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In vitro Antioxidant and Free Radical Scavenging Activity of Ethyl Acetate Extract of *Delonix regia* Rafin. Flowers

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The ethyl acetate extract of *Delonix regia* Rafin. (Leguminosae) flowers were used for the antioxidant and free radical scavenging activity by different *in vitro* antioxidant models. The ethyl acetate extract showed better antioxidant activity as compare to the standard one by DPPH radical scavenging and phenylhydrazine induced haemolysis of erythrocyte methods. IC_{50} value for DPPH radical scavenging and phenyl hydrazine induced haemolysis of erythrocyte were 29.69 ± 0.431 and 14.586 ± 0.145, respectively. Total polyphenolic and total flavonoids were 40.83 and 30.32 % w/w, respectively. Ethyl acetate extract contains quercetin, which was isolated and identified by using preparative TLC, Co-TLC and UV-Visible spectroscopy.

Key Words: Antioxidant, Free radical, *Delonix regia*, Quercetin.

INTRODUCTION

Delonix regia Rafin. (Leguminosae) is commonly known as Gulmohar, which is ornamental medium size tree planted in avenues and gardens and all part of India. It bears flowers with panicles varying in color from deep crimson through scarlet orange[1]. Free radicals are the major causative agent of many diseases and disorders like diabetes, liver cirrhosis, nephrotoxicity, arthritis, connective tissue disorders, cancer and liver related disorders. The major cause of natural aging and death is due to different type of radicals *i.e.* reactive oxygen species (ROS) such as super oxide anions (O_2), hydroxyl radical (OH) and nitric oxide (NO), which inactivate body enzymes and damage important cellular components[2,3].

Methanolic extract of the flowers shows antioxidant activity due to the polyphenolic compound present in the extract. It also contains alkaloids, tannins, flavonoids and glycosides[4].

EXPERIMENTAL

Collection and authentification of plant: Flowers of *Delonix regia* Rafin. (Leguminosae) collected from KTHM campus, Nasik. Identification has been done by Prof. D.R. Mahajan, HOD, Department of Botany, KTHM Art, Science, Commerce College, Nasik, India and Dr. S.C. Pal, HOD, Department of Phytochemistry and Pharmacognosy, College of Pharmacy, Nasik, India.

Extraction of flowers: Fresh flowers were refluxed with 2 M HCl (acid hydrolysis). Filtration was done and aqueous filtrate was collected, proceed for the extraction with petroleum ether, ethyl acetate and *n*-butanol. Ethyl acetate extract was chosen for phytochemical investigation and antioxidant activity[5].

Phytochemical investigation: Ethyl acetate extract was evaluated for preliminary chemical tests for phenol, tannins, steroids, triterpenoids, alkaloids, glycosides and flavonoids. Extract shows shinoda and ferric chloride test positive for flavonoids and tannins, respectively[6].

Isolation and identification of quercetin: Isolation was done by using preparative-thin layer chromatography and accordingly identified by using Co-TLC with authentic sample of quercetin and UV-Visible spectroscopy (Shimadzu 250-1PC)[5,7,8].

Total polyphenolic content: Total polyphenolic content was determined by Begum method[2] with some modification. One mL of different concentration (5, 10, 15, 20, 25 mcg/mL) of gallic acid was mixed with 1 mL of 95 % ethanol, 5 mL of distilled water and 0.5 mL of 50 % Folin-Ciocalteu reagent. The mixture was kept for 5 min and 1 mL of 5 % Na_2CO_3 was added. Mixture was incubated for 1 h in dark and absorbance was measured at 725 nm using UV-Visible spectrophotometer (Shimadzu 250-1PC). Gallic acid standard calibration curve was prepared. Similar procedure was carried out for ethyl acetate extract. The concentration of polyphenolic content was obtained from the standard curve.

Total flavonoid content: Aluminium chloride colorimetric method was used for flavonoid determination. 1 mL of different concentration (5, 10, 15, 20, 25 µg/mL) of quercetin was mixed with 3 mL of methanol, 0.2 mL of 10 % aluminium chloride, 0.2 mL of 1 M sodium acetate and 5.6 mL of distilled water. It was incubated at room temperature for 0.5 h and the absorbance was measured at 415 nm with UV-Visible spectrophotometer (Shimadzu 250-1PC). A quercetin standard calibration curve was obtained. Similar procedure was performed for ethyl acetate extract. The concentration of flavonoid was obtained from the standard curve[2].

