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In vitro study of antioxidant activity of *Syzygium cumini* fruit

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Abstract

Food rich in antioxidants plays an essential role in the prevention of diseases. The fruits of wild Indian *Syzygium cumini* (L.) Skeels (Myrtaceae), also known as black plum, are edible. Traditionally they are also used to cure a number of ailments. In this paper, the antioxidant activity of the fruit skin has been analysed using different assays, such as hydroxyl radical-scavenging assay, based on the benzoic acid hydroxylation method, superoxide radical-scavenging assay, based on photochemical reduction of nitroblue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system, DPPH radical-scavenging assay, and lipid peroxidation assay, using egg yolk as the lipid-rich source. Total antioxidant capacity was determined by the assay based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex. In all the systems, a significant correlation existed between concentration of the extract and percentage inhibition of free radicals or percentage inhibition of lipid peroxidation. The antioxidant property of the fruit skin may come in part from the antioxidant vitamins, phenolics or tannins and anthocyanins present in the fruit.

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Keywords: *Syzygium cumini* fruit; Antioxidant activity; Java plum; Black plum

1. Introduction

Free radicals are species that contain unpaired electrons. The oxygen radicals, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and non-free radical species, such as H_2O_2 and singlet oxygen ($^1O_2$), are various forms of activated oxygen (Gulcin, Oktay, Kufrayvioglu, & Aslan, 2002; Yildirim et al., 2000), generated in many redox processes. They are trapped and destroyed by specific enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. Overproduction of free radicals, together with A, C and E avitaminosis and a reduced level of the above mentioned enzymes, is considered to be the main contributor to oxidative stress (Ellnain-Wojtaszek, Kruczynski, & Kasprzak, 2003). These oxygen radicals may induce some oxidative damage to biomolecules such as carbohydrates, proteins, lipids and DNA (Kellog & Fridovich, 1975; Lai & Piette, 1977; Wiseman & Halliwell, 1996), thus accelerating aging, cancer, cardiovascular diseases, neurodegenerative diseases and inflammation (Ames, 1983; Stadtman, 1992; Sun, 1990). Antioxidant nutrients vitamin E, vitamin C and β-carotene, may play a beneficial role in the prevention of several chronic disorders (Diplock et al., 1998). Flavonoids, tannins, anthocyanins and other phenolic constituents present in food of plant origin are potential antioxidants (Salah et al., 1995; Saskia et al., 1996). Food rich in antioxidants plays an essential role in the prevention of cardiovascular diseases and cancers (Gerber et al., 2002; Kris-Etherton et al., 2002; Serafini, Bellocco, Wolk, & Ekstrom, 2002) and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Di Matteo & Esposito, 2003), as well as inflammation and problems caused by cell and cutaneous aging (Ames, Shigrenaga, & Hagen, 1993). So, search for natural antioxidant sources among plants used as food is necessary for their health evaluation.

The fruits of wild Indian *Syzygium cumini* (L.) Skeels (Black Plum) are edible and are reported to contain vitamin C, gallic acid, tannins, anthocyanins, includes cyanidin-, petunidin, malvidin-glucoside and other components (Martinez & Del Valle, 1981; Wealth of

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India, 1976). The juice of unripe fruits is used for preparing vinegar that is considered to be a stomachic, carminative and diuretic. The ripe fruits are used for making preserves, squashes and jellies. The fruits are astringent. A wine is prepared from the ripe fruits in Goa (Wealth of India, 1976). The leaf extract of *S. cumini* protects against radiation-induced DNA damage (Jagetia & Baliga, 2002). Extract of seed, which is traditionally used in diabetes, has a hypoglycaemic action and antioxidant property in alloxan diabetic rats (Prince, Menon, & Pari, 1998), possibly due to tannins (Bhatia, Bajaj, & Ghangas, 1971). In contrast, Teixeira et al. (1997) suggested that the teas prepared from the leaves and seeds of *S. cumini* had no antihyperglycaemic activity. Here we report on the in vitro antioxidant activity of the fruit skin of *S. cumini*.

