Antioxidant and free radical scavenging activity of *Pithecellobium dulce* (Roxb.) Benth wood bark and leaves

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ABSTRACT

Background: Several studies are going on worldwide directed towards finding natural antioxidants from plant origin which possess strong antioxidant activity. **Objective:** The aim of the present study was to study *in vitro* antioxidant and free radical scavenging activity of *Pithecellobium dulce* (Roxb.) Benth. **Method**: Methanol and 70% acetone extracts of wood bark and leaves of *Pithecellobium dulce* were evaluated for their total phenolic & flavonoid content, antioxidant and radical scavenging activity. **Results:** Total antioxidant activity (IC_{50} values) in DPPH assay by methanol extract of bark (MB), acetone extract of bark (AB), methanol extract of leaf (ML) and acetone extract of leaf (AL) shown 150.23 ± 2.8, 16.83 ± 0.38, 250.32 ± 4.8 and 18.30 ± 0.43 µg/ml respectively. The IC_{50} values for scavenging of free radicals like for hydroxyl, superoxide, nitric oxide, hydrogen peroxide, singlet oxygen and hypochlorous acid by MB, AB, ML and AL were studied. The extract was found to be a good iron chelator. The reducing power was increased with increasing amounts of extract. The MB, AB, ML & AL extracts (5 mg/ml) yielded 0.129 ± 0.11, 0.190 ± 0.14, 0.084 ± 0.24 and 0.115 ± 0.25 µg/ml gallic acid-equivalent phenolic content and 0.43 ± 0.01, 0.23 ± 0.01, 0.90 ± 0.01 and 0.25 ± 0.01 µg/ml quercetin-equivalent flavonoid content respectively. **Conclusion:** Thus, our findings provide evidence that the methanolic and 70% acetone extracts of *P. dulce* wood bark and leaves are potential source of natural antioxidants and some extent validated its medicinal potential.

Keywords: *Pithecellobium dulce,* leguminosae, antioxidant activity, polyphenols, free radicals, flavonoid.

INTRODUCTION

Pithecellobium dulce (Roxb.) Benth is an evergreen tree widely distributed in the greater part of India and is also found in Southeast Asia. *P. dulce* is now commonly grown as a hedge plant throughout India and is locally called 'Jungal jalebi'.[1] The plant is reported to be used as astringent in dysentery, febrifuge and also used as abortificient, antidiabetic, anticonvulsant, antiulcer and larvicide. It is also useful in dermatitis and eye inflammation, indigestion, intestinal disorder, ear ache, leprosy and tooth ache. The leaves can be applied as plasters for pain and veneral sores.[2] Fruits of plant are consumed as food in many part of India, because of sweet taste and medicinal properties.

Phytochemical investigation of bark and leaves had revealed the presence of β-sitosterol, saponin glycosides, oleanolic and echinocystic acids as sapogenins, echinocystic acid, bisdesmoside, dulcin, triterpenoids, acylated triterpenoid saponin, flavanoids, saccharrides, long chain aliphatic hydrocarbons, and tannins.[3–11] Oxidative stress is an important contributor to the pathophysiology of a variety of pathological conditions such as cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases.[12] Human body has multiple mechanisms especially enzymatic and non-enzymatic antioxidant systems which protect the cellular molecules against reactive oxygen species (ROS) induced damage.[13] However the

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innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in human body. The objective of present work was to verify the antioxidant activity and free radical scavenging potential of wood bark and leaves of *P. dulce* by employing various *in vitro* test models.

MATERIALS AND METHODS

Plant material

The wood bark and leaves of *P. dulce* were collected from Mumbai, India. Plant material was authenticated from Dr. G. Iyer, Ramnarain Ruia College, Mumbai. Authentic sample specimen (ICT/2010/3) was deposited in Institute of Chemical technology.

Chemicals

Acetyl acetone, acetone, ammonium acetate, ammonium thiocyanate, ascorbic acid, butylated hydroxyl toluene (BHT), catalase, *N*, *N*-dimethy l–4-nitrosoaniline (RNO), 2, 2-dipheny l-1-picryl-hydrazyl (DPPH), potassium persulfate ($K_2S_2O_8$), ethylenediamine tetraacetic acid (EDTA), ascorbic acid, 2-deoxy-2-ribose, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), sulfanilamide, naphthylethylenediamine dihydrochloride (NED), L-histidine, sodium pyruvate and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide, potassium hexacyanoferrate, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), butylated hydroxytoluene (BHT), sodium hypochlorite (NaOCl), aluminium chloride ($AlCl_3$), ammonium iron (II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_{26}H_2O$), potassium nitrite ($KNO_2$), *N*, *N* dimethyl-4-nitrosoaniline and xylenol orange were obtained from Merck, Mumbai, India. Gallic acid, quercetin, lipoic acid and curcumin were obtained from Natural Remedies, Bangaluru, India. Ferrous sulfate and catalase were obtained from Hi Media Laboratories Pvt. Ltd, Mumbai, India. All other reagents used were of analytical grade.

