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Cytoprotective and antioxidant activity studies of jaggery sugar

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ABSTRACT

Jaggery and other sugars namely white, refined and brown sugars were evaluated for cytoprotectivity on NIH 3T3 fibroblasts and erythrocytes, DPPH radical scavenging activity, reducing power and DNA protection. In addition, total phenol content and phenolic acid composition were also determined. Results indicated a total phenolic content of 26.5, 31.5, 372 and 3837 μg GAE/g for refined, white, brown and jaggery, respectively. The HPLC analysis revealed the presence of different phenolic acids in brown sugar and jaggery. On NIH 3T3 cells oxidation, at 4 mg/ml concentration, jaggery showed 97% protection compared to brown sugar, and both sugars effectively reduced erythrocyte oxidation. A dose dependent reducing power and DPPH radical scavenging activity was also observed for jaggery and brown sugar. An EC_{50} of 7.81 and 59.38 μg/ml were observed for jaggery and brown sugar in the DPPH scavenging assay. In DNA oxidation studies, higher protection was observed in jaggery followed by brown, white and refined sugar treated samples.

1. Introduction

Sugars, often called culinary sugars (used in cooking) are an important foodstuff consumed all over the world, and are manufactured either from sugarcane (70%) or sugar beet (30%). Its consumption remains high despite increase in synthetic sweeteners, and has become an essential nutrient in the world diet for its nutritional, sweetening and preservative properties (Chen & Chou, 1993). The culinary sugars are of different types based on their method of production, and also there is difference in the nature, size of the crystals, colour and taste of these sugars. The most common types of sugars that are widely consumed are white, refined, brown and raw sugar. White sugar, also called blanco directo is common in India and other south Asian countries, comes from precipitating many impurities out of the cane juice by using the phosphatation technique. Refined sugar is the most common form of sugar in North America as well as in Europe and is made by dissolving brown sugar and purifying it with a phosphoric acid method similar to that used for blanco directo. It is then further decolourised by filtration through a bed of activated carbon or bone char depending on where the processing takes place. Refined sugar is typically sold as granulated sugar, which has been dried to prevent clumping. Raw sugar is comprised of yellow to brown sugars made from clarified cane juice boiled down to a crystalline solid with minimal chemical processing, which helps in retaining more mineral salts and phytochemicals. Manufacturers sometimes prepare raw sugar as loaves called jaggery in India rather than as a crystalline powder. Brown sugar comes from late stages of sugar refining, when sugar forms fine crystals with significant molasses content or from coating refined sugar with cane molasses syrup. In terms of sucrose purity, refined sugar is more pure than blanco directo followed by brown sugar and jaggery sugars.

In recent years, plant and plant products have been the main focus in the search for nutraceuticals to combat oxidative stress induced diseases (Saxena, Venkaiah, Anitha, Venu, & Raghunath, 2007). Free radicals are generated during normal cellular metabolism and their effect is neutralised by antioxidant molecules present in the body. However, this balance between the oxidants and antioxidant molecules is disturbed by free radicals derived from exogenous sources like ozone, exposure to UV radiations and cigarette smoke (Gutteridge & Halliwell, 2000). The free radical production in cells can be significantly increased by certain toxic redox cycling compounds such as drugs and carbon tetrachloride (Wang, Ma, Liu, Tian, & Fu, 2007). Importantly, the main biomolecules like DNA, lipids and proteins are vulnerable to free radical damage resulting in cell destruction. Damaged cells lead to abnormal functioning and results in oxidative stress induced diseases. A potent scavenger or quencher of these free radical species may serve as a possible preventive measure for free radical mediated diseases.

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; EC_{50}, effective concentration for 50% radical scavenging activity; GAE, gallic acid equivalent; ABTS, [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffer saline; SD, standard deviation.