2. Materials and methods

2.1. Plant material

The fully ripe fruits of *S. cumini* were collected from plants growing in the Calcutta University garden during the months of July–August, 2003. The fruit skins were dried and stored at 40 °C for 7 days–6 months. The infusion, prepared from the dried fruit skins by boiling in distilled water for 5 min, was used for analyzing antioxidant activity in vitro. Each experiment was repeated five times. Tea (CTC HGH) was used as a reference due to its well known antioxidant properties.

2.2. Reagents and solvents

Chemicals, such as ethylenediamine tetra acetic acid (EDTA), trichloroacetic acid (TCA), butanol, ammonium molybdate, sodium dodecyl sulphate, were puschased from E. Merck (India) Limited. 1,1 Diphenyl-2-picrylhydrazyl was procured from Sigma, USA. Thiobarbituric acid (TBA) was purchased from Spectrochem Pvt. Ltd., India. Nitroblue tetrazolium was obtained from Sisco Research Laboratories Pvt. Ltd., India. All other reagents were of analytical grade.

2.3. Assay of hydroxyl radical (OH)-scavenging activity

The assay was based on the benzoic acid hydroxylation method, as described by Chung, Osawa, and Kawakishi (1997). In a screw-capped tube, 0.2 ml sodium benzoate, (10 mmol) and 0.2 ml of $FeSO_4 \cdot 7H_2O$ (10 mmol) and EDTA (10 mmol) were placed. Then the sample solution and a phosphate buffer (pH 7.4, 0.1 mol) were added to give a total volume of 1.8 ml. Finally, 0.2 ml of a H_2O_2 solution (10 mmol) was added. The reaction mixture was then incubated at 37 °C for 2 h. After that the fluorescence was measured at 407 nm emission (Em), and at 305 nm excitation (Ex).

OH-scavenging activity was expressed as follows: $[\%] = [1 - (FIs\text{–}FIo)/(FIc\text{–}FIo)] \times 100$ where FIo is fluorescence intensity at Ex 305 and Em 407 nm with no treatment, FIc is fluorescence intensity at Ex 305 and Em 407 nm of treated control, FIs is fluorescence intensity at Ex 305 nm and Em 407 nm of treated sample.

2.4. Assay of superoxide radical (O_2^-)-scavenging activity

The assay was based on the capacity of the extracts to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of the riboflavin–light–NBT system (Beauchamp & Fridovich, 1971). The method used by Martinez, Louoreiro, and OLiva (2001) for determination of superoxide dismutase was followed after modification. Each 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM) and 1 ml sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance values of the control and those of the reaction mixture containing sample solution.

2.5. Scavenging activity of DPPH radicals

The antioxidant activities of the aqueous extracts were measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical following the method described by Braca et al. (2001). Aqueous extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated as $[(Ao\text{–}Ae)/Ao] \times 100$ (Ao = Absorbance without extract, Ae = absorbance with extract).

2.6. Lipid peroxidation assay

A modified thiobarbituric acid-reactive species (TBARS) assay (Ohkowa, Ohisi, & Yagi, 1979) was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid rich media (Ruberto, Baratta, Deans, & Dorman, 2000). Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm (Janero, 1990). Egg homogenate (0.5 ml of 10% v/v) and 0.1 ml of extract were added to a test tube and made up to 1 ml with

distilled water. 0.05 ml of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 ml 20% TCA were added and the resulting mixture was vortexed and then heated at 95 °C for 60 min. The fruit skin of *S. cumini* has a high concentration of anthocyanin which also absorbs at 532 nm. To eliminate this non-MDA interference, another set of samples was treated in the same way, incubating without TBA, to subtract the absorbance for anthocyanin. After cooling, 5.0 ml of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation (%) by the extract was calculated according to $[(1 - E/C) \times 100$ where C is the absorbance value of the fully oxidised control and E is $(\mathrm{Abs532}_{+\mathrm{TBA}} - \mathrm{Abs532}_{-\mathrm{TBA}})]$.

2.7. Determination of total antioxidant capacity

The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acidic pH (Prieto, Pineda, & Aguilar, 1999). 0.1 ml extract was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against a blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid and gallic acid.

