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Research Article

# EXTRACTION OF ANTHOCYANIN AND ANALYSING ITS ANTIOXIDANT PROPERTIES FROM *PITHECELLOBIUM_DULCE* FRUIT PERICARP

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**ABSTRACT**

The present study investigates the benificial role of anthocyanin extracted from Pithecellobiumdulce fruit percarp.The pericarp which is used as a waste,itwas utilized for the extraction of anthocyanin and evaluate the antioxidant activity. The data represented in this study demonstrated that anthocyanin extracted by using acidified methanol showed better results when compared with methanol.

**Keywords**: Pithecellobiumdulce fruit pericarp;anthocyanin;antioxidant; total flavonoid; free radical scavenging effect

## INTRODUCTION

Flavanoid are common and widespread secondary plant metabolites, which have a wide range of biological and physiological activities. Flavanoid occur in plants (food products) as different glycosides, which is also preferred form for uptake in human intestine. After uptake the glycosides are converted in to aglycon and free carbohydrates in hydrolysis reaction. Flavanoid are polyphenolic compounds, they are effective antioxidants due to their capability to scavenge free radicals of fatty acids and oxygen, their flavonol content considerably decreases atherosclerotic process, inhibits cholesterol accumulation in the blood serum and enhances resistance of vascular walls. Flavanoid decreases a risk of coronary heart diseases.

### Anthocyanin

The word anthocyanin, derived from the Greek word was originally used to describe the blue pigments of the cornflower, *Centaurea cyanus* (Marquart, 1835). Anthocyanins are polyphenolic compounds responsible for cyanic colors ranging from salmon pink through red and violet to dark blue of most flowers, fruits, leaves and stems. They comprise the largest group of the water-soluble pigments in the plant kingdom (Strack and Wray, 1994), and during the last few years it has been an exponential increase in the report of new anthocyanin structures (Andersen and Jordheim, 2006). This can be explained by the use of improved analytical techniques, but the potential use of Anthocyanins as health beneficial compounds is another reason for the increased scientific interest in these pigments. They play a definite role in attracting insects in pollination and seed dispersal. They may also have a role in the mechanism of plant resistance to pest attack (Strack and Wray, 1989).

The anthocyanins consist of an aglycon (anthocyanidin), sugar(s), and, in many cases, acyl group(s). The classical anthocyanin aglycon is based on a C15 skeleton (C6–C3–C6 skeleton) (Andersen and Jordheim, 2006). Anthocyanins are positively charged at acidic pH. Even though there are around 30 different anthocyanidins, approximately 90% of all anthocyanins are based on the six most common anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin which only differ by the hydroxylation and methoxylation pattern on their B-rings. The anthocyanins will differ with respect to glycoslyation of hydroxyl groups, nature of glycosyl units, substitution pattern, and potential aliphatic and aromatic acylation (Andersen and Jordheim, 2006). The 3- deoxyanthocyanidins (non glycosides) found in Sorghum. In plants spagnorubins and rosacyanin B are the only anthocyanidins (aglycon) found in their non glycosidated form. Andersen Jordheim, (2006) indicated the presence of cyanidin, peonidin and pelargonidin in black dried beans in glycosidated form (*Phaseolus vulgaris* L.). Pyranoanthocyanins have been discovered in small amounts in wines and grape pomace (Bakker and Timberlake *et al.,* 1997; Fulcrand *et al.,* 1998; Mateus *et al.,* 2004; Cheynier, 2006). More recently, glucosides of carboxypyranocyanidin have been isolated from red onion (Fossen and Andersen, 2003), and carboxy-pyranopelargonidin 3- glucoside from strawberry (Andersen *et al.,* 2004) extracts which are all in glycosidated form.

### Antioxidants

Antioxidants are widely used as a food additive to provide protection against oxidative degradation of foods by free radicals. In order to prolong the storage of foods several synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used currently. But these substances may be inappropriate for chronic human consumption. Hence the development of alternative antioxidants from natural origin has attracted considerable attention and its development.

