Antioxidative Efficacies of Floral Petal Extracts of Delonix Regia Rafin.

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ABSTRACT

The pigment extracts and the successive extracts prepared from dried flower petals of Delonix regia were evaluated for their ability to scavenge free radicals for the first time through in vitro chemical and biological models. Petals were found to contain 5.8 μg g⁻¹ of anthocyanin, 33.5 mg g⁻¹ of total phenolics and 694 μg g⁻¹ of total carotenoids of which 367 μg g⁻¹ was β-carotene. The different extracts were screened for DPPH scavenging activity, total reduction capacity, OH⁻ scavenging power, NO scavenging ability and anti-lipid peroxidation in brain cells, kidney cells and plasma in vitro. All the extracts, except for the hexane extract, showed over 90% quenching of DPPH⁺ at 250 ppm. While hydroxyl radicals were effectively scavenged (>90%) at 25-100 ppm by all the extracts with the exception of crude pigment extract and the xanthophyll fraction, most of the extracts of D. regia were effective in countering the actions of free radicals and lipid peroxidation in the experimental models. The various antioxidant activities were compared with standard antioxidants such as BHA, BHT, gallic acid and ascorbic acid on the experimental model. The results of the present study have established that floral petals of D. regia are rich in pigments and potential anti-oxidants holding a great promise for food and pharmaceutical applications.

Keywords: anthocyanin, β-carotene, carotenoids, Gul Mohr, lipid peroxidation

INTRODUCTION

Delonix regia Rafin. Bojer ex Hook (Syn. Poinciana regia) commonly known as ‘Gul Mohr’, produces abundant clusters of orange-red flowers during early summer. Flowers are eaten fresh due to their sweet-sour-astringent taste and are reported to be used as an anthelminthic (Anonymous 1952, 1976). They are also used in culinary preparations by rural people of Asia, the far-East and Australia. Though the chemical composition (Gupta et al. 2005) and medicinal properties (Sethuraman et al. 1986) have been well established for the other species of Delonix (D. elata), only a few preliminary reports are available on the chemistry and biological potential of D. regia (Seetharam et al. 2002). The methanolic extract of D. regia Gamble was reported to have antioxidant properties (Aqil et al. 2006). Four flavanoids isolated from the flowers of D. regia have been reported to show strong antioxidant activity against various free radicals (Su et al. 1997). Saleh et al. (1976) reported the presence of two anthocyanins – cyanidin-3-glucoside and cyanidin-3-gentiobioside – in the flowers whereas the carotenoids of different floral parts of D. regia were first reported by Jungalwala et al. (1962). Carotenoids comprise a diverse class of natural pigments that are of interest for pharmaceuticals, coloring food and animal feed and nutriment supplements (Schmidt-Dannert et al. 2000) due to their high antioxidant activity (Sies et al. 1992; Serrano et al. 2005). Apart from their provitamin-A activity and antioxidative properties, carotenoids are responsible for a wide range of intercellular activities such as communication, immune response, neoplastic transformation, growth control and regulation of cellular levels of the enzymes that detoxify carcinogens (Pryor et al. 2000). Low plasma carotenoids concentration is often used as an indicator for those at risk of chronic diseases. Such indices are based on the direct association between the intake of carotenoid-rich fruit and vegetables, plasma and tissue concentration of carotenoids and the development of chronic disease states, particularly cardiovascular diseases and cancer of various organs (Southon et al. 2001). Natural antioxidants are more favorably accepted and recognized by higher animals than synthetic ones (Shi 2001). The same is true with carotenoids, the natural forms of which exist both as cis and trans isomers along with oxygenated carotenoids that have better efficacy over synthetic all trans forms (Heber 2004; Murthy et al. 2005).

Anthocyanins, the pigments also present in the flowers of D. regia, are responsible for the red, purple and blue color of many other flowers, fruits and vegetables and are chemically derived from phenyl-propanoids. There is considerable anecdotal and epidemiological evidence that dietary anthocyanins and polyphenols confer preventive and therapeutic roles in a number of human diseases. For similar rea-
Dried flowers (powdered) 
Extraction (acetone: hexane, 4:6) 
Saponified with 2% KOH at 4°C 
Extracted in ether 
Washed free of alkali 
Concentrated to dryness 
Re-dissolved in light petrol 
Stored at -80°C under N₂ 
Filtration (Whatman no. 1) 
Phase partitioned with 90% aq. MeOH 
Organic layer 
Concentrated 
Dry extract 
1) Carotene hydrocarbons and mono-hydroxy xanthophylls 
2) Mono-and di-epoxy derivatives of I. 
(Carotene hydrocarbon fraction II) 

Organic layer 
Sterols ppt 

Aq. methanol layer 
Concentrated 
Dry extract 
1) Di- and poly-hydroxy xanthophylls 
2) Epoxy derivatives of I. 
(Xanthophyll fraction III) 

Fig. 2 Steps involved in the extraction of pigments from the flowers of *D. regia*.

**MATERIALS AND METHODS**

**Chemicals**

2'-azo bis (2-methylpropionamidine) dihydrochloride (AAPH), α,α-diphenyl-β-picylhydrazyl (DPPH), gallic acid (GA) and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (Steinheim, Germany). Butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) and ascorbic acid were from Merck (Darmstadt, Germany). n-Naphthyl ethylenediamine and trichloroacetic acid (TCA) was purchased from Sisco Research Lab (Mumbai, India). Sulphanilamide was obtained from Loba Chemie (Mumbai, India). All other chemicals and solvents were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Qualigens (India).