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Apart from the nutritional and sweetening aspects of sugars, very little has been studied on their nutraceutical role. The interest in polyphenols, including flavonoids and phenolic acids, has considerably increased in recent years because of their possible role in the prevention of oxidative stress induced diseases such as cardiovascular complications, diabetes, ulcers and cancer (Halliwell, 2007; Repetto & Llesuy, 2002; Sachidanandam, Fagan, & Ergul 2005; Shah, Baliga, Rajapurkar, & Fonseca, 2007). Sugarcane (*Saccharum officinarum*) contains phenolic compounds (Fontaniella et al., 2003) and these compounds have also been found in sugar products such as syrup or molasses and in brown sugar (Palla, 1982). However, the presence of these phytochemicals in sugarcane juice is often undesirable, as they influence the quality and colour of final product sugar and hence these phytochemicals are removed through various purification procedures in the sugar industry. Jaggery and brown sugar are the least processed sugars containing polyphenols. The brown sugar was also known to possess ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (Payet, Cheong Sing, & Smadja, 2005; Takara, Matsui, Wada, Ichiba, & Nakasone, 2002). White and refined sugars undergo extensive purification procedures for the removal of phenolic compounds. The bioactivity of these sugars can be anticipated, as they contain phytochemicals to different extent depending on their manufacturing process. Jaggery is the main source of sugar in rural India and has been considered by many Ayurveda practitioners as a wholesome sugar. Indian Ayurvedic medicine considers jaggery to be beneficial in treating throat and lung infections. Sahu and Saxena (1994) have found that jaggery can prevent lung damage from particulate matter such as coal and silica dust in rats. However, there are no reports available in the literature on cytoprotective abilities of jaggery and other sugars and their comparative evaluation.

Hence, in the present investigation, the protective effect of jaggery in comparison with white, refined and brown sugars on free radical induced damage of NIH 3T3 fibroblasts, erythrocytes and DNA were assessed in addition to 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability and reducing power. Further, the total phenol content and various phenolic acids present in these sugars were also determined.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, ascorbic acid, tris–HCl, glutaraldehyde, agarose, ethidium bromide, cell culture media (RPMI 1640), fetal bovine serum, L-glutamine, penicillin, streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), *tert*-butyl hydroperoxide, phenolic acid standards such as caffeic, *p*-coumaric, ferulic, gallic, gentisic, 4-hydroxyphenylacetic acid, protocatechuic, cinnamic, syringic and vanillic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Lambda phage DNA was procured from Bangalore Geni, Bangalore, India. NIH 3T3 fibroblast cells were purchased from National Center for Cell Sciences, Pune, India. The other chemicals such as ferric chloride, trichloroacetic acid and solvents used in the experiment were purchased from Sisco Research Laboratories, Mumbai, India.

2.2. Sample

Refined, white, and brown sugars were procured from three local sugar factories (Mandya, Karnataka, India) and jaggery was purchased from a local market (Mysore, Karnataka, India). All the samples ($n = 3$) were preserved in dry condition at room temperature.

2.3. Determination of total phenol content

The total phenol content of refined, white, brown and jaggery sugars were determined colorimetrically using the Folin–Ciocalteu method (Singleton & Rossi, 1965). A sample aliquot of 100 μl was added to 900 μl of water, 5 ml of 0.2 N Folin–Ciocalteu reagent and 4 ml of saturated sodium carbonate solution (100 g/l) and mixed in a cyclo mixer. The absorbance was measured at 765 nm in Shimadzu UV-160 spectrophotometer (Kyoto, Japan) after incubation for 2 h at room temperature. The total phenolic content was expressed as micrograms of gallic acid equivalent (GAE) per gram sample.

2.4. Extraction of phenolic acids

The phenolic acids of various sugars were extracted as per the protocol followed by Liyana-Pathirana and Shahidi (2006) with slight modification. Two grams of sugar sample was solubilised in 50 ml distiled water (in triplicates, $n = 3$) at room temperature (25 ± 2 °C) with constant stirring. The solution was then centrifuged at 4000*g* for 20 min (Sigma 3-16K, USA) and supernatants were collected and combined. The solution was acidified to pH 2 with 6 M hydrochloric acid and extracted six times with diethyl ether. The ether extracts were then combined and evaporated to dryness at 30 °C under vacuum (Buchi 011, Switzerland). The extracted phenolic acids were dissolved separately in 2 ml of methanol and stored at –20 °C until used within 1 week.

