**In Vitro Antioxidant Property of Leaf Extracts of Ixora coccinea L.**

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ABSTRACT

*Ixora coccinea* L. is widely distributed throughout India and is used in the treatment of various diseases. Leaves and flowers are used in dysentery, dysmenorrhoea and while roots and fruits are used in urinary problems of females. In Indo-China, an infusion of leaves is given to treat fevers. In this study, the antioxidant capacity of extracts of *I. coccinea* leaves were assayed for their scavenging abilities against superoxide anion radicals, hydroxyl radical, nitric oxide radical, hydrogen peroxide, metal chelation and reducing power. All the extracts of *I. coccinea* inhibited all above said free radicals in a dose-dependent manner. These results clearly indicate that *I. coccinea* is effective against free radical-mediated diseases.

Keywords: hydrogen peroxide scavenging, hydroxyl radicals, metal chelation, nitric oxide radicals, rangan, superoxide anion radicals

INTRODUCTION

Most diseases are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari 2001). The most common reactive oxygen species (ROS) include superoxide (O$_2^-$) anion, hydrogen peroxide (H$_2$O$_2$), peroxyl (ROO$^-$) radicals and reactive hydroxyl (OH$^-$) radicals. The nitrogen-derived free radicals are nitric oxide (NO$^-$) and peroxynitrite anion (ONOO$^-$). ROS have been implicated in over 100 diseased states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (Joyce 1987).

In the treatment of these diseases, antioxidant therapy has gained immense importance (Buyukokuroglu et al. 2001). Flavonoids and phenolic compounds, widely distributed in plants, have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic, etc. (Miller 1996). They were also suggested to be potential iron chelator (Havsteen 1983; Boyer et al. 1988).

Herbal medicine represents one of the most important fields of traditional medicine in India. A great number of traditional medicinal plants have been used in folk medicine to treat a wide range of physical ailments. *Ixora coccinea* Linn. (Rubiaceae), commonly known as Rangan (Fig. 1), is widely distributed throughout India and is used in the treatment of various ailments. Flowers and leaves are used in dysentery, dysmenorrhoea and roots and fruits are used in females when the urine is highly coloured. In Indo-China, an infusion of leaves is given in fevers (Kirtikar and Basu 1995). Flowers and leaves of *I. coccinea* are reported to have antimicrobial, hepatoprotective, anticancer and anti-inflammatory property (Latha and Panikkar 1998; Latha and Panikkar 1999; Annapurna et al. 2003). Some preliminary phytochemical investigations reported that flavonoids are present in the flowers and leaves of the same.

Therefore, the objectives of the present study were to investigate the in vitro antioxidant activity of *I. coccinea* leaves through the free radical scavenging, superoxide anion radical scavenging, nitric oxide scavenging, metal chelation and reducing power assay.

MATERIALS AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), Sodium nitroprusside (SNP), Trichloro acetic acid (TCA) and Potassium hexacyanoferrate (K$_3$Fe(CN)$_6$)$_3$ were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant material

The leaves of *Ixora coccinea* were collected from the herbal garden, Gupta College of Technological Sciences, Asansol, India. The collected leaves were identified and authenticated by Mr. M. S. Mondal, Joint Director, Botanical Survey of India, Shibpur, Kol-
Preparation of extracts

The leaves were shade dried at room temperature for 15 days, finally powdered and used for extraction. A required quantity of powder (100 g) was extracted with 50% hydro-alcohol and ethyl acetate separately by continuous hot percolation in a soxhlet apparatus for 72 hrs. Both the extracts were concentrated in reduced pressure below 40°C by using rotary flash evaporator to get two separate hydro-alcoholic extract (27.2% w/w) and ethyl acetate extract (14.34% w/w). Separately another quantity of powder (100 g) was extracted with distilled water (600 ml) by cold maceration and concentrated the extract under reduced pressure below 40°C to get a separate aqueous extract (13.17% w/w). All the extracts were stored at 4°C until use.

