging from 200 to 1000 µg/ml at interval of 200 µg/ml between the tubes. To the tubes numbered eighth and ninth compound I was added in concentration 25 and 50 µg/ml. Absorbance of each tube was recorded at 517nm at regular intervals of 10 min up to 40 min. The scavenging activity in percentage of inhibition was calculated by comparing the control and test samples applying the Equation 2 mentioned in 7.2.3.1.

### 7.2.3.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of *C.oppositifolia* was measured by studying the competition between deoxy ribose and test compounds for hydroxyl radical generated by the $Fe^{3+}$– ascorbate – EDTA – $H_2O_2$ system according to the method of Kunchandy and Rao [10]. The reaction mixture was containing of 100 µl of 2-deoxy-ribose, 500 µl of the various concentrations of the methanol extract (200 to 1000 µg/ml) as well as compound I (25, 50 µg/ml) and standard compound (Mannitol 50 mM) in $KH_2PO_4$- KOH buffer (20mM, pH 7.4), 200 µl of 1.04 mM $H_2O_2$ and 100 µl of 1.0mM ascorbic acid. The final volume of the solution was made up to 1.0 ml by adding phosphate buffer. The tubes were incubated at 37°C for 1 hr. One ml of 1% trichloroacetic acid was added to each test tube and incubated at 100°C for 20 min. After cooling to room temperature, absorbance of the content of the tubes was measured at 532nm against a control preparation containing 2-deoxyribose and buffer. Percent inhibition was determined by comparing the results of the test and control samples applying the Equation 2 as mentioned in 7.2.3.1.

### 7.2.3.4. Superoxide scavenging activity

The superoxide scavenging activity of *C.oppositifolia* was determined by the method described by Nishimik et al [11], with slight modification. To 1.0 ml of NBT solution containing 156 µM NBT dissolved in 100 mM phosphate buffer, pH 7.4, 1.0 ml of NADH solution containing 468 µM NADH dissolved in 100mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of the methanol extract (200 to 1000 µg/ml) as well as compound I (25, 50 µg/ml) and standard compound (vitamin E) were added mixed and the reaction was started by adding 100 µl of phenazine methosulfate solution

containing 60μM phenazine methosulphate in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25 °C for 5 min and absorbance of the contents at 560 nm was measured against a control sample. Percent inhibition was determined by comparing the results of the test and control samples with the help of Equation 2 mentioned in 7.2.3.1.

### 7.2.4. Antioxidant activity of *H.nepalense* root.

The antioxidant activity of methanol extract of *H.nepalense* root and its isolated compound II was determined as per the methods described above in **7.2.3** for *C.oppositifolia* leaf.

## 7.3. Results

### 7.3.1. Antioxidant activity of *C.oppositifolia* leaf

The results presented in Table 7.1 showed the effect of methanol extract of *C.oppositifolia* leaf and compound I on ferrous sulphate induced lipid peroxidation in goat liver homogenate. The results revealed that at 1000 μg/ml concentration of methanol extract had the maximum inhibition percentage (58.67 ± 2.21) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 72.94 ± 2.12. The same results have also been presented in Figure 7.1, which revealed that compound I at 50 μg/ml showed 77.46 ± 2.8 % inhibitions, greater than the inhibition produced by vitamin E. The $IC_{50}$ value, (concentration of the test substances at which 50 % of inhibition produced) was found to be 845.68 ± 3.98 μg/ml. From the results of quantitative estimation of TBARS levels it appears that both the methanol extract at 1000 μg/ml and compound I at 25 μg/ml concentration decreases the levels of TBARS in liver homogenate to a similar level (0.71 ± 0.046), which is highly comparable to the results obtained from the well known antioxidant vitamin E ($p< 0.05$).

The effect of methanol extract and compound I on scavenging of DPPH radical is presented in Table 7.2. The results showed that the DPPH scavenging capacity of the extract was found to be 71.52 ± 2.32 % at 1000 μg/ml. The compound I at 50 μg/ml

concentration, on the other hand, exhibited 80.54 ± 1.34 % compared to 79.69 ± 1.36 % for the standard drug vitamin E at 5mM. The $IC_{50}$ value of the extract was found to be 6.8 mg/ml. The percentage of activity was also dependent on time as depicted from Figure 7.2.

