

RESEARCH ARTICLE

IN- VITRO EVALUATION OF FREE RADICAL SCAVENGING ACTIVITY OF ERYTHRINA INDICA LEAVES

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ABSTRACT

Introduction: The plant *Erythrina indica* has been widely used as traditional Indian herbal medicine against a variety of diseases including diarrhoea, liver disorders, dysentery and convulsion, as a diuretic, rheumatism, asthma, inflammation and leprosy. **Methods:** The study was aimed to evaluate the antioxidant and free radical scavenging activities of methanolic (50%) leaves extract of *Erythrina indica* by 1,1-diphenyl, 2-picrylhydrazyl assay (DPPH), reducing power, nitric oxide scavenging activity, total flavonoid content and total phenolic content. **Results:** All the parameters were found to be concentration dependent and increased with increasing amounts of sample. The mechanism underlying the protective effects was assayed in vitro and the *E. indica* extracts displayed dose dependent free radical activity by utilizing DPPH ($IC_{50} = 530 \pm 1.52 \mu\text{g/mL}$) and nitric oxide ($IC_{50} = 495 \pm 1.38 \mu\text{g/mL}$). **Conclusion:** This study indicates that *E. indica* is a potential source of natural antioxidant. Further investigation on the isolation and identification of antioxidant component(s) in the plant may lead to chemical entities with potential for clinical use.

Key-words: *Erythrina indica*, DPPH, Nitric oxide, HPTLC, Free radical scavenging.

1. INTRODUCTION

Oxidative stress acts a major role in the pathogenesis of diverse diseases such as atherosclerosis, alcoholic liver cirrhosis including cancer.¹ Oxidative stress is initiated by reactive oxygen species (ROS), such as superoxide anion (O_2^-), perhydroxy radical (HOO^\cdot) and hydroxyl radical (HO^\cdot). These radicals are formed by one electron reduction process of molecular oxygen (O_2). ROS can easily initiate the lipid peroxidation of the membrane lipids, causing damage of the cell membrane of phospholipids and many other lipoproteins by propagating a chain reaction cycle.² The abundant evidence suggested that the reacting oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical involve in the pathogenesis of the various disorder and diseases.³ Thus, antioxidants defense systems have co-evolved with aerobic metabolism to counteract oxidative damage from ROS. Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways including removal of O_2 , scavenging reactive oxygen/nitrogen species or their precursors, inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation and up-regulation of endogenous antioxidant defenses. The protective efficacy of antioxidants depends on the type of ROS that is generated, the place of generation and the severity of the damage.^{4,5}

The plant *Erythrina indica* Lam. is a middle sized tree widely distributed throughout India. It belongs to the family Papilionaceae, commonly known as Mandara (in Hindi) and Indian coral tree (in English). Traditionally its

leaves are used as laxative, diuretic, emmenagogue, galactagogue and also used in the treatment of anti-helminthic and joints pain.^{6,7,8} Phytochemically, *Erythrina indica* contain alkaloids like N-norprotosinomenine (1), protosinomenine (2), erysodienone (3), 3-erythroidine, erysopine, erythraline, erythramine, erysodine, erysotrine, erythratine, N, N-dimethyltryptophan, hyparphorine, sterols like campesterol, β -sitosterol, β -amyrin. The isoflavones named as indicanines D and E and flavonoids include apigenin, genkwanin, iso-vitexin, swertisin, saponarin, 5-O-glucosylswertisin and 5-O-glucosylisoswertisin, and a triterpene betulin have also been isolated.⁹⁻¹¹ Earlier scientific investigation of *Erythrina indica* showed that the crude extract has anti-osteoporotic¹², cytotoxic¹³, cardiovascular¹⁴, central nervous system effect¹⁵, anthelmintic¹⁶, analgesic¹⁷, antiulcer¹⁸, antioxidant^{19, 20} and diuretic activity.²¹ Present study was carried out to explore the plant *E. indica* with regard to their therapeutic potentials using an *in vitro* free radical-scavenging assay.

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2. MATERIALS AND METHODS

2.1 Collection and authentication

The fresh leaves of *Erythrina indica* were collected from the field area of Pallavaram, Chennai, India, in the month of June 2011. The plant specimen was authenticated by National Institute of Herbal Science, Plant Anatomy Research Center, Chennai, Tamilnadu (Voucher specimen no. PARC- 2011/ 955)

2.2 Chemical

All solvents, chemicals, solutions and reagents used in the study were of analytical grade procured from SD Fine Chemicals Pvt. Ltd., Mumbai, India; Fischer Inorganic and Aromatics Pvt. Ltd., Lucknow. Required chemical ascorbic acid, Aluminium Chloride, sodium nitroprusside sodium nitrite, potassium acetate, gallic acid, rutin, trichloroacetic acid, Griess reagent and 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich Co MO, USA.