*Pithecellobium dulce* Benth. (Leguminosae) is a small to medium sized, evergreen, spiny tree up to 18 m height, native of tropical America and cultivated throughout the plains of India and in the Andaman. It is known as 'Vilayati babul' in Hindi and 'Kodukkapuli' in Tamil. The bark of the plant is reported to be used as astringent in dysentery, febrifuge and it is also useful in dermatitis and eye inflammation. The leaves have been reported to possess astringent, emollient, abortifiacient and antidiabetic properties.

The presences of steroids, saponins, lipids, phospholipids, glycosides, glycolipids and polysaccharides have been reported in the seeds. The bark contains 37% of tannins of catechol type. Quericitin, kaempferol, dulcitol and afezilin have been reported from the leaves. Roots have been reported to possess estrogenic activity. Studies on alkylated resins from seed oil have been reported recently. 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 factors contributing to the disorders. In the present study the *pithecellobium dulce* fruit pericarp was used for the extraction of anthocyanins and analysis of antioxidant properties.

### Objective

The aim of the study was to determine the anthocyanin content in the *pithecellobium dulce* fruit pericarp using different solvent system and to analyses its antioxidant properties.

## MATERIALS AND METHODS

### Sample Collection

*Pithecellobium dulce* fruit were collected from the market and stored at -20°C.

### Extraction

500 mg of *Pithecellobium dulce* fruit pericarp were treated with 10 ml of 2 different solvents (methanol and acidified methanol). And

the mixture was centrifuged at 10,000 rpm for 10 min and supernatant was taken for analysis (Lachman *et., al.,* 2003).

**Analytical Procedures**

**Flavonoid confermation test (Harbone-1998)**

**A. $FeCl_3$**

1 ml of sample extraction was added with a small amount of $FeCl_3$, and results were observed.

**B. $AlCl_3$**

1 ml of sample extraction was added with 5% of $AlCl_3$ solution, and results were observed.

**Total Phenolic Assay**

Total phenolic compounds in anthocyanin samples were quantified by using Foliciocalteu's method described by Ronald *et.,al* (1998).50 µl of Folin-ciocalteu's reagent (50% v/v) were added to 10µl of sample extract. It was incubated for 5 min. After incubation 50µl of 20 % (w/v) sodium carbonate and water was added to final volume of 400 µl. Blank was prepared by replacing the reagent by water to correct for interfering compounds. After 30 min of incubation, the absorbance was measured using spectrophotometer at 760 nm.

**Stability at variable pH**

The anthocyanin stability was tested by treating 1 ml of sample with 1 ml of $P^H$ 1.0 and 4.5 solutions. The color change was observed. (Strack, 1989).

**Determinatiomn of total anthocyanin**

The total amount of anthocyanin content was determined by using $p^H$ differential method. A spectrophotometer was used for the spectral measurements at 210 nm and 750 nm. (Fuleki & Francis, 1968)[20]. the absorbance of the samples (A) was calculated as follows:

Anthocyanin pigment content (mg/liter) = (A X MW X DF X 1000)/ (ε X 1).

Where,

A= (Absorbance λ vis-max-A750) pH 1.0- (Absorbance λ vis-max-A750) pH 4.5

Molecular weight of anthocyanin (cyd-3-glu) = 449, Extraction coefficient (ε)=29, 600, DF=Diluted factor.

