Antioxidant activity and free radical-scavenging potential of *Pithecellobium dulce* Benth seed extracts

Dnyaneshwar M. Nagmoti,[1] Dharmendra K. Khatri,[2] Parikshit R. Juvekar,[3] Archana R. Juvekar[4*]

Department of Pharmaceutical Sciences and Technology (DPST), Institute of Chemical Technology (ICT), Matunga, Mumbai - 400 019, India

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ABSTRACT

Introduction: Oxidative stress resulted from free radicals and reactive oxygen species are associated with many diseases. Several studies are going on worldwide directed towards finding natural antioxidants of plant origin. Plants containing phenolic compounds have been reported to possess strong antioxidant activity. The aim of the present study was to screen phytochemical constituents and *in vitro* antioxidant activity of *Pithecellobium dulce* Benth. aqueous and methanolic seed extracts. **Methods and Materials:** The aqueous (AEPD) and methanolic extract (MEPD) of *Pithecellobium dulce* seeds were studied for antioxidant potential by using different *in vitro* assays such as inhibition of DPPH, nitric oxide, hydroxyl, superoxide anions and lipid peroxidation. The total phenolic contents and reducing power of the extracts were also determined by using standard phytochemical reaction methods. Butylated hydroxyl toluene (BHT), ascorbic acid and mannitol were taken as standards. **Results:** The aqueous and methanolic extract of *P. dulce* seeds showed good dose dependant free radical scavenging activity in all the models. The total phenolic content of the aqueous and methanolic extract was found to be 1.31 ± 0.006 and 1.74 ± 0.003 mg gallic acid equivalents/g of extract powder respectively. Reducing power was also found to increase with increase in extracts concentrations. **Conclusion:** All the results of the *in vitro* antioxidant assays revealed antioxidant and free radical scavenging potential of *P. dulce* seeds, compared with standard antioxidants. This antioxidant activity may be endorsed to its high phenolic contents. Thus, our findings provide evidence that *P. dulce* is a potential source of natural antioxidants.

Keywords: Antioxidant activity, *Pithecellobium dulce*, Free radicals, Reducing power.

INTRODUCTION

Free radicals may be generated by living cells as a result of pathophysiological and biochemical processes as well as due to environmental pollutants, radiation, chemicals and toxins.[1,2] Oxidative stress results from imbalance between formation and neutralization of prooxidants and also initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA, which leads to protein and DNA damage along with lipid peroxidation in healthy human cells. Eventually these changes lead to many chronic diseases such as cancer, diabetes, aging, atherosclerosis, cardiovascular diseases, inflammatory diseases and other degenerative diseases in human.[3,4] All human cells protect themselves by multiple mechanisms especially enzymatic and non enzymatic antioxidant systems against free radical damage. However these protective mechanisms may not be enough for severe or continued oxidative stress. Hence certain amounts of antioxidant supplements are constantly required to maintain an adequate level of antioxidants in order to balance the reactive oxygen species in human body.[5] Plants are affluent source of free radical scavenging molecules, such as vitamins, terpenoids, phenolic compounds, lignins, tannins,

***Corresponding address:**
Prof. (Mrs.) Archana R. Juvekar, Professor in Pharmacology and Physiology, Pharmacology Research Laboratory,
Department of Pharmaceutical Sciences and Technology (DPST),
Institute of Chemical Technology (ICT), Matunga,
Mumbai- 400 019, India.
Phone number: (022) 3361 2215
E-mail: arj04@rediffmail.com

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flavanoids, alkaloids, coumarins, and other metabolites, which are rich in antioxidant activity. Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants, to inhibit lipid peroxidation, or to protect against the damage of free radicals. Hence, compounds especially from natural sources capable of protecting against ROS mediated damage may have potential application in prevention and/or curing of diseases.[1,6]