2.5. HPLC analysis of phenolic acid extracts

The phenolic acid extracts of jaggery and other sugars were analysed on a HPLC (Model LC-10A. Shimadzu Corporation, Kyoto, Japan) using a reversed phase Shimpak C_{18} column (4.6 × 250 mm) using a diode array UV-detector (operating at 280 nm). A solvent system consisting of water/acetic acid/methanol (Isocratic, 80:5:15) was used as mobile phase at a flow rate of 1 ml/min (Subba Rao & Muralikrishna, 2002). Phenolic acid standards such as caffeic, *p*-coumaric, ferulic, gallic, gentisic, 4-hydroxyphenylacetic acid, protocatechuic, cinnamic, syringic and vanillic acid were used for identification of phenolic acids. The identified phenolic acids were quantified on the basis of their peak area and comparison with a calibration curve obtained with the corresponding standards.

2.6. Cytoprotective effect on cultured NIH 3T3 fibroblast cells exposed to tert-butyl hydroperoxide

Cytoprotective ability of different sugars was carried out using NIH 3T3 fibroblast cells. The cells were subjected to oxidative stress according to the method reported by Nardini et al (1998). NIH 3T3 fibroblast cells (1×10^6 cells/ml, maintained at 37 °C under 5% CO_2 and 95% air in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) were used for the assay. NIH 3T3 fibroblast cells (2.8×10^4 cells/ml) were cultured with or without sugar samples (20 μL, 0–20 μg/ml) dissolved in PBS in a 96 well microplate (180 μl suspension/well). After 30 min of incubation, cells were treated with 500 μM tert-butyl hydroperoxide and incubated for 3 h. Cell viability was assessed by microculture tetrazolium assay (Hansen, Nielsen, & Berg, 1989). Twenty-five microlitres of MTT solution (5 mg/ml) were added to each well, and the cells were incubated at 37 °C for 4 h. Then, 100 μl of lysis buffer were added to each well and the cells

were again incubated at 37 °C for about 16 h to dissolve dark blue formazan crystals. The solution in each well was completely pipetted out and then the absorption of formazan solution at 570 nm was measured using a microplate reader (Molecular Devices Spectra Max 340, Global Medical Instrumentation Inc., Minnesota, USA) and expressed as percent cell protection.

2.7. Scanning electron microscopic studies of erythrocyte oxidation

Erythrocytes were obtained from healthy, consenting donors. Heparinized blood was centrifuged at 1000*g* for 15 min. After removal of plasma and buffy coat, the erythrocytes were washed thrice with PBS (20 mM, pH 7.4, NaCl – 0.9%) at room temperature and resuspended in PBS four times its volume for subsequent analysis (Suwalsky, Orellana, Avello, & Villena, 2007). Erythrocytes were preincubated with sugar samples (20 mg/ml) for 5 min, and then hydrogen peroxide (30 mM), ferric chloride (80 μM) and ascorbic acid (50 μM) were added and incubated at 37 °C for 1 h. The reaction mixture was gently shaken while being incubated (Manna, Galletti, Cucciolla, Montedoro, & Zappia, 1999). Then the cells were fixed overnight at 4 °C with glutaraldehyde in normal saline, reaching a final fixation concentration of about 2.4%. The cells were washed in saline solution and then dehydrated using ascending grades of alcohol (10–100%). Few drops of each sample were placed on A1 glass cover slips, air dried at room temperature, gold coated and examined in a scanning electron microscope at 2000× magnification.

