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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 depending 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 anthelmintic (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 very 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 nutrient 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-

Fig. 1 Flowers of *Delonix regia* in full bloom.

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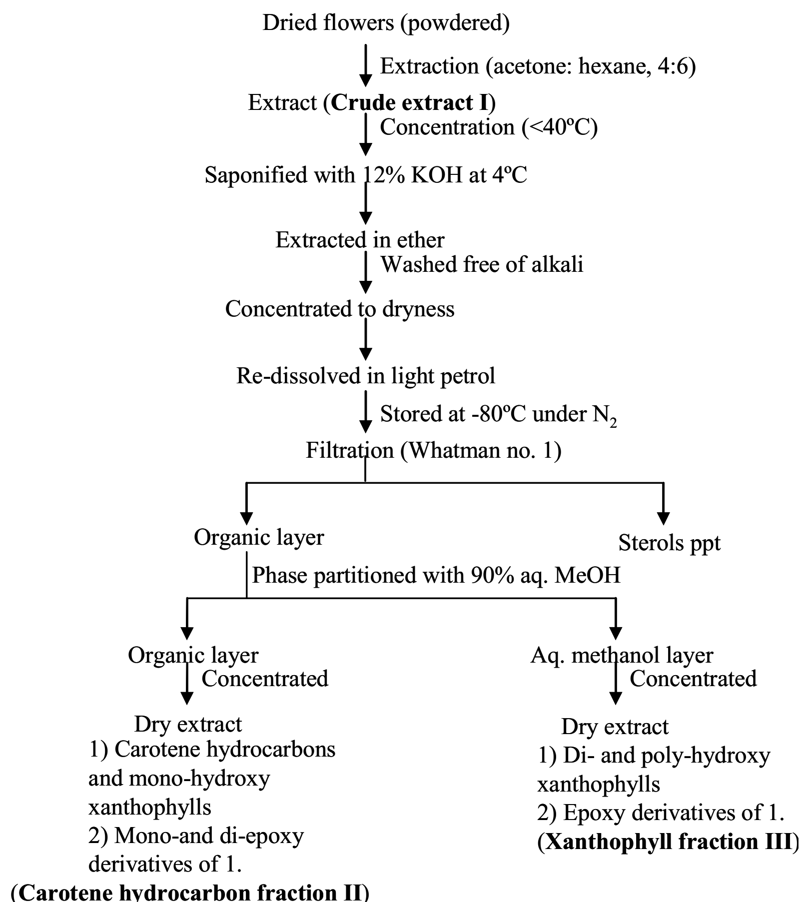

Fig. 2 Steps involved in the extraction of pigments from the flowers of *D. regia*.

sons, the consumption of red wine, rich in anthocyanins, is believed to lower cardiovascular diseases and related mortality (de Lorgeril *et al.* 2006). A growing body of literature points to the importance of natural antioxidants from many plants that may be used to reduce oxidative damage in the human body (Tsuda *et al.* 2004; Dominguez *et al.* 2005). In addition to their individual effects, the antioxidants interact in synergistic ways imparting "sparing effects" in which one antioxidant protects another against oxidative destruction (Langseth 1995). For these reasons, there is an upsurge of research in finding newer sources of effective natural antioxidants. The present study focuses on the antioxidant capacity of pigments and other phytochemicals extracted using different solvents from dried flowers of *D. regia* by chemical and biological models.

MATERIALS AND METHODS

Chemicals

2'azo bis (2-methylpropionamidine) dihydrochloride (AAPH), α,α-diphenyl-β-picrylhydrazyl (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*-Napthylethylenediamine and trichloroacetic acid (TCA) was purchased from Sisco Research Lab (Mumbai, India). Sulphanilamide was obtained from Loba Chemie (Mumbai, Inida). 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 -20ºC 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 (H_2O). 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 -20ºC till further use.