Pithecellobium dulce Benth. (Manila Tamarind) belongs to the Mimosaceae family, mostly grown in India for hedges, street trees and for ornament because of its handsome foliage and curious pods. It is locally called as 'Jungal jalebi' and also known as 'Vilayati babul' in Hindi and 'Vilayati chinch' in Marathi. The seeds are stated to be eaten raw or in curries and seed oil is suitable for edible purposes and for soap manufacture.[7] The bark of the plant contains tannins (up to 37%) of a catechol type and is reported to be used as astringent in dysentery, febrifuge, is also useful in dermatitis and eye inflammation. Polyphenols from bark extract of *P. dulce* was also reported for their antivenomous activity.[8] The constituents of *P. dulce* fruits have been isolated and characterized, and were also studied for their anti-inflammatory activity due to saponin fraction, free radical scavenging, H+, K+-ATPase inhibition, gastroprotective and hepatoprotective effect.[9-13] Presence of steroids, saponins, lipids, phospholipids, glycosides, glycolipids and polysaccharides has been reported in the seeds.[14] Studies on free radical-scavenging properties, antimycobacterial activity of afzelin (kaempferol-3-O-a-L-rhamnopyranoside) isolated from the alcoholic extracts of leaves, anti-inflammatory, analgesic and antidiabetic activity of leaves of *P. dulce* were recently reported.[15,16] From the literature, it is evident that the plant has great potentials in treating a number of ailments where the free radicals have been reported to be the major contributing factor. The present study, therefore investigated the *in vitro* antioxidant and free radical scavenging potential, which can throw light on the medicinal property of *P. dulce* seeds.

MATERIALS AND METHODS

Plant material

Seeds of *P. dulce* were collected during the month of August 2011 from Dhule District, Maharashtra, India. The plant material was authenticated by Dr. Ganesh Iyer, Botanist at Ruia College, Matunga, Mumbai. Freshly collected seeds were cleaned to remove adhering dust and then dried under shade. The dried samples were powdered in mixer grinder and used for solvent extraction.

Preparation of plant extract

The air dried powdered plant material were extracted in soxhlet extractor successively with pet ether followed by methanol and distilled water. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. The extracts were concentrated by rotary vacuum evaporator and then dried. The extracts thus obtained were used directly for the estimation of total phenolic and also for the assessment of antioxidant potential through various chemical assays.

Chemicals

Folin-Ciocalteu reagent, Na_2CO_3, glacial acetic acid, nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS), DPPH (2,2-diphenyl-1-picrylhydrazyl), 2-deoxy-2-ribose, $FeSO_4$, EDTA, ascorbic acid, Trichloro acetic acid (TCA), sodium dodecyl sulphate (SDS), sodium nitroprusside, naphthyl ethylenediamine dihydrochloride, sulphanilamide, phosphoric acid, trichloro acetic acid (TCA), Potassium ferricyanide, ferric chloride, hydrogen peroxide, mannitol, butylated hydroxyl toluene (BHT) were obtained from Merck, SD Fine Chemicals, Himedia or Sigma. All other reagents used were of analytical grade.

Determination of total phenolic content

The total phenolic content in the extracts was determined with Folin-Ciocalteu reagent using the method of Siddu-raju and Becker.[17] 100 µL of the extracts (10 mg/ml) were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm using a spectrophotometer (Shimadzu 1650, Japan) against the reagent blank. The analysis was performed in triplicate and the results were expressed as the gallic acid equivalents.

DPPH radical scavenging activity

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to method of

Blios.[18] A methanol solution of the sample extracts at various concentrations (50-1000 μg/ml) was added to 0.5 ml of 0.1 mM methanolic solution of DPPH and allowed to stand for 30 min at 25°C. The absorbance of the sample was measured at 517 nm. A 0.1 mM solution of DPPH in methanol was used as control, whereas BHT was used as reference standard. All tests were performed in triplicate. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{test})]/(Abs_{control})] \times 100 \quad (1)$$

Nitric oxide ($NO^{\bullet}$) scavenging activity

Nitric oxide scavenging activity AEPD and MEPD was determined in terms of $NO^{\bullet}$ generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction.[19] One milliliter of sodium nitroprusside (10 mM) in phosphate-buffered saline (pH 7.4) was mixed with 1 ml of test extracts at various concentrations (50-1000 μg/ml) dissolved in methanol and a control without a test compound, but with an equivalent amount of methanol. The mixture was incubated at 25°C for 30 min. After 30 min, 1 ml of the incubated solution was withdrawn and mixed with 1 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the pink chromophore formed during the diazotization of the nitrite with sulphanilamide and the subsequent coupling with naphthyl ethylenediamine dihydrochloride was measured at 546 nm. All the tests were performed in triplicate. Percentage inhibition was calculated using Equation 1.