The effect of leaf extract and compound I on scavenging of hydroxyl radical is presented in Table 7.3. The results revealed that the extract significantly inhibited (79.12 ± 3.8 %) degradation of deoxy-ribose mediated by hydroxyl radical at the concentration of 1000 µg/ml, compared to that of known scavenger mannitol (88.14 ± 1.42 %). Here the compound I at 50 µg/ml concentration exhibited inhibition of 81.42 ± 1.36 % compared to 88.14 ± 1.42 % for the standard drug at a concentration of 50 mM. The concentration of the methanol extract needed for 50 % inhibition was 593.64 µg/ml.

The effect of leaf extract and compound I on superoxide scavenging model is presented in Table 7.4. It was found that the methanol extract at concentration of 1000 µg/ml caused significant increase of the inhibition up to 58.46 ± 1.28 %. The compound I at 50 µg/ml concentration exhibited 68.23 ± 1.8 % inhibition of superoxide radicals. $IC_{50}$ was found to be 8.75 mg/ml. Inhibition was found directly proportional to the amount of the extract added.

**Table 7.1** Effect of methanol extract of *C.oppositifolia* and compound I on ferrous sulphate induced lipid peroxidation in goat liver homogenate.

| Dish Number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ value and confidence interval (µg/ml) | TBARS (n moles/mg tissue) |
|---|---|---|---|---|
| 1 | Control | – | – | – |
| 2 | Vitamin E (5 mM) | 72.94 ± 2.12 | | 0.70 ±0.02[a] |
| 3 | 200 | 14.02 ± 1.84 | | 1.06 ± 0.012[a] |
| 4 | 400 | 25.34 ± 1.67 | | 1.04 ± 0.026[a] |
| 5 | 600 | 34.18 ± 1.52 | | 1.00 ± 0.016[a] |
| 6 | 800 | 48.54 ± 1.21 | 845.68 ± 3.98 | 0.92 ± 0.038[a] |
| 7 | 1000 | 58.67 ± 2.21 | | 0.71 ± 0.12[a] |
| | Compound I | | | |
| 8 | 25 | 72.12 ± 4.3 | | 0.71 ± 0.046[a] |
| 9 | 50 | 77.46 ± 2.8 | | 0.71 ± 0.036[a] |

Values are mean ± SEM of 3 replicates [a]$p < 0.05$.

**Table 7.2** Free radical scavenging activity of *C.oppositifolia* leaf extract and compound I by DPPH reduction.

| Tube number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ and confidence interval (µg/ml) |
|---|---|---|---|
| 1 | Control | – | |
| 2 | Vitamin E (5 mM) | 79.69 ± 1.36 | |
| 3 | 200 | 03.42 ± 1.45 | |
| 4 | 400 | 24.16 ± 2.12 | |
| 5 | 600 | 45.51 ± 3.31 | 685.08 ± 3.67 |
| 6 | 800 | 64.14 ± 1.82 | |
| 7 | 1000 | 71.52 ± 2.32 | |
| | Compound I | | |
| 8 | 25 | 78.52 ± 2.32 | |
| 9 | 50 | 80.54 ± 1.34 | |

Values are mean ± SEM of 3 replicates

**Table 7.3** Hydroxyl radical scavenging activity of *C.oppositifolia* leaf extract and compound I.

| Tube number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ and confidence interval (µg/ml) |
|---|---|---|---|
| 1 | Control | – | |
| 2 | Mannitol (50 mM) | 88.14 ± 1.42 | |
| 3 | 200 | 24.28 ± 1.42 | |
| 4 | 400 | 39.46 ± 1.82 | 593.64 ± 1.16 |
| 5 | 600 | 49.54 ± 2.14 | |
| 6 | 800 | 59.68 ± 1.32 | |
| 7 | 1000 | 79.12 ± 3.8 | |
| | Compound I | | |
| 8 | 25 | 79.34 ± 3.14 | |
| 9 | 50 | 81.42 ± 1.36 | |