Superoxide anion scavenging activity assay

The scavenging activity of various extracts of *I. coccinea* leaves towards superoxide anion radicals was measured by colorimetric method (Liu et al. 1997). Superoxide anion was generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 μM) solution, 0.75 ml of NADH (936 μM) solution and 0.3 ml of different concentrations of each extract. The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

\[
\% \text{ inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \( A_0 \) was the absorbance of the control (blank, without extract) and \( A_1 \) was the absorbance in the presence of the extract.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured (Yu et al. 2004). Reaction mixture contained 60 μl of 1.0 mM FeCl₃, 90 μl of 1 mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 μl of 0.17 M H₂O₂ and 1.5 ml of extract at various concentrations. Adding H₂O₂ started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the following equation:

\[
\% \text{ inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \( A_0 \) was the absorbance of the control (blank, without extract) and \( A_1 \) was the absorbance in the presence of the extract.

Nitric oxide scavenging activity assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess illosvoy reaction (Garrat 1964; Bhatt and Baek 2007). 2 ml of 10 mM sodium nitroprusside in 0.5 ml of phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml of sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer. The NO radicals scavenging activity was calculated according to the following equation:

\[
\% \text{ inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \( A_0 \) was the absorbance of the control (blank, without extract) and \( A_1 \) was the absorbance in the presence of the extract.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide (H₂O₂) scavenging activity of the extract was estimated by replacement titration (Zhang 2000). Aliquot of 1.0 ml of 0.1 mM H₂O₂ and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 M H₂SO₄ and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM NaS₂O₃ until yellow colour disappeared. Percentage of scavenging of H₂O₂ was calculated as:

\[
\% \text{ inhibition} = \left( \frac{V_0 - V_1}{V_0} \right) \times 100
\]

where \( V_0 \) was volume of NaS₂O₃ solution used to titrate the control sample in the presence of H₂O₂ (without extract), \( V_1 \) was the volume of NaS₂O₃ solution used in the presence of extract.

Fe²⁺ chelating activity assay

To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of FeCl₃ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min of room temperature, the absorbance of the Fe²⁺-Ferrozine complex was measured at 562 nm (Dinis et al. 1994). The chelating activity of the extract for Fe²⁺ was calculated as:

\[
\text{Chelating rate} (\%) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \( A_0 \) was the absorbance of the control (blank, without extract) and \( A_1 \) was the absorbance in the presence of the extract.

Reducing power assay

The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate (K₃[Fe(CN)₆]) (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 800 g for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power (Oyaizu 1986). Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis

Tests were carried out in triplicate. The amount of extract needed to inhibit free radicals concentration by 50%, IC₅₀, was graphically determined and means, standard deviation and correlation were computed by using Microsoft Excel.

RESULTS AND DISCUSSION

The phytochemical analysis of *I. coccinea* leaf extracts contain rich source of flavonoids. Polyphenol and flavonoids used for the prevention and cure of various diseases which is mainly associated with free radicals.

Superoxide anion scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Oikawa et al. 1979). The superoxide anion scavenging activity of the extracts from *I. coccinea* – assayed by the PMS-NADH system is shown in Table 1. The superoxide anion scavenging activity of all the extracts of *I. coccinea* increased markedly with the concentrations. Now in between three extracts...
### Table 1