**Total Flavonoid Content**

The flavanoid content was determined according as the aluminum chloride colorimetric method described by Chang, Yang and Chern (2002).Briefly, aliquots of 0.1g of *Pithecellobium dulce* fruit pericarp sample was dissolved in 1 ml of deionized water. This solution (0.5 ml) was mixed with 1.5 ml of 95% alcohol, 0.1 ml of 10 % aluminium chloride hexahydrate (AlCl3), 0.1 ml of 1 M potassium acetate (CH3COOK), and 2.8 ml of deionized water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415 nm against a deionized water blank on a spectrophotometer. Quericitin was used as a standard. Using a seven point standard curve (0-50mg/l), *Pithecellobium dulce* fruit pericarp the levels of total flavanoid contents in was determined in triplicate, respectively. The data was expressed as milligram quercetin equivalents (QE)/100 g fresh matter from fresh the *pithecellobium dulce* fruit pericarp

**Antioxidant Assays**

**Quantification of ascorbic acid**

This assay was carried out by the method of Sadasivam and Manickam (1997). 0.1 ml of brominated sample extract was added with 2.9 ml of distilled water. Then 1 ml of 2 % DNPH reagent and 1-2 drops of Thiourea was added with sample. After incubation at $37^0$c for 3 hours, the range-red osazone crystals that were formed were dissolved by the addition of 7 ml of 80% Sulphuric acid. Again incubated for 5 minutes. After incubation absorbance was measured at 450 nm. Vitamin C concentration was expressed in terms of mg/g of sample

**Scavenging activity of dpph radicals**

Scavenging activity of Anthocyanins against DPPH radicals was assessed according to the method of Larrauri, Sanchez-Moreno, and Saura-Calixto (1998) with some modifications. Briefly, 0.1 mM DPPH-methanol solution was mixed with 1 ml of 0.1mM DPPH methanol solution. After the solution was incubated for 30 min at 25º C in dark, the decrease in the absorbance was measured at 517nm. Methanol was used as a Control instead of antioxidant. Ascorbic acid and BHT were used as positive controls. The inhibition of DPPH radicals by the samples was calculated according to the following equation:

DPPH-scavenging activity (%) = [1-(absorbance of the sample-absorbance of blank)/absorbance of the control] ×100

**Hydroxyl radical scavenging activity**

The hydroxyl radical scavenging activity was determined according to the method described by Singh *et., al*,(2002). 0.1 ml of the anthocyanin sample extracts was taken in test tubes. 1.0 ml of iron-EDTA solution (0.1% ferrous ammonium sulfate and0.26% EDTA), 0.5 ml of DMSO (0.85% v/v in 0.1 M Phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 ml 0f 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80-$90^0$ C for 15 min. The reaction was terminated by the addition of 1 ml of ice cold TCA (17.5 %w/v), 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for the color development. The intensity of the yellow color formed was measured spectrophotometrically at 412 nm against the reagent blank. The percentage of hydroxyl radical scavenging activity is calculated by using the formula:

% of hydroxyl radical scavenging activity = 1-absorbance of sample/absorbance of blank×100

**Determination of redising power**

The reducing power was determined according to the method of Oyaizu (1986). A 0.25 aliquot of various concentrations of anthocyanins was mixed with 2.5 ml of 200mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50 º C for 20 min. after 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 650g for 10 min. a 5ml aliquot of the upper layer was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride at 700nm was measured.

**Determination of super oxide radical Scavengin activity**

Superoxide radicals were generated by the method of Ginnopolites and Ries (1977), described by Siddhurajuna et.,al (2000), with some modifications all solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo induced reactions were performed in aluminium foil-lined box with two 30W fluorescent lamps. The distance between the reaction solution and the lamp was adjusted until the intensity of illumination reached about 4000 lux. A 30µL aliquot of various concentrations of anthocycanins was mixed with 3ml of reaction buffer solution (1.3 mm riboflavin, 13 mM methionine, 63 µM nitro blue tetrazolium and 100µM EDTA, pH 7.8). The reaction solution was illuminated for 15 min at 25 º C. The reaction mixture, without sample, was used as a control. The scavenging activity was calculated as follows: scavenging activity (%) = (1-absorbance of the sample/absorbance) ×100.