Values are mean ± SEM of 3 replicates

**Table 7.4** Superoxide radical scavenging activity of *C.oppositifolia* leaf extract and compound I.

| Tube number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ and confidence interval (µg/ml) |
|---|---|---|---|
| 1 | Control | – | |
| 2 | Vitamin E (5mM) | 69.23 ± 1.45 | |
| 3 | 200 | 12.28 ± 1.42 | |
| 4 | 400 | 26.42 ± 2.13 | |
| 5 | 600 | 32.14 ± 1.32 | 875.95 ± 3.74 |
| 6 | 800 | 44.14 ± 2.82 | |
| 7 | 1000 | 58.46 ± 1.28 | |
| | Compound I | | |
| 8 | 25 | 66.34 ± 3.2 | |
| 9 | 50 | 68.23 ± 1.8 | |

Values are mean ± SEM of 3 replicates

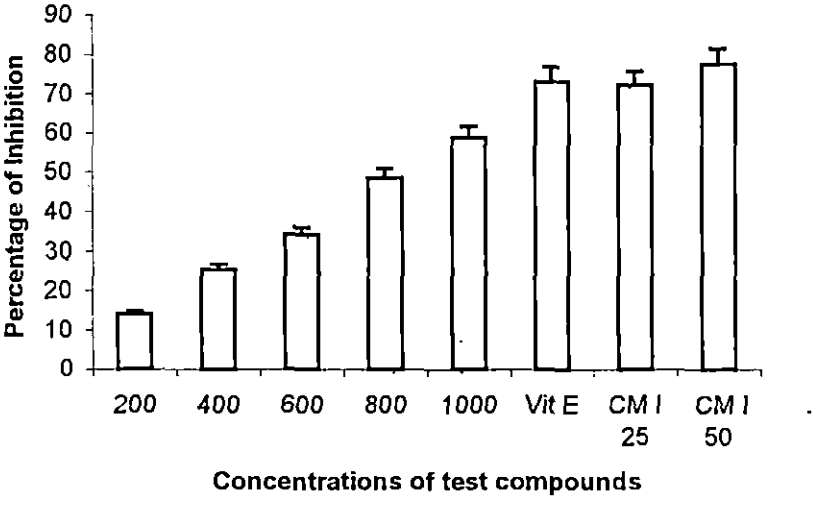


**Fig 7.1** Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *C. oppositifolia* leaf, Compound I (CM I) and Vitamin E (Vit E).

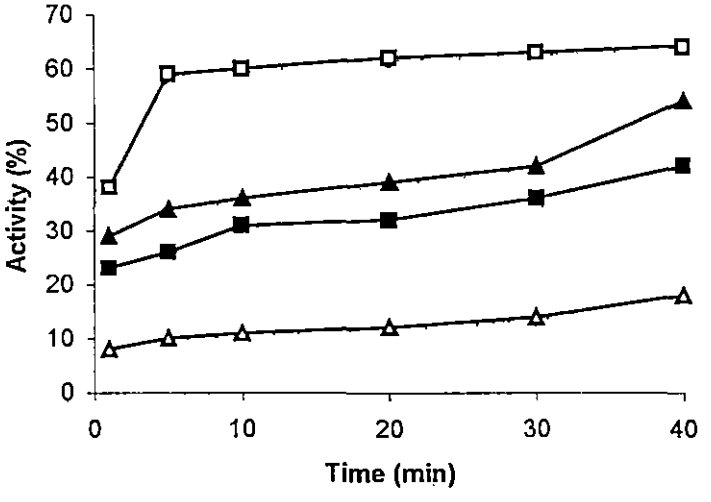


**Figure 7.2** DPPH scavenging activity of methanol extract of *C.oppositifolia* leaf, 10mg/ml (□), 7.5mg/ml (▲), 5mg/ml (■) and 2.5 mg/ml (Δ).

### 7.3.2. Antioxidant activity of *H.nepalense* root

The results presented in Table 7.5 showed the effect of methanol extract of *H.nepalense* root and compound II on ferrous sulphate induced lipid peroxidation in goat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition (69.25 ± 1.21 %) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 73.42 ± 2.3. The same result have also been presented in Figure 7.3 which revealed that compound II at 50 µg/ml showed 72.38 ± 1.9 % inhibitions, which is almost equal to that of the inhibition produced by vitamin E. The $IC_{50}$ value was found to be 745.5 ± 3.16 µg/ml. From the results of quantitative estimation of TBARS levels it appears that the methanol extract at 1000 µg/ml concentration decreases the levels of TBARS in liver homogenate from 0.99 ± 0.09 to 0.63 ± 0.11 nmoles/mg of tissue and its compound II at 25 µg/ml concentration showed nearly similar level of TBARS (0.63 ± 0.034 nmoles/mg of tissue), which is much higher rate of decrease than that of the results obtained from the well known antioxidant vitamin E ($p< 0.05$).