Superoxide ($O_2^{\bullet -}$) radical scavenging activity

Measurement of superoxide radical scavenging activity of AEPD and MEPD was done by using method of Palash Mandal followed by slight modification.[20] The reaction mixture contained 1 ml of NBT solution (150 μM prepared in phosphate buffer, pH 7.4), 1 ml of NADH solution (468 μM prepared in phosphate buffer, pH 7.4) and methanol diluted sample extracts at various concentrations (50-800 μg/ml) were added. Finally reaction were accelerated by adding 100 μL PMS solution (60 μM prepared in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance was measured at 560 nm against methanol as control. All the tests were performed in triplicate. Percentage inhibition was calculated by comparing the results of control and test samples (Equation 1).

Hydroxyl ($OH^{\bullet}$) radical scavenging activity

The hydroxyl radical-scavenging activity of AEPD and MEPD was assessed by the method of Elizabeth and Rao with a slight modification.[21] The assay was based on quantification of the degradation product of 2-deoxy-2-ribose by condensation with thiobarbituric acid (TBA). Hydroxyl radical was generated by the Fe^{3+}–ascorbate–EDTA–H_2O_2 system (the Fenton reaction). The reaction mixture containing 0.1 ml of 2-deoxy-2-ribose (2.8 mM); 0.1 ml of phospahte buffer (20 mM, pH 7.4); 0.1 ml of $FeCl_3$ (100 μM); 0.1 ml of EDTA (100 μM); 0.1 ml of H_2O_2 (1.0 mM); 0.1 ml of ascorbic acid (100 μM) and various concentration of the extracts (50– 800 μg/ml) in water were added and incubated for 1 h at 37°C. After incubation, 0.5 ml of the reaction mixture was added with 1 ml of 2.8% TCA, 1 ml of 1% aqueous TBA and incubated at 90°C for 15 min to develop the pink chromagen and cooled. After cooling, the absorbance was measured at 532 nm against control preparation containing deoxyribose and buffer. Mannitol was used as reference standard. The assay was performed in triplicates and the results were averaged. Percentage inhibition was calculated by comparing the results of control and test samples (Equation 1).

Determination of lipid peroxidation

The lipid peroxidation level is measured as the thiobarbituric acid reactive substance (TBARS), by using rat liver homogenate for induction of lipid peroxidation, mediated by $FeSO_4$ as pro-oxidant and assessed by the method of Okhawa, Oishi, and Yagi.[22] Reaction mixture containing rat liver homogenate 0.1 ml (25% w/v in Tris–HCl buffer (20 mM, pH 7.0); 0.1 ml of $FeSO_4.6H_2O$ (0.16 mM); 0.1 ml ascorbic acid (0.06 mM) and various concentrations of AEPD and MEPD (100–1000 μg/ml) in water were incubated at 37°C for 1 h. After the incubation period, reaction mixture was treated with 0.2 ml SDS (8.1%); 1.5 ml TBA (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95–100°C for 30 min. After cooling, 1.0 ml of distilled water, 5.0 ml of n-butanol and pyridine mixture (15:1, v/v) was added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance was measured. The inhibition of *in vitro* lipid peroxidation by the measurement of thiobarbituric acid reactive substances (TBARS) in the extracts was measured at

532 nm. The assay was performed in triplicates. Ascorbic acid was taken as reference standard. The percentage of inhibition of lipid peroxidation was calculated using Equation 1.

Determination of reducing power

The reducing power of the AEPD and MEPD was determined by the method reported by Oyaizu.[23] Various concentrations of the extracts (100-1000 μg/ml) in distilled water were added with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), 2.5 ml of 1% potassium ferricyanide solution and incubated at 50°C for 20 min. After incubation 2.5 ml of 10% TCA was added to the reaction mixture. The content was then centrifuged at 1000 rpm for 10 min. After centrifugation, the upper layer of the supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Then the absorbance of the reaction mixture was measure at 700 nm. Ascorbic acid (10-100 μg/ml) was used as positive control. The higher the absorbance of the reaction mixture the greater is the reducing power.

Statistical analysis

Data were expressed as mean ± SD of triplicate determinations. Linear regression analysis was used to calculate IC_{50} values.

RESULTS AND DISCUSSION

Preliminary phytochemical screening showed the presence of steroids like triterpenoids, glycosides, saponins and flavonoids (Table 1). The antioxidants from natural sources are the only alternative to synthetic antioxidants in counteracting the free radicals associated diseases. A great number of naturally occurring substances have been recognized to have antioxidant abilities and various *in vitro* methods have been used to assess their fee radical scavenging and antioxidant activity. Therefore, in the present study AEPD and MEPD in graded concentrations were assessed for their free radical scavenging and antioxidant activity in various *in vitro* models. It was observed that the test extracts scavenged free radicals in a dose dependent manner and antioxidant activity of AEPD and MEPD was compared to standards such as BHT, Mannitol and ascorbic acid.