**Metal chelating activity**

The chelation of ferrous ions by the extract was estimated by the method of Dinis *et.,al.*(1994) with slight modification and compared with that EDTA, BHT and that of ascorbic acid. The chelation test initially includes the addition of ferrous chloride. The antioxidants present in the samples chelates the ferrous ions from the ferrous chloride. The remaining ferrous combine with ferrozine to form ferrous-ferrozine complex. The intensity of the ferrous-ferrozin

complex formation depends on the chelating capacity of the sample and the colour formation was measured at 562 nm (Shimadzu UV-Vis 2450). Different concentrations of standard and extracts (100-500 µg/ml) were added to a solution of 100 µl FeCl2 (1mM).The reaction was initiated by the addition of 250 µl ferrozine (1 mM). The mixture was finally quantified to 1.3 ml with methanol, shaken vigorously and left standing at room temperature for 10 min. after the mixture had reached equilibrium, the absorbance of the solution was analysis were done in duplicate and average values were taken. The percentage inhibition of ferrous-ferrozine complex formation was calculated using the formula; %= 1-As/Ac X 100. Where, 'Ac' is the absorbance of the control, 'As' is the absorbance of the sample.

**Determination of inhibitory effect on deoxyribose degradation**

Inhibitory effect of the anthocyanins on deoxyribose degradation was determined by measuring the reaction activity between either antioxidants or hydroxyl radicals (referred to as non-site-specific scavenging assay) or antioxidants and iron ions (referred to as site-specific scavenging deoxyribose and 100µM L-Ascorbic acid pH 7.4) and measured spectrophotometrically. The entire test and incubated for 1 h at $37^0$ C. A ml of 0.5% 2-thiobarbituric acid in 0.025 M NaOH and 1 ml of 2.8% trichloroacetic acid were added to the mixture and it was heated for 30 min at $80^0$ C. The mixture was cooled on ice and the absorbance was measured at 532nm. Site- specific scavenging activity, which represented the ability of anthocyanins to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA. % inhibition of degradation was calculated as %=( 1-absobance of sample/absorbance of control) ×100.

**RESULT AND DISCUSSION**

**Anthocyanin extraction and quantification**

The extraction of anthocyanin from *pithecellobium dulce* fruit pericarp was done by using methanol and acidified methanol solvent system. A spectrum of the extract, especially the peek in visible region was recorded at 400 nm, where a single peek was observed in acidified methanol extract and the absorbance was also high in the case of methanol extract (Tsushida *et al* 1995).

**Table 1: Total Anthocyanin, Flavonoid And Phenol Content In *Pithecellobium Dulce* Fruit Pericarp**

| *Pithecellobium dulce* fruit pericarp | | |
|---|---|---|
| **Assays** | **Solvents** | |
| | **Methanol** | **Acidified methanol(1% HCl)** |
| Total anthocyanin (mg/g) | 29 ± 0.2 | 32 ±0.3 |
| Total flavanoid (mg/g) | 2.03 ± 0.01 | 6.2 ± 0.01 |
| Total phenol (mg/g) | 204 ± 0.3 | 200±0.3 |

Value are mean (n=3) ± SD (n=3, P<0.05)

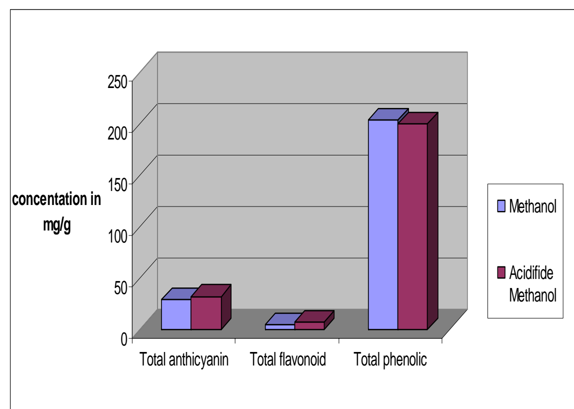



**Fig. 1: Total Anthocyanin, Flavonoid And Phenol Content In *Pithecellobium Dulce* Fruit Pericarp**

**Flavonoid confermation test**

In the presence of $FeCl_3$ methanol and acidified methanol extract showed brown color in the presence of $AlCl_3$ red color was observed in acidified methanol and pale yellow color was observed in methanol extract of *pithecellobium dulce* fruit pericarp. (Sellappan. *et.al*., (2002)

**Total phenolic assay**

Phenolic content in *pithecellobium dulce* fruit pericarp varies in different solvent system. The Figure 1 shows highest content was observed in acidified methanol (204mg/g) extract. Prasenjit manna *et al,* (2010) reported the presence of phenols in aqueous extract of *pithecellobium dulce* fruit.

