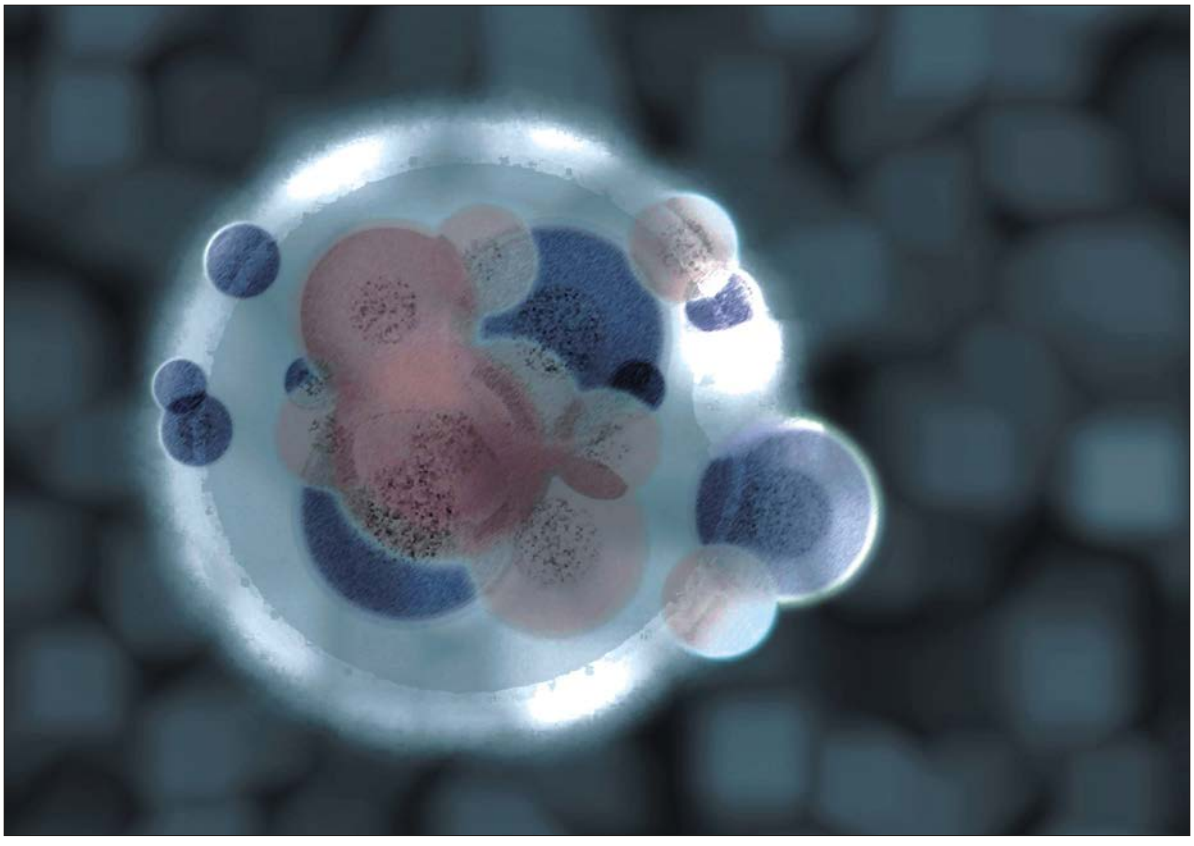

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Antioxidant Potential of *Eugenia jambolana* Lam. Seeds

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Abstract: Antioxidant potential of *Eugenia jambolana* seeds was carried out using various *in vitro* models. Ethanolic (50%) extract at 1000 μg mL^{-1} showed maximum scavenging of the radical cation, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) observed upto 98.92% followed by scavenging of nitric oxide radical (96.75%), ferric ion radical (94.43%), 1,1- diphenyl, 2-picryl hydrazyl (DPPH) (92.25%) and antilipid peroxidation potential (81.13%). The antioxidant activity of the seeds, help in preventing the oxidative damages caused and also possess α glucosidase inhibitory activity, which are very vital in the management of Diabetes mellitus. The finding justifies the therapeutic application of the plant in the indigenous system of medicine, augmenting its therapeutic value.