2.8. Antioxidant activity

2.8.1. DPPH radical scavenging assay

The effect of different sugar varieties on DPPH radical was estimated according to the method of Lai, Chou, and Chao (2001). Sugar samples (0–62.5 μg/ml) in 200 μl aliquot was mixed with 100 mM Tris–HCl buffer (800 μl, pH 7.4) and then added to 1 ml of 500 μM DPPH in ethanol (final concentration of 250 μM). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge DPPH radical was calculated using the following equation. An effective concentration for 50% DPPH radical scavenging activity was also calculated (EC_{50}).

$$\text{Scavenging effect } (\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

2.8.2. Measurement of reducing power

The reducing power of sugar samples were determined according to the method of Yen and Chen (1995). The sugar sample (0–20 mg/ml) was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then an equal volume of 10% trichloroacetic acid was added to the mixture and then centrifuged at 5000*g* for 10 min. The upper layer of solution was mixed with distiled water and 0.1% ferric chloride at a ratio of 1:1:2 and the absorbance were measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.8.3. DNA protection assay

DNA protection ability of sugar samples were performed using lambda phage DNA (Suresh Kumar, Harish Nayaka, Shylaja, & Salimath, 2006). Briefly, λ phage DNA (0.6 μg) was subjected to oxidation using Fenton's reagent (0.3 mM hydrogen peroxide, 0.5 μM ascorbic acid and 0.8 μM ferric chloride) in presence and absence of sugar samples (0.8 mg/16 μl) for 2 h at 37 °C. The samples were subjected for electrophoresis (Submarine electrophoresis system, Bangalore Geni, Bangalore, India) on 1% agarose for 2 h at 50 volts DC. Gels were stained with ethidium bromide (0.5 μg/ml) and documented (Herolab, Germany).

Table 1
Total phenol content and phenolic acid composition of jaggery and other sugar varieties.[a]

Compound	Refined sugar	White sugar	Brown sugar	Jaggery
Total phenol (μg GAE/g)	26.5 ± 3.79	31.5 ± 1.44	372 ± 1.44	3837 ± 154
Phenolic acids (μg/g)	Refined sugar	White sugar	Brown sugar	Jaggery
Gallic acid	Trace	Trace	14.6 ± 0.29	122 ± 6.07
Protocatechuic acid	–	–	1.98 ± 0.07	60.0 ± 3.47
Gentisic acid	–	Trace	35.2 ± 0.13	130 ± 5.49
4-Hydroxyphenylacetic acid	–	–	1.75 ± 0.07	29.5 ± 2.08
Vanillic acid	–	–	5.08 ± 0.20	25.6 ± 1.82
Caffeic acid	–	–	–	–
Syringic acid	–	–	11.2 ± 0.26	0.75 ± 0.05
p-Coumaric acid	–	–	6.25 ± 0.17	13.0 ± 0.51
Ferulic acid	–	–	1.2 ± 0.08	34 ± 1.26
t-Cinnamic acid	–	–	–	–

[a] Values are expressed as mean ± SD ($n = 3$).

2.9. Statistical analysis

All the experiments were carried out in triplicates ($n = 3$) and the results are expressed as mean ± standard deviation (SD) using Microsoft Excel software.

3. Results

3.1. Total phenol content

Total phenolic content as quantified by Folin–Ciocalteu method indicated (Table 1) higher total phenolics in jaggery followed by brown, white and refined sugars. Approximately 10-fold higher phenolic content was observed in jaggery (3837 μg GAE/g) compared to brown sugar (372 μg GAE/g). The total phenolic content of white and refined sugars was found to be 31.5 and 26.5 μg GAE/g, respectively.