**Total Flavonoid**

The anthocyanins are major class of flavanoids in plants. The total flavanoid content results were similar to total phenolic content where the acidified methanol extract showed higher content than methanol. Figure 1 shows total flavanoid content in acidified methanol was (6.2mg/g) pithecellobium *dulce* fruit pericarp extract and (1mg/g) was observed in methanol extract. Sascina *et al* (1999) reported a new flavanoid from *Pithecellobium dulce* stem.

**Determination of total anthocyanin**

Acidified methanol extract of pithecellobium dulce fruit pericarp resulted significantly higher values than the methanol extracts. The Figure 1 shows total anthocyanin content in acidified methanol was found to be 32mg/g and 29mg/g in methanol extracts respectively. Several authors reported that acidified methanol preserves the extracted anthocyanin in their original form better than the other solvent system. Adinarayana et al, 1985 and Zasochnaja *et al,* 1980 reported the anthocyanin like Quericitin, kaempferol in the leaves of *Pithecellobium dulce*.

**Stability at variable Ph**

The samples appear in red color at pH 1 and the color disappears at pH 4.5 Giusti, (2003) reported that the anthocyanin are stable in low pH. The same result was observed in methanol and acidified methanol.

**Antioxidant Assays**

**Scavenging activity of DPPH radicals**

The ability of phenolic compound quench reactive species by hydrogen donation was measured through the DPPH radical scavenging activity assay. Activity is measured as the reactive decrease in absorbance at 517nm as the reaction between DPPH and antioxidant progresses. (Huang *et., al.,* and Singh & Ragini, 2004). Antioxidant activity was evaluated with percentage inhibition values, the concentration and the radical scavenging activity as listed in the table-2. The results indicates the percentage of inhibited value of *Pithecellobium dulce* fruit pericarp extractranging from 40%-66%. The acidified methanol extracts showed a highest scavenging activity than the methanol. This implies that the acidified methanol is a more powerful solvent than the methanol in extracting *Pithecellobium dulce* fruit pericarp antioxidant.

**Hydroxyl radical scavenging activity**

The hydroxyl radical is extremely reactive free radicals formed in biological system and has been implicated as a higher damaging species in free radical pathology. Capable of damaging almost every molecule found in living cells. This species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991) (Figure 3).

The hydroxyl radical scavenging activity of the extract of two different solvent systems was given in the table 2. Methanol and acidified methanol extract of anthocyanin exhibited a similar activity 94.6% and 94.3% respectively (Chang *et., al., 2002,*). The ability of methanol and acidified methanol extract of anthocyanin to quench hydroxyl radical seems to be directly related in to prevention of propagation of the process of lipid peroxidation. The methanol

extract seems to be a good scavenger of active oxygen species, thus reducing rate of chain reaction. A high positive correlation was observed between the polyphenol content and hydroxyl radical scavenging activity of total phenolic content of *Pithecellobium dulce* fruit pericarp extracts.

**Redusing Power**

The potassium ferric cyanide reduction method was used to measure the ability of phenolic compounds to quench radicals through electron donation. The activity of total antioxidants of is measured by spectrophotometer and in which the change in absorbance is measured when the antioxidant reduces the ferric ion cyanide complex to the ferrous form and resulted with higher absorbance value Figure 2 shows reducing power of two different anthocyanin extract *Pithecellobium dulce* fruit pericarp.