Key words: *Eugenia jambolana*, antioxidant, free radicals

INTRODUCTION

Importance of reactive oxygen and free radicals in cellular injury and the aging process has attracted increased attention over the past twenty years. It is increasingly being realized that a majority of the disease of today are due to the shift if the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body (Lee *et al*., 2003). Pro-oxidant conditions dominate either due to increased generation of free radicals or due to excess oxidative stress of depletion of the dietary antioxidant (Tiwari, 2001). Together with other derivatives of oxygen, they are inevitable by products of biological redox reaction (Arora *et al*., 2002). Reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radical (OH), ferric ion and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation (Geesin *et al*., 1990) and thus have been shown to augment collagen synthesis and fibrosis. The increased production of toxic oxygen derivatives is considered a universal feature of stress conditions. Plants and other organisms have evolved a wide range of mechanisms to contend with this problem, with a variety of antioxidant molecules and enzymes. Natural antioxidant derived plants are of considerable interest (Halliwell, 1978).

Eugenia jambolana Lam. (Syn. *Syzygium cumini* Skeels or *Syzynium jambolana* DC). (Myrtaceae) is one such plant widely used for Diabetes (Ravi *et al*., 2004) and widely distributed through out India, Ceylon-Malaya and Australia and known as Jamun, Jam, Jambul in India. It has been valued in Ayurveda and Unani system of medication for possessing variety of therapeutic properties (Chougale *et al*., 2009). A detailed review of literature afforded no information on the antioxidant potential of the plant. It was therefore worthwhile to investigate the antioxidant potential of *E. jambolana*.

MATERIALS AND METHODS

Chemicals: Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide were purchased from Sigma Chemicals Co. (USA). Gallic acid, quercetin, Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Plant material and preparation of extract: The seeds of *E. jambolana* were collected from the local areas of Tirunelveli District, Tamilnadu, India during May 2009 and were authenticated by the coauthor. A voucher specimen of the same is being deposited in the department for future reference. The shade dried seeds (500 g) were coarsely powdered and were exhaustively extracted with 50% ethanol using a soxhlet apparatus (Ravi *et al*., 2004). The extract was concentrated in a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal (yield 64.8 g).

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Determination of total phenolic compounds and flavonoid content: Total phenols in each extract were determined by Folin Ciocalteu reagent (Ebrahimzadeh *et al*., 2008; Dehpour *et al*., 2009). About 0.5 mg of extract dissolved in 1 mL of methanol were mixed with 5 mL of Folin Ciocalteu reagent (a 10% v/v in distilled water) and 4 mL of 1 M aqueous Na_2CO_3. The mixtures were kept for 15 min and the total phenol content were determined by colorimeter, at 765 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (UV-Visible EZ201, Perkin Elmer: USA). The standard curve was prepared using 25-300 μg mL^{-1} solutions of Gallic acid in methanol: water (50:50). Total phenol values are expressed in terms of Gallic acid, equivalent (mg g^{-1} of dry mass) which is a common reference compound.

Flavonoid content of each extract was determined by following a colorimetric method (Dehpour *et al*., 2009). 0.5 mg of extract dissolved in 1 mL of methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations of 12.5 to 100 μg mL^{-1} in methanol. Total flavonoid contents were calculated as quercetin equivalent (mg g^{-1} of dry mass) from a calibration curve.

DPPH radical-scavenging activity: Stable 1,1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract (Dehpour *et al*., 2009). Different concentrations of each extract (50-1000 μg mL^{-1}) were added, at an equal volume, to methanol solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated a total of three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals.

