CHAPTER - 5

STUDIES ON ANTIOXIDANT ACTIVITY

5.1. Introduction

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxy nitrite radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system; however, oxidative stress is generated when equilibrium favors free radical generation as a result of a depletion of antioxidant levels. Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, such as cancer, cardiovascular diseases, neurodegenerative disorders and ageing[1]. Nitric oxide (NO) a diatomic free radical, is synthesized in biological systems by constitutive and inducible nitric oxide synthase (cNOS) and (iNOS)[2]. Excess generation of NO by NOS has been found to contribute too many diseases, such as carcinogenesis, septic shock, cerebral injury, atherosclerosis, rheumatoid arthritis, cell apoptosis and necrosis[3]. Moreover, under pathological conditions, macrophages can greatly increase their production of both NO and superoxide anion simultaneously to form a peroxy nitrite anion ($ONOO^-$), which is more toxic than O^{-2} or NO to biological systems, by causing modification of proteins[4] or nucleic acid[5]. Thus, reducing the NO generation in excess amounts is now accepted widely as an important goal for the chemoprevention of various diseases, e.g., cancer. Search for natural antioxidants, especially from plant sources, as a potential preventive intervention for free-radical mediated diseases has already turned into an attractive research field and a very important social issue for improvement of the quality of human life. Polyphenols, including phenolic acids, flavonoids, tannins, lignins and others are widespread in plant foods and in different medicinal plants. Plant phenolics may function as potent free radical scavengers, reducing agents, quenchers of ROS and protect against lipid peroxidation[6].

Free radicals and oxygen derivatives are constantly generated *in vitro* both by accident of chemistry and specific metabolic purposes. The reactivity of free radicals varies with many to cause inflammation or even severe damage to biological molecule, especially to DNA, lipids and proteins. The generation of free radicals during the metabolic process is now observed to be responsible for wide range of human condition such as ageing, cancer, atherosclerosis, arthritis, viral infection, stroke, myocardial infraction, pulmonary

condition, inflammatory bowel disease, neurodegenerative disease and others may be produced by reactive oxygen species (e.g., oxygen free radicals, hydrogen peroxide). The action of free radicals is counteracted by free radicals either endogenous or exogenous[7].

The term oxygen free radical refers to the form of oxygen exhibiting high reactivity and having at least one unpaired electron. However, other reactive forms of O_2 are also known as non-free radical. Both these forms are collectively referred to as active Oxygen Species (ROS) and include singlet oxygen, hydrogen peroxide, hydroxyl radical[8]. They are produced continuously by ionizing radiation as well as by the byproduct of cellular metabolism, which are toxic and are important factor for several pathological conditions such as diabetes, inflammation and cancer[9,10].

Free radicals are a normal product of metabolism; the body produces its own antioxidants (e.g., the enzyme superoxide dismutase) to keep them in balance. However, stress, ageing, and environmental sources such as polluted air and cigarette smoke can add to the number of free radicals in the body, creating an imbalance. The highly reactive free radicals can damage healthy DNA and have been linked to changes that accompany ageing (such as age-related muscular degeneration, a leading cause of blindness in older people) and with disease processes that lead to cancer, heart disease and stroke[11].

Antioxidants act as a major defense against radical mediated toxicity by protecting the damage caused by free radicals. Potential antioxidant therapy should therefore include either natural antioxidant enzymes or agents, which are capable of augmenting the function of this oxidative free radical scavenging enzymes[12].

Antioxidants are molecules that slow or prevent the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols[13].

In biological systems, the normal processes of oxidation produce highly reactive free radicals. Antioxidants work by binding to the free radicals, they transforms them into non-damaging compounds or repairs cellular damage. They are able to easily donate electrons to molecules that need of an electron, such as free radicals, before they steal one

from some place else, thus stabilize and prevent a damaging chain reaction. The antioxidant molecules interact with the oxygen free radicals and halt the spread of cancer causing cells with damaged DNA before other vital molecules are damaged and they neutralize the production of free radicals which are chemical complexes that cause harm to our cells and play a major role in the disease process.[14]

