Comparative Study of *In Vitro* Free Radical Scavenging Activity of Different Leaf Extracts of *Ixora coccinea* L.

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This article is available online at www.ssjournals.com

ABSTRACT

In this study free radical scavenging activity of chloroform, methanolic and aqueous leaf extracts of *Ixora coccinea* L. (Rubiaceae) were determined and compared by *in vitro* assay models such as DPPH free radical, nitric oxide radical, hydroxyl radical scavenging assay, reductive ability and total antioxidant capacity. Ascorbic acid was used as reference standard. IC_{50} of all the exracts in all the method were determined. Among all the three extracts, methanolic extract have shown better scavenging and antioxidant property compared to other extracts in all the assay methods evaluated thereby leading it into the modern system of medicine in future.

KEY WORDS: Free radical, Antioxidant, *Ixora coccinea*, DPPH.

INTRODUCTION

A free radical is defined as any atom or molecule that possesses an unpaired electron. In biological and related fields the major free radical species of interest have been those of oxygen that is oxygen free radicals (OFRs). OFRs are part of a greater group of molecules often called reactive oxygen species (ROS) that are all more strongly oxidizing than molecular oxygen itself. However, it is in the biological arena where the study of OFR species has attracted by far the greatest interest in recent years. OFRs are potentially very toxic to cells. Due to their highly reactive nature they can readily combine with other molecules, such as enzymes, receptor and ion pumps, causing oxidation directly and inactivating or inhibiting their normal function. Some of the products of OFRs can interfere with nucleic acid function, generating alterations in the base sequence with the potential for mutations, leading in extreme pathological situations to cancers or germ-line mutations. OFRs and other free radicals are generated by normal metabolic process such as the reduction of oxygen to water by the mitochondrial electron transport chain [1].

Antioxidants are the substances that when present in body at low concentration compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate. They have also been of interest to biochemist and health professionals because they may help the body protect itself against damage caused by reactive oxygen species and degenerative diseases [2].

Ixora coccinea L. belonging to the family Rubiaceae is common flowering shrub with few brunches and ovate leaves found in India especially in west coast and also cultivated as ornamental plant.

The roots, flowers and leaves are associated with treatment of several physiological disorders such as diarrhoea, dysentery, dysmenorrhoea and leucorrhoea. Additionally these parts are

also indicated as sedative, antiseptic and astringent [3].

The leaves have shown antimicrobial, antinociceptive activity [4,5] and also anti-inflammatory and antioxidant property [6].

The literature review has shown that there are very few investigations have been carried out in detail on the phytochemical and free radical scavenging nature of the leaves. The objective of this study is to compare the antioxidant and free radical scavenging properties of chloroform, methanol and aqueous leaf extracts of *I. coccinea*. For better comparison the evaluation has been performed maintaining the same concentration range of all the extracts in different *in vitro* antioxidant assays i.e DPPH radical scavenging activity, reductive ability, nitric oxide scavenging activity, hydroxyl radical scavenging activity and total antioxidant capacity.

MATERIALS AND METHODS

Reagents and Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), Sodium nitropruside, Ascorbic acid, Thiobarbituric acid (TBA), Potassium ferricyanide, N-1-Naphthyl ethylele diamine dihydrochloride and Ammonium molybdate were procured from Loba Chemie Pvt. Ltd. Mumbai, India.

Ehylene diamine tetra acetic acid (EDTA), Trichloro acetic acid (TCA) and all other solvents were procured from Merck Specialties Pvt. Ltd. Mumbai, India. All the chemicals and solvents used in the study were of analytical grade.

Plant Materials

The plant materials (leaves of I. coccinea) were collected from the medicinal plant garden of BCDA College of Pharmacy and Technology in the month of March, 2010 and Identified from Botanical survey of India, West Bengal. A voucher specimen (BCDA/2010/0101) was deposited in the department of pharmacognosy, BCDA College of Pharmacy and Technology for further reference.