DPPH Radical scavenging assay[3,9]: DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity was measured by spectrophotometric method.

Preparation of DPPH stock solution: 1.3 mg of DPPH was dissolved in 1mL of methanol.

DPPH stock solution (75 µL) added to different concentrations of (25, 30, 35, 40, 50 µg/mL) of ethyl acetate extract of *Delonix regia* and make up volume upto 3 mL by methanol. Equal amount of methanol is added to control. After 20 min absorbance of mixture was taken at 517 nm and percentage of inhibition was calculated.

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Ascorbic acid was used as positive control.

Nitric oxide radical scavenging activity[2,9]

Preparation of nitric oxide stock solution: Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. One mL sodium nitroprusside (5 mM) in 0.5 mL standard phosphate buffer (pH-7.4) solution.

Preparation of ethyl acetate extract sample: Different concentrations (25, 50, 75, 100, 125 µg/mL) of ethyl acetate extract dissolved in phosphate buffer.

Incubate stock solution with different concentrations of sample at 25 ºC for 2.5 h. Control experiment without the test sample but with equivalent amount of buffer was conducted in an identical manner.

After incubation take 0.5 mL of solution and add 0.5 mL of Griess reagent (1 % sulphanilamide, 2 % *o*-phosphoric acid and 0.1 % naphthyl ethylene diamine dihydrochloride). The absorbance was measured at 535 nm by using UV-visible spectrophotometer (Shimadzu 250-1PC) and percentage of inhibition calculated.

Ascorbic acid used as positive control.

Assay for phenylhydrazine induced haemolysis of erythrocytes (membrane stabilization study): 20 % Packed cell volume of erythrocyte suspension (from human blood) was prepared according to procedure of Hill & Thornally (1983). Assay was carried out according to procedure described by Ravishankara *et al.*[10] The incubation mixture comprises of 1 mL of phenylhydrazine hydrochloride (0.5 mM). Different concentrations of plant extract and 0.1 mL of 20 % erythrocyte suspension made to a total volume of 3 mL with phosphate buffered saline (PBS) solution. The mixture was incubated at 37 ºC for 1 h and centrifuged at 1000 g for 10 min. The extent of haemolysis was measured by reading the absorbance of the supernatant at 540 nm, control was taken without the extract and α-tocopherol acetate was used as positive control and percentage inhibition of induced haemolysis of erythrocyte is measured.

Hydrogen peroxide scavenging activity: Hydrogen peroxide scavenging activity was measured with titrimetric method of estimation 4.1 mL of extract was mixed with 1 mL of 0.1 mM of H_2O_2, 2 drops of 3 % ammonium molybdate indicator, 10 mL of sulphuric acid and 7 mL of 2 M KI. The mixed solution was titrated with 5 mM sodium thiosulphate until yellow colour disappeared. Ascorbic acid used as positive control and percentage hydrogen peroxide scavenging was determined.

Statistical analysis: Inhibition of concentrations and total phenolic and flavonoids were determined by linear regression analysis method. Results were expressed as mean ± SD (standard deviation) n = 3.

RESULTS AND DISCUSSION

The chemical tests show that the ethyl acetate extract of *Delonix regia* flower contains phenolic and flavonoid compounds, which give blue colour with ferric chloride and pink red colour with Shinoda reagent, respectively. The total polyphenolic content of extract is 40.83 % w/w and total flavonoid content is 30.32 % w/w *i.e.* good source of flavonoid. Flavonoid and polyphenolic compounds have good anti-

oxidant activity and used in many disease conditions[11]. From the ethyl acetate extract, quercetin is isolated by using the TLC and column chromatography. Primary separation was done by TLC using ethyl acetate:methanol (7:3) as mobile phase. For column chromatography same solvent was used and different fractions were collected and separation was checked by TLC[5,7,8]. Ethyl acetate extract and authentic sample of quercetin were run on TLC and compared[5] visually, R_f value, colour of spot after spraying with alc. ferric chloride solution as well as shows λ_{max} at band I-255 nm and band II -374 nm.

DPPH radical scavenging assay: DPPH is a stable free radical at normal temperature. It shows the specific absorbance at 517 nm due to colour of methanolic solution of DPPH. Body also contains the many free radicals, which assumed same as DPPH[3,12]. Decrease in the absorbance of mixture indicates that scavenging of free radicals. *Delonix regia* extract and standard ascorbic acid show decrease in absorbance *i.e.* it has the radical scavenging activity; which is measured in terms of IC_{50} (29.69 ± 0.431) (Table-1). This value is determined by using plot of concentration *vs.* percentage inhibition (Fig. 1).