**Plant material and extraction**

The flowers of *D. regia* in full bloom (Fig. 1) were collected from the trees available at the Institute campus during May-June and the flowers were separated, dried at 45°C ± 1°C until they reached constant weight and either used immediately or stored in an air-tight container in the dark for 2-3 months at -20°C during which time all the analyses were completed.

The oven-dried petals were powdered using a kitchen blender and 20 g (dry weight, dw) were subjected to successive extraction in a Soxhlet extractor using solvents in increasing order of their polarity starting from hexane followed by ethyl acetate (EtOAc), acetone, methanol (MeOH) and water (H₂O). The individual extracts were concentrated at 40 ± 1°C under reduced pressure (as per manufacturer’s instruction for the respective solvent) using Buchi rotavapour (Flawil, Switzerland), dried in a vacuum oven and stored at -20°C till further use.

A flow chart for the preparation of pigment extracts is given in Fig. 2.

**Total carotenoid determination**

Carotenoid content was determined by the method described in AOAC International (1993). Briefly, 2.5 g of the dried sample was blended for 5 min with acetone:hexane (4:6) containing 0.1 g of magnesium carbonate and centrifuged at 8000 rpm (10,600 × g) for 10 min. The residue was washed with two 25 mL portions of acetone followed by n-hexane. The extracts were combined and washed with water to remove acetone. The upper layer was placed in a 100.0 mL volumetric flask containing 9 mL acetone and the volume was adjusted with hexane. The optical density (OD) of the suitably diluted solution was measured at 436 nm. Concentration of β-carotene was calculated from the formula:

\[
C = \frac{(OD \text{ at } 436 \text{ nm} \times 454)}{(196 \times L \times W)}
\]

where C represents concentration of β-carotene (mg lb⁻¹), L represents the path length (cm) and W represents the weight of the sample in g mL⁻¹ of final dilution. (C × 2.2 gives the concentration in mg kg⁻¹).

**Total anthocyanin content**

Total anthocyanins (Tacy) content in the flowers was determined
by a pH differential method (Lee et al. 2005). One gram of dried flowers was powdered and extracted in methanol acidified with 1% HCl at room temperature on an orbital shaker set at 100 rpm for 6 hrs. The mixture was centrifuged at 7000 rpm (9300 × g) for 10 min and the supernatant decanted into a 50.0 mL volumetric flask. The pellet was re-extracted thrice, the supernatants combined and the volume was made up with methanol. One mL each of the solution was diluted with pH 1.0 buffer (1.86 g KCl dissolved in distilled water and adjusted to pH 1.0 ± 0.05 with HCl and diluted to 1.0 L with distilled water in a volumetric flask) and pH 4.5 buffer (0.4 M sodium acetate adjusted to pH 4.5 ± 0.05 with HCl) respectively and the OD of the two solutions was measured at 520 and 700 nm against a distilled water blank. The concentration of Tacy was expressed as cyanidin-3-glucoside equivalents using the following formula:

\[
Tacy (% w/w) = \left( \frac{A - MW \times DF \times V \times 100}{\varepsilon \times 1 \times W} \right)
\]

where: \(A = (A_{200nm} - A_{700nm})\) pH 1.0 - (\(A_{200nm} - A_{700nm}\)) pH 4.5; MW = 449.2 g mol\(^{-1}\) for cyanidin-3-glucoside; DF = dilution factor; \(V = \) total volume of the extract; \(\varepsilon = 26900\) molar extinction coefficient, \(\lambda = m\) mol\(^{-1}\) cm\(^{-1}\), for cyanidin-3-glucoside; \(I = \) path length in cm; \(W = \) sample weight (mg).

Estimation of total phenolics

Total phenolics were determined as per the method described by Velioglu et al. (1998) with a slight modification. Briefly, 1 g of the sample was extracted with 80% methanol containing 1% HCl at room temperature on an orbital shaker set at 100 rpm for 6 hrs. The mixture was centrifuged at 7000 rpm (9000 × g) for 10 min and the supernatant decanted into a 50.0 mL volumetric flask. The pellet was re-extracted thrice, the supernatants were pooled and the volume was made up with methanol. An aliquot of the extract (25 mL) was mixed with 225 μL methanol and 1.0 mL Folin-Ciocalteu reagent (0.2 M) and allowed to stand at room temperature (25°C ± 0.5°C) for 5 min. One mL of 6% w/v sodium carbonate solution was added to the mixture followed by incubation for 90 min at room temperature and the absorbance was measured at 725 nm. A calibration curve of gallic acid was prepared (ranging from 0.001-0.01 mg mL\(^{-1}\)). Results were determined from regression equation of the calibration curve (\(y = 0.0965x\); \(R^2 = 0.9726\)) and expressed as mg gallic acid equivalents per gram (mg GAE g\(^{-1}\)) of the sample. The method was applied to the successive extracts also, to select the solvent that was most suitable for the extraction of polyphenols.

Antioxidant assays

Free radical scavenging activity

The antioxidant activities of pigments as well as successive extracts of D. regia were measured on the basis of the ability of the extracts to scavenge the stable DPPH\(^*\). Different concentrations of the extracts in methanol (2.0 mL) were treated with 0.5 mL of 0.5 mM solution of DPPH\(^*\) in methanol. Absorbance at 517 nm was determined after 20 min and the percentage scavenging activity was calculated against a reagent blank (Murthy et al. 2002).