ABTS radical cation decolorization assay: ABTS radical cation (ABTS) was produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium per sulfate and the mixture was allowed to stand in dark at room temperature for 12-16 h before use. For the study, different concentration (50-1000 μg mL^{-1}) of the different extracts (0.5 mL) were added to 0.3 mL of ABTS solution and the final volume was made up with ethanol and double distilled water to make 1 mL. The absorbance was read at 745 nm 10 and the percentage inhibition calculated by using the formula:

$$\text{Percentage of inhibition} = \frac{\text{Control-test}}{\text{Control}} \times 100$$

Scavenging of nitric oxide radical: Nitric oxide was generated from sodium nitroprusside and measured by Griess's reaction (Marcocci *et al*., 1994). Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with different concentrations (50-1000 μg mL^{-1}) of the extracts dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 h control experiments without the compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 h, 0.5 mL of incubation solution was removed and diluted with 0.5 mL of Griess's reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. Quercetin was used as a positive control (Dehpour *et al*., 2009).

Reducing power determination: Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim *et al*., 2001). The reducing power of SE was determined according to the method of Yen and Chen (Dehpour *et al*., 2009). Different amounts of each extracts (50-1000 μg mL^{-1}) in water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as a positive control.

Determination of antioxidant activity by FTC method: Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (Liangli *et al*., 2001). The inhibitory capacity of extract was tested against oxidation of linoleic acid by FTC method. This method was adopted from Ebrahimzadeh *et al*. (2008). About 20 mg mL^{-1} of samples dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in a screw cap container at 40°C in the

dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely, 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured and it was measured again every 24 h, until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as:

$$\text{Inhibition (\%)} = 100 - \frac{\text{Absorbance increase of the sample}}{\text{Absorbance increase of the control}} \times 100$$

Vit C and BHA used as a positive control.

***In-vitro* "-glucosidase inhibitory activity:** α-glucosidase is one of the number of glucosidases located in the brush-border surface membrane of intestinal cells and is a key enzyme of carbohydrate metabolism (Caspary, 1978). The study was carried out as per the methodology of Chougale *et al.* (2009). α-Glucosidase inhibitory assay is based on the breakdown of maltose to glucose and measured by GOD-POD method at 546 nm. Briefly, the procedure is as follows, 200 µL of α-glucosidase solution (0.6 U mL^{-1}) was pre-incubated with the test sample (dissolved in MeOH) or vehicle for 5 min. The reaction was started by adding 200 mL of 37 mM sucrose and terminated after 30 min incubation at 37°C by heating at 90-100°C. The formation of glucose was determined. IC_{50} values were determined as the concentration of the sample that gave a 50% decrease in the absorbance from a blank test.

***In-vitro* antioxidant studies:** Aliquots of 0.1 mL of sample solution/vitamin E (equivalent to 500 µg) was combined with 1 mL of reagent solution (0.6 M Sulphuric acid, 28 mM Sodium Phosphate and 4 mM Ammonium molybdate) and in blank 0.1 mL of methanol was used instead of sample. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. after the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in Perkin Elmer-UV-visible spectrophotometer (Milton Roy, New York, USA). The antioxidant activity was expressed as equivalents of vitamin E (Sanchez-Moreno, 2002).

To 1 mL of extract (test sample) a drop of thiourea (10%) and 0.25 mL of 2% Dinitro phenyl hydrazine (in 9 N H_2SO_4) were added and incubated at 37°C for 3 h. Methanol was used as blank. After incubation, 1.25 mL of 85% H_2SO_4 was added under ice cold condition and kept at room temperature for 30 min. The absorbance was measured at 540 nm against blank in Perkin Elmer-UV-visible spectrophotometer (Milton Roy, New York, USA). The Reducing power was expressed as equivalents of vitamin C.

Statistical analysis: Experimental results are expressed as Mean±SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p<0.05$) and the means separated by Duncan's multiple range tests. The EC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of the extract revealed presence of phenolic compounds, reducing sugars, flavones, saponins, proteins and tannins and absence of glycosides, alkaloids, anthroquinones and quinones.