Total phenolic content of extracts

Total phenolic content of aqueous and methanolic extracts obtained from *P. dulce* seeds, as determined by Folin-Ciocalteu method, are reported as gallic acid equivalents. The total phenolic content of AEPD and MEPD were found to be 1.31±0.0062 is and 1.74±0.0035 mg gallic acid equivalent/g of extract, respectively.

DPPH radical scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical at room temperature which acts as an acceptor of electrons or hydrogen radicals to become a stable diamagnetic molecule[21] and used as substrate to evaluate the free radical scavenging activity of the extracts. It involves the reaction of specific antioxidant with a stable free radical and results in decrease in absorbance which can be detected at 517 nm, is due to scavenging of the radical by hydrogen donation which is measured by the change in color from purple to yellow. The scavenging effect of AEPD and MEPD at the concentration of 1000 μg/ml was 81.95%, 85.41% respectively, when compared to the scavenging effect of BHT (78.48%) at the same concentration with their IC_{50} values 502.3, 473.42 and 458.74 μg/ml. The present investigation has shown that both the extracts exhibited DPPH radical-scavenging activity in a concentration dependant manner and results were compared with the standard compound as shown in Figure 1a, indicating their abilities to act as radical scavengers.

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator generated from amino acid L-arginine by vascular endothelial cells, phagocytes and certain cells of brain. The toxicity of NO becomes adverse when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion ($ONOO^-$).[24] The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitrite ions diazotize

Table 1 Phytochemical components of *Pithecellobium dulce* based on the preliminary aqueous and methanolic extract screening

Phytochemical compounds	Presence	
	Aqueous	Methanolic
Steroids (Triterpenoids)	+	+
Saponins	+	+
Flavanoids	+	+
Alkaloids	—	—
Glycosides	+	+