Total reduction capacity

The reduction of ferric to ferrous ion by the extracts is an indication of the potential antioxidant property. The reducing power of the extracts was determined by the method of Gulcin (2006). Different concentrations of the extracts (1-10 ppm to the final concentration) in methanol (1.0 mL) were diluted with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and mixed with 2.5 mL 1% potassium ferricyanide. After incubation at 50°C for 20 minutes, 2.5 mL of 10% TCA were added to the mixture. 2.5 mL of the reaction mixture was diluted with an equal amount of distilled water and absorbance was measured at 700 nm after treatment with 0.5 mL of 0.1% FeCl\(_3\). Increased absorbance of the reaction mixture indicates an increase in reduction capability.

Assay of hydroxyl radical scavenging activity

This test is based on the oxidation of 2-deoxyribose by OH\(^*\) (formed by the Fenton reaction) and its degradation to malondialdehyde (MDA) (Chung et al. 1997). Different concentrations of the extracts were made up to 1.2 mL with 0.1 M phosphate buffer (pH 7.4) in test tubes and treated with 0.2 mL each of Fe-EDTA solution (10 mM each of FeSO\(_4\), HEDTA and EDTA), 2-deoxyribose (10 mM), H\(_2\)O\(_2\) (10 mM) and ascorbic acid (10 mM). The reaction mixture was incubated at 37°C for 1 hr and the reaction was stopped by adding 1 mL of ice cold 2% trichloroacetic acid. One mL of 1% TBA solution was added to the reaction mixture and placed in a boiling water bath for 15 min, cooled on ice, centrifuged and absorbance of the supernatant was measured at 535 nm.

Nitric oxide scavenging activity

Aqueous sodium nitroprusside (SNP) at physiological pH spontaneously generates nitric oxide, a very unstable species which reacts with oxygen to produce nitrite ions. The extent of nitrite ions formed is measured using Griess reagent. In the present study, 0.5 mL of 10 mM SNP in phosphate buffered saline (PBS, pH 7.4) was incubated with different concentrations of extract (1-100 ppm) in PBS (2.0 mL) for 180 min. After the incubation period, 0.5 mL of the reaction mixture was removed and diluted with 0.5 mL of Griess reagent (1:1 mixture of 1% sulphanilamide in 2% phosphoric acid and 0.1% of n-naphthyl ethylenediamine dihydrochloride) (Marocci et al. 1994). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with n-naphthyl ethylenediamine (Ilavarasan et al. 2005) was checked by measuring OD at 546 nm after 10 min incubation.

Antioxidant assay using β-carotene linoleate model system (BCLAMS)

The antioxidant activity of the successive extracts was evaluated by β-carotene linoleate model system (Singh et al. 2002). Briefly, β-carotene (0.2 mg) was dissolved in 0.2 mL of chloroform and mixed with linoleic acid (20 mg) and Tween-40 (200 mg). Chloroform was removed by flushing with nitrogen gas and the resulting mixture was diluted with 0.5 mL of oxygenated water. Four mL aliquots of the resulting emulsion were pipetted into test tubes containing different concentrations of standard and extracts in methanol. The reaction mixture without the sample served as control and the one without β-carotene served as blank. The reaction mixture was incubated at 50°C for 15 min. The OD was measured at 470 nm at zero time and continued till the color of β-carotene disappeared in control tubes (t = 180 min) at 15 min intervals. The antioxidant activity (AA) of the extracts was calculated in terms of bleeding of β-carotene using the formula: AA = 100 [\(1 - \frac{A_n - A_1}{(A_{n} - A_0)}\)], where \(A_0\) and \(A_n\) are the absorbance values measured at zero time for test sample and control respectively; \(A_1\) and \(A_{n}\) are the absorbance values measured in the test and control after 180 min.

Iron-induced lipid peroxidation in rat kidney and brain homogenates

Thiobarbituric acid reactive substance (TBARS), which forms a pink colored diadduct with the lipid peroxidation product MDA, can be measured spectrophotometrically at 532 nm (Halliwell et al. 1981). Kidney and brain were obtained from healthy untreated rats (control), washed in ice-cold saline and stored in PBS, pH 7.4, at -20°C until further use. A 10% w/v homogenate of the kidney was prepared in ice-cold phosphate buffer (0.1 M, pH 7.4) with a Potter-Elvehjem glass homogeniser. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was used for analysis. Aliquots of the tissue homogenates were treated with different concentrations of the extracts in methanol (0.5 mL) followed by 1.0 mL each of 10 μM FeSO\(_4\) and 0.1 M ascorbic acid and incubated at 37°C for 60 min. One mL each of 28% TCA and 1% TBA were added to the reaction mixture and heated for 15 min at 95°C. After cooling on ice and centrifuging the samples, the absorbance of the supernatant was read at 532 nm. The reaction mix-
ture without the extract served as a control. Similar procedure was followed for analyzing the brain tissue homogenate.

### AAPH induced plasma oxidation

AAPH induced plasma oxidation and its inhibition by the extracts of *D. regia* was monitored by the method described by Ljubuncic et al. (2005) with minor modification. Human plasma from healthy donors (obtained from a local hospital/health center) was diluted (1:2) with PBS, pH 7.4. Aliquots (1.0 mL) of diluted plasma were treated with different concentrations (1-25 ppm) of the extracts in PBS and incubated with 0.25 mL of freshly prepared 200 mM AAPH at 37°C for 2 h. At the end of the incubation period, plasma oxidation was stopped by the addition of 0.5 mL of ice-cold 20% TCA and 0.5 mL 1% TBA was added to each tube followed by heating at 100ºC for 20 min. After cooling on ice, the samples were centrifuged at 10,000 rpm (13000 × g) for 5 min and absorbance of the supernatant was read at 532 nm. Blanks of each sample were prepared and assessed in the same way to exclude any contribution due to background. Results were expressed in terms of spectrophotometric readings of the samples at 532 nm against a reagent blank.