3.2. Analysis of phenolic acids in jaggery and other sugars

The phenolic acids present in refined, white, brown and jaggery was determined using HPLC. Results indicated (Table 1) the pres-

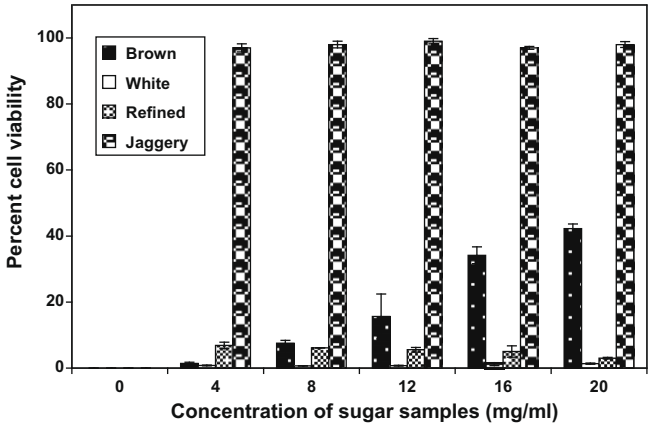

Fig. 1. Cytoprotective effect of jaggery and other sugar samples on *t*-butyl hydroperoxide induced cell damage in NIH 3T3 cells. Values are mean ± SD ($n = 3$).