Preparation of Extract

The leaves were shade dried and powdered. The dried leaves (100 gm each) were extracted in soxhlet extractor with 3 L each of chloroform, methanol and water separately. The extracts were filtered and dried under reduced pressure to get the concentrated extracts. The yields for Chloroform extract, Methanolic extract and Aqueous extract were found to be 1.8% w/w, 2.3% w/w and 3.9% w/w respectively. These were used as test substances and designated as CLE, MLE, ALE respectively for Chloroform extract, Methanol extract and Aqueous extract.

Antioxidant assay [7-12]

The antioxidant activity of CLE, MLE, ALE were determined by different *in vitro* method such as DPPH radical scavenging activity, Reduction ability, Nitric oxide scavenging activity, Hydroxyl radical (OH^-) scavenging activity and total antioxidant capacity. All the assays were carried out in triplicate and average values were considered.

DPPH radical scavenging activity [7]

The free radical scavenging activity of the extracts was determined using DPPH. DPPH solution (0.1 mM) was prepared in 95% methanol. Methanol extract of I. coccinea was mixed with 95% methanol to prepare the stock solution (0.5 mg/mL). Freshly prepared 1 mL of DPPH solution

(0.1 mM) was added to 3 mL of various concentrations (50 - 350 µg/mL) of CLE, MLE and ALE. After 30 min, absorbance was measured at 517 nm. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Ascorbic acid was used as a reference compound. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extracts) using the following formula- absorbance of the control minus absorbance of the test sample divided by absorbance of the control multiplied by 100 [8].

Reductive ability [9]

1 mL of different concentrations of the various extracts (CLE, MLE and ALE; 50 - 350 µg/mL) were mixed with potassium ferricyanide (2.5 mL, 1%) and 2.5 mL of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. 2.5 mL TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 mL of supernatant was taken out and to this 2.5 mL water and 0.5 mL $FeCl_3$ (0.1%) were added and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Nitric oxide scavenging activity [10]

In aqueous solution at physiological pH, sodium nitroprusside generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be measured by the Griess reaction. 100 mM sodium nitroprusside (SNP) was prepared in phosphate buffer saline (PBS) pH 7.4. The reaction mixture (2 mL) containing 100mM SNP (0.2 mL, final conc.10 mM) and different concentrations of various extracts (CLE, MLE and ALE, 50-350 µg/mL) in PBS (1.8mL) were incubated at 25°C for 150 min. From the incubated mixture, 1 mL was taken out and 1 mL of Griess reagent (1% sulphanilic acid in 20% glacial acetic acid and 0.1% naphthylethylene diamine dihydrochloride in 20% glacial acetic acid) was added to it. Absorbance was read at 540 nm and percentage inhibition was calculated by comparing the results of the test with those of the control (not treated with extracts) using the formula as mentioned above in the DPPH method.

Hydroxyl radical scavenging activity [11]

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and different extracts in respective solvents for hydroxyl radical generated by Fe^{3+}-Ascorbate–EDTA–H_2O_2 system (Fenton reaction). The reaction mixture contained in a final volume of 1.0 mL, 100 µL of 28 mM 2-deoxy-2-ribose in 20 mM KH_2PO_4-KOH buffer of pH 7.4, 500 µL of the selected concentrations various extracts (CLE, MLE and ALE, 50-350 µg/mL) in KH_2PO_4-KOH buffer (20 mM, pH 7.4), 100 µL of 1.04 mM EDTA, 100 µL 200 mM $FeCl_3$, 100 µL of 1.0 mM H_2O_2 and 100 µL of 1.0 mM ascorbic acid was incubated at 37 ºC for 1 hour. Then 1.0 mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8 %) were added to the test tubes and were incubated at 100 ºC for 20 min. After cooling, absorbance was measured at 532nm against control containing deoxyribose and buffer (not treated with drug). Ascorbic acid was used as a positive control. The percentage inhibition was determined by comparing the results of the test and control compounds. The hydroxyl radical scavenging activity of the extract is calculated as % inhibition of deoxyribose degradation and is calculated

according to formula used in DPPH method.

Determination of total antioxidant capacity [12]

The antioxidant activity of the extracts of *I. coccinea* was evaluated by the phosphomolybdenum method. 0.3 mL each of various extracts (CLE, MLE and ALE, 50-350 µg/mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solutions was measured at 695 nm against blank after cooling to room temperature.