The effect of methanol extract and compound II on scavenging of DPPH radical is presented in Table 7.6. The result showed that the DPPH scavenging capacity of the extract was found to be 72.38 ± 3.92 % at 1000 µg/ml concentration. The compound II at 50 µg/ml concentration, on the other hand, exhibited 76.38 ± 5.12 % compared to 80.46 ± 4.62 % for the standard drug vitamin E at 5mM. The $IC_{50}$ value of the extract was found to be 6.0 mg/ml. The percentage of activity was also dependent on time as depicted from Figure 7.4.

The effect of root extract and compound II on scavenging of hydroxyl radical is presented in Table 7.7. The results revealed that the extract significantly inhibited (80.38 ± 2.28 %) degradation of deoxy-ribose mediated by hydroxyl radical at the concentration of 1000 µg/ml, compared to that of known scavenger mannitol (89.64 ± 4.62 %). Here the compound II at 50 µg/ml concentration exhibited inhibition of 79.68 ± 2.62 % compared with 89.64 ± 4.62 % for the standard drug at a concentration of 50 mM. The concentration of the methanol extract needed for 50 % inhibition was 615.57 µg/ml.

The effect of root extract and compound II on superoxide scavenging model is presented in Table 7.8. It was found that the methanol extract at 1000 µg/ml concentration caused significant increase of the inhibition up to 60.57 ± 2.34 %. The compound II at 50 µg/ml exhibited 68.24 ± 1.86 % inhibition of superoxide radicals. $IC_{50}$ was found to be 8.91 mg/ml. Inhibition was found to be directly proportional to the amount of the extract added.

**Table 7.5** Effect of methanol extract of *H.nepalense* and compound II on ferrous sulphate induced lipid peroxidation in goat liver homogenate.

| Tube number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ value and confidence interval (µg/ml) | TBARS (n moles / mg tissue) |
|---|---|---|---|---|
| 1 | Control | – | – | – |
| 2 | Vitamin E (5mM) | 73.42 ± 2.3 | | 0.69 ± 0.02[a] |
| 3 | 200 | 16.08 ± 4.3 | | 0.98 ± 0.014[a] |
| 4 | 400 | 26.57 ± 3.8 | | 0.97 ± 0.017[a] |
| 5 | 600 | 36.36 ± 1.84 | | 0.95 ± 0.015[a] |
| 6 | 800 | 51.74 ± 1.92 | 747.5 ± 3.16 | 0.89 ± 0.018[a] |
| 7 | 1000 | 69.25 ± 1.21 | | 0.63 ± 0.11[a] |
| | Compound II | | | |
| 8 | 25 | 70.15 ± 1.64 | | 0.63 ± 0.034[a] |
| 9 | 50 | 72.38 ± 1.9 | | 0.61 ± 0.016[a] |

Values are mean ± SEM of 3 replicates [a]$p < 0.05$.

**Table 7.6** Free radical scavenging activity of *H.nepalense* root extract and compound II by DPPH reduction.

| Tube number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ and confidence interval (µg/ml) |
|---|---|---|---|
| 1 | Control | – | |
| 2 | Vitamin E (5mM) | 80.46 ± 4.62 | |
| 3 | 200 | 8.47 ± 1.83 | |
| 4 | 400 | 36.46 ± 2.32 | |
| 5 | 600 | 45.52 ± 3.73 | 600.52 ± 3.46 |
| 6 | 800 | 69.42 ± 3.86 | |
| 7 | 1000 | 72.38 ± 3.92 | |
| | Compound II | | |
| 8 | 25 | 71.32 ± 1.85 | |
| 9 | 50 | 76.38 ± 5.12 | |