### Data analysis

All experiments were done in triplicates and the data presented as average of mean of three independent experiments with standard deviation. The IC<sub>50</sub> was graphically estimated using a non-linear regression algorithm.

### RESULTS AND DISCUSSION

#### Quantification of total phytochemicals

The present study has established that the dried petals of the tropical tree, *D. regia*, are rich in carotenoids and anthocyanins along with other compounds like polyphenols, flavonoids, etc. Since these compounds are believed to be effective as free radical scavengers/antioxidants (Sies et al. 1992; Schmidt-Dannert et al. 2000; Serrano et al. 2005), a thorough analysis using various assay models was addressed in the present study. Upon chemical analysis, total carotenoid content in petals was found to be 694 μg g⁻¹ on a dry weight basis (dw) of which about 367 μg g⁻¹ was β-carotene. Total phenolics as high as 19 mg per gram Gallic acid equivalent (GAE) and anthocyanin content of 5.8 mg g⁻¹ (dw) were recorded in the floral petals of *D. regia* where the anthocyanins contributed to 1/5th of the total phenolics of the petals (Table 1). The yield in methanol was highest (31.8 g % dw) having a total phenolic content of 24.6 mg GAE g⁻¹ which is probably because of the exhaustive nature of Soxhlet extraction, resulting in maximum removal of the phenolic compounds from the material (Table 1). It was also noted that when the phenolic contents of all the successive extracts were summed up, the value rose to 33.5 mg GAE g⁻¹ (dw), which is equivalent to 1/4 of that reported in tea (131.9 mg g⁻¹) (Turkmen et al. 2006). In this context, it is worth recalling that tea polyphenols have recently gained enormous commercial importance in various anti-oxidant formulations. Though a recent report claimed the presence of nearly 170 mg/g of total phenolics in flower powder of *D. regia* (Aqil et al. 2006) our repeated extraction of floral petal resulted in the yield of only 33.5 mg GAE g⁻¹ (dw). The higher value in the former study may probably be due to the use of entire flower powder (including the calyx) and additional phenolic compounds might also have formed during sample storage.

### Table 1 The extract yield in different solvents and the total phenolic content in each of the extracts of *D. regia*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Yield (g % dw)</th>
<th>TP (mg GAE g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>3.3</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td>EtOAc</td>
<td>3.1</td>
<td>1.3 ± 0.01</td>
</tr>
<tr>
<td>Acetone</td>
<td>3.9</td>
<td>2.3 ± 0.02</td>
</tr>
<tr>
<td>MeOH</td>
<td>31.8</td>
<td>24.6 ± 0.03</td>
</tr>
<tr>
<td>Water</td>
<td>5.2</td>
<td>4.8 ± 0.03</td>
</tr>
<tr>
<td>II</td>
<td>4.6</td>
<td>nd</td>
</tr>
<tr>
<td>III</td>
<td>1.2</td>
<td>nd</td>
</tr>
</tbody>
</table>

Total phenolics are determined in triplicates for each extract. Data are expressed as mean ± SD. TP = total phenolics; nd = not determined; I = crude extract; II = carotene hydrocarbon fraction; III = xanthophylls fraction.

### Table 2 A comparison of the total phenolics (TP) and DPPH radical scavenging activity between different plants.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Part used</th>
<th>TP (mg GAE g⁻¹)</th>
<th>Conc. (ppm)</th>
<th>DPPH scavenging (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delonix regia</td>
<td>Flowers</td>
<td>169.67</td>
<td>100</td>
<td>71.93</td>
<td>Aqil et al. 2006;</td>
</tr>
<tr>
<td></td>
<td>Petals</td>
<td>33.5</td>
<td>100</td>
<td>84.17</td>
<td>Present study</td>
</tr>
<tr>
<td>Camellia sinensis</td>
<td>Leaves</td>
<td>130.6</td>
<td>4.14</td>
<td>50</td>
<td>Turkman et al 2006</td>
</tr>
<tr>
<td>Punica granatum</td>
<td>Fruit peel</td>
<td>44.0</td>
<td>100</td>
<td>95</td>
<td>Singh et al 2002</td>
</tr>
<tr>
<td>Vitis vinifera</td>
<td>Pomace</td>
<td>35.7</td>
<td>100</td>
<td>90</td>
<td>Murthy et al 2002</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Extract</td>
<td>45.1</td>
<td>--</td>
<td>50</td>
<td>Silva et al 2005</td>
</tr>
<tr>
<td>Panax notoginseng</td>
<td>Root</td>
<td>3000</td>
<td>--</td>
<td>50</td>
<td>Zhao et al 2006</td>
</tr>
</tbody>
</table>