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

Total carotenoid determination

Carotene 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:

$$C = (\text{OD at 436 nm} \times 454) / (196 \times L \times W)$$

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 were 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:

$$\text{Tacy (\% w/w)} = (A \times MW \times DF \times V \times 100) / \varepsilon \times l \times W$$

where: $A = (A_{520nm} - A_{700nm})$ pH 1.0 − $(A_{520nm} - A_{700nm})$ pH 4.5; MW = 449.2 g mol^{-1} for cyanidin-3-glucoside; DF = dilution factor; V = total volume of the extract; ε = 26900 molar extinction coefficient, in L × mol^{-1} × cm^{-1}, for cyanidin-3-glucoside; l = 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 µL) was mixed with 225 µL methanol and 1.0 mL Folin-Ciocalteu reagent (0.2 M) and allowed to stand at room temperature (25ºC ± 0.5ºC) 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 $FeSO_4.7H_2O$ and EDTA), 2-deoxyribose (10 mM), H_2O_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) (Marcocci *et al*. 1994). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with *n*-naphthyl ethylenediamide (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 chlorofom 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 10 mL distilled water followed by 40 mL 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 bleaching of β-carotene using the formula: $AA = 100\,[1-(A_0-A_t)/(A°_0-A°_t)]$, where A_0 and $A°_0$ are the absorbance values measured at zero time for test sample and control respectively; A_t and $A°_t$ 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 clear 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 37ºC 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_{50} 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, flavanoids, etc. Since these compounds are believed to be effecttive 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 ⅓rd 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 ¼ 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 antioxidant 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*.

Extract	Yield (g% dw)	TP (mg GAE g^{-1})
Hexane	3.3	0.5 ± 0.01
EtOAc	3.1	1.3 ± 0.01
Acetone	3.9	2.3 ± 0.02
MeOH	31.8	24.6 ± 0.03
Water	5.2	4.8 ± 0.03
I	6.3	nd
II	4.6	nd
III	1.2	nd

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.

Plant species	Part used	TP (mg GAE g^{-1})	Conc. (ppm)	DPPH scavenging (%)	Reference
Delonix regia	Flowers	169.67	100	71.93	Aqil *et al*. 2006;
	Petals	33.5	100	84.17	Present study
Camellia sinesnsis	Leaves	130.6	4.14	50	Turkman *et al*. 2006
Punica granatum	Fruit peel	44.0	100	95	Singh *et al*. 2002
Vitis vinifera	Pomace	35.7	100	90	Murthy *et al*. 2002
Ginkgo biloba	Extract (EGb761)	--	45.1	50	Silva *et al*. 2005
Panax notoginseng	Root	--	3000	50	Zhao *et al*. 2006

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

Assay system	Conc. (ppm)	% activity AA	GA	BHA	BHT
DPPH• model	1	17.4 ± 0.9	41.5 ± 0.1	22.5 ± 0.6	13.2 ± 2.9
	5	68.2 ± 3.2	93.8 ± 0.2	68.4 ± 1.1	45.7 ± 2.1
	10	96.1 ± 0.4	94.1 ± 0.1	88.5 ± 0.7	71.4 ± 0.7
	25	96.5 ± 0.2	94.3 ± 0.1	91.6 ± 0.4	88.7 ± 0.5
	50	96.5 ± 0.1	94.8 ± 0.2	91.3 ± 0.2	91.3 ± 0.5
	100	96.5 ± 0.1	95.2 ± 0.1	91.3 ± 0.6	91.7 ± 0.1
AAPH-induced plasma oxidation	5	39.9 ± 1.0	51.7 ± 1.0	34.6 ± 1.5	66.8 ± 3.5
	10	50.0 ± 0.5	63.6 ± 1.0	59.8 ± 0.5	71.7 ± 0.5
	25	61.2 ± 3.5	74.1 ± 2.0	76.9 ± 2.4	76.6 ± 0.5
	50	63.9 ± 0.5	71.4 ± 0.2	83.1 ± 1.6	79.7 ± 1.0
	100	67.8 ± 1.0	71.7 ± 1.5	86.5 ± 0.0	83.2 ± 1.0
OH• scavenging assay	5	*	69.9 ± 4.1	77.6 ± 0.7	69.1 ± 0.3
	10	*	81.6 ± 0.5	84.1 ± 0.2	85.9 ± 0.1
	25	*	92.1 ± 0.3	94.8 ± 0.7	95.2 ± 0.7
	50	*	97.2 ± 1.0	98.7 ± 0.3	99.4 ± 0.1
	100	*	101.5 ± 1.0	103.2 ± 0	106.4 ± 0.2

