

In-vitro free radical scavenging activity of *Ixora coccinea* L

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Abstract

Antioxidant activity of the methanol extract of *Ixora coccinea* L. was determined by DPPH free radical scavenging assay, reducing power and total antioxidant capacity using phosphomolybdenum method. Preliminary phytochemical screening revealed that the extract of the flower of *I. coccinea* possesses flavonoids, steroids and tannin materials. The extract showed significant activities in all antioxidant assays compared to the standard antioxidant in a dose dependent manner and remarkable activities to scavenge reactive oxygen species (ROS) may be attributed to the high amount of hydrophilic phenolics. In DPPH radical scavenging assay the IC₅₀ value of the extract was found to be 100.53 µg/mL while ascorbic acid had the IC₅₀ value 58.92 µg/mL. Moreover, *I. coccinea* extract showed strong reducing power and total antioxidant capacity.

Introduction

In living systems, free radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthine oxidase activity, atmospheric pollutants and from transitional metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage. Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders. For instance in diabetes, increased oxidative stress which co-exist with reduction in the antioxidant status has

been postulated. Oxygen free radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in the long-term complication of diabetes (Sabu and Kuttan, 2002; Boynes, 1991; Collier et al., 1990). Although synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, and tertbutylhydroquinone have been commonly used as antioxidants in foods for years, their safety has long been questioned (Branen, 1975; Ito et al., 1983). This has led to an increased interest in natural antioxidants (Lim et al., 2002; Kayano et al., 2002; Gyamfi and Aniya, 2002; Braca et al., 2002). Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the



chemoprevention of diseases that has their etiology and pathophysiology in reactive oxygen species. The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, but may also be influenced by other organic and inorganic compounds such as coumarins, phenolic acids and antioxidant micronutrients, e.g., Cu, Mn, Zn (Repetto and Llesuy, 2002). *Ixora coccinea* L. belongs to the family *Rubiaceae*, is a common flowering shrub native to Asia including Bangladesh, Southern India, and Sri Lanka (Ghani, 2003). Leaves are given in diarrhea (Ghani, 2003). Flowers are used in the treatment of dysentery, leucorrhoea, dysmenorrhoea, hemoptysis and catarrhal bronchitis (Ghani, 2003). Roots possess stomachic and sedative properties.

I. coccinea flowers showed chemoprotective effects on cyclophosphamide-induced toxicity by increasing the life span of treated mice (Latha and Panikkar, 1999). Ether and methanol extracts of *I. coccinea* dry leaves have antimicrobial activity (Annapurna et al., 2003). Flowers have cytotoxic and antitumor activity in mice (Latha and Panikkar, 1998). Aqueous leaf extract of *I. coccinea* leaves showed antinociceptive activity in mice (Ratnasooriya et al., 2005a,b). The extract of *I. coccinea* flowers contains triterpenoid, ursolic acid (Latha and Panikkar, 1999). The air-dried flowers of *I. coccinea* afforded two new cycloartenol esters (1a and 1b), lupeol fatty ester, lupeol, ursolic acid, oleanolic acid, and sitosterol. The structures of 1a and 1b were elucidated by extensive 1D and 2D NMR spectroscopy and MS (Ragasa et al., 2004).

A review of literature did not reveal any information on the antioxidant study of this plant. As a part of our ongoing investigations about natural antioxidants from local medicinal plants of Bangladesh (Alam et al., 2008a, Alam et al., 2008b), in this paper, we have reported the antioxidant activity of the flowers of *I. coccinea*. The evaluation of antioxidant power was performed *in vitro* by the DPPH (1, 1-diphenyl, 2-picrylhydrazyl), reducing power and total antioxidant capacity assays.

Materials and Methods

Chemicals: DPPH, trichloroacetic acid and ferric chloride were obtained from Sigma Chemical Co. USA. Ascorbic acid was obtained from SD Fine

Chem. Ltd., Biosar, India. Sodium nitroprusside was obtained from Ranbaxy Lab., Mohali, India. Potassium ferricyanide was obtained from May and Backer, Dagenham, UK. Ammonium molybdate was purchased from Merck, Germany.

Plant material: Flowers of *I. coccinea* were collected from Gazipur, Bangladesh in June 2007, and identified by Professor Abdul Ghani (Stamford University, Dhaka, Bangladesh); a voucher specimen (SU-MAA-2007-7) for this collection has been retained in the Pharmacognosy Laboratory, Stamford University, Dhaka, Bangladesh.

Extraction: The shade-dried flowers were coarsely powdered and extracted with mixture of methanol: water (7:3 ratios) by a soxhlet apparatus at 45° C. The solvent was completely removed by rotary evaporator and obtained greenish gummy exudates. This crude extract was used for further investigation for potential antioxidant properties.