<table>
<thead>
<tr>
<th>Conc. (μg/ml)</th>
<th>Hydro-alcoholic extract</th>
<th>Aqueous extract</th>
<th>Ethyl acetate extract</th>
<th>Hydro-alcoholic extract</th>
<th>Aqueous extract</th>
<th>Ethyl acetate extract</th>
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<tbody>
<tr>
<td>10</td>
<td>31.3 ± 0.48</td>
<td>37.77 ± 0.60</td>
<td>51.26 ± 0.12</td>
<td>18.25 ± 0.23</td>
<td>23.63 ± 1.13</td>
<td>49.16 ± 0.81</td>
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<td>50</td>
<td>34.61 ± 0.45</td>
<td>51.22 ± 1.12</td>
<td>71.31 ± 1.01</td>
<td>22.09 ± 0.24</td>
<td>48.75 ± 0.43</td>
<td>54.63 ± 0.00</td>
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<td>100</td>
<td>51.27 ± 0.46</td>
<td>72.67 ± 0.70</td>
<td>75.58 ± 0.64</td>
<td>47.05 ± 1.66</td>
<td>52.55 ± 0.39</td>
<td>67.73 ± 0.005</td>
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<tr>
<td>250</td>
<td>70.85 ± 0.17</td>
<td>77.16 ± 0.82</td>
<td>81.64 ± 0.54</td>
<td>58.19 ± 0.99</td>
<td>65.23 ± 0.02</td>
<td>71.86 ± 1.00</td>
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<td>500</td>
<td>73.77 ± 1.77</td>
<td>85.68 ± 0.02</td>
<td>89.47 ± 0.13</td>
<td>65.39 ± 0.50</td>
<td>69.23 ± 0.05</td>
<td>84.2 ± 1.76</td>
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<td>750</td>
<td>81.34 ± 1.05</td>
<td>88.62 ± 0.02</td>
<td>91.77 ± 0.65</td>
<td>79.31 ± 0.18</td>
<td>82.68 ± 0.47</td>
<td>85.32 ± 3.01</td>
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<td>1000</td>
<td>81.12 ± 1.19</td>
<td>93.23 ± 0.47</td>
<td>94.72 ± 0.57</td>
<td>86.99 ± 1.52</td>
<td>90.66 ± 0.54</td>
<td>91.94 ± 1.67</td>
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Values are means ± SD (n=3), IC50 = 50% Inhibition Concentration.

### Table 2

<table>
<thead>
<tr>
<th>Conc. (μg/ml)</th>
<th>Hydro-alcoholic extract</th>
<th>Aqueous extract</th>
<th>Ethyl acetate extract</th>
<th>Hydro-alcoholic extract</th>
<th>Aqueous extract</th>
<th>Ethyl acetate extract</th>
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</thead>
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<tr>
<td>10</td>
<td>9.19 ± 0.04</td>
<td>14.12 ± 0.54</td>
<td>6.97 ± 0.67</td>
<td>5.42 ± 0.33</td>
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<td>50</td>
<td>12.36 ± 0.00</td>
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<td>9.21 ± 0.00</td>
<td>11.25 ± 0.96</td>
<td>15.31 ± 0.21</td>
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<tr>
<td>100</td>
<td>15.13 ± 0.11</td>
<td>32.12 ± 0.96</td>
<td>13.10 ± 0.005</td>
<td>21.27 ± 0.005</td>
<td>21.18 ± 1.10</td>
<td>15.58 ± 0.005</td>
</tr>
<tr>
<td>250</td>
<td>28.15 ± 0.17</td>
<td>39.25 ± 0.11</td>
<td>27.31 ± 0.00</td>
<td>33.23 ± 1.11</td>
<td>39.69 ± 0.00</td>
<td>23.31 ± 0.01</td>
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<tr>
<td>500</td>
<td>34.94 ± 4.50</td>
<td>46.07 ± 0.05</td>
<td>32.52 ± 0.01</td>
<td>42.42 ± 0.00</td>
<td>45.37 ± 0.29</td>
<td>31.48 ± 1.18</td>
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<tr>
<td>750</td>
<td>44.62 ± 2.05</td>
<td>54.69 ± 3.18</td>
<td>38.61 ± 0.56</td>
<td>49.23 ± 0.03</td>
<td>51.14 ± 0.02</td>
<td>41.94 ± 2.01</td>
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<tr>
<td>1000</td>
<td>55.10 ± 1.24</td>
<td>64.38 ± 1.89</td>
<td>45.15 ± 0.00</td>
<td>53.19 ± 0.00</td>
<td>63.14 ± 0.54</td>
<td>46.55 ± 0.00</td>
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Values are means ± SD (n=3), IC50 = 50% Inhibition Concentration.