Solvent extraction

Collected plant materials were dried in tray dryer at 55 ^oC for 24 hours, finely powdered and defatted by petroleum ether. After defatting, dried plant powder was extracted by Soxhlet apparatus successively with methanol followed by 70% acetone for 3 hour each. Each time before extracting with the next solvent, the plant material was dried in hot air oven at 40 ^oC for appropriate time. The extracts were concentrated till dryness by rotary vacuum evaporator. The dry methanol extract of bark (MB) & leaves (ML) and 70% acetone extract of bark (AB) & leaves (AL) were weighed and the percentage yield was expressed in terms of air dried weight of plant materials. The extracts thus obtained were used directly for the estimation of total phenolic & flavonoid content, antioxidant and free radical scavenging activity through various *in vitro* methods. For all assays extracts were dissolved by sonication or stirring at room temperature.

Free radical scavenging activity on DPPH

The antioxidant activity of the methanol and 70% acetone extract was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH.[14,15] A methanol and 70% acetone extract of samples at various concentrations (0–250 μg/ml) were added to 5 ml of a 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at 27 ^oC. The absorbance of the sample was measured at 517 nm. All experiments were repeated three times. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula

$$\%\ \text{DPPH radical scavenging activity} = \{(\text{control OD} - \text{sample OD})/\text{control OD}\} \times 100$$

Antioxidant activity in linoleic acid emulsion system

Peroxyl radicals are formed by a direct reaction of oxygen with alkyl radicals. Decomposition of alkyl peroxides also results in peroxyl radicals. Peroxyl radicals are good oxidising agents having more than 1000 mV of standard reduction potential.[16] They can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation.[17] The antioxidant activity of methanol and 70% acetone extract was determined using the earlier described thiocyanide method.[14] Each sample (500 μg) in 0.5 ml of absolute ethanol was mixed with 0.5 ml of 2.51% linoleic acid in absolute ethanol, 1 ml of 0.05 M phosphate buffer (pH 7.0), and 0.5 ml of distilled water and placed in a screw capped tube. The reaction mixture was incubated in an oven 40 ^oC. Aliquots of 0.1 ml were taken at every 12 h during incubation and the degree of oxidation was measured by sequentially adding

ethanol (9.7 ml, 75%), ammonium thiocyanate (0.1 ml, 30%) and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl). After the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. All experiments were repeated three times. The antioxidant activity was calculated as percentage of inhibition relative to the control.

AA = 100 − (sample absorbance at 48 h − sample absorbance at 0 h/control absorbance at 48 h − control absorbance at 0 h) × 100

Hydroxyl radical scavenging

This was assayed as described by Elizabeth and Rao.[18] The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); EDTA (100 μM); H_2O_2 (1.0 mM); ascorbic acid (100 μM) and various concentrations (0.0–200.0 μg/ml) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol, a classical $OH^{\cdot}$ scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

Superoxide radical scavenging

This activity was measured by the reduction of NBT according to a previously reported method by Fontana.[19] The nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations (0.0–100.0 μg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. All tests were performed three times. Quercetin was used as positive control.

Nitric oxide scavenging

The nitric oxide-scavenging activity of the methanol and 70% acetone extract was determined according to the earlier described method.[20,21] In this experiment, 1 ml of sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with *P. dulce* extract (2 mg/ml) dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extract but with an equivalent volume of water, served as control. Following the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read spectrophotometrically at 546 nm. All experiments were repeated three times. Curcumin was used as positive control.

Hydrogen peroxide scavenging

The hydrogen peroxide scavenging activity of the methanol and 70% acetone extracts was determined according to the earlier described method.[20,21] The hydrogen peroxide scavenging of the extract may be attributed to its phenolic contents as well as other active components such as anthocyanins, tannins and flavonoids which can donate electrons to hydrogen peroxide, thus neutralizing it to water.[22] 1.4 ml of each extract at various concentrations (0.0–50.0 μg/ml) in distilled water was added to 0.6 ml of the hydrogen peroxide solution (40 mM in phosphate buffer pH 7.4). The absorbance of mixture was noted at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide solution. The results were compared with ascorbic acid (control) as μg/g dry weight. All experiments were repeated three times. Percentage of hydrogen peroxide scavenging by the extracts and standard was calculated by following formula

$$\%\ \text{scavenged of hydrogen peroxide} = (A_o - A_1)/A_o \times 100$$

Where A_o is the absorbance of the control and A_1 the absorbance of the mixture containing either the extract or standard.

Singlet oxygen scavenging

The production of singlet oxygen ($^1O_2$) was determined by monitoring *N*, *N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method.[23,24] Singlet oxygen was generated by a reaction between NaOCl and H_2O_2, and the bleaching of RNO was monitored at 440 nm. The reaction mixture

contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2, 50 mM histidine, 10 μM RNO and various concentrations (0.0–200.0 μg/ml) of sample in a final volume of 2 ml. It was incubated at 30 °C for 40 min and the decrease in RNO absorbance was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed three times.