### Table 3 Antioxidant properties of standard antioxidants as tested by different model systems.

<table>
<thead>
<tr>
<th>Extract system</th>
<th>Conc. (ppm)</th>
<th>AA</th>
<th>GA</th>
<th>BHA</th>
<th>BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH* model</td>
<td>1</td>
<td>17.4 ± 0.9</td>
<td>41.5 ± 0.1</td>
<td>22.5 ± 0.6</td>
<td>13.2 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>68.2 ± 3.2</td>
<td>93.8 ± 0.2</td>
<td>68.4 ± 1.1</td>
<td>45.7 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96.1 ± 0.4</td>
<td>94.1 ± 0.1</td>
<td>88.5 ± 0.7</td>
<td>71.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>96.5 ± 0.2</td>
<td>94.3 ± 0.1</td>
<td>91.6 ± 0.4</td>
<td>88.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>96.5 ± 0.1</td>
<td>94.8 ± 0.2</td>
<td>91.3 ± 0.2</td>
<td>91.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>96.5 ± 0.1</td>
<td>95.2 ± 0.1</td>
<td>91.3 ± 0.6</td>
<td>91.7 ± 0.1</td>
</tr>
<tr>
<td>AAPH-induced plasma oxidation</td>
<td>1</td>
<td>39.9 ± 1.0</td>
<td>51.7 ± 1.0</td>
<td>34.6 ± 1.5</td>
<td>66.8 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50.0 ± 0.5</td>
<td>63.6 ± 1.0</td>
<td>59.8 ± 0.5</td>
<td>71.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>61.2 ± 3.5</td>
<td>74.1 ± 2.0</td>
<td>76.9 ± 2.4</td>
<td>76.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>63.9 ± 0.5</td>
<td>71.4 ± 0.2</td>
<td>83.1 ± 1.6</td>
<td>79.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>67.8 ± 1.0</td>
<td>71.7 ± 1.5</td>
<td>86.5 ± 0.0</td>
<td>83.2 ± 1.0</td>
</tr>
<tr>
<td>OH• scavenging assay</td>
<td>5</td>
<td>*</td>
<td>69.9 ± 4.1</td>
<td>77.6 ± 0.7</td>
<td>69.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>*</td>
<td>81.6 ± 0.5</td>
<td>84.1 ± 0.2</td>
<td>85.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>*</td>
<td>92.1 ± 0.3</td>
<td>94.8 ± 0.7</td>
<td>95.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>*</td>
<td>97.2 ± 1.0</td>
<td>98.7 ± 0.3</td>
<td>99.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>*</td>
<td>101.5 ± 1.0</td>
<td>102.0 ± 2.0</td>
<td>106.4 ± 0.2</td>
</tr>
</tbody>
</table>

* Three independent experiments were performed as triplicate at each concentration. Data are expressed as mean ± SD. AA = ascorbic acid; GA = gallic acid; BHA = butylated hydroxy anisole; BHT = butylated hydroxy toluene; * since the assay system involves the use of ascorbic acid, the same was not used as standard.
Radical scavenging activity

The activities of the extracts against various free radicals were analysed using different in vitro chemical and cellular models. The DPPH• scavenging assay was carried out first to check the antioxidant property of the different extracts. DPPH•, a stable free radical with an unpaired electron, shows a strong absorption band at 517 nm, its solution appearing deep violet color. As the electron becomes paired off, which happens in the presence of an antioxidant (electron/hydrogen donor), the absorption vanishes. The resulting decolorization is stoichiometric with respect to the number of electrons taken up (Blois 1958). The total phenolic content and DPPH• scavenging property of methanol extracts of few well-known plants are presented in Table 2, in order to provide a comparative picture. Though the methanol extract (100 ppm) of the flowers of D. regia showed about 72% DPPH• scavenging activity in an earlier study (Aqil et al. 2006), our analysis showed that the acetone, methanol and water extracts exhibit more than 80% DPPH• scavenging. However, the pigment extracts were active only at concentrations above 250 ppm (Fig. 3). As compared to standard antioxidants such as ascorbic acid, gallic acid, BHA and BHT (Table 3), the water extract of D. regia at 100 ppm was equivalent to 50 ppm of almost all the standards. The IC50 of the hexane, EtOAc, acetone, MeOH, H2O, crude extract, carotene hydrocarbon fraction and xanthophyll fraction were respectively 213.6, 53.0, 72.9, 55.9, 29.5, 103.4, 228.7 and 366.3, all in ppm, when compared with BHA (IC50 3.6 ppm). All the samples inhibited radical formation in a concentration dependent manner with the water extract being the most active followed by EtOAc, MeOH and acetone extract (Fig. 3). The high antioxidant activity of water extract may be attributed to the presence of water-soluble antioxidants like phenolic acids. The relationship between the antioxidant activity of methanol extract and total phenolics of D. regia is represented in Fig. 4. There is a strong correlation between the antioxidant activities as measured by DPPH• radical scavenging and total polyphenol content (R2 = 0.9998) suggesting that polyphenols are likely to contribute to the antiradical activity. This was especially so in methanol and water extracts, as both are highly polar. In another similar study on black tea there was a strong correlation between antioxidant activity and polyphenol content (R2 = 0.989)(Miliauskas et al. 2004).

Total reduction capacity

The reducing property is generally associated with the presence of reductones. Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. The reduction capacity of the extracts is directly proportional to the green/blue color produced due to the reduction of Fe3+/ferricyanide complex to the ferrous form. The Fe2+ was monitored by measuring the formation of Perl’s Prussian blue at 700 nm (Gulcin 2006). Fig. 5 represents the reducing power of the successive extracts and pigment extracts. The Fe2+/Fe3+ transformation in the presence of the extracts increased in a concentration dependent manner. The reducing power of the standard/extracts is in the following order: EtOAc > xanthophyll fraction > ascorbic acid > MeOH > hexane > acetone > H2O > carotene hydrocarbon fraction > crude extract. The reducing power of the extracts may be due to their hydrogen donating ability (Shimada et al. 1992).
Hydroxyl radical scavenging