Values are mean ± SEM of 3 replicates

**Table 7.7** Hydroxyl radical scavenging activity of *H.nepalense* root extract and compound II.

| Tube number | Concentration of methanol extract (µg/ml) | Inhibition (%) | $IC_{50}$ and confidence interval (µg/ml) |
|---|---|---|---|
| 1 | Control | – | |
| 2 | Mannitol (50mM) | 89.64 ± 4.62 | |
| 3 | 200 | 32.14 ± 2.24 | |
| 4 | 400 | 46.28 ± 1.89 | |
| 5 | 600 | 65.37 ± 2.26 | 615.57 ± 2.16 |
| 6 | 800 | 69.42 ± 3.86 | |
| 7 | 1000 | 80.38 ± 2.28 | |
| | Compound II | | |
| 8 | 25 | 72.35 ± 2.93 | |
| 9 | 50 | 79.68 ± 2.62 | |

Values are mean ± SEM of 3 replicates

**Table 7.8** Superoxide radical scavenging activity of *H.nepalense* root extract and compound II.

| Tube number | Concentration of methanol extract (μg/ml) | Inhibition (%) | $IC_{50}$ and confidence interval (μg/ml) |
|---|---|---|---|
| 1 | Control | – | |
| 2 | Vitamin E (5mM) | 68.36 ± 2.73 | |
| 3 | 200 | 12.23 ± 1.42 | |
| 4 | 400 | 24.12 ± 1.38 | |
| 5 | 600 | 40.26 ± 2.16 | 891.9 ± 14.42 |
| 6 | 800 | 52.27 ± 3.18 | |
| 7 | 1000 | 60.57 ± 2.34 | |
| | Compound II | | |
| 8 | 25 | 68.36 ± 2.73 | |
| 9 | 50 | 68.24 ± 1.86 | |

Values are mean ± SEM of 3 replicates

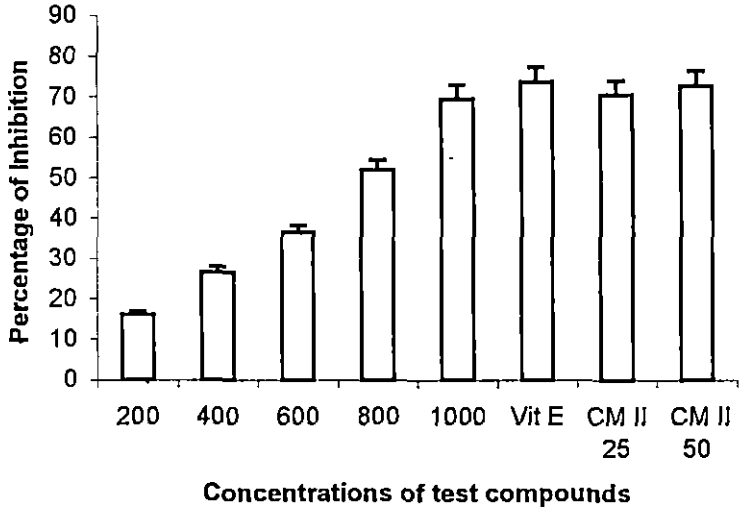


**Fig 7.3** Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *H.nepalense* root, Compound II (CM II) and Vitamin E (Vit E).

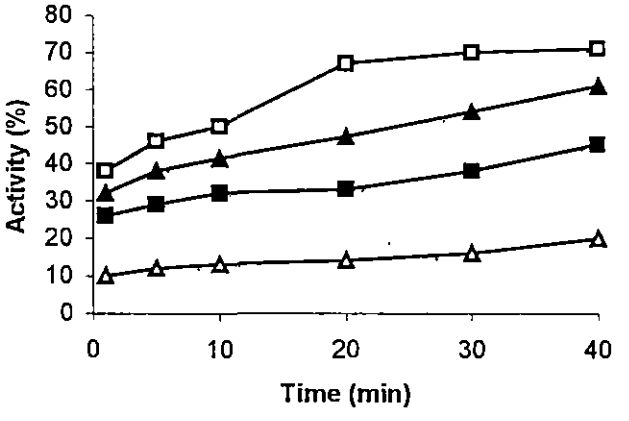


**Figure 7.4.** DPPH scavenging activity of *H.nepalense*. 10mg/ml (□), 7.5mg/ml (▲), 5mg/ml (■) and 2.5 mg/ml (Δ).

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