RESULTS AND DISCUSSION

The utility of antioxidant therapies in many diseases is well recognized. Cellular damage arising from an imbalance between free radical generating and scavenging systems has been implicated in the pathogenesis of a wide range of disorders including cardiovascular diseases, cancer and aging [13].

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in Absorbance [14]. Comparison of the antioxidant activity of different extracts and ascorbic acid is shown in Figure-1. This activity was increased by increasing the concentration of the sample extract. The methanolic extract of *I. coccinea* exhibited a significant antioxidant activity in dose dependent manner. The IC_{50} values of CLE, MLE, ALE and ascorbic acid were found to be at 225 µg/mL, 158 µg/mL, 192 µg/mL and 98 µg/mL respectively.

The reductive ability of a compound generally depends on the presence of reductants which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom. The presence of reductants (i.e. antioxidants) in *I. coccinea* extracts causes the reduction of the Fe^{3+} ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm [14]. Figure-2 shows the comparison of the reductive capability of various extracts of *I. coccinea* leaves with ascorbic acid. The reducing power of *I. coccinea* extracts was increased with the concentration of extracts.

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes [15]. Excess concentration of NO is associated with several diseases [16, 17]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals [18,19]. In this study the extracts compete with oxygen to react with nitric oxide and thus inhibit generation of the anions.Figure-3 shows the NO scavenging capabilities of various extracts of *I. coccinea*. The IC_{50} values of CLE, MLE, ALE and ascorbic acid were found to be at 342 µg/mL, 200 µg/mL, 282 µg/mL, and 150 µg/mL respectively.

The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxides are produced. Lipid hydroperoxide can be

decomposed to produce alkoxy and peroxy radical and they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases. Thus the decrease in the MDA level with the increase in the concentration of the extracts indicates the role of the extracts as antioxidant [20]. Figure 4 shows comparison among the different extracts of *I. coccinea* and ascorbic acid in a dose dependent manner. The IC_{50} values of CLE, MLE, ALE and ascorbic acid were found to be at 350 µg/mL, 260 µg/mL, 321 µg/mL, and 225 µg/mL respectively.

Total antioxidant capacity of the *I. coccinea* extracts, expressed as the number of equivalents of ascorbic acid, is shown in Figure-5. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm [21].

So, from this comparative analysis it has been observed that among all the extracts of *I. coccinea*, methanolic extract (MLE) exhibits better scavenging activity against free radicals and having considerable reduction capability compared to aqueous extract (ALE) and chloroform extract (CLE) as well. All the methods have proven the effectiveness of the methanolic extract compared to the reference standard ascorbic acid. In some methods antioxidant activity of methanolic extract in certain concentrations is very much comparable to standard reference ascorbic acid.

More over it is also noticeable that free radical scavenging activity of aqueous extract is almost closer to the free radical scavenging activity of methanolic extract.

In conclusion, this comperative study scientifically demonstrates promising antioxidant activity of methanolic leaf extract of *I. coccinea* which may be responsible for its anti-inflammatory and chemoprotective principle.

Further investigations on the isolation, identification, structural determination of antioxidant constituent(s) of methanolic leaf extract may lead to chemical entities having remarkable medicinal and economical value.

ACKNOWLEDGEMENT

The authors wish to thank the authority of BCDA College of Pharmacy and Technology for continuous encouragement and support.