Hypochlorous acid scavenging

Hypochlorous acid (HOCl) was prepared immediately before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 M^{-1} cm^{-1}. The assay was carried out as described by Aruoma and Halliwellwith minor changes.[25] The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 μM), HOCl (8.4 mM) and increasing concentrations (0.0–250.0 μg/ml) of plant extract. The assay mixture was incubated at 25 °C for 20 min and the absorbance was measured against an appropriate blank. All tests were performed three times. Ascorbic acid, a potent HOCl scavenger, was used as a reference.[26]

Fe^{2+} chelation

The ferrous ion chelating activity was evaluated by a standard method with minor changes.[21] The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations (0.0–80.0 μg/ml) of plant extract were added to 12.5 μM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 μM). The mixture was shaken vigorously and incubated for 20 min at room temperature and then the absorbance was measured at 562 nm. All tests were performed three times. EDTA was used as a positive control.

Reducing power

The reducing power of the methanol and 70% acetone extract was determined by the method reported by Siddhuraju, Mohan, and Becker.[27] 0.0–40.0 μg/ml of extracts in 1 ml of phosphate buffer with 5 ml of 0.2 M phosphate buffer (pH 6.6) and 5 ml of 1% potassium ferric cyanide solution were incubated at 50 °C for 20 min. After the incubation, 5 ml of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5 ml) was mixed with 5 ml of distilled water and 0.5 ml of 0.1% ferric chloride was added. Then the absorbance of reaction mixture was noted spectroscopically at 700 nm. All tests were performed three times.

Determination of total phenolic content

The total phenolic content was determined by spectrophotometrically (Jasco, spectrophotometer model 1575) under UV 760 nm.[28] 20 μl (5 mg/ml) of extract solution was mixed with 1.6 ml distilled water and 100 μl of Folin-Ciocalteu reagent, followed by addition of 300 μl of Na_2CO_3 solution (20%) after 1 minute. Subsequently, the mixture was incubated at 40 °C for 30 min. Gallic acid was used as a standard for calibration curve. The total phenolic content was expressed as gallic acid equivalents using the linear equation method. All tests were performed three times.

Determination of total flavonoid content

Total flavonoid was estimated as according to the earlier described method.[29] Briefly, 0.5 ml solution of the extract in methanol (5 mg/ml) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water, and then left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in a double beam spectrophotometer (Jasco V-530). Total flavonoid content was calculated as quercetin from a calibration curve. All tests were performed three times.

Statistical analysis

Results were evaluated by Prism software. Statistical analyses were carried out by one way ANOVA (Graph Pad Prism 5.01 Software). The results were expressed (where appropriate) as mean ± standard deviation of three analysis. The IC_{50} values were compared by student t tests. $P < 0.05$ was considered significant.

RESULTS

DPPH radical scavenging

The results of free radical scavenging activity of the methanol and 70% acetone extract of bark and leaves of *P. dulce* are shown in Figure 1. The decrease in absorbance of the DPPH radical was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow. A lower value of IC_{50} indicates a higher antioxidant activity DPPH radical scavenging activity of each extracts is directly proportional

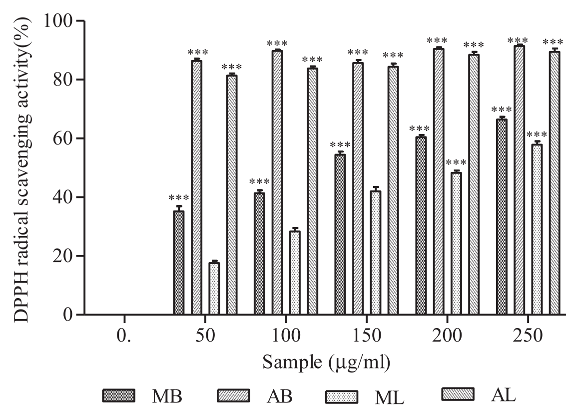

Figure 1. Free radical scavenging activity on DPPH.
Free radical scavenging activity on DPPH of the *P. dulce* plant extracts. The data represents the percentage inhibition of DPPH radical. The results are mean ± S.D. of three parallel measurements. $^{***}p < 0.001$ vs 0 μg/ml. IC_{50} of MB, AB, ML and AL = 150.23 ± 2.8, 16.83 ± 0.38, 250.32 ± 4.8 and 18.30 ± 0.43 μg/ml respectively.