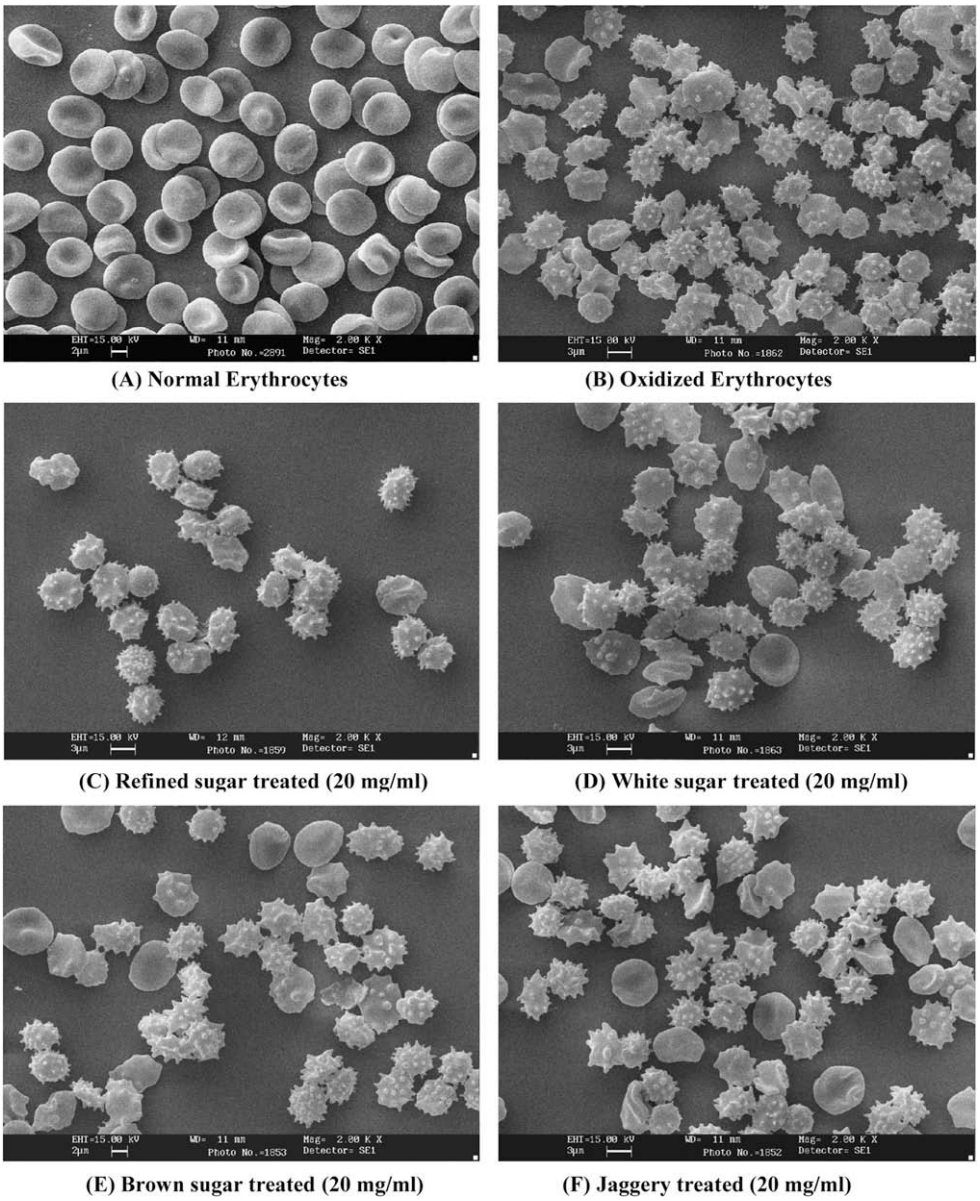

Fig. 2. Scanning electron microscopic studies of erythrocyte oxidation and their protection by jaggery and other sugar samples (at 20 mg/ml concentration).

ence of gallic, protocatechuic, gentisic, 4-hydroxyphenylacetic, vanillic, syringic, *p*-coumaric and ferulic acids in both brown sugar and jaggery. Comparatively, jaggery had higher phenolic acid content than brown sugar. White sugar showed the presence of gallic acid and gentisic acid in trace amounts and the remaining phenolic acids tested for were totally absent. Refined sugar had only trace amounts of gallic acid, while the rest of phenolic acids were absent.

3.3. Cytoprotective effect of jaggery and other sugars on NIH 3T3 fibroblasts

The cytoprotective effect of jaggery, white, refined and brown sugars on NIH 3T3 fibroblasts indicated (Fig. 1) 97% protection by jaggery at 4 mg/ml concentration against tert-butyl hydroperoxide induced cell death. At higher jaggery concentration, there was no toxic effect of the sugar on the cells and the cytoprotection remained constant up to 20 mg/ml. Brown sugar showed dose dependent cytoprotection with maximum activity at 20 mg/ml concentration. However, white and refined sugars showed very less activity and were not statistically significant.

To substantiate the results of cytoprotectivity on NIH 3T3 fibroblasts, the effect of various sugars (20 mg/ml) on erythrocyte oxidation was also studied. The scanning electron micrographs (Fig. 2) show the protective ability of various sugars on erythrocyte membrane oxidation. As compared to normal erythrocytes (A), erythrocytes treated with hydrogen peroxide showed the appearance of echinocytes indicating damage to the cell membrane. In jaggery and brown sugar treated samples the presence of normal cells can be seen in addition to oxidised cells indicating the protective role of these sugars.

3.4. Antioxidant activities of jaggery and other sugars

Further, antioxidant activity of jaggery and other sugars was evaluated by DPPH radical scavenging, reducing power and DNA protection assays. The free radical scavenging ability of sugars as evaluated by DPPH scavenging model system indicated free radical scavenging ability of jaggery, brown, white and refined sugars (Fig. 3A). Both, jaggery and brown sugars showed free radical scavenging ability with an EC_{50} of 7.81 and 59.4 μg/ml, respectively.

