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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_2O_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 through out 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.

Fig. 1 Leaves and flowers of *Ixora coccinea* L.

MATERIALS AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), Sodium nitroprusside (SNP), Trichloro acetic acid (TCA) and Potassium hexacyanoferrate ($K_3Fe(CN)_6$) 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-

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kata. A voucher specimen (Specimen No. CNH/I-I/(201)/2007/ Tech.II/2) has been deposited at the office of the Central National Herbarium, Botanical Survey of India, Shibpur, Kolkata.

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 each) 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:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100$$

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 $FeCl_3$, 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 H_2O_2 and 1.5 ml of extract at various concentrations. Adding H_2O_2 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:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100$$

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 lllosvoy 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:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100$$

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 (H_2O_2) scavenging activity of the extract was estimated by replacement titration (Zhang 2000). Aliquot of 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 M H_2SO_4 and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM NaS_2O_3 until yellow colour disappeared. Percentage of scavenging of H_2O_2 was calculated as:

$$\% \text{ inhibition} = [(V_0-V_1)/V_0] \times 100$$

where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of H_2O_2 (without extract), V_1 was the volume of NaS_2O_3 solution used in the presence of extract.

Fe^{2+} chelating activity assay

To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (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 Fe^{2+}-Ferrozine complex was measured at 562 nm (Dinis *et al*. 1994). The chelating activity of the extract for Fe^{2+} was calculated as:

$$\text{Chelating rate } (\%) = [(A_0-A_1)/A_0] \times 100$$

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 ($K_3Fe(CN)_6$) (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%, IC_{50}, 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 (Okhawa *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 Superoxide and hydroxyl radical scavenging activity of *I. coccinea* at different concentrations.

Conc. (µg/ml)	Superoxide radical scavenging %			Hydroxyl radical scavenging %		
	Hydro-alcoholic extract	Aqueous extract	Ethyl acetate extract	Hydro-alcoholic extract	Aqueous extract	Ethyl acetate extract
10	31.3 ± 0.48	37.77 ± 0.60	51.26 ± 0.12	18.25 ± 0.23	23.63 ± 1.13	49.16 ± 0.81
50	34.61 ± 0.45	51.22 ± 1.12	71.31 ± 1.01	22.09 ± 0.24	48.75 ± 0.43	54.63 ± 0.00
100	51.27 ± 0.46	72.67 ± 0.70	75.58 ± 0.64	47.05 ± 1.66	52.55 ± 0.39	67.73 ± 0.005
250	70.85 ± 0.17	77.16 ± 0.82	81.64 ± 0.54	58.19 ± 0.99	65.23 ± 0.02	71.86 ± 1.00
500	73.77 ± 1.77	85.69 ± 0.82	89.47 ± 0.13	65.39 ± 0.50	69.23 ± 0.05	84.2 ± 1.76
750	81.34 ± 1.05	88.62 ± 0.02	91.77 ± 0.65	79.31 ± 0.18	82.68 ± 0.47	85.32 ± 3.01
1000	87.12 ± 1.91	92.33 ± 0.47	94.72 ± 0.57	86.99 ± 1.52	90.66 ± 0.54	91.94 ± 1.67
IC_{50}	95	45	< 10	120	55	20

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

Table 2 Nitric oxide and Hydrogen peroxide radical scavenging activity of *I. coccinea* at different concentrations.

Conc. (µg/ml)	Nitric oxide radical scavenging %			Hydrogen peroxide scavenging %		
	Hydro-alcoholic extract	Aqueous extract	Ethyl acetate extract	Hydro-alcoholic extract	Aqueous extract	Ethyl acetate extract
10	9.196 ± 0.04	14.12 ± 0.54	6.97 ± 0.67	5.42 ± 0.33	8.14 ± 0.12	8.15 ± 0.06
50	12.36 ± 0.00	21.88 ± 0.49	9.21 ± 0.00	11.25 ± 0.96	15.31 ± 0.21	10.10 ± 0.005
100	15.13 ± 0.11	32.12 ± 0.96	13.10 ± 0.005	21.27 ± 0.005	21.18 ± 1.10	15.58 ± 0.005
250	28.15 ± 0.17	39.25 ± 0.11	27.31 ± 0.00	33.23 ± 1.11	39.69 ± 0.00	23.31 ± 0.01
500	34.94 ± 4.50	46.07 ± 0.05	32.52 ± 0.01	42.42 ± 0.00	45.37 ± 0.29	31.48 ± 1.18
750	44.62 ± 2.05	54.69 ± 3.18	38.61 ± 0.56	49.23 ± 0.03	51.14 ± 0.02	41.94 ± 2.01
1000	55.10 ± 1.24	64.38 ± 1.89	45.15 ± 0.00	53.19 ± 0.00	63.14 ± 0.12	46.55 ± 0.00
IC_{50}	920	620	>1000	800	720	>1000

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

ethyl acetate and aqueous extracts showed better result than hydro-alcoholic extract. The half inhibition concentration (IC_{50}) of hydro-alcoholic, aqueous and ethyl acetate extracts ware 95 µg/ml, 45 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.