2.8. Determination of anthocyanin content

Total anthocyanin concentration in the dried material, extracted with methanol containing 1% HCl for 24 h at 4 °C, was measured at 530 nm and calculated as cyanidin equivalents, using $\in$ 29500 (Madhusudan & Ravisankar, 1996).

2.9. Determination of total phenol content

Phenol was determined by the Folin–Ciocalteau reagent in alkaline medium and was expressed as gallic acid equivalents (Sadasivam & Manikam, 1992). Phenol content was calculated from the regression equations prepared from a range of concentrations of extract versus optical density for such concentrations and the regression equation prepared from different concentrations of gallic acid and optical densities for the concentrations

3. Results and discussion

IC_{50} values (concentration of sample required to scavenge 50% of free radicals or to prevent lipid peroxidation by 50%) were calculated from the regression equations prepared from the concentrations of the extracts and percentage inhibition of free radical formation/percentage inhibition of lipid peroxidation in different systems of assay, e.g. DPPH assay, superoxide radical-scavenging assay, hydroxyl radical-scavenging assay and lipid peroxidation assay. IC_{50} values were compared with the IC_{50} value of tea leaves in each system to assess the antioxidant property of *S. cumini* fruit skin (Table 1). A lower IC_{50} value indicates greater antioxidant activity.

Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules (Gutteridge, 1984). *S. cumini* fruit skin extract was found to be a powerful scavenger of the hydroxyl radical (Fig. 1). There is a linear correlation ($r = 0.98034$; $p = 0.001$) between concentrations of fruit skin extract and $^{\cdot}OH$-scavenging activity within the applied concentrations. The IC_{50} value amounted to 428 μg/ml. The scavenging power of the fruit skin is about one half that of the tea.

The superoxide radical ($O_2^{\cdot-}$) radical is a highly toxic species which is generated by numerous biological and photochemical reactions. Our results show that *S. cumini* fruit skin contains water-soluble scavengers of superoxide radicals (Fig. 2) and that these react in a dose-dependent manner ($r = 0.99056$; $p = 0.001$). The IC_{50} value is 260 μg/ml. In tea, $O_2^{\cdot-}$ radical scavenging activity is 9.9 times higher than that of fruit skin.

DPPH is a stable free radical. Antioxidants, on interaction with DPPH, either transfer electrons or hydrogen atoms to DPPH, thus neutralising free radical

Table 1
Comparison of IC_{50} values

Sample	Hydroxyl radical-scavenging (IC_{50} value) (μg/ml)	Superoxide radical-scavenging (IC_{50} value) (μg/ml)	DPPH radical-scavenging (IC_{50} value) (μg/ml)	Prevention of lipid peroxidation (IC_{50} value) (μg/ml)
S. cumini fruit skin				
7 days after drying	428	260	168	222
6 months after drying				268
Tea	221	26.20	22.7	15.9

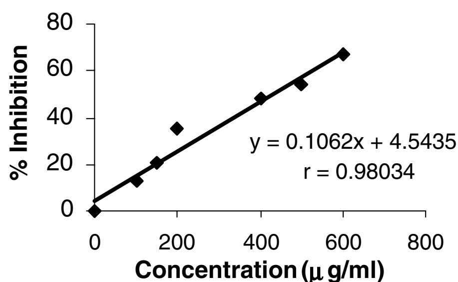

Fig. 1. Hydroxy radical-scavenging activity of *S. cumini* fruit skin.

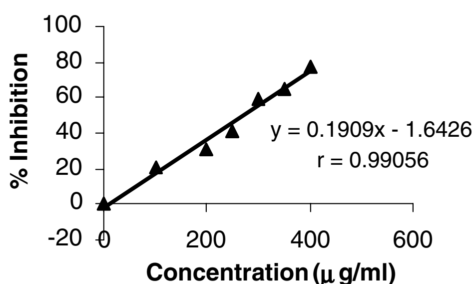

Fig. 2. Superoxide radical-scavenging activity of *S. cumini* fruit skin.