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

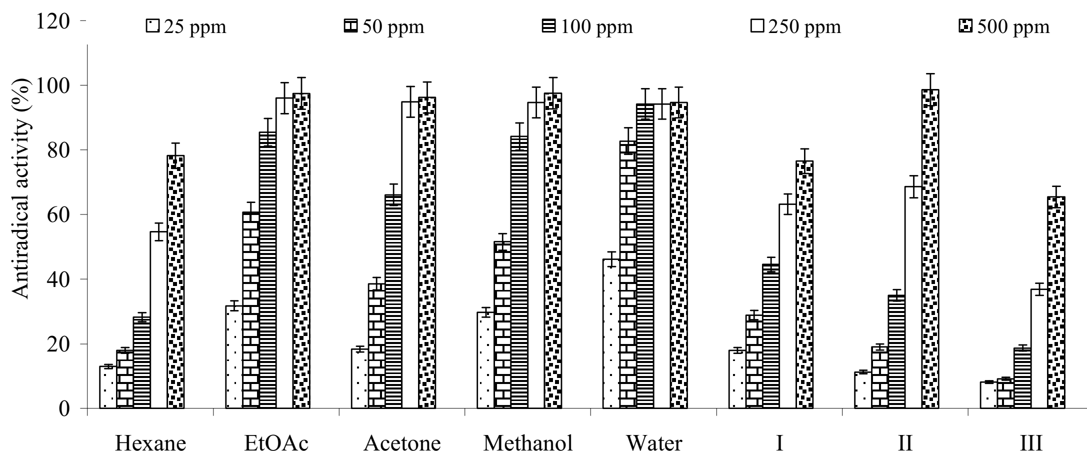

Fig. 3 DPPH• scavenging activity of extracts of *D. regia* flowers. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$. I = crude extract; II = carotene hydrocarbon fraction; III = xanthophylls fraction.

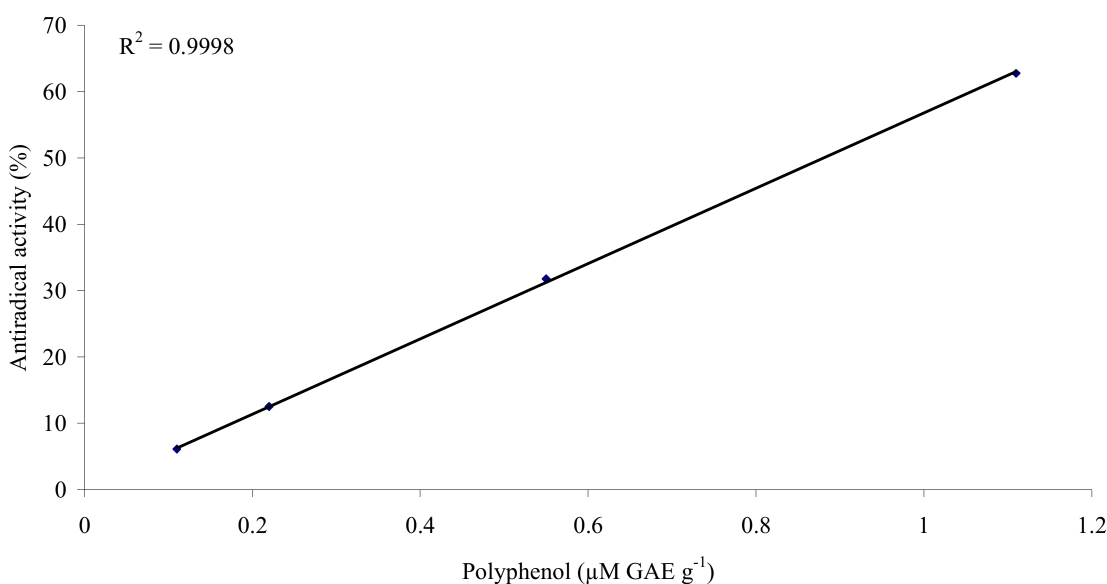

Fig. 4 Relationship between the polyphenol content and antioxidant activity of *D. regia*. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$. The polyphenol content is expressed as μM GAEg^{-1} of flowers.

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 IC_{50} of the hexane, EtOAc, acetone, MeOH, H_2O, 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 (IC_{50} 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 ($R^2 = 0.9998$) 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 ($R^2 = 0.989$)(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 Fe^{3+}/ferricyanide complex to the ferrous form. The Fe^{2+} 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 Fe^{3+}-Fe^{2+} 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 > H_2O > carotene hydrocarbon fraction > crude extract. The reducing power of the extracts may be due to their hydrogen donating ability (Shimada *et al.* 1992).