2.3 Preparation of the extract

500 g of the coarsely dried powdered material were packed in muslin cloth and subjected to a Soxhlet extractor for continuous hot extraction with methanol (50%) for 72 hrs at 30 °C. Thereafter methanolic extracts of *E. indica* were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried at temperature 40 °C and the pressure 760 torr to 1 bar. The yield of the methanolic extract was 12.5 % w/w.

2.4 Preliminary phytochemical screening

The preliminary phytochemical investigation of the methanolic extract of *E. indica* showed the presence of alkaloids, flavonoids, terpenoid, saponins and tannins as the major phytoconstituents. A distinct chemoprofile of the methanolic extract of *E. indica* was obtained using HPTLC. Precoated TLC plates of silica-gel 60 F254 (E. Merck, India), 0.2 mm thick, were used. Ten microliters of the methanolic extract was spotted in the form of a band using a CAMAG Linomat-V Automatic Spotter (CAMAG, Switzerland) (Fig. 1). The TLC pattern of the methanolic extract was developed using chloroform: methanol: formic acid (7:2.7:0.3) as a solvent system.²²⁻²⁴ Plates were scanned in the CAMAG TLC Scanner-III and the peaks were recorded at a wavelength of 254 nm. The TLC and HPTLC fingerprinting studies of the methanolic extract showed the presence of various phytoconstituents with their respective R_f values. The observed R_f values from TLC agreed with the HPTLC fingerprints.

2.5 Estimation of total phenols

The total phenolic content was determined according to the method described by Singleton (1999).^[25] A suitable aliquot of the (50 %) methanolic extract of *E. indica* was placed in test tubes and made up to 1 ml with distilled water. Then, 0.5 ml Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml sodium carbonate solution (20 %) were added sequentially to each tube. Then, the tubes were vortexed for 2 min, kept in the dark for 40 min and the absorbance was recorded at 725 nm. The amount of

total phenolics was calculated as gallic acid equivalents /mg of extract.

2.6. Estimation of total flavonoids

Total flavonoids content was estimated by aluminum chloride colorimetric method.²⁶ From the (50%) methanolic extract, 1mg/ml concentration of stock solution was prepared. Then 0.5 ml of above stock solution (1mg/ml) of extract was taken in 1.5 ml of methanol, and 0.1 ml of potassium acetate (1M) was added to reaction test tubes and volume was made up 5 ml with distilled water. After incubation at room temperature for 30 min with intermittent shaking and absorbance was measured at 514 nm. The calibration curve was prepared by using rutin solutions as standard at concentrations (10-100 µg/ml) in methanol. The concentration of total flavonoids in the extract was obtained by extrapolating the absorbance of rutin through graph. The total flavonoid content was expressed as rutin equivalent % w/w of dry extracts.

2.7. In vitro antioxidant assay

2.7.1 Assay for DPPH radical scavenging capacity

The assay was performed according to the method of Lim *et al.*, (2003)²⁷ with minor modification. The radical scavenging activities of the leaves extracts *E. indica* against 1, 1-Diphenyl-2-picrylhydrazyl were determined by UV spectrophotometer at 517 nm. An aliquot of (0.05, 0.1, 0.5, 1.0, 1.25 and 1.50 mg/ml) of extract was mixed in a test tube containing 3 ml of methanol and 0.5 ml of 1 mM DPPH. Ascorbic acid was used as the antioxidant standard at concentrations of 0.05 and 1.0 mg/ml. A blank solution was prepared containing the same amount of methanol and DPPH. The reaction mixture was incubated at 37 °C for 30 min. The radical scavenging activity was calculated using the following equation:

$$\% \text{ Scavenging} = [\text{AbsControl} - \text{AbsSample}] / \text{AbsControl} \times 100$$