Antioxidants help alleviate the symptoms and side effects of many of these diseases. According to the free radical theory, radicals damage cells in an organism, causing ageing. Antioxidants break the free radical chain reaction by sacrificing electrons, and then humbly existing without stealing more. The body naturally circulates many nutrients for their antioxidant properties, and creates antioxidant enzymes just for the purpose of controlling free radicals and their chain reactions. Antioxidants are thought to thwart heart disease by preventing oxidation. Antioxidants combat chronic inflammation. Vitamin E suppresses platelet stickness, acting as an anticoagulant to discourage the formation of clots that lead to heart attacks. Vitamin C decreases a blood factor needed to build clots. Antioxidants fight cancer by neutralizing DNA-damaging free radicals. Antioxidants neutralize free radicals as the natural by-product of normal cell processes and prevent injury to blood vessel membranes, helping to optimize blood flow to the heart and brain, defend against cancer-causing DNA damage, and help to lower the risk of cardiovascular disease and dementia, including Alzheimer's disease. Antioxidants can cancel out the cell-damaging effects of free radicals[15] and people who eat fruits and vegetables rich in polyphenols and anthocyanins have a lower risk of cancer, heart disease and some neurological diseases[16]. This observation suggested that these compounds might prevent conditions such as macular degeneration[17], suppressed immunity due to poor nutrition[18] and neurodegeneration, which are caused by oxidative stress[19].

Antioxidants are found in varying amounts in foods such as vegetables, fruits, grain cereals, legumes and nuts. Some antioxidants such as lycopene and ascorbic acid can be destroyed by long-term storage or prolonged cooking[20,21]. Other antioxidant compounds are more stable, such as the polyphenolic antioxidants in foods, whole-wheat cereals and tea[22,23]. In general, processed foods contain less antioxidant than fresh and uncooked foods, since the preparation processes may expose the food to oxygen[24].

Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxy nitrite[25,26]. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Several authors demonstrated that antioxidant intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks[27-29]. As well as antioxidant defense system of our body, antioxidants that are mainly supplied as dietary consumptions can also impede carcinogenesis by scavenging oxygen radicals or interfering with the binding of carcinogens to DNA which includes vitamin C, vitamin E (tocopherol), carotenoids (carotene, cryptoxanthin, lutein, zeaxanthin, lycopene) and several polyphenolic compounds including flavonoids (catechins, flavonols, flavones, isoflavonoids[30,31]). In particular, phenolic compounds have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals. The commercial development of plants as sources of antioxidants to enhance health and food preservation is of current interest[32].

Plant extract screening studies pointed out further investigations, as several promising plant materials with strong free radical scavenging properties showed up. Plants are the rich source of compounds, which may protect organism from free radical injury and disease[33].

The aim of the present study is to determine the antioxidant activity of the methanol extract of *K. rotunda* Linn. and *E. cannabinum* Linn. For this purpose, plant extracts were tested for antioxidant activity and their capacity to reduce lipid peroxidation by using malonaldehyde (MDA) and 4-hydroxy 2-nolnel (4-HNE) as the model molecule.

5.2. Materials and methods

5.2.1. Plant materials

Methanol extracts of *K. rotunda* rhizomes and *E. cannabinum* leaves were used as test drug in these experiments. The crude extracts were mixed with DMSO to prepare the stock solution (10µg/10ml). The test samples were prepared from stock solution by serial dilution to attain the concentrations of 100, 200, 500 and 1000µg/ml.

5.2.2. Drugs and chemicals

Thiobarbituric acid (TBA) was obtained from Loba Chemie, India. 2, 4 dinitrophenyl hydrazine (DNPH) and 1,1,3,3-tetramethoxy propane (TEP) were obtained from Sigma Chemicals, USA. Dimethyl sulphoxide (DMSO), ethylenediamine tetra acetic acid (EDTA), ferrous sulphate, trichloroacetic acid (TCA), hydrogen peroxide, ascorbic acid, potassium dihydrogen phosphate, potassium hydroxide, hexane, methanol and HCl were of analytical grade and obtained from Ranbaxy Fine Chemicals. 4-HNE (4 hydroxy- 2 noneal) was obtained from Ranbaxy Ltd. as gift sample. All other chemicals and reagents used were of analytical grade.

- **Preparation of 5 mM DNPH**

100 mg of DNPH was dissolved in 100 ml of 0.5 M HCl and stirred for approximately 1 hour at room temperature. It was filtered to remove undissolved DNPH and stored in an amber coloured bottle.

- **Preparation of 0.5 M HCl**

21ml conc. HCl was added to 479ml of water to produce 500ml of 0.5 M HCl.