Total phenol compound reported as Gallic acid equivalents, with reference to standard curve (y = 0.0054±0.0628). Total phenolic content was 27.37±0.18 mg Gallic acid equivalent/g and total flavonoid content 14.70±0.09 quercetin equivalent/g of extract, respectively, by reference to standard curve (y = 0.0063x).

Several concentrations, ranging from 50-1000 µg mL^{-1} of the ethanolic (50%) extract of *E. jambolana* seeds were tested for their antioxidant activity *in vitro* models. It was observed that the test compounds scavenged free radicals in a concentration dependent manner up to the given concentration in all models (Table 1). The maximum percentage inhibition of DPPH was 53.47% at 800 µg, ABTS and nitric oxide at 1000 µg (98.92 and 52.46%), ferric ion at 800 µg (94.43%), respectively.

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing. Antioxidant may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent disease (Lee *et al*., 2003).

Table 1: Effect of aqueous alcoholic extract of *E. jambolana* on free radical scavenging activity and α-glucosidase inhibitory activities

Concentration (µg mL^{-1})	Scavenging of free radicals (Z%)				α-Glucosidase inhibition
	DPPH	ABTS	Nitric oxide	Ferric ion	
50	2.01	7.65	-59.94	58.14	40.64
100	5.69	13.69	-46.59	64.80	41.70
200	9.51	18.54	-39.48	74.22	46.59
400	23.89	40.82	18.94	81.77	47.98
600	35.76	65.69	27.39	92.32	52.41
800	53.47	98.88	43.84	94.43	50.59
1000	51.53	98.92	52.46	94.43	48.93

Results are mean of triplicates and expressed in percentage

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through OH radical by Fenton reaction thereby initiating a cascade of oxidative reactions. The results obtained in the present studies may be attributed to several reasons viz., the inhibition of ferryl-perferryl complex formation; scavenging of OH or superoxide of OH or superoxide radical or by changing the ratio of $Fe3^{+}/Fe2^{+}$; reducing the rate of conversions of ferrous to ferric or by chelation of the iron itself (Sanchez-Moreno, 2002). The moderate activity of the extract may probably be due to the rapid and extensive degradation of the antioxidant principles in an *ex vivo* state thereby corroborating the finding that was observed in the study carried out in Australia with a group of human volunteers (Tiwari, 2001). It is also known that the OH radical which initiates lipid peroxidation has a very short lifetime (10-9 sec at 37°C) and hence very difficult to investigate by conventional method.

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$, which has a characteristic long wavelength absorption spectrum. The results obtained imply the activity of the extract either by inhibiting or scavenging properties of antioxidants towards $ABTS^{+}$ radicals have been reported earlier (Lee *et al*., 2003). Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. In the present study, the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by the ethanolic and aqueous extract of *E. jambolana*. This may be due to the antioxidant principles in the extract (Ravi *et al*., 2004), which complete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite.

DPPH is a reactively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. From the present results, it may be postulated the *E. jambolana* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (Ravi *et al*., 2004). DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses color spectrophotometrically depending on the number of electrons taken up. Reduction of ferric ions by ortho- phenanthroline, ortho-substituted phenolic compounds were found more active than unsubstituted phenol (Marcocci *et al*., 1994). Hence, these compounds may exert pro-oxidant effect by interacting with iron. In the presence of scavenger, reduction of ferric ions will occur which is to be measured.

The total antioxidant activity of *E. jambolana* seeds were 2.09 g equivalent to Vitamin E. The reducing power was found to be 28.94 g equivalent to Vitamin C. The antioxidant activity further confirms its *in vitro* antioxidant activity, which further might be useful for the prevention of cellular injury (Geesin *et al*., 1990). The *in vitro* α-glucosidase inhibitory activity also strengthens the activity of *E. jambolanum* to be an eligible candidate towards the management of Diabetes (Caspary, 1978).

Further investigation based upon *in vivo* antioxidant and different antioxidant mechanisms would explain in detail about the potential of this plant.

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