**Table 2: Antioxydant Analysis Of Different Solvent Extracts From Pithecellobium Dulce Fruit Pericarp**

| ***Pithecellobium dulce* fruit pericarp** | | |
|---|---|---|
| **Antioxidant assays** | **Solvents** | |
| | **Methanol** | **Acidified methanol** |
| Ascorbic acid (mg/g) | 32 ±0.1 | 60±0.1 |
| DPPH radicals (%) | 40.33% | 66.16% |
| Hydroxyl radical scavenging activity (%) | 94.6% | 94.3% |
| Reducing power (OD) | 0.084 | 0.108 |
| Superoxide radical-Scavenging activity (%) | 55.42% | 56.62% |
| Metal chelating activity (%) | 11.08% | 92.95% |
| Inhibitory effect on deoxyribose degradation (%) | 78% | 31% |

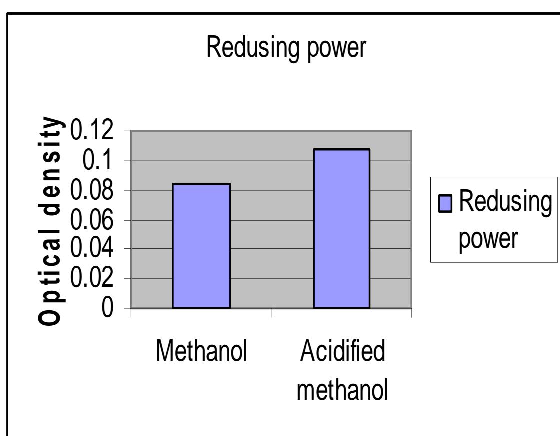
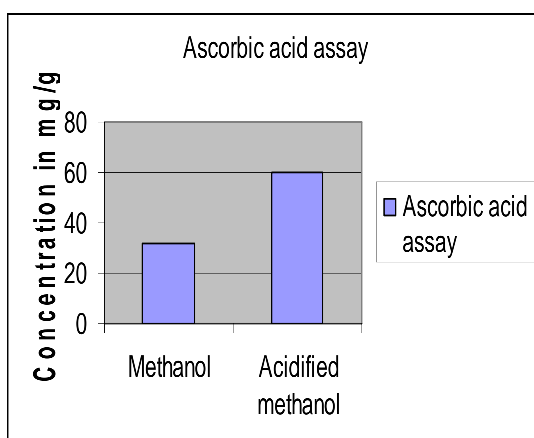



**Fig. 2: Antioxydant Analysis Of Different Solvent Extracts From Pithecellobium Dulce Fruit Pericarp**



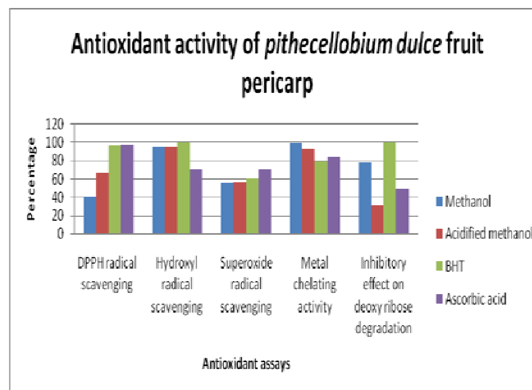




**Fig. 4: Antioxydant Analysis Of Different Solvent Extracts From *Pithecellobium_Dulce* Fruit Pericarp**

In the present study, the acidified methanol extract of anthocyanin has revealed the highest reducing power as 0.108 of absorbance at 700nm followed by methanol extract of 0.084. The reducing property are generally associated with the presence of reductones (Lee,J,C.,Kim, *et.,al.*,2002) which have been shown to exert antioxidant action by breaking free radical chain by donating a hydrogen atom.