+ Present, — Absent

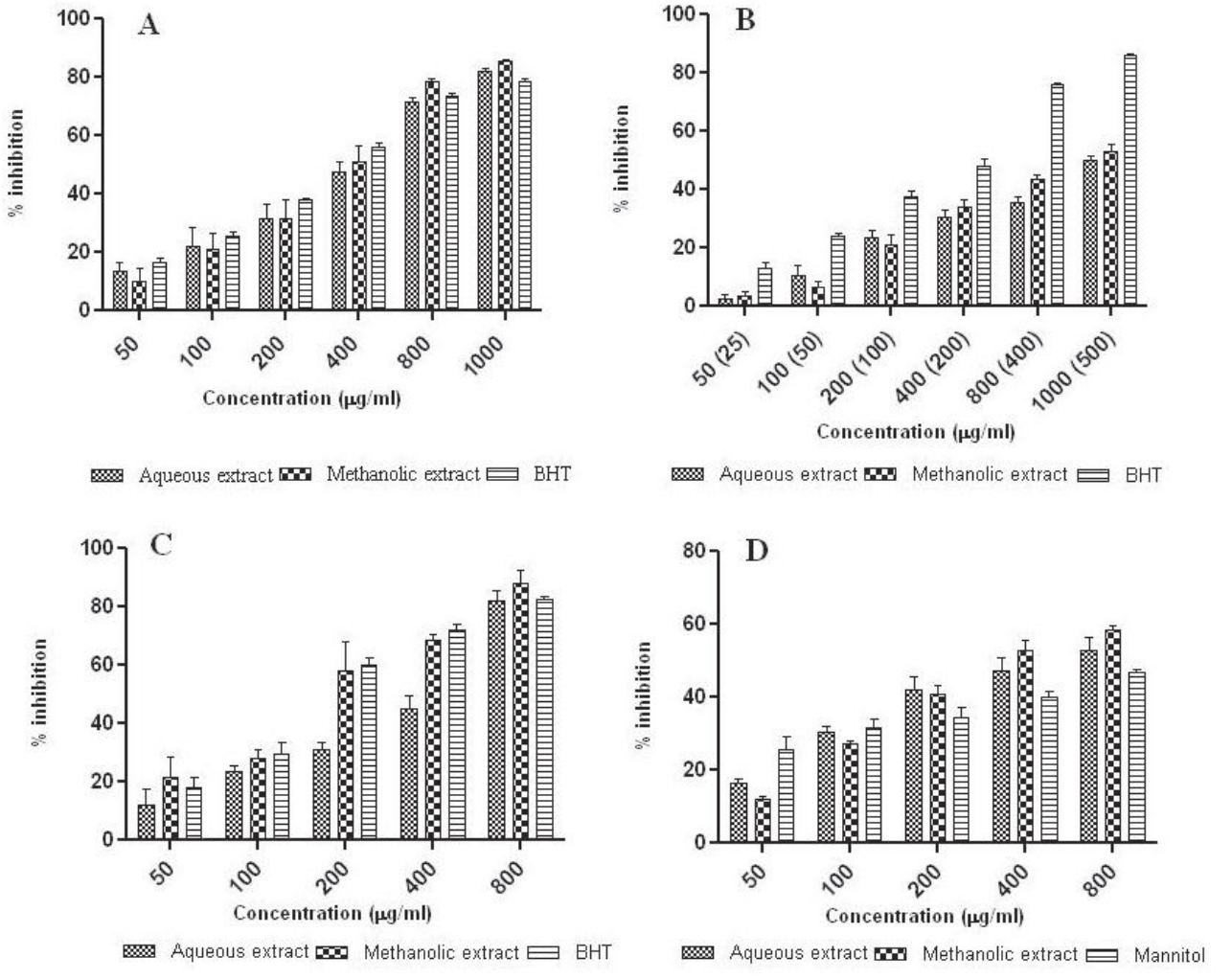

Figure 1. (A-D) Free radical scavenging activity of aqueous and methanolic extracts of *P. dulce* and standards at various concentrations. (a) DPPH radical-scavenging activity. Values are means of triplicate determinations (n=3) ± SD. (b) Nitric oxide radical scavenging activity. Each value represents mean ± SD (n=3). Concentration of BHT (25-500 µg/ml) in parenthesis. BHT: Butylated hydroxyl toluene. (c) Superoxide anion radical scavenging activity. Each value represents mean ± SD (n=3). (d) Hydroxyl radical scavenging activity. Each value represents mean ± SD (n=3).

with sulphanilic acid and couple with naphthylethylenediamine forming pink colour, which can be measured at 546 nm. Figure 1b illustrates the percentage inhibition of NO by AEPD, MEPD and BHT. At the concentration of 1000 µg/ml the percentage inhibition of AEPD, MEPD and BHT was found to be 49.89%, 52.97% and 86.02%, respectively, with their respective IC_{50} value were found to be 1027.32, 893.61 and 262.55 µg/ml. The results show that MEPD is more potent scavenger of NO than AEPD.

Superoxide anion scavenging activity

Superoxide anion radical is one of the strongest reactive oxygen species and is also very harmful to cellular components.[25] In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease in absorbance at 560 nm with the plant extracts indicates their abilities to quench superoxide radicals in the reaction mixture. Figure 1c illustrates that MEPD at 800 µg/ml exhibited (88%) superoxide radical scavenging activity, which is higher than AEPD (82.19%) and standard compound BHT, which exhibited 82.3%. The IC_{50} values of AEPD, MEPD and BHT was found to be 437.81, 276.88 and 280.13 µg/ml. The results suggest that the MEPD is more potent than AEPD.

Hydroxyl radical scavenging activity

Hydroxyl radicals have a short half-life and are the most reactive and damaging oxygen species causing lipid peroxidation and cellular damage.[26,27] Hydroxyl radicals were produced in this study by incubating ferric-EDTA with ascorbic acid

and H_2O_2 at pH 7.4 (Fenton reaction), and made to react with 2- deoxy-2-ribose to generate malondialdehyde like product. This compound forms a pink chromogen upon heating with TBA at low pH.[28] Figure 1d illustrates the hydroxyl radical scavenging potential of AEPD and MEPD in comparison with the standard Mannitol. When the aqueous, methanolic extract and standard Mannitol (1000 μg/ml) were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented the degradation of 2- deoxy-2-ribose. MEPD at 800 μg/ml exhibited (58.35%) greatest scavenging effect of $OH^{\bullet}$ than AEPD (52.68%) and standard Mannitol (46.97%). The IC_{50} value of AEPD and MEPD was found to be 855.76 and 753.56 μg/ml, in comparison with standard Mannitol (1215.87 μg/ml). The results suggest that MEPD is a more potent scavenger of hydroxyl radicals than AEPD and mannitol. Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membranes and they bring peroxidic reactions of lipids. It is thus anticipated that methanolic extract would show antioxidant effects against lipid peroxidation on biomembranes and would scavenge $OH^{\bullet}$ radicals at the stages of initiation and termination.