Living tissues are constantly subjected to oxidative insults by various means resulting in the formation of oxygen radicals. In biochemical systems, \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) react together to form the hydroxyl radical, \( \text{OH}^- \). This is the most reactive of all the free radicals and can attack and destroy almost all known biomolecules (Halliwell et al. 1989; Chung et al. 1997). The 2-deoxyribose oxidation method was used for the determination of hydroxyl radical scavenging ability of the extracts of \( D. \) regia. The addition of low concentrations of Fe (II) salts to deoxyribose causes rapid degradation of the sugar into a malondialdehyde like compound, which forms a chromogen with TBA. 2-deoxyribose is oxidized by \( \text{OH}^- \) that is formed by the Fenton reaction and degraded to malondialdehyde (Halliwell 1978). The extent of malondialdehyde formed and its inhibition by the extracts is measured at 520 nm. The \( \text{OH}^- \) scavenging efficacy of \( D. \) regia extracts were studied at different concentrations (5-100 ppm). The successive extracts were nearly as effective as BHA at 5 ppm against \( \text{OH}^- \)-mediated damage while carotene hydrocarbon and xanthophyll fractions were needed at a higher concentration (above 25 ppm) to bring about the same effect (Fig. 6). The crude pigment extract showed negative results at the concentrations tested. However, at 1.0 ppm it showed 94.5% activity (data not shown). The effective quenching of hydroxyl radicals by \( D. \) regia extracts may be attributed to the prevention of propagation of lipid peroxidation thereby reducing the rate of chain reaction, almost as efficiently as the vastly used anti-oxidants such as gallic acid, BHA and BHT (Table 3).

Nitric oxide scavenging

Nitric oxide (NO) or some related reactive nitrogen species accounts for the activity of endothelium derived relaxing factor (EDRF) responsible for vascular smooth muscle relaxation, acts as a neurotransmitter, prevents platelet aggregation and is a defense molecule of immune system against tumor cell, parasites and bacteria (Gulcin 2006). Despite these positive effects, it is also a potentially toxic gas with free radical properties. NO has been reported to cause mutagenesis and deamination of DNA bases to form carcinogenic \( n \)-nitroso compounds. NO reacts rapidly and spontaneously with a superoxide anion (\( \text{O}_2^- \)) to form a peroxynitrite anion (ONOO\(^-\)), which is more toxic than \( \text{O}_2^- \) or NO to biological systems and causes modification of proteins or nucleic acids causing deleterious health effects (Yen et al. 2006). In the present study NO scavenging effect of the different extracts of \( D. \) regia (1-50 ppm) was investigated. Of the eight extracts tested, hexane extract and crude pig-

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**Fig. 5** Iron reducing capacity of extracts of \( D. \) regia flowers. Three independent experiments were performed in triplicate at each concentration and data presented as the average where the deviation was within 5%. I = crude extract; II = carotene hydrocarbon fraction; III = xanthophylls fraction.

**Fig. 6** Hydroxyl radical scavenging activity of extracts of \( D. \) regia flowers. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at \( p<0.05 \). I = crude extract; II = carotene hydrocarbon fraction; III = xanthophylls fraction.
Table 4 Nitric oxide scavenging activity of extracts of *D. regia*.

<table>
<thead>
<tr>
<th>Conc. (ppm)</th>
<th>BHA</th>
<th>Hexane</th>
<th>EtOAc</th>
<th>Acetone</th>
<th>MeOH</th>
<th>Water</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.1 ± 0.2</td>
<td>16.8 ± 1.07</td>
<td>52.9 ± 1.6</td>
<td>18.0 ± 2.1</td>
<td>42.8 ± 1.3</td>
<td>60.9 ± 2.1</td>
<td>22.6 ± 0.6</td>
<td>29.8 ± 0.9</td>
<td>31.7 ± 1.6</td>
</tr>
<tr>
<td>5</td>
<td>49.9 ± 2.6</td>
<td>54.1 ± 0.5</td>
<td>70.0 ± 0.0</td>
<td>63.5 ± 1.1</td>
<td>70.5 ± 1.3</td>
<td>71.7 ± 2.4</td>
<td>48.1 ± 1.9</td>
<td>58.3 ± 0.6</td>
<td>52.1 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>52.2 ± 0.0</td>
<td>76.5 ± 0.5</td>
<td>70.0 ± 0.0</td>
<td>72.1 ± 0.8</td>
<td>75.9 ± 1.3</td>
<td>73.8 ± 0.0</td>
<td>70.8 ± 3.2</td>
<td>78.7 ± 0.6</td>
<td>74.1 ± 1.9</td>
</tr>
<tr>
<td>25</td>
<td>54.1 ± 0.0</td>
<td>80.4 ± 1.8</td>
<td>70.5 ± 0.8</td>
<td>74.7 ± 0.2</td>
<td>77.2 ± 0.0</td>
<td>74.3 ± 0.2</td>
<td>83.7 ± 2.6</td>
<td>81.0 ± 0.6</td>
<td>83.3 ± 1.3</td>
</tr>
<tr>
<td>50</td>
<td>56.7 ± 1.0</td>
<td>93.9 ± 0.5</td>
<td>67.9 ± 2.4</td>
<td>71.2 ± 1.3</td>
<td>77.4 ± 0.2</td>
<td>73.6 ± 0.3</td>
<td>93.1 ± 0.65</td>
<td>75.9 ± 1.3</td>
<td>73.1 ± 0.6</td>
</tr>
</tbody>
</table>

Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD.

*I* = crude extract; *II* = carotene hydrocarbon fraction; *III* = xanthophylls fraction.