**Determination of super oxide radical scavengin activity**

Superoxide anion radicals are produced by a number of cellular reactions, including various enzymes systems such as lipooxygenase, peroxidase, NADPH oxidase and xanthain oxidase. Superoxide anions place an important role in plant tissue and are involved in the formation of other cell damaging free radicals. (Bloknina *e.,al.*, 2003). In the present study, superoxide radical can be generated by illuminating a solution containing riboflavin. Based on the results obtained as represented in the table -2 is clear that acidified methanol extracts of *Pithecellobium dulce* fruit pericarp has better super oxide scavenging activity as compare to the methanol extracts, which may be again due to the higher amount of total phenol content. Further superoxides are also known to indirectly initiate lipid peroxidation as a result of $H_2O_2$ formation, creating precursor of hydroxyl radicals (Meyar and Isakser, 1995). Our result clearly shows that antioxidant activity of anthocyanin extracted from *Pithecellobium dulce* fruit pericarp is also related to ability to scavenge superoxides.

**Metal chelating activity**

The ability of antioxidants to form insoluble metal complexes with ferrous ion or to generate steric hindrance that prevent interaction between metal and lipid is evaluated using the ion chelating capacity assay (Hsu *et., al.,* 2003). The activity is measured by monitoring the decrease in absorbance of the red ferric ($Fe^{2+}$) - ferrozin complex as antioxidants complete with ferrozin in chelating ferrous ion (Elmastas *et.,al.,* 2003). Figure-2 shows the metal chelating power of Pithecellobium *dulce* fruit pericarp. It is clear that chelating power of methanol extracts of *Pithecellobium dulce* fruit pericarp was higher as compared with acidified methanol extract (Table 2). So the metal chelating activity of anthocyanin extracted from *Pithecellobium dulce* fruit pericarp is due to phenolic and non phenolic compounds. Non phenolic metal chelators include phosphoric acid, ascorbic acid, carnosin, some amino acids, peptides and proteins such as transferrin ovotransferrin are also responsible for metal chelation (Lee *et., al* 2004).

**Quantification of ascorbic acid**

It was observed that the vitamin C content was to be higher in acidified methanol extract (60mg/g) were as in methanol extract it was found to be 32mg/g. (Figure-2) Foyer, 1993 has reported that ascorbate has been found in chloroplast, vacuole and extra cellular compartments of plant cell and shown to function as a reluctant for many free radicals.

**Determination of inhibitory effect on deoxyribose degradation**

Hydroxyl radicals can be formed by the Fenton's reaction in the presence of reduced transition metal such as Fe2+ and H202, which is known to be the most reactive of all reduced from of dioxygen and thought to initiate all damage *in vivo* (Rollet-Labella.,*et.,al*-1998). To determine whether anthocyanins reduce hydroxyl radical generation by chelating metal ions or by directly scavenging hydroxyl radical. The effects of the anthocyanin hydroxyl radicals generated by determining the degree of deoxyribose degradation. Table-2 shows the inhibition of hydroxyl radicals induced deoxyribose degradation by anthocyanin through site specific and non-site specific analysis. Relatively greater antioxidant activity was observed in the methanol extract (table 2) in the presence study, methanol extract shows 78% inhibition on non-site –specific deoxyribose degradation the acidified methanol extract. Similar results were reported for extracts of Opuntia ficus-indica varieties (Lee*. et.,al.,* 2002).

Antioxidant activity of *Pithecellobium dulce* fruit pericarp is well known and was first reported in the literature. The data represented the study demonstrated the amount of anthocyanin and phenolic compounds differ significantly between two different extracts and determining the free radical scavenging activity of *Pithecellobium dulce* fruit pericarp.

**CONCLUSION**

The *Pithecellobium dulce* fruit pericarp could be evaluated as a major source of anthocyanin, flavanoids and poly phenol antioxidants. Antioxidant activity of *Pithecellobium dulce* fruit pericarp is well known and was first reported in the literature. The study here indicated the amount of anthocyanin and phenolic significantly between two different extracts and determining the free radical scavenging activity of *Pithecellobium dulce* fruit pericarp.

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