2.7.2 Nitric oxide anion scavenging activity.

The procedure was made on the principle that sodium nitroprusside solution at physiological PH suddenly generates nitric oxide which interacts with oxygen to generate nitric ion that was estimated by using Geiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylendiamine dihydrochloride).²⁸ Scavenger of nitric oxide competes with oxygen leading to reduce production of nitric ion. For the experiment, an aliquot (1ml) of different concentrations (0.05-5) of methanolic extracts of *E. indica* leaves were dissolved in Phosphate buffer solution (PBS) and added 1 ml of sodium nitroprusside (10 mM) and incubated at room temperature for 150 min. The reaction without the extracts sample but equivalent amount of methanol served as control. After incubation period, 0.5ml of Griess was added. The absorbance was determined by UV spectrophotometer at 546 nm. The radical scavenging activity was calculated using the following equation:

$$\% \text{ Scavenging} = [\text{AbsControl} - \text{AbsSample}] / \text{AbsControl} \times 100$$

2.7.3 Reducing power

The total reducing power of the extract was measured according to the Oyaizu method²⁹. Extract (10, 25, 50 and 100 mg/ml) in 1 ml of distilled water was mixed with 2.5 ml phosphate buffer (0.2 mM, pH 6.6) and 1% of 2.5 ml potassium ferricyanide. The mixture was incubated at 55°C for 25 min. Subsequently 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of the solution (1.5 ml) was mixed with 1.5 ml distilled water and FeCl₃ (0.3 ml, 0.1%) and the absorbance was measured at 680 nm using UV spectrophotometer. Elevated absorbance of the reaction mixture indicated better reducing power.

8. Statistical analysis

All the *in vitro* experiments were performed in triplicate. The IC₅₀ values were calculated by linear regression analysis.

3. RESULTS

3.1 Preliminary phytochemical screening

Phytochemical testing of the methanolic extract of *E. indica* leaves showed the presence of proteins, reducing sugar, flavonoids, steroids, terpenoids and phenolic compounds. The preliminary HPTLC studies revealed that the solvent system, chloroform: methanol: formic acid (7:2.7:0.3), was ideal and gave well resolved sample peaks. The spots on the chromatogram were visualized at 254 nm (Fig. 1) with a 500 k filter at *R_f* values of 0.12, 0.16, 0.25, 0.34, 0.43, 0.46, 0.49, 0.57, 0.78 and 0.88. The densitometric scanning at 254 nm gives six major spots with an area of 14.87 %, 18.16 %, 12.26 %, 12.33 %, 11.40 % and 22.34 % at an *R_f* value of 0.25, 0.34, 0.43, 0.46, 0.49 and 0.57.

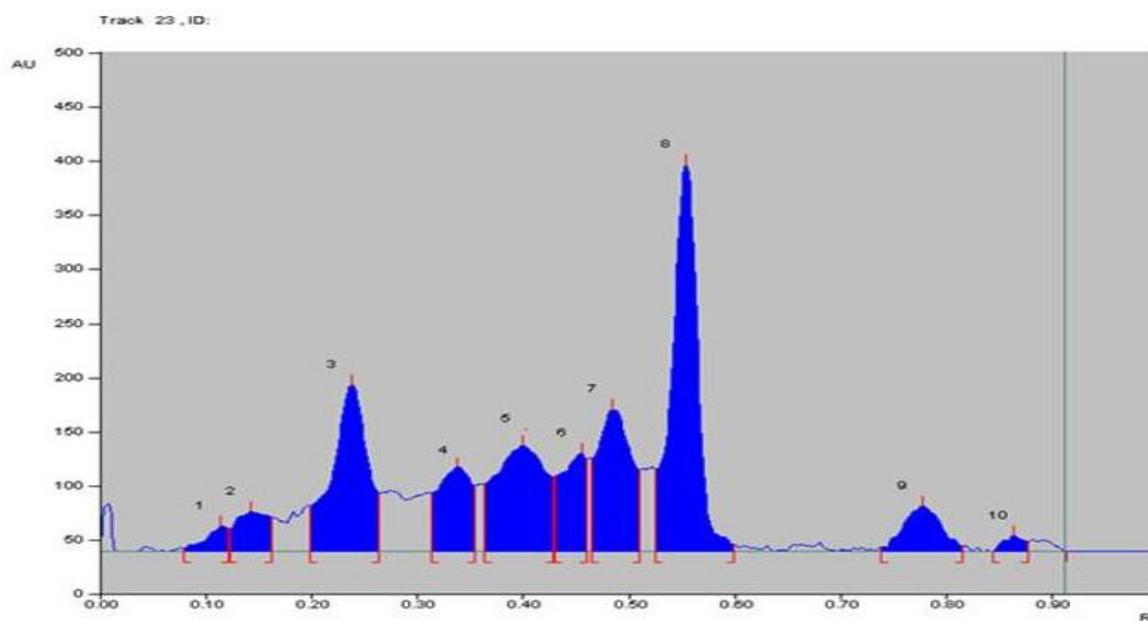


Figure 1: Chromatogram of HPTLC Finger printing of methanolic extract of *E.indica* scan at wavelength 254 nm.