TABLE-1
% INHIBITION CONCENTRATION OF ETHYL ACETATE EXTRACT OF *Delonix regia* AND ASCORBIC ACID FOR DPPH RADICAL SCAVENGING ASSAY

Ethyl acetate extract (EAE)		Ascorbic acid (AA)	
Concentration (µg/mL)	% Inhibition* (EAE)	Concentration (µg/mL)	% Inhibition* (AA)
25	43.03 ± 0.513	5	47.86 ± 0.404
30	50.43 ± 0.450	10	94.06 ± 0.208
35	56.83 ± 0.300	15	96.05 ± 0.132
40	68.43 ± 0.550	20	96.21 ± 0.000
50	77.70 ± 0.360	25	96.21 ± 0.000
IC_{50}	29.69 ± 0.431	IC_{50}	07.20 ± 0.216

*Values are mean ± SD, n = 3.

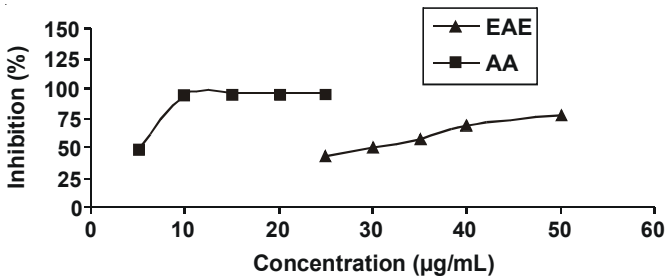

Fig. 1. Plot of % inhibition *vs.* concentration of ethyl acetate extract (EAE) of *Delonix regia* and ascorbic acid (AA) for DPPH radical scavenging assay

Nitric oxide radical scavenging activity: Nitric oxide (NO) is a free radical produced in mammalian cells, which is mediator of many physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity[3]. Sodium nitroprusside generate nitric oxide radical in presence of physiological buffer solution at 25 ºC. Nitric oxide reacted with Griess reagent and diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine form colour complex. Decrease in colour intensity is directly proportional to nitric oxide radical scavenging.

Delonix regia extract and standard ascorbic acid show decrease in absorbance *i.e.* it has the radical scavenging activity; which is measured in terms of IC_{50} (56.81 ± 1.200) (Table-2). This value is determined by using plot of concentration *vs.* percentage inhibition (Fig. 2).

TABLE-2
% INHIBITION CONCENTRATION OF ETHYL ACETATE EXTRACT OF *Delonix regia* AND ASCORBIC ACID FOR NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

Concentration (µg/mL)	% Inhibition*	
	Ethyl acetate extract (EAE)	Ascorbic acid (AA)
25	43.00 ± 1.053	50.00 ± 0.800
50	46.93 ± 1.404	57.56 ± 0.600
75	49.86 ± 0.830	62.10 ± 1.050
100	65.96 ± 1.006	81.31 ± 0.830
125	73.00 ± 0.400	87.50 ± 0.390
IC_{50}	56.81 ± 1.200	27.92 ± 0.500

*Values are mean ± SD, n = 3.

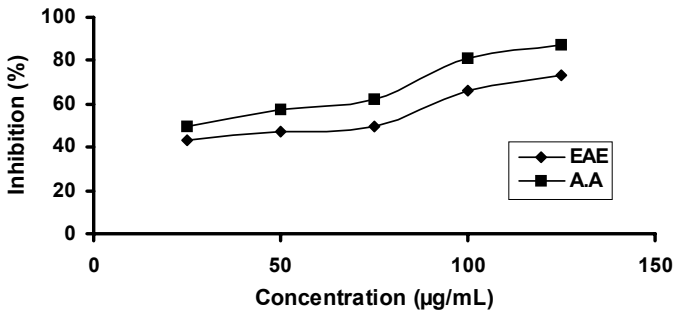

Fig. 2. Plot of % inhibition vs concentration of ethyl acetate extract (EAE) of *Delonix regia* and ascorbic acid (AA) for nitric oxide radical scavenging activity

Assay for phenylhydrazine induced haemolysis of erythrocytes (membrane stabilization study): Phenylhydrazine is one of the autooxidizable substance, which under aerobic conditions may react with molecular oxygen to form a reactive species *e.g.*, superoxide radical, hydrogen peroxide, hydroxyl radical and phenyl radical

each with the capacity to initiate the peroxidation of unsaturated fatty acids in endogenous membrane phospholipid. These reactions may give rise to alteration in red blood cell membrane structure and function[10]. Extract shows good phenylhydrazine scavenging activity and ultimately decrease in haemolysis of erythrocyte.