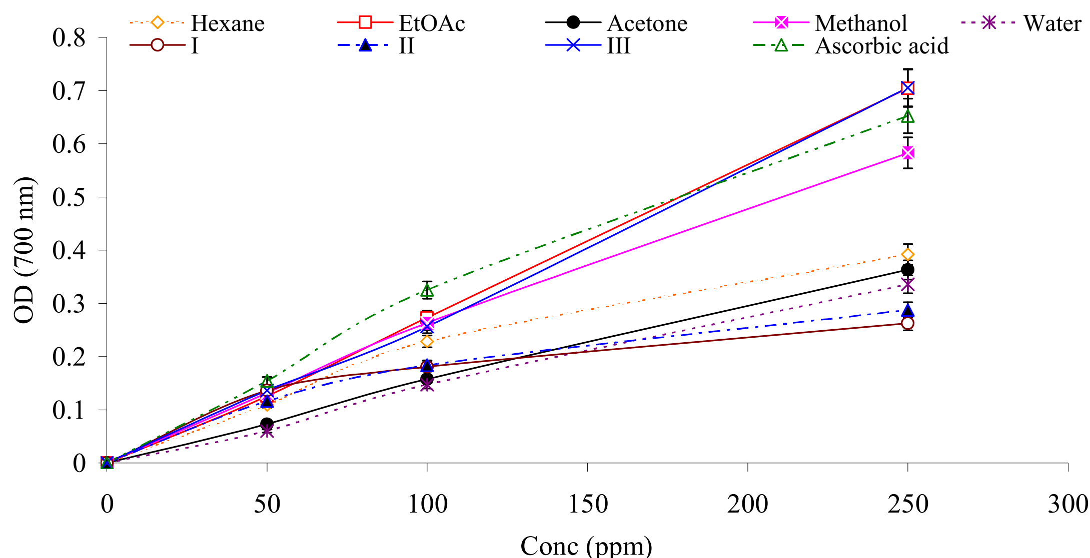

Fig. 5 Iron reducing capacity of extracts of *D. regia* flowers. Three independent experiments were performed in triplicate at each concentration and data presented as the average where the deviation was within 5%. I = crude extract; II = carotene hydrocarbon fraction; III = xanthophylls fraction.

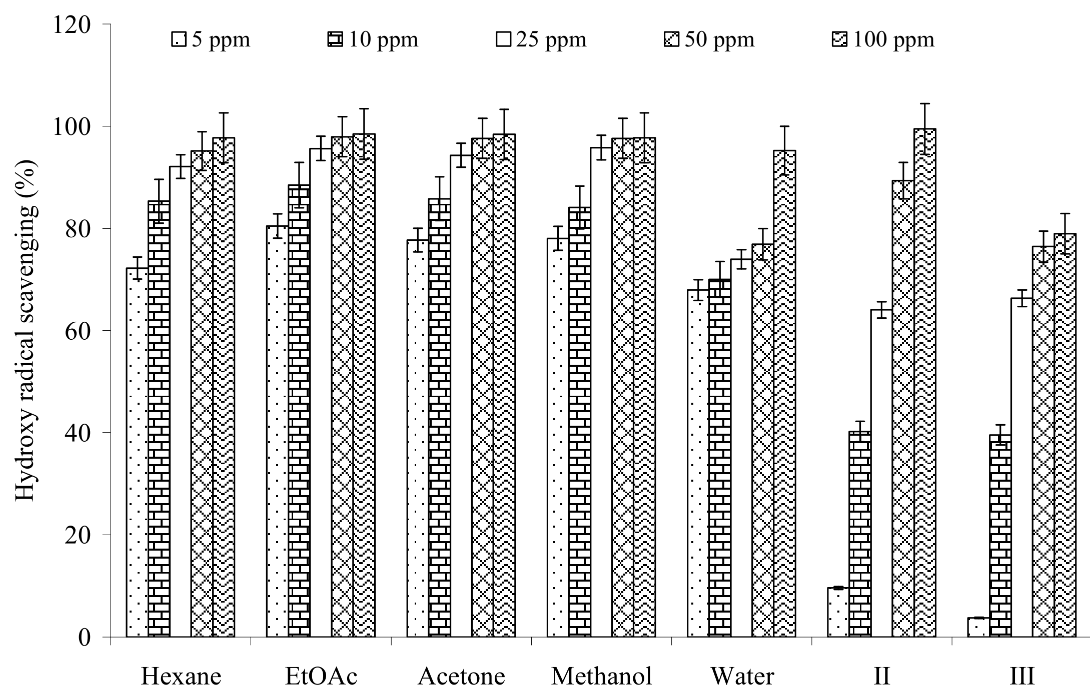

Fig. 6 Hydroxyl radical scavenging activity of extracts of *D. regia* flowers. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$. I = crude extract; II = carotene hydrocarbon fraction; III = xanthophylls fraction.