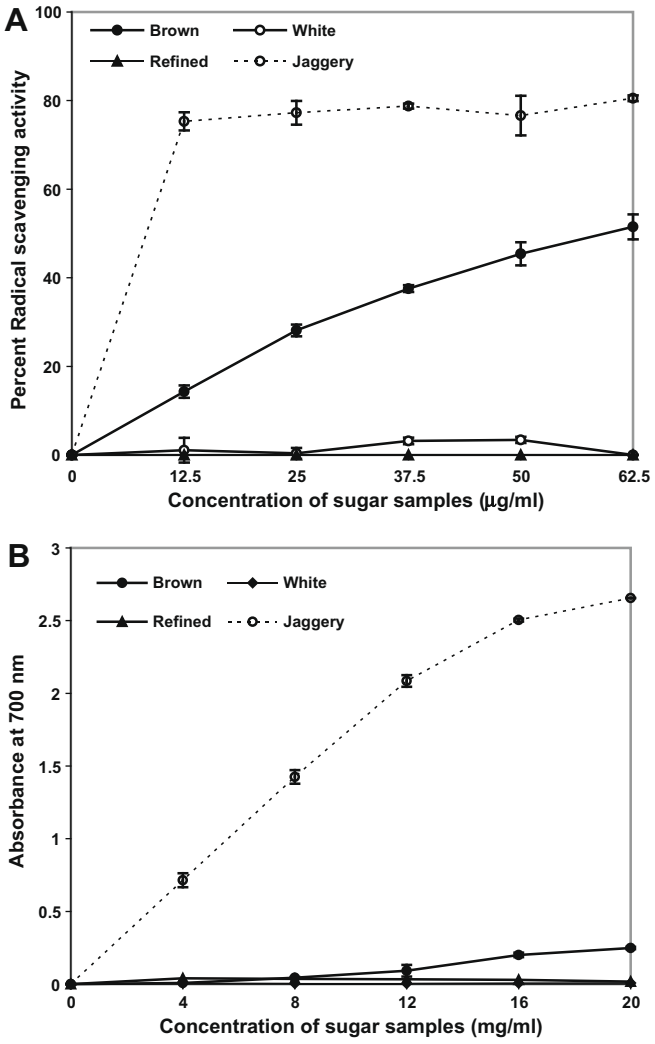

Fig. 3. (A) DPPH Radical scavenging activity of jaggery and other sugar samples. (B) Reducing power of jaggery and other sugar samples. Values are mean ± SD ($n = 3$).

These results indicate the potential electron donating ability of jaggery and brown sugars.

In addition, reducing power of jaggery and other sugars was also evaluated by their ability to reduce ferric chloride and potassium ferricyanide complex. Fig. 3B indicated a dose dependent increase in absorbance for jaggery and brown sugar. The increased absorbance at 700 nm indicated the presence of reducing power. At 20 mg/ml concentration, an absorbance unit of 2.66 and 0.248 was observed for jaggery and brown sugars, respectively compared to white (0.008) and refined sugars (0.018).

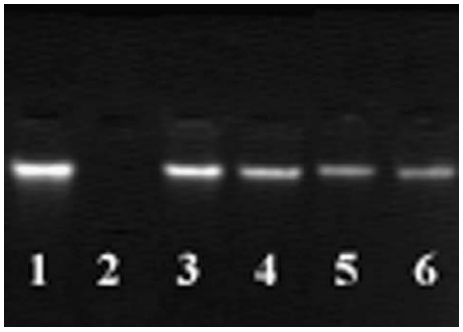

Fig. 4. Electrophoretic analysis of DNA protection by jaggery and other sugar samples (at 0.8 mg/16 µl concentration). Lane 1- native DNA; lane 2-DNA + oxidant; lane 3-DNA + jaggery + oxidant; lane 4-DNA + brown sugar + oxidant; lane 5- DNA + white sugar + oxidant and lane 6- DNA + refined sugar + oxidant.

Also, DNA protective ability of jaggery and other sugars was evaluated on lambda phage DNA oxidation (Fig. 4). The hydroxyl radical generated by Fenton's reagent caused DNA fragmentation with increase in its electrophoretic mobility (lane 2). This DNA fragmentation was recovered with the treatment of jaggery and other sugars (0.8 mg/16 µl) to varying extent. As evidenced by gel documentation analysis, higher protection (70%) was observed in jaggery treated samples, while 31%, 15% and 18% protection was observed for brown, white and refined sugar treated samples, respectively.