Phytochemical screening: The freshly prepared extract of *I. coccinea* was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorffs reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce suds. Gum was tested using Molish reagents and concentrated sulfuric acid. These were identified by characteristic color changes using standard procedures (Trease and Evans, 1983).

DPPH radical scavenging activity: The free radical scavenging capacity of the extracts was determined using DPPH (Hasan et al., 2006). DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanol extract of *I. coccinea* was mixed with 95% methanol to prepare the stock solution (5 mg/mL). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and *I. coccinea* extracts was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 mL and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (5 mg/mL). Control sample was prepared containing the same volume without any

extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation: absorbance of the control minus absorbance of the test sample divided by absorbance of the control multiplied by 100.

The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition \pm standard deviation. IC_{50} values were obtained by probit analysis (Viturro et al., 1999).

Reducing power: The reducing power of *I. coccinea* was determined according to the method previously described (Oyaizu, 1986). Different concentrations of *I. coccinea* extract (100 μ g - 1,000 μ g) in 1 mL of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments were taken was expressed as mean \pm standard deviation.

Determination of total antioxidant capacity: The antioxidant activity of the extracts of *I. coccinea* was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al., (1999). The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract was used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Results and Discussion

Preliminary phytochemical screening of the extract of *I. coccinea* revealed the presence of various bioactive components of which flavonoids and tannins were the most prominent and the result of phytochemical test has been summarized in the Table I. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans et al., 1997; Jorgensen et al., 1999). Literature reviews about the plant also confirm the presence of terpenoids group. Many naturally occurring triterpenoids exhibited a good anti-inflammatory activity have been isolated from various plants (Fernandez et al., 2001; Ismaili et al., 2002). Pentacyclic triterpenoids have a wide spectrum of biological activities and some of them may be useful in medicine. There is growing interest in natural triterpenoids caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of biological activities, they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anti-inflammatory, anti-cancer and antiallergic (Patocka, 2003).

In this present study the antioxidant activity of the methanol extracts of the flowers of *I. coccinea* were investigated by using DPPH scavenging assay, reducing power of the extract and by determining total antioxidant capacity of the extract. All the three methods have proven the effectiveness of the methanol extract compared to the reference standard antioxidant ascorbic acid.

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. Comparison of the antioxidant activity of the extract and ascorbic acid is shown in Figure 1. The methanol extract of *I. coccinea* exhibited a significant dose dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 100.53 μ g/mL. The IC_{50} value of the extract was

Table I: Phytochemical screening of *Ixora coccinea* methanol extracts

	Methanol extract
Alkaloids	++
Steroids	++
Gum	-
Flavonoids	++
Tannins	+++
Saponins	++

found to be comparable to reference standard ascorbic acid (IC_{50} 58.92 $\mu\text{g}/\text{mL}$).

The reducing ability of a compound generally depends on the presence of reductants (Duh, 1999), which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom (Gordon, 1990). The presence of reductants (i.e. antioxidants) in *I. coccinea* extract 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. Figure 2 shows the reductive capabilities of the plant extract compared to ascorbic acid. The reducing power of extract of

I. coccinea was very potent and the power of the extract was increased with quantity of sample.

The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the reference standard of ascorbic acid.

Total antioxidant capacity of the methanol extract is given in Figure 3. Total antioxidant capacity of *I. coccinea* is expressed as the number of equivalents of ascorbic acid. 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. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts (Prieto *et al.*, 1999). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract exhibits increasing trend with the increasing concentration of the plant extract.

This *in vitro* antioxidant activity of the methanol extract is further supported by other workers who reported that the extract of *I. coccinea* significantly protect the experimental animal during *cis* platinum and cyclophosphamide-induced toxicity (Latha and Panikkar, 2001; Latha and Panikkar, 1999).