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 IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 120 µg/ml, 55 µg/ml and 20 µg/ml respectively. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups (Korycka-Dahl and Richardson 1978).

Nitric oxide scavenging activity

NO is a potent pleiotropic mediator of physiological process 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, vasodilation and antimicrobial and antitumor activities (Hagerman *et al*. 1998). All three extracts of *I. coccinea* moderately inhibited NO in a dose-dependent manner (**Table 2**). The IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts were 920, 620 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.

Hydrogen peroxide scavenging activity

H_2O_2 is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. H_2O_2 can cross cell membranes rapidly, and, once inside the cell, can probably react with Fe^{2+}, and possibly Cu^{2+} 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 H_2O_2 that is allowed to accumulate. All three extracts of *I. coccinea* moderately inhibited H_2O_2 in a dose-dependent manner (**Table 2**). The IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 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.

Table 3 Ferrous ion chelating activity of *I.coccinea* at different concentrations.

Conc. (µg/ml)	Fe^{2+} chelating activity %		
	Hydro-alcoholic extract	Aqueous extract	Ethyl acetate extract
10	15.04 ± 0.64	30.33 ± 0.27	35.33 ± 0.00
50	22.27 ± 1.00	38.41 ± 0.005	41.16 ± 1.05
100	34.32 ± 4.54	44.62 ± 1.75	53.12 ± 0.00
250	52.38 ± 0.79	55.12 ± 0.00	63.07 ± 2.30
500	65.16 ± 3.02	62.36 ± 0.00	72.77 ± 0.40
750	76.03 ± 1.70	69.57 ± 0.05	78.93 ± 0.70
1000	84.30 ± 0.24	85.34 ± 1.18	86.52 ± 0.00
IC_{50}	225	160	80

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

Fe^{2+}-chelating activity

All three extracts of *I. coccinea* inhibited ferrous ion in a dose-dependent manner (**Table 3**). The IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 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-

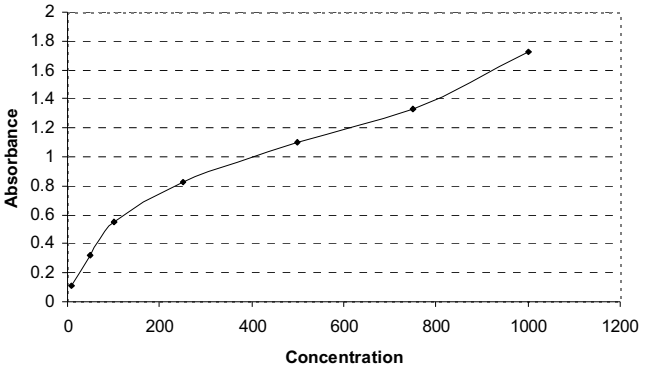

Fig. 2 Reducing power of hydro-alcoholic extract of *I. coccinea* at different concentrations. ♦: Absorbance of hydro-alcoholic extract.

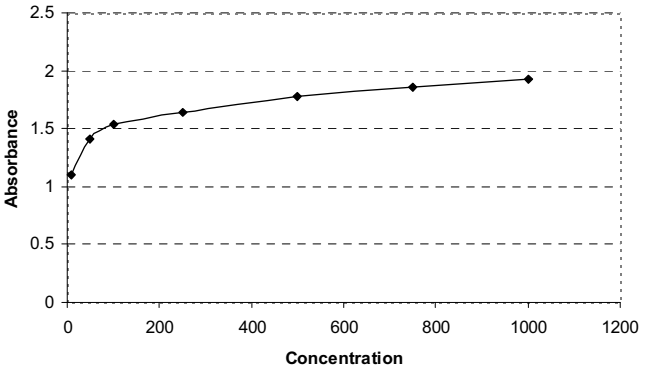

Fig. 3 Reducing power of aqueous extract of *I. coccinea* at different concentrations. ♦: Absorbance of aqueous extract.

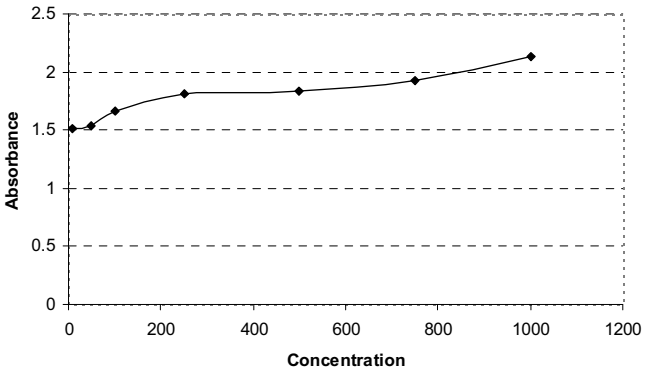

Fig. 4 Reducing power of ethyl acetate extract of *I. coccinea* at different concentrations. ♦: Absorbance of ethyl acetate extract.

dent manner. These results clearly indicate that *I. coccinea* is effective against free radical-mediated diseases. Further 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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