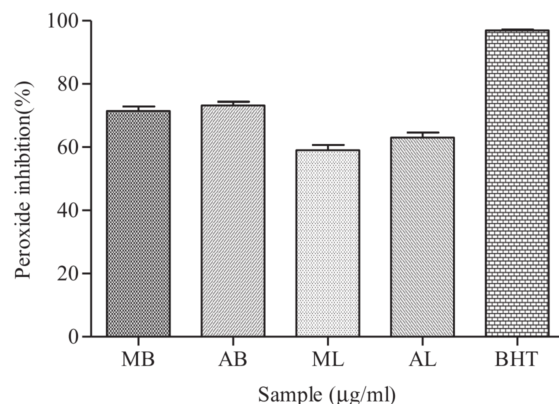

Figure 2. Antioxidant activity in linoleic acid emulsion system.
Antioxidant activity in linoleic acid emulsion system of the *P. dulce* plant extracts and the reference compound BHT. The data represent the percentage inhibition of peroxyl radicals. The results are mean ± S.D. of three parallel measurements. $^{***}p < 0.001$ vs BTH μg/ml. At 16 μg/ml concentration in the final reaction mixture of MB, AB, ML, AL extracts and BHT standard exhibited 70.4, 72.8, 59.3, 63.7 and 97% respectively.

to the concentration of total phenolics including tannins of respective extracts. Percentage DPPH radical scavenging activities of methanol extracts of bark and leaves are dose dependent. IC_{50} values for MB, AB, ML and AL were 150.23 ± 2.8, 16.83 ± 0.38, 250.32 ± 4.8 and 18.30 ± 0.43 μg/ml respectively. At 100 μg/ml radical scavenging for MB, AB, ML and AL were 41.5, 89.3, 28.9 and 83.2% respectively. This radical scavenging activity of extracts could be related to the nature of phenolics.

Antioxidant activity in linoleic acid emulsion system

As lipid oxidation of cell membranes increases, the polarity of lipid phase surface charge and formation of protein oligomers increase; and molecular mobility of lipids, number of SH groups, and resistance to thermal denaturation decreases. Malonaldehyde, one of the lipid oxidation products, can react with free amino group of proteins, phospholipid, and nucleic acids leading to structural modification, which induce dysfunction of immune systems. The antioxidant effect of each extracts and BHT on the peroxidation of linoleic acid was investigated and the results are shown in Figure 2. At a concentration 16 μg/ml in the final reaction mixture of MB, AB, ML and AL extracts exhibited 70.4, 72.8, 59.3 and 63.7% respectively peroxidation of linoleic acid after incubation for 48 h. However, those values were significantly lower ($P < 0.001$) than those of the positive controls BHT (97%).

Hydroxyl radical scavenging

This assay shows the abilities of the extract to inhibit hydroxyl radical-mediated deoxyribose degradation in an Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in Figure 3. The IC_{50} values (Table 1) of extract of MB, AB, ML, AL and standard were 175.87 ± 11.4, 110.43 ± 9.56, 200.12 ± 14.97, 135.04 ± 10.61 and 565.05 ± 18.12 μg/ml respectively. At 100 μg/ml, the percentage inhibition of MB, AB, ML and AL were 35.23, 49.03, 29.97 and 43.97% respectively whereas that of manitol was 20.6%. The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of chain reaction.

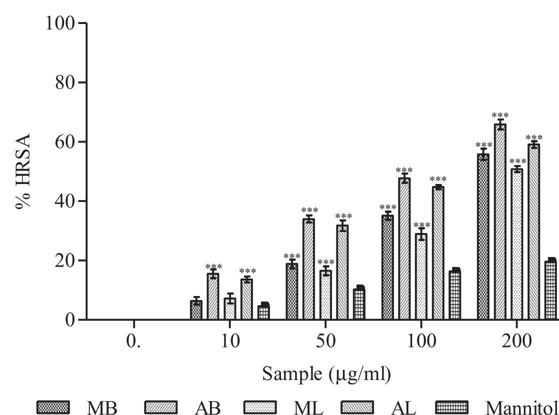

Figure 3. Hydroxyl radical scavenging assay.
Hydroxyl radical scavenging activities of the *P. dulce* plant extracts and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation. The results are mean ± S.D. of three parallel measurements. $^{***}p < 0.001$ vs 0 μg/ml. IC_{50} of MB, AB, ML and AL = 175.87 ± 11.4, 110.43 ± 9.56, 200.12 ± 14.97 and 135.04 ± 10.61 μg/ml respectively. The IC_{50} value of the standard is 20.01 ± 0.10 μg/ml.

Table 1 Scavenging of reactive oxygen species and iron chelating activity (IC_{50} values) of *Pithecellobium dulce* and reference compounds