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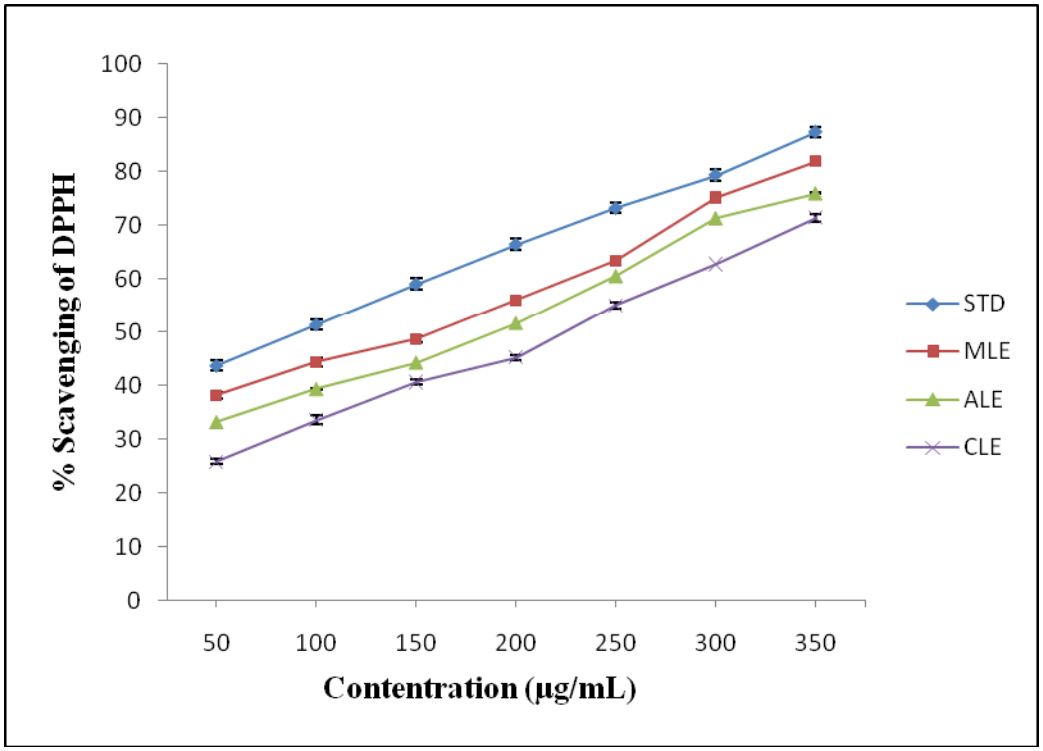

Figure-1: DPPH radical scavenging activity of ascorbic acid and different extracts of *I. coccinea*. Values are the average of triplicate experiments and represented as mean ± standard deviation.

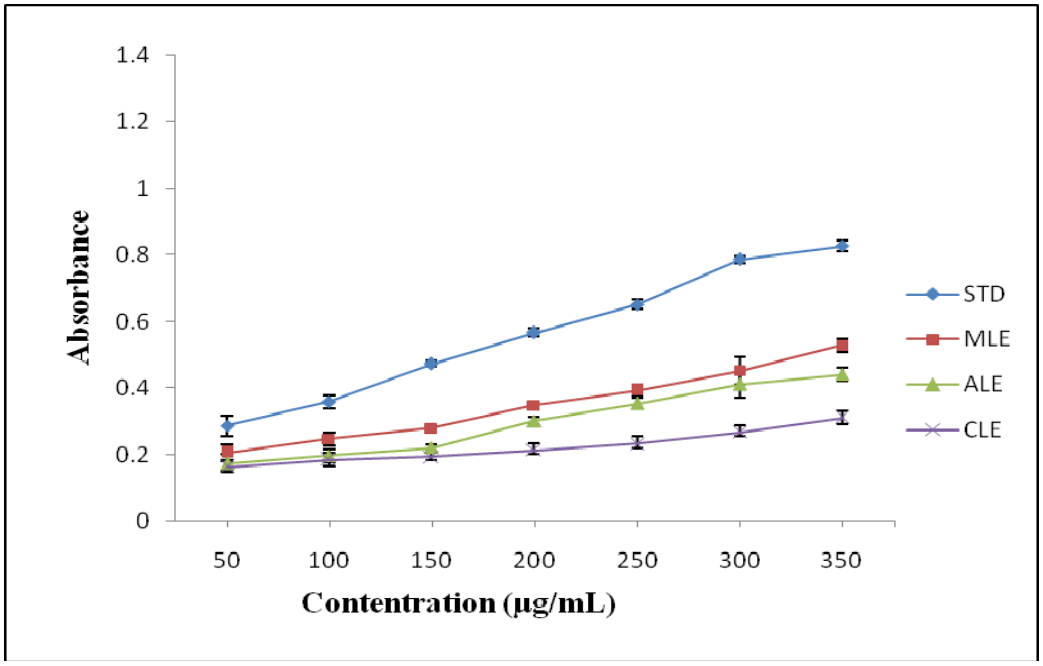

Figure-2: Reductive ability of different extracts of *I. coccinea* compared to ascorbic acid. Values are the average of triplicate experiments and represented as mean ± standard deviation.