3.2 Assessments of total phenolic and flavonoids

Total phenolics and flavonoids content in the methanolic extract of *E. indica* was found to be 53.44±7.73 µg/mg equivalent to gallic acid and 51.10±9.49 µg/mg

equivalent to rutin respectively. The results indicate well-built association between anti-oxidatives activities of phenolic and flavonoids compounds are responsible for the anti-oxidant activity of *E. indica* (Table- 1).

Table 1: Estimation of content of total phenolic and flavonoids

Extract	Phenolics content	Flavonoidal content
Methanolic Extract of <i>E. indica</i>	53.44 ± 7.73 µg/mg	51.10 ± 9.49 µg/mg

All the values are mean ± SEM. (n=4)

3.3 Effect on the DPPH radical scavenging capacity

Effect on the DPPH radical scavenging capacity: The percentage inhibition of *E.indica* leaves extract at concentrations 0.05, 0.1, 0.5, 1.0, 1.25 and 1.50 mg/mL were 32.21, 37.55, 48.65, 82.59, 88.18 and 92.07% and by ascorbic acid standard 36.58, 42.31, 55.12, 87.58,

95.26 and 98.17% respectively (Figure 2). The standard ascorbic acid presented a high scavenging effect of 98.17% at the concentration of 1.50 mg/mL. The IC₅₀ of the methanolic leaves extract of *E. indica* was found to be 530±1.52µg/mL, which is comparable with the IC₅₀ of standard ascorbic acid 345±2.11 µg/mL.

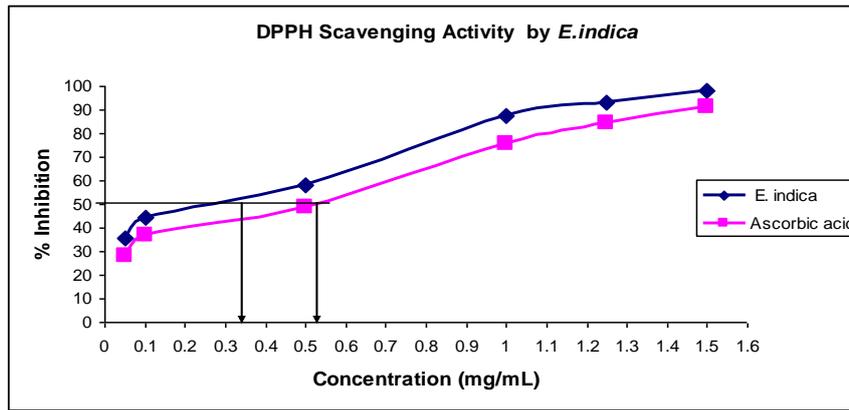


Figure 2: Effects of methanolic extract of leaves of *E. indica* on DPPH radical scavenging activity. Results are Mean ± SEM three parallel measurements.

3.4 Nitric oxide scavenging assay

The leaves of *E.indica* extracts illustrated significant free radical scavenging action against nitric oxide (NO) induced release of free radicals. The percentage inhibitions at concentrations 0.05, 0.10, 0.5, 1.0, 1.25 and 1.5 mg/mL of standard ascorbic acid illustrated 35.50, 48.64, 57.27, 74.25, 88.53 and 95.32% inhibition of

respectively and by extract 33.24, 38.56, 49.95, 67.45, 78.16 and 89.84% respectively at same concentration (Figure 3). The standard ascorbic acid presented a high scavenging effect of 95.32% at the concentration of 1.5 mg/ml The IC₅₀ of the methanolic extract of *E .indica* was 495±1.38 µg/mL, which is comparable with the IC₅₀ of standard ascorbic acid 140±2.54 µg/mL.