- **Preparation of 4-HNE (4 hydroxy-2 noneal) primary standard**

1mg of 4-HNE (6.4 µmole) was dissolved in 6.4 ml of methanol to give a 1mM solution. Diluting 100µl of the solution to 5ml in methanol in a volumetric flask the solution was standardized and the absorbance was measured at 220nm using methanol as blank. The concentration of 4-HNE in the primary standard was calculated using the following equation.

4-HNE= (A_{220} / 13750) X 50

Where A_{220} is the absorbance at 220nm

'13750' is the molar extinction co-efficient for 4-HNE in methanol and 50 is the dilution factor.

5.2.3. Preparation of goat liver homogenate

The liver was chosen to estimate the markers of lipid peroxidation because the metabolites from liver may diffuse into various extra hepatic tissue causing lipid peroxidation and cellular damage[34]. The goat liver was purchased from local market and collected in phosphate buffer (pH-7.4). The liver was homogenized in 100ml of

phosphate buffer and filtered to get clear homogenate. This homogenate was used to estimate the lipid peroxidation markers. Necessary steps were taken as much as possible to simulate the conditions of *in vitro* system.

5.3. *In Vitro* antioxidant activity of rhizomes of *K. rotunda* Linn.

5.3.1. Lipid peroxidation model on MDA marker suppression activity

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[35]. Goat liver was purchased from local market. 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 10min. To study the comparative response, the experiment was performed in six conical flasks. Liver homogenate (3ml) was aliquoted to each of the flask. The flask number one and six were treated as control and standard where buffer and vitamin C were added respectively. To the no. two to five flasks different concentrations of methanol extract (100, 200, 500 and 1000µg/ml) were added respectively. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate in six flasks[36]. After 30min, 1.5ml of each reaction mixture was taken into centrifuge tubes containing 1.5ml of 10% TCA. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% TBA. The mixture was heated in a water bath at 85°C for 30min and in a boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 530nm in a spectrophotometer (Shimadzu 1601). The TBARS concentration was calculated by using standard graph[37,38].

5.3.2. Lipid peroxidation model on 4-HNE suppression activity

The liver supernatant was prepared as the procedure mentioned in 5.2.6. The liver homogenate was divided into six conical flasks containing 3ml in each. The flask number one and six were treated as control and standard where buffer and vitamin C were added respectively. To the no. two to five flasks, different concentrations of methanol extract (100, 200, 500, 1000µg/ml) were added. All the conical flasks were placed in mechanical shaker. After two hours the liver sample was transferred to the centrifuge tubes (3 for

each set i.e. control and so on). 2ml of TCA was added to all the centrifuge tubes and centrifuged for 30 minutes then the supernatant was filtered and 2ml of filtrate was taken in a 13x100mm glass tube with cap. 1ml of DNPH was added to all the tubes containing liver supernatant, mixed and set aside for 1h to react with 4-HNE. Then the formed adduct of 4-HNE and DNPH was extracted by hexane, which was evaporated under argon at 40°C. After cooling, 2ml of methanol was added to all the samples and the absorbance was measured at 350nm in the spectophotometer[39]. The quantity of 4-HNE was calculated by linear regression analysis[40].

5.3.3. Preparation of standard calibration curves

- **Standard curve for the estimation of MDA**

Different aliquots from TEP solutions were taken graduated stopper (10ml.) test tube at volume of each solution was made up to 5ml. TBA reagent (5ml) was added and the mixtures were heated in a steam bath for 30 min. to develop pink coloured solution. The solutions were cooled to room temperature and absorbances were noted at 530nm using TBA reagent as blank. A curve was obtained by taking conc. vs absorbance (Fig. **5.5**).

The quantity of MDA was calculated by using the following equation:
μM of MDA = 6.578×10^{-3}/A. where A is the absorbance at 530nm.

- **Standard curve for the estimation of 4-HNE**

2ml of 4-HNE sample (300, 400, 500 and 600μl) was pipette out and transferred into a 13x100 mm glass tube with cap. 3.1ml of DNPH reagent was added to all the samples and mixed by cyclomixer and allowed to react at room temperature for 1h. The samples were extracted with 2ml aliquots of hexane combining the extracts in a glass tube. Hexane portion was evaporated under argon at 40°C and reconstituted with methanol. The absorbance was measured at 350nm. The standard calibration curve was plotted between absorbance and concentration of 4-HNE, which is shown in fig. **5.6**. A linear regression analysis was performed to determine the slope and intercept of the calibration line.