Hydroxyl radical scavenging

Living tissues are constantly subjected to oxidative insults by various means resulting in the formation of oxygen radicals. In biochemical systems, $O_2^{\bullet}$ and H_2O_2 react together to form the hydroxyl radical, $OH^{\bullet}$. 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 $OH^{\bullet}$ 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 $OH^{\bullet}$ 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 $OH^{\bullet}$-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 ($O_2^{\bullet -}$) to form a peroxynitrite anion ($ONOO^-$), which is more toxic than $O_2^{\bullet}$ 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-

Table 4 Nitric oxide scavenging activity of extracts of *D. regia*.

Conc. (ppm)	% NO scavenging activity								
	BHA	**Hexane**	**EtOAc**	**Acetone**	**MeOH**	**Water**	**I**	**II**	**III**
1	39.1 ± 0.2	16.8 ± 1.07	52.9 ± 1.6	18.0 ± 2.1	42.8 ± 1.3	60.9 ± 2.1	22.6 ± 0.6	29.8 ± 0.9	31.7 ± 1.6
5	49.9 ± 2.6	54.1 ± 0.5	70.0 ± 0.0	63.5 ± 1.1	70.5 ± 1.3	71.7 ± 2.4	48.1 ± 1.9	58.3 ± 0.6	52.1 ± 0.3
10	52.2 ± 0.0	76.5 ± 0.5	70.0 ± 0.0	72.1 ± 0.8	75.9 ± 1.3	73.8 ± 0.0	70.8 ± 3.2	78.7 ± 0.6	74.1 ± 1.9
25	54.1 ± 0.0	80.4 ± 1.8	70.5 ± 0.8	74.7 ± 0.2	77.2 ± 0.0	74.3 ± 0.2	83.7 ± 2.6	81.0 ± 0.6	83.3 ± 1.3
50	56.7 ± 1.6	93.9 ± 0.5	67.9 ± 2.4	71.2 ± 1.3	77.4 ± 0.2	73.6 ± 0.3	93.1 ± 0.65	75.9 ± 1.3	73.1 ± 0.6

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.

ment extract showed similar results (**Table 4**) exhibiting 93.9% and 93.1% NO scavenging respectively at 50 ppm. At this concentration, the inhibitory activity of BHA, EtOAc, acetone, MeOH, H_2O, carotene hydrocarbon fractions and the xanthophyll fraction were 56.7, 67.9, 71.2, 77.4, 73.6, 75.9 and 73.1%, respectively. The activity profile of the extracts almost matched at all the concentrations (**Table 4**). Thus the present study shows that all the extracts effectively suppress NO production under the prevailing conditions. The activity of the extracts against NO production was significantly better than that of BHA. The effect of the extracts on the accumulation of nitrite can also be a consequence of the reaction of the extract with other oxides of nitrogen, i.e. NO_2, N_2O_3, N_2O_4 and $OONO^-$, which are the possible intermediates in the oxidation of nitric oxide to nitrite (Marcocci *et al*. 1994). Non-polar extracts such as that from EtOAc, acetone and carotene hydrocarbon fraction as well as the xanthophylls fraction showed a retarded free radical scavenging activity at the highest levels (50 ppm) tested. This behavior is probably because of the dual nature of diazotization and coupling reactions that fluctuate based on the availability of phenolic groups. NO scavenging properties of the extracts suggest that they can be potential therapeutic agents for the control of oxidative and non-oxidative damage caused by nitric oxide.