Determination of lipid peroxidation

In vitro lipid peroxidation was assessed by means of an assay system that determines the production of malondialdehyde and related compounds in rat liver homogenate. Malondialdehyde (MDA) is one of the major degradative products of lipid peroxidation and serves as a marker for oxidative stress. Thiobarbituricacid reactive species (TBARS), the byproducts of lipid peroxidation that occur in non-polar region of the biological membranes and involve in the free radical induced cellular damage that lead to many human diseases.[29] Figure 2 illustrates the antilipid peroxidation activity of *P. dulce* aqueous and methanolic extracts in Fe^{2+}–ascorbate system by inhibiting the formation of MDA in comparison with the standard ascorbic acid. At the concentration of 1000 μg/ml the inhibition effect of AEPD and MEPD in the formation of MDA was 42.98% and 67.31%, respectively. Standard ascorbic acid at the same concentration showed 84.32% inhibition. The concentration of AEPD, MEPD and ascorbic acid needed for 50% inhibition was found to be 1158.72, 535.42 and 23.44 μg/ml, respectively. The results suggest that MEPD is more potent scavenger of TBARS than AEPD but lower than ascorbic acid.

Determination of reducing power

The reducing properties are generally depends on the presence of reductones, which have been shown to

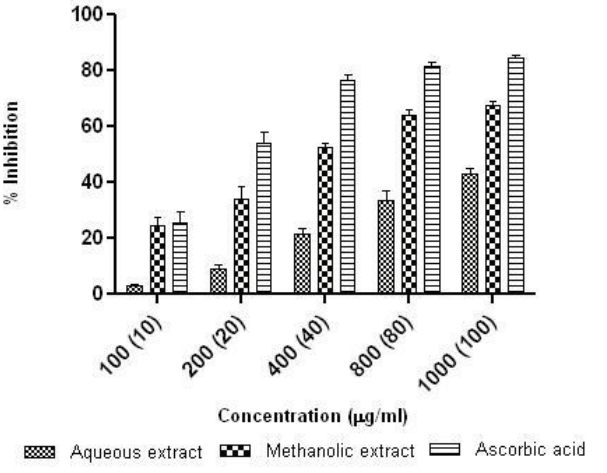

Figure 2. Lipid peroxidation activity of aqueous and methanolic extract of *P. dulce*. Values are means of triplicate determinations (n=3) ± standard deviation. Concentration of ascorbic acid (10-100 μg/ml) in parenthesis.

exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom.[30] Figure 3 shows the reducing capacity of AEPD and MEPD compared to standard ascorbic acid (10-100 μg/ml) at 700 nm. Like the scavenging activity, the reducing power of

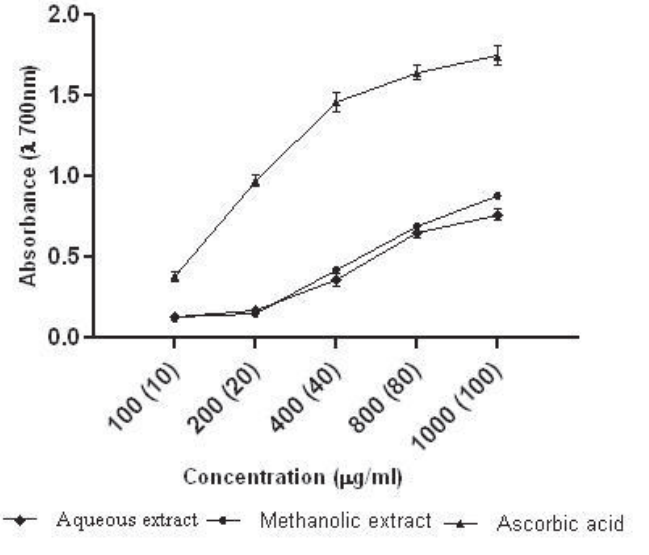

Figure 3. Reducing power of aqueous and methanolic extract of *P. dulce*. Values are means of triplicate determinations (n=3) ± standard deviation. Concentration of ascorbic acid (10-100 μg/ml) in parenthesis.

both the extracts increased with increasing concentration. The result suggest that MEPD has more reducing power than AEPD but not as efficient as standard ascorbic acid.

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