Activity	Extract/Standard	IC_{50} (#)
Hydroxyl radical ($OH^{\cdot}$) scavenging	MB	175.87 ± 11.4
	AB	110.43 ± 9.56
	ML	200.12 ± 14.97
	AL	135.04 ± 10.61
	Mannitol	20.01 ± 0.10
Superoxide anion (O_2-) scavenging	MB	50.21 ± 1.21
	AB	61.78 ± 1.50
	ML	53.45 ± 1.94
	AL	60.09 ± 2.98
	Quercetin	41.71 ± 1.98
Nitric oxide radical (NO) scavenging	MB	110.00 ± 2.10
	AB	96.70 ± 1.35
	ML	118.60 ± 1.21
	AL	105.00 ± 2.12
	Quercetin	91.81 ± 3.70
Hydrogen peroxide (H_2O_2) scavenging	MB	35.09 ± 0.30
	AB	40.08 ± 0.29
	ML	38.07 ± 0.81
	AL	45.06 ± 0.47
	Ascorbic acid	23.01 ± 0.01
Singlet oxygen ($^1O_2$) scavenging	MB	125.89 ± 3.98
	AB	155.12 ± 4.98
	ML	120.02 ± 2.90
	AL	200.09 ± 4.82
	Lipoic acid	46.09 ± 1.07
Hypochlorous acid (HOCl) scavenging	MB	150.45 ± 2.97
	AB	195.01 ± 3.07
	ML	200.91 ± 1.97
	AL	247.34 ± 3.76
	Ascorbic acid	155.98 ± 1.84
Iron Chelating	MB	80.29 ± 1.92
	AB	85.10 ± 1.45
	ML	100.08 ± 2.04
	AL	105.87 ± 1.76
	EDTA	2.50 ± 0.13

 # Units of IC_{50} for all activities are μg/ml, except Nitric oxide radical (NO) scavenging, where the units are mg/ml. Data are expressed as mean ± S.D. Data in parenthesis indicate number of independent assays. EDTA, Ethylenediamine tetraacetic acid.
**** $p < 0.001$ vs Pithecellobium dulce.*

Superoxide radical scavenging

The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. As shown in Figure 4, the IC_{50} values (Table 1) of the plant extracts and quercetin on superoxide scavenging activity were 50.21 ± 1.21 to 61.78 ± 1.50 μg/ml and 42.06 ± 1.35 μg/ml, respectively. The IC_{50} value of the extract was higher than that of the reference compound. At 20 μg/ml, the percentage inhibition of MB, AB, ML and AL were 34.97, 28.97, 31.43 and 28.21% respectively whereas that of quercetin was 38.6%.

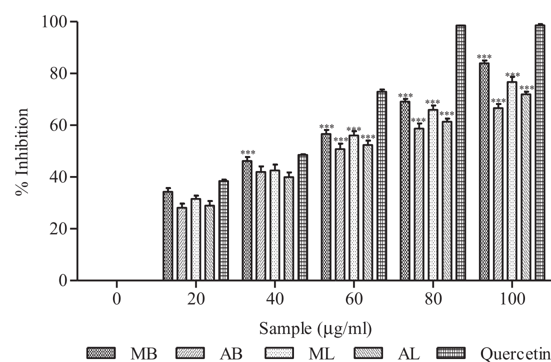

Figure 4. Superoxide radical scavenging assay.
Scavenging effect of *P. dulce* plant extracts and the standard quercetin on superoxide radical. The data represent the percentage superoxide radical inhibition. All data are expressed as mean ± S.D. (n = 3). ***$p < 0.001$ vs 0 μg/ml. IC_{50} of MB, AB, ML and AL = 50.21 ± 1.21, 61.78 ± 1.50, 53.45 ± 1.94 and 60.09 ± 2.98 μg/ml respectively. The IC_{50} value of the standard is 41.71 ± 1.98 μg/ml.

Nitric oxide scavenging

P. dulce extracts also caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} (Figure 5). Curcumin was used as a reference compound and 91.81 ± 3.70 µg/ml curcumin was needed for 50% inhibition (Table 1). The IC_{50} value of the extract was less than that of the standard. At 60 µg/ml, the percentage inhibition of the MB, AB, ML and AL extract were 35.91, 45.87, 30.92 and 41.72% respectively whereas that of curcumin was 44.78%.

Hydrogen peroxide scavenging

The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner but showed weaker activity than control (ascorbic acid). Figure 6 shows that the plant extract is good scavenger of H_2O_2 (IC_{50} values of MB, AB, ML, AL and ascorbic acid were 38.09 ± 0.081, 35.08 ± 0.030, 45.07 ± 0.047, 40.06 ± 0.029 and 23.01 ± 0.10 µg/ml respectively. At 50 µg/ml the percent inhibition for MB, AB, ML and AL were 70.3, 73.4, 61.09 and 78.3% whereas for ascorbic acid standard was 90.4% (Table 1).

Singlet oxygen scavenging

P. dulce extracts were an effective scavenger of singlet oxygen (Figure 7) and this activity was comparable to that of lipoic acid. The IC_{50} value (Table 1) of the test samples ranging from 120.02 ± 2.90 to 200.09 ± 4.82 µg/ml whereas that of lipoic acid was 46.09 ± 1.07 µg/ml. The IC_{50} value of the extract was higher than that of the reference compound. At 200 µg/ml, the percentage scavenging of MB, AB, ML and AL were 58.73, 53.01, 57.82 and 50 % respectively whereas that of lipoic acid was 78.30%.