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 related to the thermally induced formation of linoleic acid hydroperoxides. Hexane extract and H_2O extract inhibited β-carotene bleaching by 57.7% and 70.4% respectively at 50 ppm. Other extracts were nearly half as active as water extract at the same concentration. This suggests that the activity may be partly due to the water-soluble components of *D. regia*. The inhibition of β-carotene consumption by BHA after 180 min of incubation was 91.6% at 2.5 ppm (**Fig. 7**). The mechanism of bleaching of β-carotene 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 (Brighthope 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

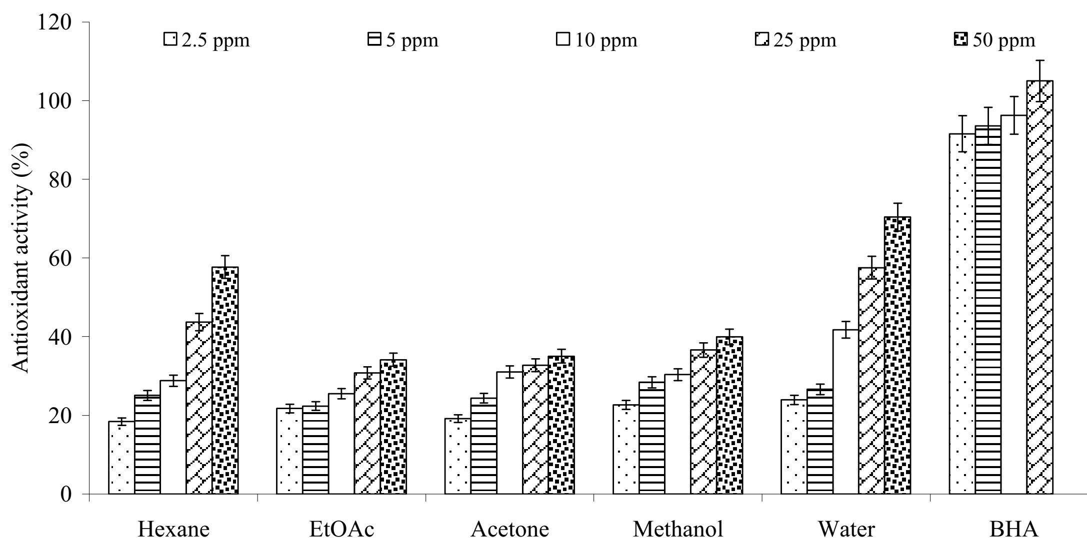

Fig. 7 Antioxidant activity of successive extracts of *D. regia* flowers by BCLAMS. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$.

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

Conc. (ppm)	% inhibition of brain lipid peroxidation								
	BHA	**Hexane**	**EtOAc**	**Acetone**	**MeOH**	**Water**	**I**	**II**	**III**
1	85.2 ± 0.2	83.1 ± 1.7	58.3 ± 2.3	59.6 ± 1.7	57.6 ± 1.1	43.3 ± 2.8	20.4 ± 1.6	33.7 ± 4.2	25.4 ± 0.7
5	87.3 ± 0.1	76.8 ± 1.9	69.8 ± 2.2	68.9 ± 0.7	69.8 ± 1.9	51.4 ± 2.1	33.7 ± 0.8	66.0 ± 0.9	40.1 ± 3.9
10	87.7 ± 0.1	67.3 ± 1.1	73.6 ± 0.6	71.7 ± 1.0	73.7 ± 2.4	64.8 ± 3.5	45.4 ± 2.5	75.5 ± 0.7	45.6 ± 0.1
25	88.8 ± 0.3	42.9 ± 2.7	79.4 ± 3.2	80.8 ± 0.8	81.9 ± 2.8	77.8 ± 2.1	53.0 ± 2.3	77.2 ± 2.2	70.8 ± 2.9

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.

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

Conc. (ppm)	% inhibition of kidney lipid peroxidation								
	BHA	**Hexane**	**EtOAc**	**Acetone**	**MeOH**	**Water**	**I**	**II**	**III**
1	85.8 ± 0.1	5.1 ± 0.4	26.5 ± 1.1	19.3 ± 0.1	24.9 ± 1.1	11.4 ± 0.1	26.4 ± 2.9	46.3 ± 1.1	39.7 ± 0.7
5	86.2 ± 0.4	--	40.8 ± 0.1	24.2 ± 0.2	33.4 ± 1.3	13.1 ± 0.4	38.8 ± 8.5	67.6 ± 2.2	59.7 ± 0.8
10	86.5 ± 0.2	--	57.3 ± 1.1	32.8 ± 2.4	53.4 ± 2.8	18.3 ± 0.7	53.2 ± 1.7	80.9 ± 0.1	71.1 ± 3.5
25	86.6 ± 0.0	--	71.9 ± 0.0	58.1 ± 0.1	74.1 ± 0.6	27.4 ± 0.1	60.2 ± 2.1	83.2 ± 0.0	78.3 ± 0.8

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.