### Table 3

<table>
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<tr>
<th>Conc. (μg/ml)</th>
<th>Fe²⁺ chelating activity %</th>
<th>Hydro-alcoholic extract</th>
<th>Aqueous extract</th>
<th>Ethyl acetate extract</th>
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</thead>
<tbody>
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<td>10</td>
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<td>35.33 ± 0.00</td>
<td>41.16 ± 1.05</td>
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<tr>
<td>50</td>
<td>38.41 ± 0.00</td>
<td>53.12 ± 0.00</td>
<td>63.07 ± 2.30</td>
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<tr>
<td>100</td>
<td>44.62 ± 1.75</td>
<td>55.12 ± 0.00</td>
<td>72.77 ± 0.40</td>
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</tr>
<tr>
<td>250</td>
<td>52.38 ± 0.79</td>
<td>62.36 ± 0.00</td>
<td>83.93 ± 0.70</td>
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<tr>
<td>500</td>
<td>65.16 ± 3.02</td>
<td>72.36 ± 0.00</td>
<td>86.52 ± 0.00</td>
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<tr>
<td>750</td>
<td>76.03 ± 1.70</td>
<td>89.57 ± 0.05</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>84.30 ± 0.24</td>
<td>85.34 ± 1.18</td>
<td>160</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD (n=3), IC50 = 50% Inhibition Concentration.

In conclusion, these results indicate that all the extracts of *I. coccinea* leaf had important superoxide radical scavenging effect.

### Hydroxyl radical scavenging activity

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. Table 1 shows the above three extracts of *I. coccinea* leaves exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC50 of hydro-alcoholic, aqueous and ethyl acetate extracts were 120 μg/ml, 55 μg/ml and below 10 μg/ml respectively. These results suggested that all the extracts of *I. coccinea* leaf had important superoxide radical scavenging effect.

### Nitric oxide scavenging activity

Nitric oxide is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell-mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems, including neuronal messenger, vasodilatation and antimicrobial and antitumour activities (Hagerman et al. 1998). All three extracts of *I. coccinea* moderately inhibited NO in a dose-dependent manner (Table 2). The IC50 of hydro-alcoholic, aqueous and ethyl acetate extracts were 95 μg/ml, 45 μg/ml and below 10 μg/ml respectively. Among the three extracts, the aqueous extract showed a better result than hydro-alcoholic and ethyl acetate extracts.

### Hydrogen peroxide scavenging activity

H₂O₂ is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. H₂O₂ can cross cell membranes rapidly, and, once inside the cell, can probably react with Fe²⁺, and possibly Cu²⁺ ions to form a hydroxyl radical and this may be the origin of many of its toxic effects (Miller 1993). It is therefore biologically advantageous for cells to control the amount of H₂O₂ that is allowed to accumulate. All three extracts of *I. coccinea* moderately inhibited H₂O₂ in a dose-dependent manner (Table 2). The IC50 of hydro-alcoholic, aqueous and ethyl acetate extracts were 800, 720 and more than 1000 μg/ml, respectively. Among the three extracts, the aqueous extract showed a better result than hydro-alcoholic and ethyl acetate extracts.

### Fe²⁺-chelating activity

All three extracts of *I. coccinea* inhibited ferrous ion in a dose-dependent manner (Table 3). The IC50 of hydro-alcoholic, aqueous and ethyl acetate extracts were 225, 160 and 80 μg/ml, respectively. Among the three extracts, the ethyl acetate extract scavenged ferrous ion more effectively than hydro-alcoholic and aqueous extracts.

### Reducing power activity

Figs. 2-4 depict the reductive effect of *I. coccinea*, and similar to the antioxidant activity, the reducing power of *I. coccinea* increased with increasing dosage. High absorbance at 700 nm indicates high reducing power.

### CONCLUSION

In conclusion, these results indicate that all the extracts of *I. coccinea* inhibited all tested free radicals in a dose-depen-
investigations should be done on isolation of active constituent(s) and their in vivo antioxidant activity and are necessary to determine the specific mechanisms.

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