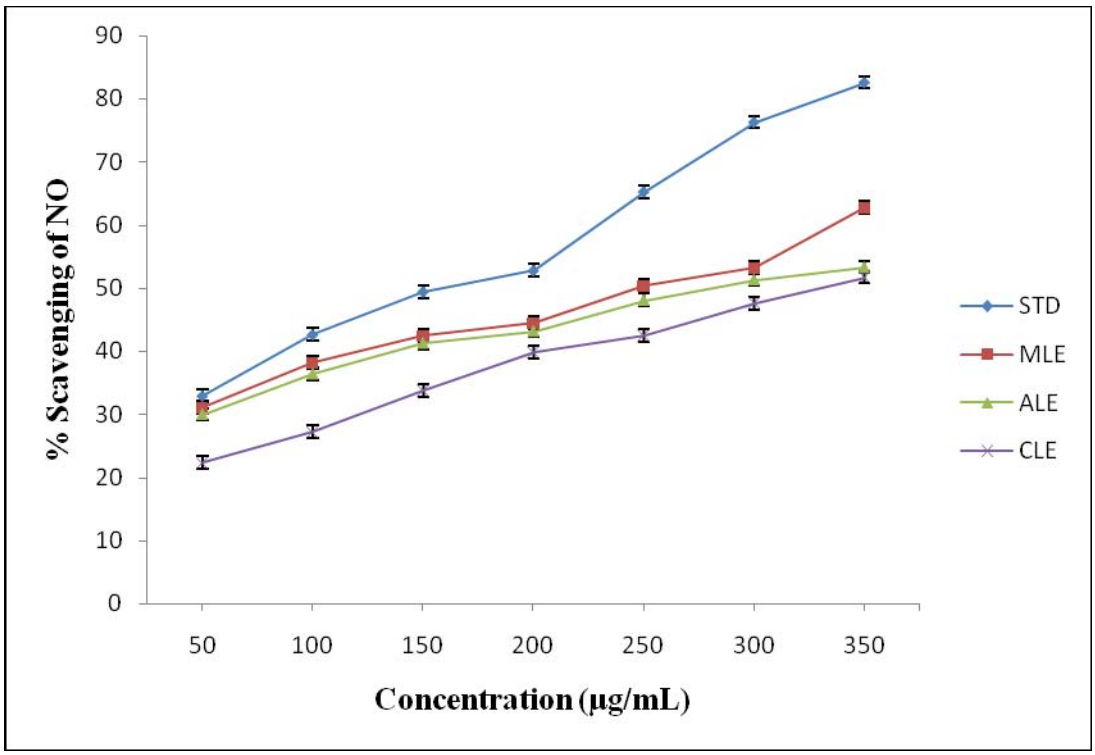

Figure-3: Nitric oxide scavenging activity of ascorbic acid and different extracts of *I. coccinea*. Values are the average of triplicate experiments and represented as mean ± standard deviation.