Delonix regia extract and standard α-tocopherol show decrease in absorbance *i.e.* it has the radical scavenging activity; which is measured in terms of IC_{50} (14.586 ± 0.145) (Table-3). This value is determined by using plot of concentration *vs.* percentage inhibition (Fig. 3).

TABLE-3
% INHIBITION CONCENTRATION OF ETHYL ACETATE EXTRACT OF *Delonix regia* AND α-TOCOPHEROL FOR PHENYLHYDRAZINE INDUCED HAEMOLYSIS OF ERYTHROCYTES

Concentration (µg/mL)	% Inhibition*	
	Ethyl acetate extract	α-Tocopherol
5	20.630 ± 0.065	32.24 ± 0.597
10	33.740 ± 1.002	42.63 ± 0.549
15	49.460 ± 0.548	61.75 ± 0.487
25	72.560 ± 0.648	81.30 ± 1.020
30	95.700 ± 0.254	96.76 ± 0.948
IC_{50}	14.586 ± 0.145	10.98 ± 0.548

*Values are mean ± SD, n = 3.

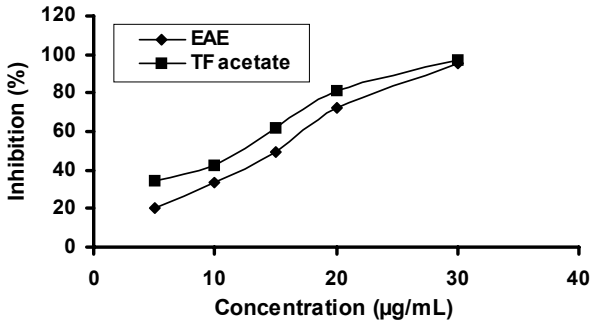

Fig. 3. Plot of % inhibition vs concentration of ethyl acetate extract (EAE) of *Delonix regia* and α-tocopherol (TF acetate) for phenylhydrazine induced haemolysis of erythrocytes

Hydrogen peroxide scavenging activity: Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiols (-SH) groups. Hydrogen peroxide cross cell membrane and react with ferric and copper ions, which shows toxic effects[2]. Extract have the good hydrogen peroxide scavenging activity.

Delonix regia extract and standard ascorbic acid show decrease in absorbance *i.e.* it has the radical scavenging activity; which is measured in terms of IC_{50} (372.23 ± 0.200) (Table-4). This value is determined by using plot of concentration *vs.* percentage inhibition (Fig. 4).

TABLE-4
% INHIBITION CONCENTRATION OF ETHYL ACETATE EXTRACT OF *Delonix regia* AND ASCORBIC ACID FOR HYDROGEN PEROXIDE RADICAL SCAVENGING ASSAY

Concentration (µg/mL)	% Inhibition*	
	Ethyl acetate extract (EAE)	Ascorbic acid (AA)
25	0.90 ± 0.000	18.20 ± 0.000
100	18.20 ± 0.000	27.30 ± 0.600
200	27.30 ± 0.600	45.50 ± 1.050
300	45.50 ± 1.050	54.50 ± 0.830
400	54.50 ± 0.830	63.30 ± 0.390
500	63.30 ± 0.390	72.70 ± 0.690
IC_{50}	372.23 ± 0.200	281.28 ± 0.500

*Values are mean ± SD, n = 3.

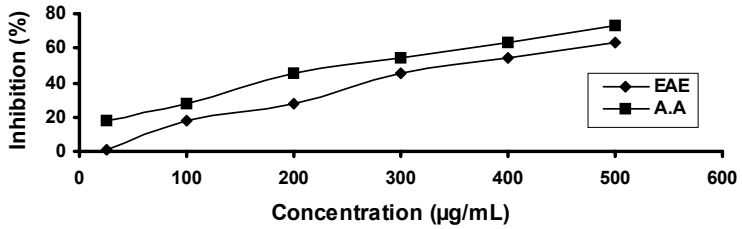

Fig. 4. Plot of % inhibition vs concentration of ethyl acetate extract (EAE) of *Delonix regia* and ascorbic acid (AA) for hydrogen peroxide radical scavenging assay

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