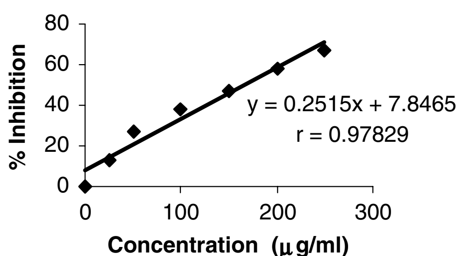

Fig. 3. DPPH radical-scavenging activity of *S. cumini* fruit skin.

character (Naik et al., 2003). The colour of the reaction mixture changes from purple to yellow and its absorbance at wavelength 517 nm decreases. Aqueous extract of *S. cumini* fruit skin extracts quenched DPPH free radical (Fig. 3) and % inhibition was proportional to the concentration of the extract ($r = 0.97829$; $p = 0.001$). IC_{50} values of fruit skin extract and tea are 168 μg/ml and 22.7 μg/ml, respectively. The scavenging effect of tea is 7.4 times greater than fruit skin.

The effect of *S. cumini* fruit skin on non-enzymatic peroxidation of lipids when incubated in the presence of ferrous sulphate is shown in Fig. 4. To study the effect of storage on activity, extracts of fruit skin, 7 days after drying and 6 months after drying, were tested. Both the extracts of *S. cumini* inhibited lipid peroxidation in a concentration-dependent manner, $r = 0.99726$ ($p = 0.001$) in fruit skin 7 days after drying; $r = 0.995516$ ($p = 0.001$) in fruit skin 6 months after drying. IC_{50} values for the inhibition of lipid peroxidation were

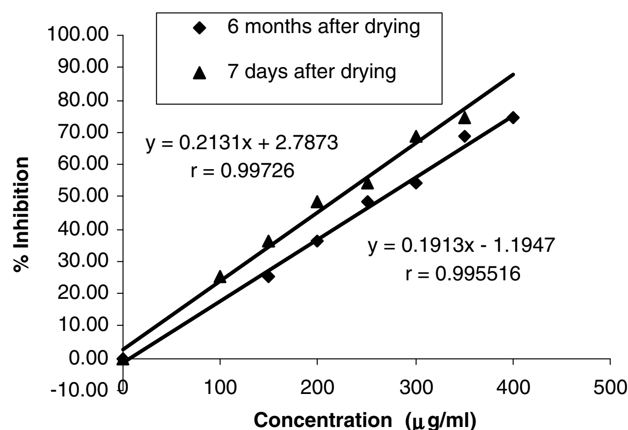

Fig. 4. Inhibition of lipid peroxidation by *S. cumini* fruit skin.

Table 2
Total antioxidant capacity of *S. cumini* fruit skin

Ascorbic acid equivalents/mg dried skin	Gallic acid equivalents/mg dried skin
0.179 mg	0.047 mg

222 μg/ml in fruit skin 7 days after drying and 268 μg/ml in fruit skin 6 months after drying. The results indicate a slight decrease in activity on storage for 6 months.

Total antioxidant capacity of *S. cumini* fruit skin was expressed as the number of equivalents of ascorbic acid and gallic acid (Table 2). The antioxidant capacity was estimated from the regression equations prepared from concentration versus optical density of sample ($y = 0.0021x + 0.0127$), ascorbic acid ($y = 0.0114x + 0.0664$) and gallic acid ($y = 0.0446x - 0.0076$).

The fruits are reported to contain vitamin C, gallic acid, tannins, anthocyanins cyanidin glucoside, petunidin, malvidin and other components (Wealth of India, 1976). The percentage anthocyanin content and total phenol content in the fruit skin are depicted in Table 3. There is much decrease in anthocyanin content 6 months after drying. Slight decrease in total phenol content 6 months after drying is probably due to decrease in anthocyanin content. Effect of storage after drying on antioxidant activity was determined only by