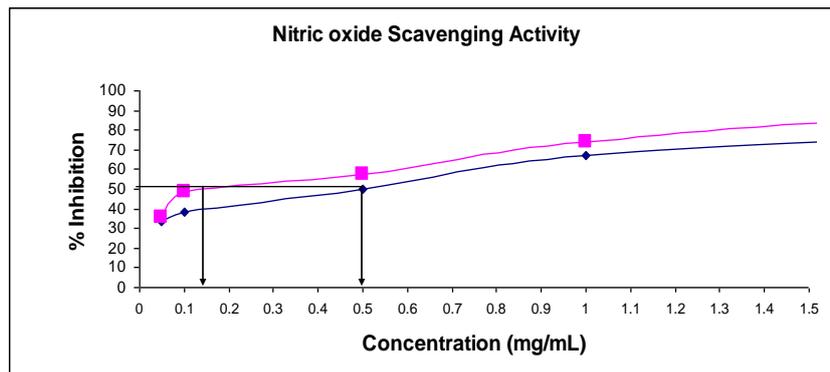


Figure 3: Effects of methanolic extract of leaves of *E. indica* on nitric oxide anion radical scavenging activity. Results are Mean ± SEM three parallel measurements.

3.5 Reducing activity of E.indica leaves extract

The reducing capabilities of methanolic extract of *E.indica* leaves at different concentration ranging from

(10–100 mg/ml) revealed reducing significant capacity in terms of ascorbic acid equivalent. The reducing power of extract was found to increase as the amount of extract increased (Fig.4).

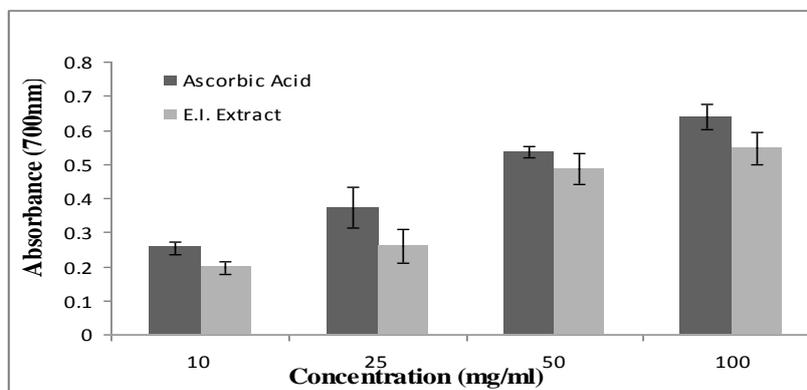


Figure 4: Effects of methanolic extract of leaves of *E. indica* on reducing power ability. Results are Mean ± S.D three parallel measurements.

4. DISCUSSION

There is a various evidence indicates that increased consumption of antioxidants from fruits and vegetables minimises the risk of degenerative diseases associated with aging and may contribute to improvement in quality of life by delaying the onset of various diseases.³⁰ The present results demonstrate that crude (50%) methanolic extract of *E. indica* posses free radical scavenging and antioxidant capacity tested in *in-vitro*. The *in-vitro* free radical scavenging capacity of the methanolic extract of leaves of *E. indica* demonstrates effect polyvalent phytophore and its scavenging capacity in *in-vitro* assays.³¹ In addition to phenolic compounds, natural flavonoids and terpenes from herbs or plant extracts are considered as most important antioxidant components and direct correlation has been established between total phenolic content and flavonoidal content of the extracts and antioxidant capacity *in-vitro*.³² HPTLC is a simple, rapid, and accurate method for analyzing plant material.³³ HPTLC fingerprint has better resolution and estimation of active constituents is done with reasonable accuracy in a shorter time. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants, with increasing demand for herbal products as medicines and cosmetics there is an urgent need for standardization of plant products.³⁴ HPTLC finger print analysis of

methanolic extract of *E. indica* suggest that six distinct peaks corresponding with different Rf values. From the HPTLC finger print it may assure that the methanolic extract has six different phytoconstituents. However this HPTLC finger prints analysis given simple data. Free radical scavenging capacity of extract was investigated by DPPH, nitric oxide and reducing power. The results from DPPH assay reveals that crude methanolic extract showed efficient quenching of DPPH and nitric oxide and thus contain free radical quenching compounds, which act as primary radical scavengers that react with DPPH by providing a hydrogen atom or electron donating ability.³⁵ Methanolic of *E. indica* extract showed high IC₅₀ values which are comparable to that of standard ascorbic acid. The reducing potential of a compound may refer as a important marker of its possible antioxidant activity.³⁶ However, the antioxidant activity of presumed antioxidants have been allowed to various mechanisms, among which are anticipation of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides. Prevention continued hydrogen abstraction and radical scavenging.³⁷ Reductive capabilities of samples of *E. indica* were compared with standard ascorbic acid.³⁸⁻⁴⁰

Conflict of interest: We declare that we have no conflict of interest.

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