**Protection of β-carotene**

The β-carotene-linoleic acid model system (BCLAMS) is an effective technique in evaluating the antioxidant activity of various plant extracts. The consumption of β-carotene is an effective technique in evaluating the antioxidant activity. The β-carotene-linoleic acid model system (BCLAMS) is a free radical mediated phenomenon resulting from the linoleic acid hydroperoxides. β-carotene, in this model system, undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene loses the double bonds by oxidation, the compound loses its chromophore and the characteristic orange color and therefore, can be monitored spectrophotometrically (Singh *et al.* 2002). The pigment extracts of *D. regia* were not subjected to this test because of their highly colored nature, which being rich in carotenoids, are themselves susceptible for bleaching in the presence of oxygenated water and thus interfere with the results.

**Protection against lipid peroxidation in brain and kidney tissues**

Oxidative processes are essential for the production of energy in every living cell, tissue and organ. However, excessive oxidation leads to breakdown of systems like lipids, proteins and DNA. As lipids breakdown through oxidative processes, they are fragmented into smaller molecules, which then become increasingly water-soluble. These free radical oxidation products have been shown to be extraordinarily powerful and behave as potentially lethal molecules leading to complications like cardiovascular diseases, cancer and other life-threatening diseases (Brigthhope 1994). In the past few decades, several epidemiological studies have revealed an inverse correlation between consumption of antioxidant rich foods and risk of several chronic diseases including cardiovascular and photosensitivity diseases, cataracts, age-related macular degeneration and some cancers (Palozza 1998; Johnson 2001; Virgili *et al.* 2001). There are no evidences of toxic symptoms of β-carotene in normal subjects even under long-term administration at pharmacological doses. Conversely, it is known to act as a tumor promoter in subjects with pre-existing cancer lesions induced by chronic tobacco smoking (Diplock 1997). According to Langseth (1995), carotenoids and vitamin C are the major contributors to the apparent cancer-protective effects of the foods. Lipid peroxidation has been recognized as a potential mechanism of cell injury. The use
of homogenized liver/brain tissue works as a rapid and convenient bench top bio-assay method for preliminary analysis of the activity of the compounds of interest which can be further confirmed using in vitro cell culture and in vivo models. In the present study, the MDA formation, an index of lipid peroxidation, significantly reduced when successive extracts were incubated with the tissue homogenates prepared from brain and kidney, in a concentration dependent manner with all the extracts except for hexane.

Table 5 Brain lipid peroxidation inhibition by extracts of D. regia.

<table>
<thead>
<tr>
<th>Conc. (ppm)</th>
<th>BHA</th>
<th>Hexane</th>
<th>EtOAc</th>
<th>Acetone</th>
<th>MeOH</th>
<th>Water</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85.2 ± 0.2</td>
<td>83.1 ± 1.7</td>
<td>58.3 ± 2.3</td>
<td>59.6 ± 1.7</td>
<td>57.6 ± 1.1</td>
<td>43.3 ± 2.8</td>
<td>20.4 ± 1.6</td>
<td>33.7 ± 4.2</td>
<td>25.4 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>87.3 ± 0.1</td>
<td>76.9 ± 1.8</td>
<td>69.8 ± 2.2</td>
<td>68.9 ± 0.7</td>
<td>69.8 ± 1.9</td>
<td>51.4 ± 2.1</td>
<td>33.7 ± 0.8</td>
<td>66.0 ± 0.9</td>
<td>40.1 ± 3.9</td>
</tr>
<tr>
<td>10</td>
<td>87.7 ± 0.1</td>
<td>67.3 ± 1.1</td>
<td>73.6 ± 0.6</td>
<td>71.7 ± 1.0</td>
<td>73.7 ± 2.4</td>
<td>64.8 ± 3.5</td>
<td>45.4 ± 2.5</td>
<td>75.5 ± 0.7</td>
<td>45.6 ± 0.1</td>
</tr>
<tr>
<td>25</td>
<td>88.8 ± 0.3</td>
<td>42.9 ± 2.7</td>
<td>79.4 ± 3.2</td>
<td>80.8 ± 0.8</td>
<td>81.9 ± 2.8</td>
<td>77.8 ± 2.1</td>
<td>53.0 ± 2.3</td>
<td>77.2 ± 2.2</td>
<td>70.8 ± 2.9</td>
</tr>
</tbody>
</table>

Three independent experiments were performed in triplicate at each concentration.

Data are expressed as mean ± SD.

I = crude extract; II = carotene hydrocarbon fraction; III = xanthophyll fraction.

Table 6 Kidney lipid peroxidation inhibition by extracts of D. regia.

<table>
<thead>
<tr>
<th>Conc. (ppm)</th>
<th>BHA</th>
<th>Hexane</th>
<th>EtOAc</th>
<th>Acetone</th>
<th>MeOH</th>
<th>Water</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85.8 ± 0.1</td>
<td>5.1 ± 0.4</td>
<td>26.5 ± 1.1</td>
<td>19.3 ± 0.1</td>
<td>24.9 ± 1.1</td>
<td>11.4 ± 0.1</td>
<td>26.4 ± 2.9</td>
<td>46.3 ± 1.1</td>
<td>39.7 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>86.3 ± 0.4</td>
<td>--</td>
<td>40.8 ± 0.1</td>
<td>24.2 ± 0.2</td>
<td>33.4 ± 1.3</td>
<td>13.1 ± 0.4</td>
<td>38.8 ± 8.5</td>
<td>67.6 ± 2.2</td>
<td>59.7 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>86.5 ± 0.2</td>
<td>--</td>
<td>57.3 ± 1.1</td>
<td>32.8 ± 2.4</td>
<td>53.4 ± 2.8</td>
<td>18.3 ± 0.7</td>
<td>53.2 ± 1.7</td>
<td>80.9 ± 0.1</td>
<td>71.1 ± 3.5</td>
</tr>
<tr>
<td>25</td>
<td>86.6 ± 0.0</td>
<td>--</td>
<td>71.9 ± 0.0</td>
<td>58.1 ± 0.1</td>
<td>74.1 ± 0.6</td>
<td>27.4 ± 0.1</td>
<td>60.2 ± 2.1</td>
<td>83.2 ± 0.0</td>
<td>78.3 ± 0.8</td>
</tr>
</tbody>
</table>

Three independent experiments were performed in triplicate at each concentration.