4. Discussion

In the present investigation, the bioactivity studies of jaggery in comparison with refined, white and brown sugars were investigated. The results include, total phenolic content, cytoprotection of NIH 3T3 fibroblasts and human erythrocyte, protection to DNA oxidation, DPPH radical scavenging activity and reducing power. Previously, the presence of phenolic compounds in sugarcane juice as well as in brown sugar has been reported (Payet et al., 2005; Takara et al., 2002). The presence of high total phenolic content (Table 1) in jaggery and brown sugar compared to white and refined sugar as per our results may be due to minimal chemical processing in the manufacture of jaggery and brown sugar which retains more polyphenols. The phenolic compounds impart colour as well as taste to the sugar and its removal is an important problem associated with sugar manufacture (Godshall, Vercellotti, & Triche, 2002). The different techniques used in cane processing to remove colour and impurities affect the amount of polyphenols in different sugars and this may explain the low phenolic content of white and refined sugars.

Further, both jaggery and brown sugar indicated cytoprotective abilities against tert-butyl hydroperoxide and hydrogen peroxide induced oxidative damage of NIH 3T3 fibroblasts and human erythrocytes, respectively. Since sucrose is a non reducing sugar, it is less obvious to have free radical quenching abilities and protect cells from oxidative damage. Hence, the cytoprotective ability may be attributed to the presence of polyphenolic components in jaggery and brown sugars. There are no reports to indicate cytoprotective abilities of polyphenols in culinary sugars. However, reports are available in the literature from other plant sources to indicate cytoprotective role of polyphenols in cell lines (Lima et al., 2007). Erythrocytes have been extensively used to study oxidative stress, which represent a simple cell model. Oxidants produce alterations in the erythrocyte membrane as manifested by a decreased cytoskeletal protein content and production of high molecular weight proteins which leads to abnormal erythrocyte shape (Battistelli et al., 2005). Hydrogen peroxide and ascorbate/Fe^{2+} induce an echinocytic type of shape alteration, characterised by protuberances over the cell membrane (Fig. 2B), indicative of oxidative damage (Srour, Bilto, Juma, & Irhimeh, 2000). From our results (Fig. 2 E and F) it is evident that jaggery and brown sugars were effective in bringing down the oxidative stress induced erythrocyte damage.

The antioxidant activity as evaluated by DPPH radical scavenging ability, reducing power and protection to DNA damage induced by hydroxyl radicals also showed the dominant antioxidant potential of the jaggery and brown sugar. The correlation co-efficient between total phenolic content and cytoprotective activity ($r = 0.9809$), DPPH radical scavenging activity ($r = 0.9187$), reducing power ($r = 0.9978$) and protection to DNA damage

($r = 0.9814$) also suggests the contribution of polyphenols in bioactivity. The literature data on the availability of phenolic components in sugarcane juice and their antioxidant activity (Duarte-Almeida, Novoa, Linares, Lajolo, & Genovese, 2006) also substantiates the bioactivity observed in different sugar varieties in the present study. To analyse the role of phenolic components present in jaggery and other sugars, and the exhibited cytoprotectivity and antioxidant activity of these sugars, the presence of phenolic acids and their content in different sugars were determined (Table 1). The results indicated various phenolic acids with proven antioxidant activity (Siddaraju & Dharmesh, 2007) in brown sugar and jaggery signifying their role in bioactivity. The higher amount of various phenolic acids in jaggery compared to brown sugar is also an indication of higher bioactivity in jaggery compared to brown sugar. Further the difference in total phenol content and the phenolic acids content (Table 1) indicates the possibility of other polyphenolic compounds being present in culinary sugars apart from the observed phenolic acids. In addition, the presence of Maillard reaction products involved in the colour and aroma of the sugar products, which are formed during sugar manufacture may also interfere with total phenol estimation in Folin–Ciocalteu method and can add up to the total phenolic content. The Maillard reaction products may also contribute to the observed antioxidant activity (Dittrich et al., 2003), but to a lesser extent.

In all the experiments, white and refined sugars showed low activity and almost negligible in case of erythrocyte oxidation, reducing power and DPPH radical scavenging assays. But in general, the use of white and refined sugar is more preferred than brown sugar and jaggery because of their colour as well as purity. From our investigation, the presence of cytoprotective and antioxidant activity in jaggery and brown sugar may encourage their use for sweetening as well as for nutraceutical benefits.

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