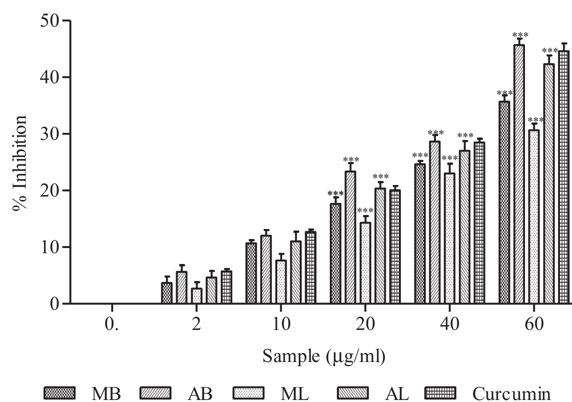

Figure 5. Nitric oxide radical scavenging assay.
The nitric oxide radical scavenging activity of *P. dulce* plant extracts and the standard curcumin. The data represent the percentage nitric oxide inhibition. Each value represents mean ± S.D. (n = 3). ***p < 0.001 vs 0 µg/ml. IC_{50} of MB, AB, ML and AL = 110.00 ± 2.10, 96.70 ± 1.35, 118.60 ± 1.21 and 105.00 ± 2.12 µg/ml respectively. The IC_{50} value of the standard is 91.81 ± 3.70 µg/ml.

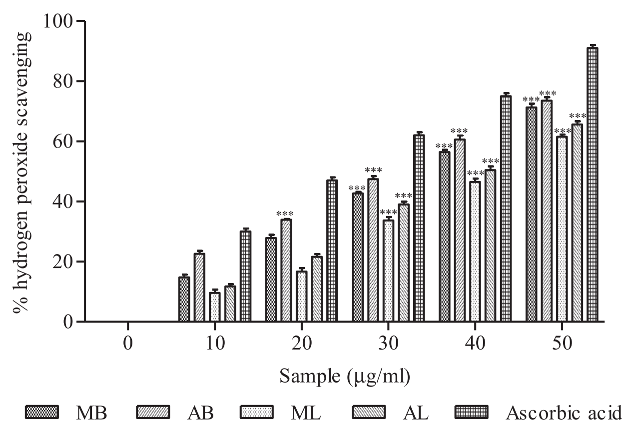

Figure 6. H_2O_2 scavenging assay.
Effects of *P. dulce* plant extracts and the standard sodium ascorbic acid on the scavenging of H_2O_2. The data represent the percentage H_2O_2 scavenging. All data are expressed as mean ± S.D. (n = 3). ***p < 0.001 vs 0 mg/ml. IC_{50} of MB, AB, ML and AL = 35.09 ± 0.30, 40.08 ± 0.29, 38.07 ± 0.81 and 45.06 ± 0.47 µg/ml respectively. The IC_{50} value of the standard is 23.01 ± 0.01 µg/ml.

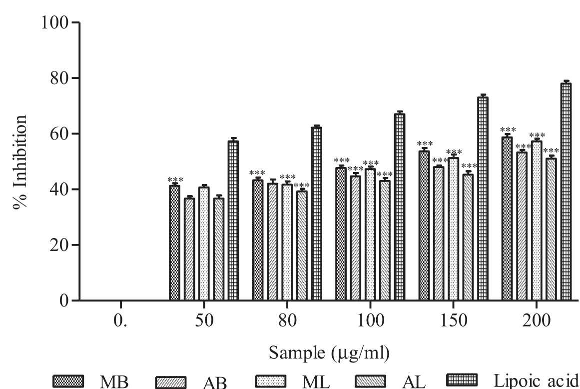

Figure 7. Singlet oxygen scavenging assay.
Effects of the *P. dulce* plant extracts and the standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of three parallel measurements. ***p < 0.001 vs 0 µg/ml. IC_{50} of MB, AB, ML and AL = 125.89 ± 3.98, 155.12 ± 4.98, 120.02 ± 2.90 and 200.09 ± 4.82 µg/ml respectively. The IC_{50} value of the standard is 46.09 ± 1.07 µg/ml.

Hypochlorous acid scavenging

Figure 8 shows the dose-dependent hypochlorous acid scavenging activity of *P. dulce* extract compared to that of ascorbic acid. The results indicate that the extracts scavenged hypochlorous acid more efficiently (IC_{50} ranges from 150.45 ± 2.97 to 247.34 ± 3.76 µg/ml) than ascorbic acid (IC_{50} = 155.98 ± 1.84 µg/ml) up to 150 µg/ml after which standard showed more inhibition than extracts (Table 1). At 100 µg/ml, the percentage scavenging of the MB, AB, ML and AL extract was 45.9, 41.5, 40.2 and 34.8% whereas that of ascorbic acid was 35.7%.