Table 3
Phenol content in *S. cumini* fruit skin

Plant material	Total phenol content (gallic acid equivalents) (mg/mg plant material)	Anthocyanin content (cyanidin equivalents) (%)
7 days after drying	0.096	0.67 ± 0.05
6 months after drying	0.089	0.08 ± 0.03

lipid peroxidation assay. Decrease in activity to prevent lipid peroxidation in the material after storage is also probably due to decrease in anthocyanin content.The results suggest that the phenolic compounds other than anthocyanin may be the major contributors to the antioxidant activity of *S. cumini* fruit skin. *S. cumini* and some other fruits have peviously been reported to have high antioxidant capacity (assessed by trolox equivalent antioxidant capacity) and high levels of total phenolics (Luximon-Ramma, Bahrun, & Crozier, 2003)

Plant phenolics present in the fruit and vegetables have received considerable attention because of their potential antioxidant activity (Lopez-Velez, Martinez-Martinez, & Del Valle-ribes, 2003) Phenolic compounds are the major contributors of antioxidant activity in vegetable juices (Gardner, White, McPhail, & Duthie, 2000), apple (Lee, Kim, Kim, Lee, & Lee, 2003) and cranberries (Vinson, Su, Zubik, & Bose, 2001). Phenolic compounds are effective hydrogen donors, which make them good antioxidant (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Gallic acid, present in *S. cumini*, is a strong antioxidant (Sroka & Cisowski, 2003). Antioxidant capacity of gallic acid, determined by both ABTS and DPPH scavenging assays, is more than that of vitamin C and other phenolic constituents such as quercetin, epicatechin, catechin, rutin and chlorogenic acid (Kim, Lee, Lee, & Lee, 2002).

Tannins are water-soluble polyphenols present in many foods. They have been recognized as antioxidants. Polyphenols and tannins have been reported to have protective action against DNA damage (Casalini et al., 1999; Giovannelli et al., 2000). Tea polyphenols and many tannin components were suggested to be anticarcinogenic. Many tannin molecules have been shown to reduce the mutagenic activity of a number of mutagens. Many carcinogens and/or mutagens produce oxygen free radicals for interaction with cellular macromolecules. The anticarcinogenic and antimutagenic potentials of tannins may be related to their antioxidative properties, which are important in protecting against cellular oxidative damage. The generation of superoxide radicals was reported to be inhibited by tannins and related compounds (Chung, Wong, Wei, Huang, & Lin, 1998). But toxic effects have also been observed. Tannins could decrease viability of cells and contribute to formation of DNA strand breaks (Labieniec & Gabryelak, 2003). Incidences of certain cancers, such as oesophageal cancer, have been reported to be related to consumption of tannin-rich foods (Chung et al., 1998).

Gracia-Alonso, Pascual-Teresa, Santos-Buelga, and Rivas-Gonzalo (2004) analysed 28 fruits for antioxidant activities. They found that the fruits which demonstrated greater antioxidant activity were all rich in anthocyanins, suggesting that these pigments could be contributing to this activity. As pigments, the anthocyanins are responsible for the red, blue and purple colours in fruits. Anthocyanins have free radical-scavenging properties (Saint-Crick de Gaulejac, Glories, & Vivas, 1999). Cyanidin is the most common anthocyanidin (Wang, Cao, & Prior, 1997) but its 3-glucoside is not necessarily the most active anthocyanin (Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002). Anthocyanin glycosides remain intact when passing from digestive tract into the blood circulation of mammals (Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999).

As an electron donor, vitamin C is also a potent water-soluble antioxidant in humans (Padayatty et al., 2003). Vitamin C is an essential dietary nutrient required as a co-factor for many enzymes, and humans are among the few animals that lack the ability to synthesize this compound from glucose. Epidemiological studies show that individuals with high intakes of vitamin C have lower risk of a number of chronic diseases, including heart disease, cancer, eye diseases and neurodegenerative conditions. (Jacob & Sotoudeh, 2002).

From the results, using different free radical-scavenging systems, it can be said that the fruit skin of *S. cumini* have significant antioxidant activity. In each case, lower antioxidant values, in comparison to tea, might be due to drying condition; through which some of antioxidants are presumably degraded. The antioxidant property of the fruit skin may come in part from antioxidant vitamins, phenolics or tannins and/or anthocyanins. Consumption of *S. cumini* fruit may supply substantial antioxidants which may provide health promoting and disease preventing effects.

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