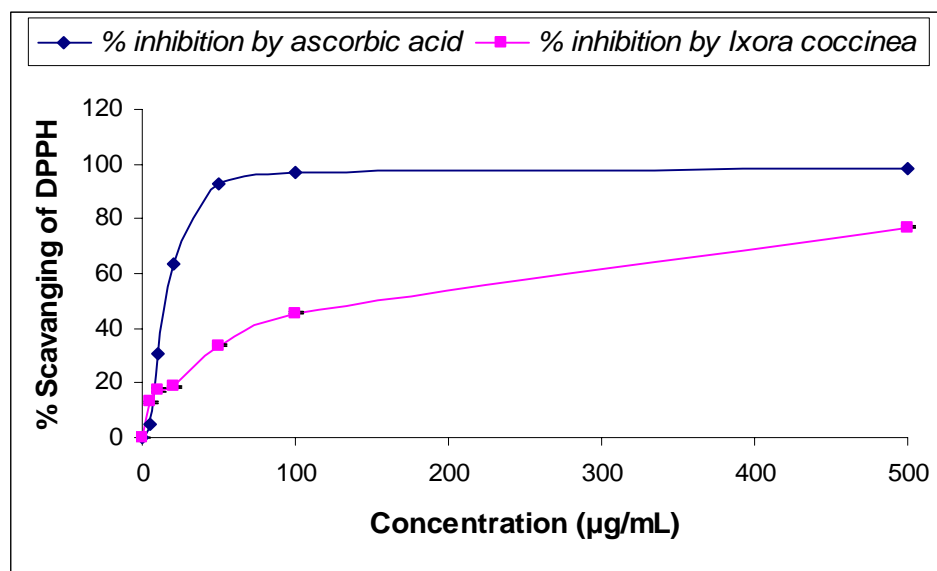


Figure 1: DPPH radical scavenging activity of ascorbic acid and methanol extract of *Ixora coccinea* flowers. Values are the average of duplicate experiments and represented as mean \pm standard deviation

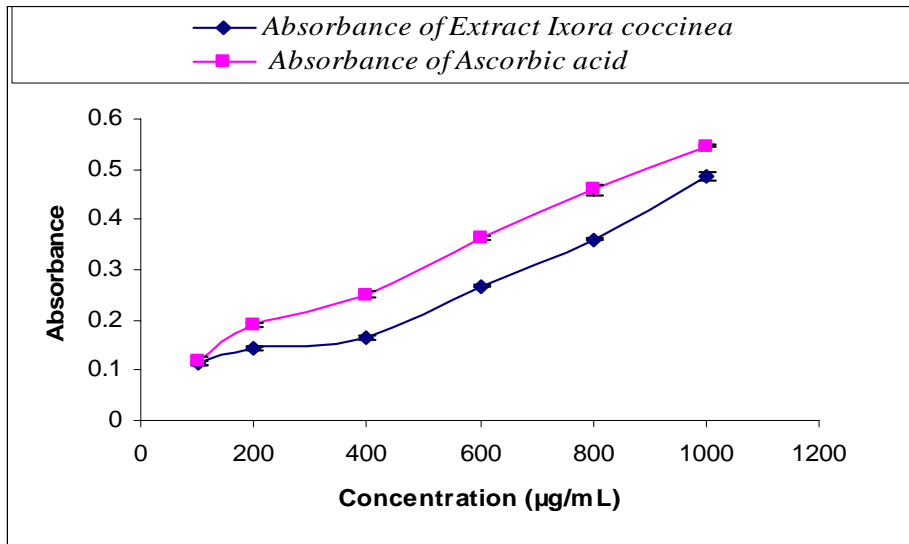


Figure 2: Reducing power of the leaves of *Ixora coccinea* extract compared to ascorbic acid. Values are the average of duplicate experiments and represented as mean ± standard deviation

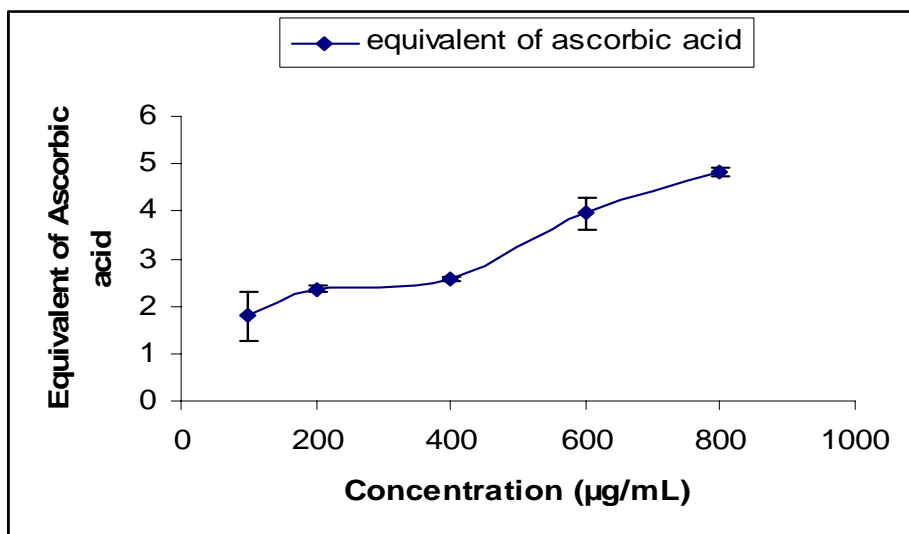


Figure 3: Total antioxidant capacity of the flowers of *Ixora coccinea* extracts. Value s are the average of duplicate experiments and represented as mean ± standard deviation

In conclusion, it can be concluded that the methanol extract of *I. coccinea* possesses the antioxidant substances which may be potentially responsible for its anti-inflammatory and chemoprotective mechanism as well as using this plant's extract as folkloric remedies.

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