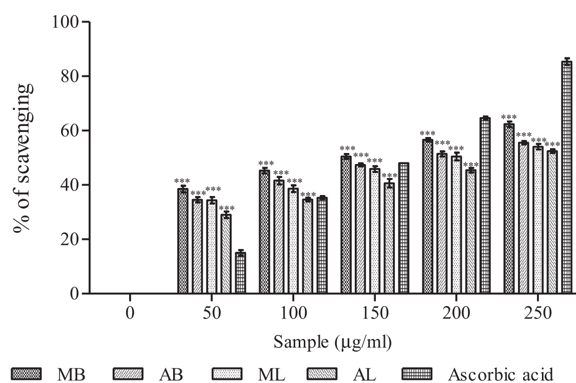

Figure 8. HOCl scavenging assay.
Hypochlorous acid scavenging activities of *P. dulce* plant extracts and the standard ascorbic acid. All data are expressed as mean ± S.D. (n = 3). ***p < 0.001 vs 0 µg/ml. IC_{50} of MB, AB, ML and AL = 150.45 ± 2.97, 195.01 ± 3.07, 200.91 ± 1.97 and 247.34 ± 3.76 µg/ml respectively. The IC_{50} value of the standard is 155.98 ± 1.84 µg/ml.

Fe^{2+} chelation

Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased. The results [Figure 9(a) and Figure 9(b)] demonstrated that formation of the ferrozine-Fe^{2+} complex is inhibited in the presence of the test and reference compounds. The IC_{50} values (Table 1) of the plant extracts ranges from 80.29 ± 1.92 to 105.87 ± 1.76 µg/ml and EDTA was 1.27 ± 0.05 µg/ml. At 120 µg/ml, the percentage inhibition of the MB, AB, ML and AL extract were 64.40, 63.51, 57.98 and 53.80% respectively whereas at 50 µg/ml that of EDTA was 99.7%.

Reducing power assay

The results of antioxidant potential of the methanol and 70% acetone extract of bark and leaves estimated using potassium ferric cyanide reduction method are shown in Figure 10. The yellow color of the test solution changes to various shades of green and blue color depending upon the reducing power of each extract. The presence of antioxidants in the herbal extracts causes the reduction of Fe^{3+}/Ferric cyanide complex to ferrous form. Therefore the Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[26] Methanol and 70% acetone extract of bark showed the higher reducing power and the values were comparable to that of tannic acid. Methanol and 70% acetone extract of leaves exhibited lower reducing power activity as compare to tannic acid. All extract shown dose dependant effect. Phenolic contents of all the extracts appears to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. At 10 µg/ml, the absorbances of the MB, AB, ML & AL extract and TA were 0.4160, 0.4982, 0.0821, 0.0962 and 0.2201 respectively.

Determination of total phenolic content

The total extractive value and total phenolic content of extracts obtained from bark and leaves using methanol and 70% acetone solvents are shown in Table 2. The total extractive values were high for both extract of leaf compared to extracts of bark. The total phenolic content of

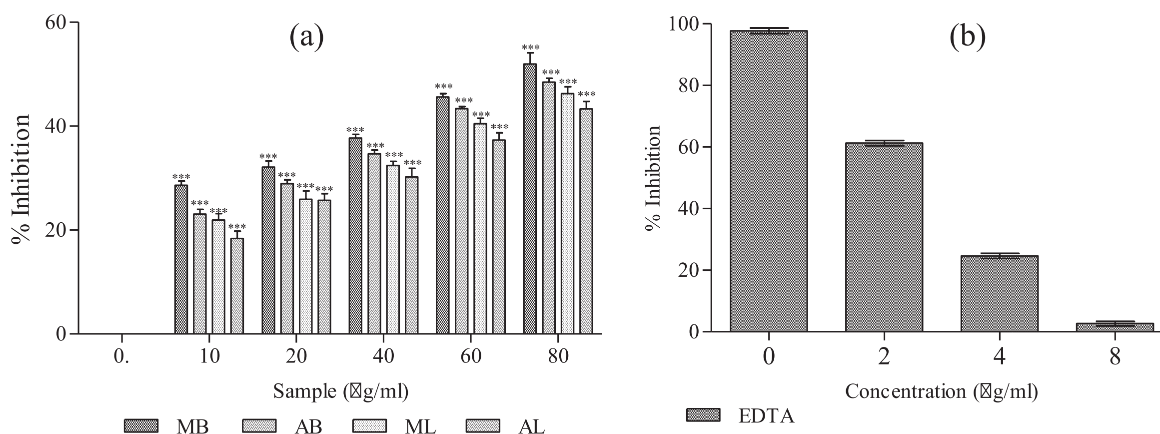

Figure 9. Iron chelation assay.
Effects of (a) *P. dulce* plant extracts and (b) standard EDTA on ferrozine-Fe^{2+} complex formation. The data are expressed as percentage inhibition of chromogen formation. The results are mean ± S.D. of three parallel measurements. ***p < 0.001 vs 0 µg/ml. IC_{50} of MB, AB, ML and AL = 80.29 ± 1.92, 85.10 ± 1.45, 100.08 ± 2.04 and 105.87 ± 1.76 µg/ml respectively. The IC_{50} value of the standard is 2.50 ± 0.13µg/ml.