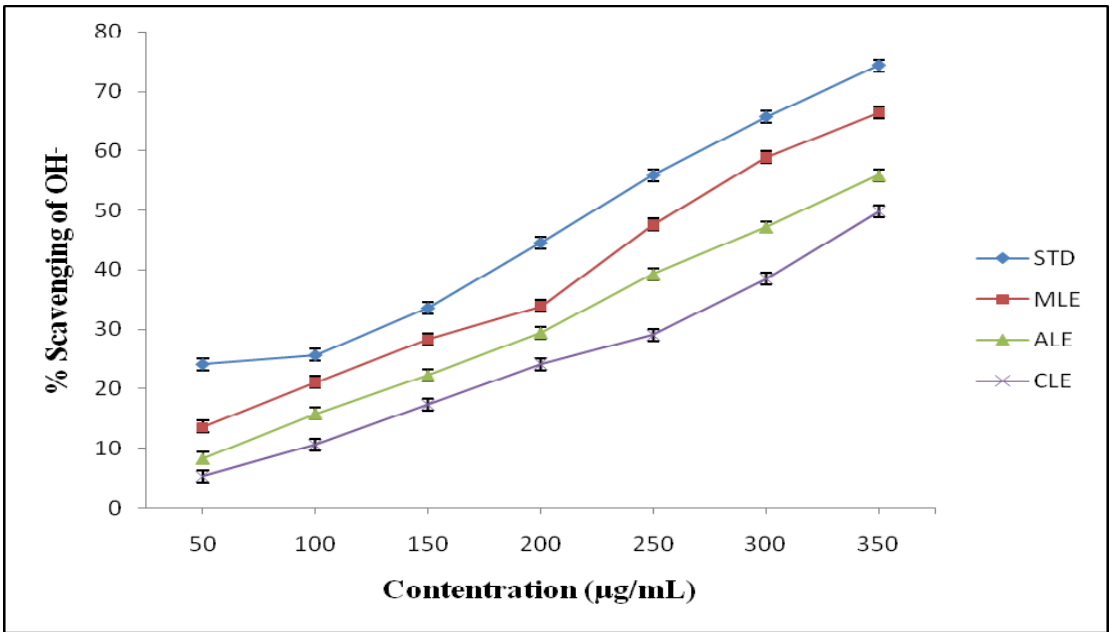

Figure-4: Hydroxyl radical (OH ˙) scavenging activity of ascorbic acid and different extracts of *I. coccinea*. Values are the average of triplicate experiments and represented as mean ± standard deviation.

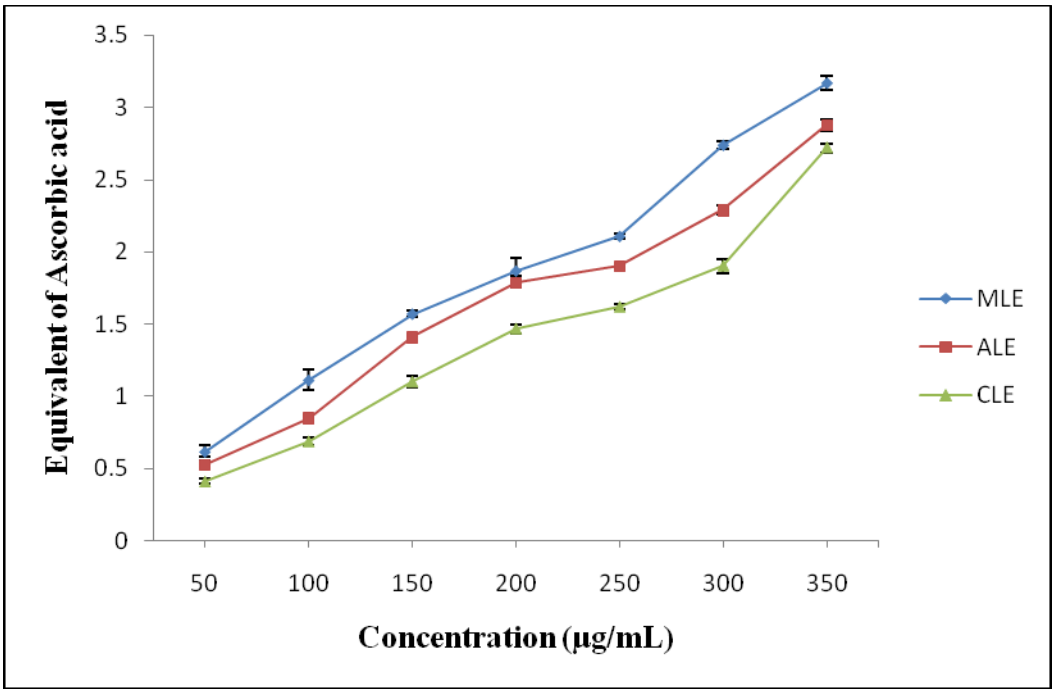

Figure-5: Total antioxidant capacity of different extracts of *I. coccinea*. Values are the average of triplicate experiments and represented as mean ± standard deviation.