The quantity of 4HNE is calculated based on the results of the linear regression analysis using the following equation.

nmole of 4-HNE = (4-HNE peak area – intercept) / slope

5.4. *In vitro* antioxidant activity of leaves of *E. cannabinum* Linn.

The antioxidant activity of methanol extract of *E. cannabinum* leaves was determined as per the methods described above in **5.3.1** and **5.3.2** for *K. rotunda* Linn.

5.5. Results

The experimental study was based on the estimation of MDA and 4-HNE and their suppression by the extracts of *K. rotunda* Linn. and *E. cannabinum* Linn. which is presented in table 5.1, 5.2, 5.3, 5.4 respectively. From the experimental results, it has been proved that the methanolic extract of both the plant has significant antioxidant activity. This study also confirmed that the lipid peroxidation was significantly controlled at low concentration of extracts of *K. rotunda* Linn. The quantification of MDA and 4-HNE can be directly correlated with the lipid peroxidation inhibition capacity of the extract. The toxic radicals quantification is also an indicator to monitor the overall progress of polyunsaturated fatty acid oxidation. The antioxidant activity of *K. rotunda* Linn. was studied by dose dependent manner. From the fig. **5.1** and **5.2**, it was concluded that the significance of antioxidant activity has inverse relationship with dose i.e. high at low dose and vice versa. The extract at 100µg/ml and 200µg/ml has significant and moderate antioxidant activity respectively but 500 and 1000µg/ml has insignificant activity. From the fig. **5.3** and **5.4** the antioxidant activity has been observed in higher doses in case of *E. cannabinum* Linn. The dose of 100µg/ml and 200µg/ml has insignificant activity whereas 500µg/ml and 1000µg/ml has moderate and significant antioxidant activity respectively. The antioxidant activity of extracts was compared with standard antioxidant (ascorbic acid). The results were analyzed statistically and found significant at $P<0.05$ levels.

From the clinical point of view, the free radicals and lipid derived metabolites have been strongly implicated in the pathology of various diseases such as myocardial infarction, diabetes mellitus, hepatic injury, atherosclerosis, rheumatoid arthritis and cancer. The free radicals i.e. MDA and 4-HNE have strong link with the above-mentioned diseases. The suppression of the radicals by the methanolic extract of rhizomes of *Kaempferia rotunda* and leaves of *Eupatorium cannabinum* in the above study has thus been correlated with the antioxidant potential of the plant and this information can be used to control the age dependent diseases mentioned above.

Table 5.1. *In vitro* antioxidant activity of *K. rotunda* Linn. on lipid peroxidation by MDA model

Flask no.	Concentration of extract (µg/ml)	Concentration of MDA (µM)	Average of MDA ($\bar{x}$ ± S.E)
1	Control (Phosphate buffer pH 7.4)	18.97 19.13 18.52	18.87±0.18
2	100	14.02 14.59 14.71	14.44±0.21
3	200	16.96 16.57 17.05	16.86±0.15
4	500	17.28 17.47 17.13	17.29±0.09
5	1000	17.97 17.81 18.08	17.95±0.08
6	Standard Ascorbic acid (250mg/kg body wt.)	13.95 13.17 13.59	13.57±0.22

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$

Table 5.2. *In vitro* antioxidant activity of *K. rotunda* Linn. by 4-HNE model

Flask no.	Concentration of extract (µg/ml)	Concentration of 4-HNE (nM)	Average of 4-HNE ($\bar{x} \pm$ S.E)
1	Control (Phosphate buffer pH 7.4)	97.29 97.92 97.56	97.59±0.18
2	100	82.45 83.09 83.17	82.90±0.23
3	200	89.18 88.72 89.05	88.98±0.13
4	500	93.12 94.09 93.53	93.58±0.28
5	1000	95.32 94.87 95.09	95.09±0.13
6	Standard Ascorbic acid (250mg/kg body wt.)	75.52 75.86 76.08	75.82±0.16

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$

Table-5.3. *In vitro* antioxidant activity of *E. cannabinum* Linn. on lipid peroxidation by MDA model

Concentration of extract (µg/ml)	Concentration of MDA(µM)	Average of MDA ($\bar{x}$ ± S.E)
Control (Phosphate buffer pH 7.4)	18.97 19.13 18.52	18.87±0.18
100	18.01 17.75 17.85	17.87±1.89
200	16.95 17.01 17.09	17.01±0.63
500	16.32 16.35 16.32	16.33±0.036
1000	15.20 15.21 15.22	15.21±0.56
Standard Ascorbic acid (250mg/kg body wt.)	13.95 13.17 13.59	13.57±0.22

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$.