Data are expressed as mean ± SD.

I = crude extract; II = carotene hydrocarbon fraction; III = xanthophyll fraction.

Table 7 Inhibition of AAPH induced plasma oxidation by extracts of D. regia.

<table>
<thead>
<tr>
<th>Conc. (ppm)</th>
<th>BHA</th>
<th>Hexane</th>
<th>EtOAc</th>
<th>Acetone</th>
<th>MeOH</th>
<th>Water</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>76.9 ± 2.4</td>
<td>32.0 ± 0.8</td>
<td>28.1 ± 1.6</td>
<td>34.3 ± 0.8</td>
<td>50.5 ± 1.6</td>
<td>50.1 ± 0.8</td>
<td>26.2 ± 2.6</td>
<td>44.7 ± 4.1</td>
<td>26.2 ± 2.5</td>
</tr>
<tr>
<td>50</td>
<td>83.1 ± 1.6</td>
<td>37.6 ± 0.8</td>
<td>74.7 ± 4.0</td>
<td>56.5 ± 0.8</td>
<td>70.2 ± 0.8</td>
<td>73.0 ± 1.6</td>
<td>34.8 ± 1.6</td>
<td>55.8 ± 0.0</td>
<td>29.6 ± 0.8</td>
</tr>
<tr>
<td>100</td>
<td>86.5 ± 0.0</td>
<td>42.1 ± 0.8</td>
<td>92.6 ± 0.8</td>
<td>81.5 ± 0.8</td>
<td>73.0 ± 1.6</td>
<td>93.3 ± 0.0</td>
<td>23.7 ± 2.4</td>
<td>62.8 ± 1.6</td>
<td>34.3 ± 0.8</td>
</tr>
<tr>
<td>250</td>
<td>87.6 ± 0.0</td>
<td>32.0 ± 0.8</td>
<td>95.5 ± 1.5</td>
<td>93.2 ± 1.6</td>
<td>74.7 ± 0.8</td>
<td>96.6 ± 1.6</td>
<td>2.9 ± 0.2</td>
<td>65.7 ± 0.8</td>
<td>38.4 ± 1.6</td>
</tr>
</tbody>
</table>

Three independent experiments were performed in triplicate at each concentration.

Data are expressed as mean ± SD.

I = crude extract; II = carotene hydrocarbon fraction; III = xanthophyll fraction.

AAPH-induced lipid peroxidation

AAPH, a water-soluble azo-compound, thermally decomposes generating peroxyl radicals in the presence of oxygen at a constant rate thereby oxidizing plasma lipids (Cherubini et al. 1999; Abajo et al. 2004). The extent of plasma oxidation was measured by thiobarbituric acid reactive substance assay. Increasing concentrations of EtOAc, acetone, MeOH and H₂O extracts caused a significant concentration-dependent inhibition of AAPH-induced plasma oxidation reaching above 90% in case of EtOAc, acetone and H₂O at the highest concentration tested (Table 7). Minimum inhibition of plasma oxidation was observed with hexane extract. The activity increased from 32.0% (25 ppm) to 42.1% (100 ppm) and reduced to 32.0% at 250 ppm. Similar behavior was noticed in crude pigment extract where the activity slightly increased from 25 ppm (26.2%) to 30 ppm (34.9%) and plunged to 2.9% at 250 ppm. This behavior of crude pigment extract was also observed in the OH scavenging assay. Unlike in the kidney and brain models, the methanol extract was less effective compared to the others.

CONCLUSION

The results of the present study demonstrate that D. regia is a rich source of potential antioxidants and such effects may be related to the biochemical constituents in the flowers, the major ones being carotenoids, flavonoids, anthocyanins and other polyphenolic compounds. Though the flowers are successively extracted under high temperature conditions, persistence of high anti-oxidative activity indicates the pre-
sence of heat stable antioxidant phyto-constituents apart from the heat labile carotenoids and anthocyanins. Unlike tea plants (one of the most sought-after polyphenol reser* ves) that require specific agro-climatic conditions to grow, D. regia is naturally abundant in tropics and consists of a variety of radical scavenging phyto-constituents. The latter compounds can be employed in a range of hydrophilic and lipophilic formulations of food, pharmaceutical and cosmetic products requiring antioxidants. The unique combination of high amounts of both anthocyanins and carotenoids makes it an even more interesting source of highly effective antioxidants.

Fig. 8 Avenue of D. regia at the author’s institute illustrating the abundance of the raw material available.

ACKNOWLEDGEMENTS

Authors are grateful to Dr. V. Prakash, Director, CFTRI, for his encouragement for the work and Dr. Anjali Arun for kindly providing human plasma. JMV and KNC are grateful to Council for Scientific and Industrial Research, Govt., of India, for the fellowship grant.

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