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

Conc. (ppm)	% Inhibition of AAPH-induced plasma oxidation								
	BHA	**Hexane**	**EtOAc**	**Acetone**	**MeOH**	**Water**	**I**	**II**	**III**
25	76.9 ± 2.4	32.0 ± 0.8	28.1 ± 1.6	34.3 ± 0.8	50.5 ± 1.6	50.1 ± 0.8	26.2 ± 2.46	44.7 ± 4.1	26.2 ± 2.5
50	83.1 ± 1.6	37.6 ± 0.8	74.7 ± 4.0	55.6 ± 0.8	70.2 ± 0.8	73.0 ± 1.6	34.8 ± 1.64	55.8 ± 0.0	29.6 ± 0.8
100	86.5 ± 0.0	42.1 ± 0.8	92.6 ± 0.8	81.5 ± 0.8	73.0 ± 1.6	93.3 ± 0.0	27.3 ± 2.46	62.8 ± 1.6	34.3 ± 0.8
250	87.6 ± 0.0	32.0 ± 0.8	95.5 ± 1.5	93.2 ± 1.6	74.7 ± 0.8	96.6 ± 1.6	2.9 ± 0.82	65.7 ± 0.8	38.4 ± 1.6

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.

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 extract. *n*-Hexane extract showed an inverse relation to the activity against brain lipid peroxidation (**Table 5**). The activity markedly reduced from 83.1% (at 1 ppm) to 42.9% (at 25 ppm) in brain homogenates. This trend was also noticed with kidney tissue where absolutely no activity was observed beyond 1 ppm (5.1%), which was marked by the very intense color owing to high concentration of MDA diadduct formation. This behavior can probably be correlated to the pro-oxidant activity of the extract which in turn may be due to its lipid rich nature especially when the response is non-linear with the concentration. However, it was observed that below 1 ppm the hexane extract exerted a significant effect against kidney lipid peroxidation. Maximum protection (81.9%) towards brain lipid peroxidation was offered by the methanol extract (81.9%) at 25 ppm, which was similar in effect to that of BHA at 1 ppm. This was followed by acetone (80.8%), ethyl acetate (79.4%) and water (77.8%). The carotene hydrocarbons fraction showed 83.3% protection against kidney lipid peroxidation followed by xanthophyll fraction (78.3%) and crude pigment extract (60.2%). Similarly, 77.3%, 70.8% and 53.0% respectively were registered for the three pigment extracts in the case of brain lipid peroxidation (**Tables 5, 6**). While the successive extracts showed higher activity against brain lipid peroxidation, the pigment extracts exerted a greater protection against kidney lipid peroxidation. This is possibly due to the involvement of different mechanisms in the two tissue systems. Similarly, the water extract did not offer a promising protection against kidney lipid peroxidation unlike in other models. However, the exact reason for this behavior of the extracts in different systems needs further studies.

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 H_2O extracts caused a significant concentration-dependent inhibition of AAPH-induced plasma oxidation reaching above 90% in case of EtOAc, acetone and H_2O 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 50 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. That is to say that there is no correlation between the total phenols and the activity in this system, indicating the possible involvement of compounds other than phenolics in bringing about inhibition of plasma oxidation. The carotene hydrocarbon fraction showed 65.7% activity at 250 ppm and the xanthophyll fractions did not significantly inhibit AAPH-induced plasma oxidation as compared to BHA. Summarized, the activity of hexane, EtOAc, acetone, crude pigment extract and xanthophyll fraction was nearly ⅓rd of that of BHA at 25ppm while that of MeOH, H_2O and carotene hydrocarbon fraction was ⅔^{rds} of that of BHA.

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-

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

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 reserves) 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 antioxidants and natural colors. The results of this study can be further exploited to know the mechanism of action of individual constituents, which helps in utilizing this naturally abundant antioxidant/natural colorant resource. Since the flowers of *D. regia* are produced in abundance (**Fig. 8**) seasonally with no or very few leaves, collection and separation of the same appears practically feasible for the extraction of pigments at a commercial scale.

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