Table-5.4. *In vitro* effect of *E. cannabinum* by 4-HNE model

Concentration of extract (µg/ml)	Concentration of 4-HNE(nM)	Average of 4-HNE ($\bar{x}$ ± S.E)
Control (Phosphate buffer pH 7.4)	97.29 97.92 97.56	97.59±0.18
100	81.04 81.66 82.27	81.65±0.35
200	79.82 80.33 80.74	81.04±0.35
500	79.20 79.82 80.43	79.81±0.35
1000	77.67 77.06 77.36	77.36±0.17
Standard Ascorbic acid (250mg/body wt.)	75.52 75.86 76.08	75.82±0.16

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$.

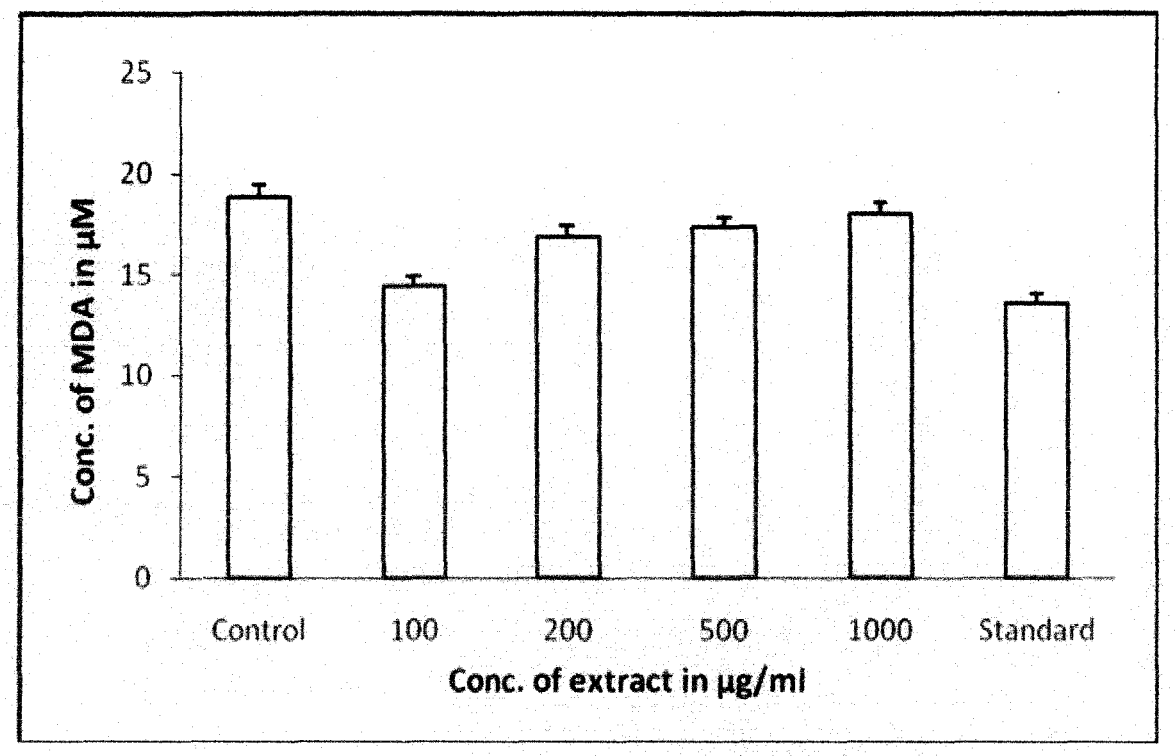

Fig.5.1. Antioxidant activity of *K. rotunda* Linn. by MDA suppression model

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

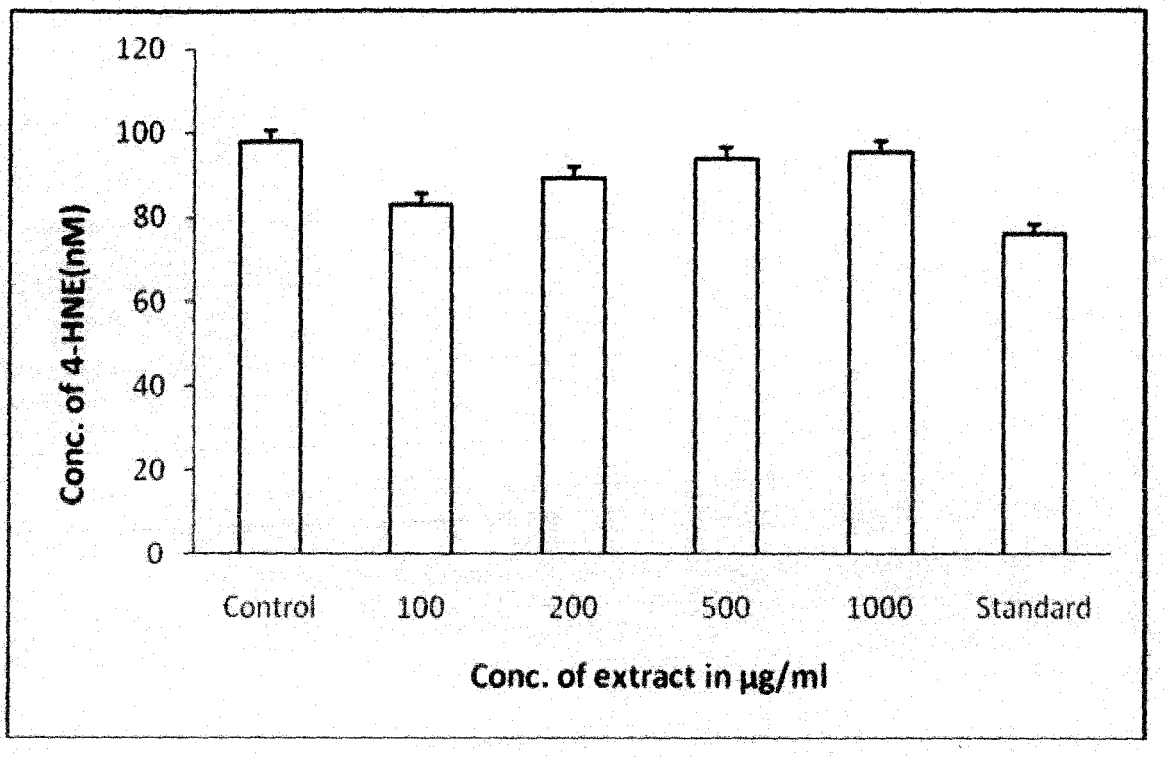