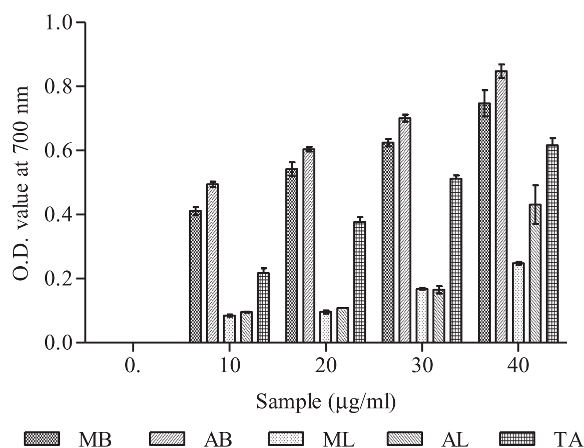

Figure 10. Reducing power assay.
The reductive abilities of *P. dulce* plant extracts and the standard tannic acid. The absorbance (A700) was plotted against concentration of sample. Each value represents mean ± S.D. (n = 3). *** $p < 0.001$ vs 0 mg/ml.

Table 2 Percentage yield, total phenolic and flavonoid content of methanol and acetone extract

Sample	% yield of extract	Gallic acid equivalent (µg/mg)[a]	Quercetin equivalent (µg/mg)[a]
MB	14.80	0.129 ± 0.11	0.43 ± 0.01
AB	11.60	0.190 ± 0.14	0.23 ± 0.01
ML	28.00	0.084 ± 0.24	0.90 ± 0.01
AL	28.20	0.115 ± 0.25	0.25 ± 0.01

[a]*Data are expressed as means ± SD of three determinations.*

MB, AB, ML & AL extracts were found to be 0.129 ± 0.11, 0.190 ± 0.14, 0.084 ± 0.24, 0.115 ± 0.25 respectively as µg quercetin equivalent/mg extract.

Determination of total flavonoid content

Total flavonoid content of extracts obtained from bark and leaves using methanol and 70% acetone solvents are shown in Table 2. The total flavonoid content of MB, AB, ML & AL were 0.43 ± 0.01, 0.23 ± 0.01, 0.90 ± 0.01 and 0.25 ± 0.01 respectively as µg quercetin equivalent/mg extract.

DISCUSSION

In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis.[31] Many synthetic drugs used to protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements, nutraceuticals and traditional medicines.[32,33] Recent advancement and wide spread research has leads to identification and isolation of many natural antioxidants from different plant materials. The antioxidant activity has been studied by the inhibition of ascorbic acid-induced lipid peroxidation and whereas in the present study, the antioxidant capacity of bark and leaves extract were measured by DPPH assay and gallic acid was used as the standard for phenolic content measurement; both studies showed promising results. Therefore, it is clear that both the leaf and stem bark extracts of the plant have good antioxidant activities as well as high polyphenolic contents.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage.[34] They were produced in this study by incubating ferric-EDTA with ascorbic acid and H_2O_2 at pH 7.4, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with TBA at low pH.[35] When *P. dulce* extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. The IC_{50} value indicates that the plant extracts are good hydroxyl radical scavenger.

Superoxide anion is also very harmful to cellular components.[36] Figure 4 shows the superoxide radical scavenging activities of the plant extract and the reference compound are increased with increasing concentrations. The results suggest that the plant extracts are good scavenger of superoxide radical.

It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.[37] The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-).[38] The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The present study shows that the extract studied has comparable nitric oxide scavenging activity with the standard curcumin.

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with

Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects.[39] From the results, it appeared that the H_2O_2 scavenging activity of the plant extract less compared to that of the standard ascorbic acid.

Singlet oxygen is generated in the skin by ultraviolet radiation. It is a high energy form of oxygen and is known as one of the ROS. Singlet oxygen induces hyperoxidation and oxygen cytotoxicity and decreases antioxidative activity.[40] The present study indicates that the *P. dulce* extract has good scavenging activity for singlet oxygen but is not as efficient as the standard lipoic acid. At sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of another harmful ROS, hypochlorous acid.[12]

HOCl has the ability to inactivate the antioxidant enzyme catalase through breakdown of the heme prosthetic group. Catalase inactivation is inhibited in the presence of the extract, signifying its HOCl scavenging activity. The results suggest that *P. dulce* is an efficient scavenger.

Iron can stimulate lipid peroxidation by the Fenton reaction ($H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH^{\cdot}$) and can also accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction.[41] Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation.[42] According to the results, the plant extract is less effective than the standard EDTA; but the decrease in color formation based on concentration dependent in the presence of the extracts indicates that it has iron chelating activity.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging.[43] Figure 10 shows that the reducing power of the plant extracts was lower compared with the standard BHT.

The results indicate that *P. dulce* plant extract contains significant amounts of flavonoids and phenolic compounds. Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability.[44] The mechanism of action of flavonoids is through scavenging or chelation.[44]

CONCLUSION

The present investigation suggests that the bark and leaf extracts of *P. dulce* contains good amounts of flavonoids and phenolic compounds, exhibits high antioxidant and free radical scavenging activities. It also posses iron chelating and reducing power. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the plant extract.

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