Fig. 5.2. Antioxidant activity of *K. rotunda* Linn. by 4-HNE suppression model

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

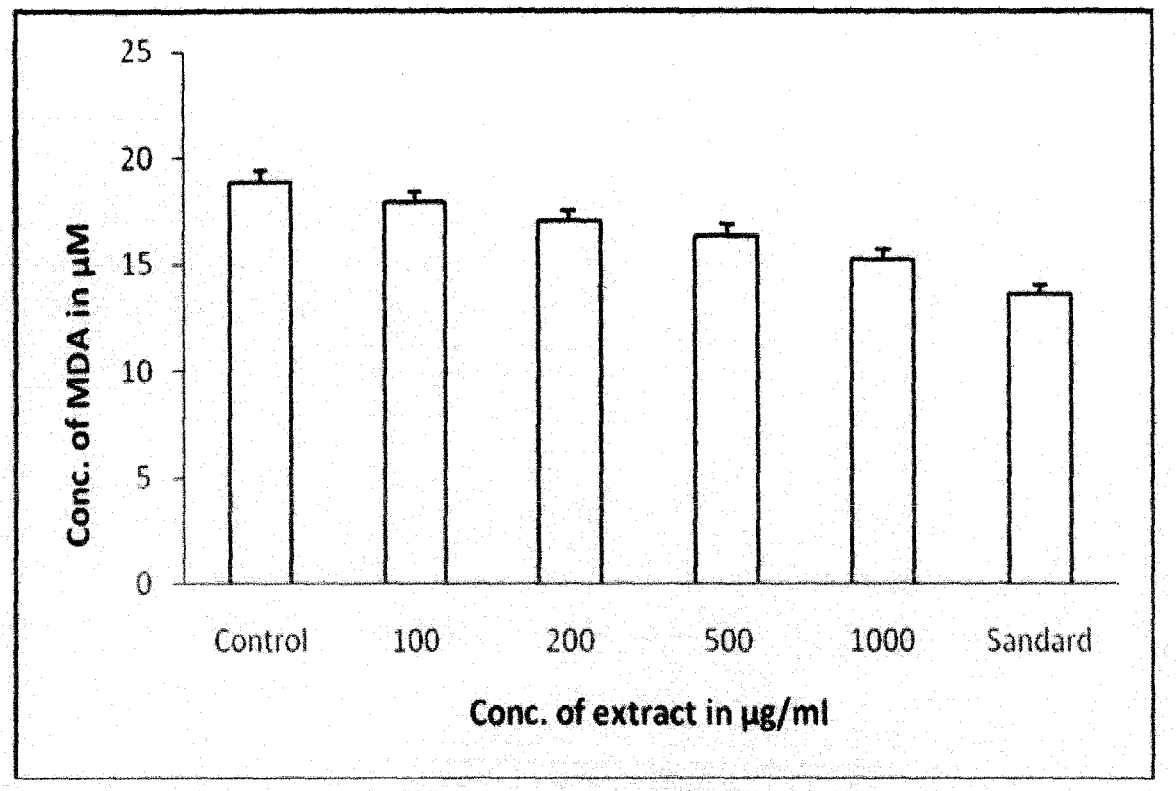

Fig.5.3. MDA suppression activity of *Eupatorium cannabinum*

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

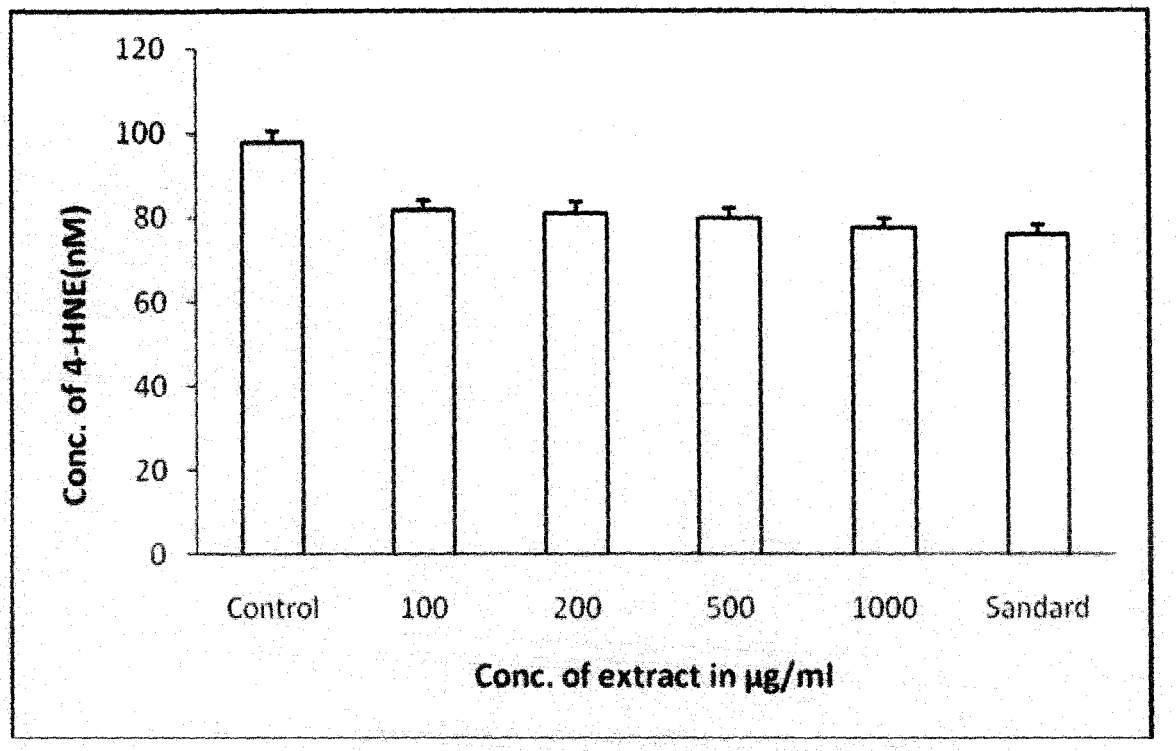

Fig.5.4. 4-HNE suppression activity of *Eupatorium cannabinum*

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

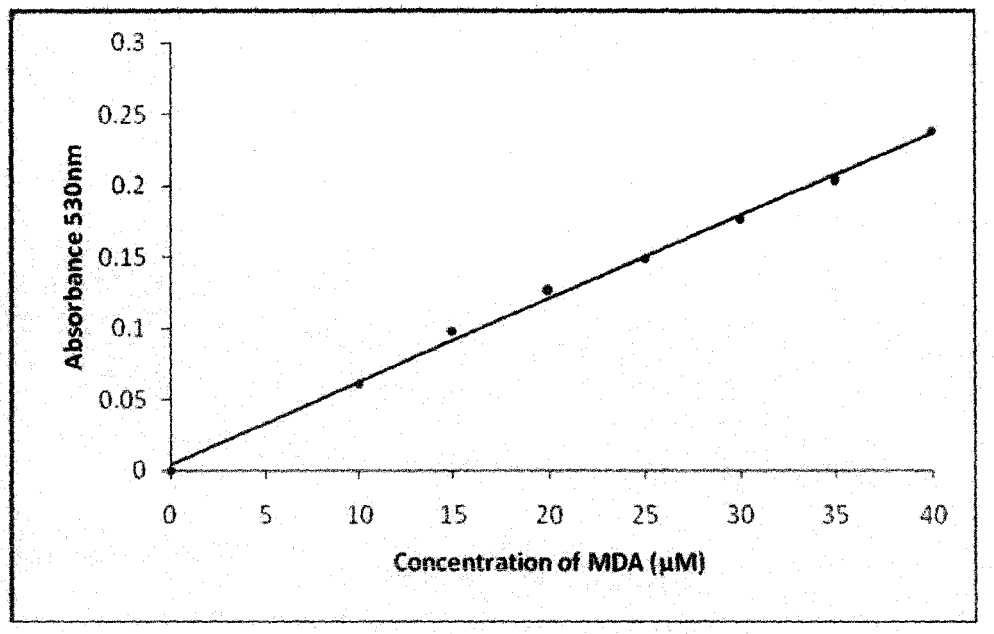

Fig.5.5. Standard calibration curve of MDA

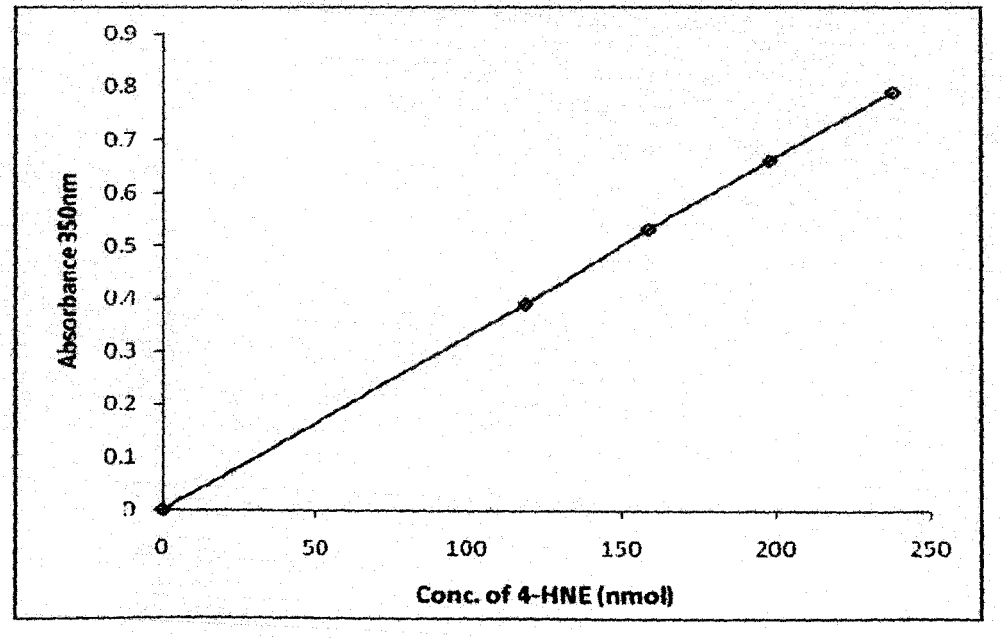

Fig.5.6. Standard calibration curve of 4-HNE

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