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Diospyros melanoxylon (Roxb.): A tribal fruit that maintains euglycemic state after consumption and cools oxidative stress

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Tendu, *Diospyros melanoxylon* Roxb. (Family: Ebenaceae) fruit is indigenous to the Indian subcontinent. The ripe fruit of tendu is eaten by tribal people. Both, unripe, as well as ripe fruits, have been used in folk-medicine by tribal communities. Aqueous methanol extract of unripe fruit displayed potent free radicals scavenging properties and also mitigated free radicals induced DNA damage. Furthermore, this extract also alleviated the development of oxidative stress induced due to a hyper physiological concentration of H_2O_2 and glucose in NIH 3T3 cells. FACS analysis revealed that extracts significantly (p <0.001) prevented the build-up of reactive oxygen species in NIH 3T3 cells generated due to a hyper physiological concentration of H_2O_2. Total polyphenols, flavonoids, and anthocyanins were present in unripe fruit were observed radically decreased when the fruit ripened. Presence of pancreatic α-amylase, intestinal α-glucosidase, and pancreatic lipase inhibitory activities in fruit extracts were also recorded. Postprandial glycemic excursion of unripe as well as ripe fruits pulp were significantly (p <0.05) less than that induced due to oral sucrose administration. Results suggest for the first time that fruit of *D. melanoxylon* may become an economic beverage fully equipped to counter free radicals and resultant oxidative stress. Furthermore, fruit may serve as a true euglycemic sweetener against sucrose.

Keywords: Antioxidant activity, Antioxidative stress potential, *Diospyros melanoxylon* Roxb., Glycemic excursion, Pancreatic lipase inhibition, α-amylase inhibition, α-glucosidase inhibition.

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Introduction

Wild fruits serve as a substitute to staple food during periods of scarcity to remote and underprivileged people. Although such fruits can be seen in the local market during their peak seasons, they find rare visibility in developed markets and struggle hard to compete with exotic invasion. *Diospyros melanoxylon* Roxb. (Family: Ebenaceae) known as *Tendu* in Hindi, Malabar ebony in English and *Tinduka* in Sanskrit is such tribal fruit. It is popular among tribal communities of Orissa, Madhya Pradesh, Chattisgarh, Andhra Pradesh, Telangana and other states of central and Southern parts of India. Although this fruit is sold by tribal communities in local markets, it could not become popular in cities. In folk medicine, tendu fruit is advocated as a cooling and astringent agent[1]. Forest people use this fruit against intense summer heat[2]. Tribal people use this fruit for extra vigour and efficiency to work for long hours without exhaustion[2]. *Tendu-Seeta* pudding is a famous recipe for Chattisgarh[3]. Unripe fruit is useful in relieving flatulence and ripe fruit is used to check excessive bile secretion[4]. Fruit extract relieves fistula problems and is also used as skincare agent[5]. In ethnomedicine, it is also used against rheumatoid arthritis and abdominal pain[6]. Methanolic extract of *D. melanoxylon* is reported to possess saponins, tannins, terpenoids, flavonoids, alkaloids and essential oils[7].

Tendu fruits ripen during the month of May-June. Fruit is ovoid or globose. It turns yellow to light orange when ripe. The pulp is yellow, glutinous, soft,

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mildly sweet and slightly astringent. These fruits are rich in sugars, proteins, fibre and vitamin C. Proximate analysis of this tribal fruit reveal that it contains approximately 81% carbohydrate, 2 % protein, 2 % fat, and 11 % fibre with the caloric value of 349 Kcal. It is also rich source of minerals like calcium (11.8 %), magnesium (62 %), iron (3.4 %), zinc (1.28 %) and copper (0.2 %). Furthermore, vitamin C (49 mg %), and β-carotene (260 µg %) are also reported to be present in ripe fruit of *D. melanoxylon*[8].

Tendu fruit wine is popular among the tribal community. An analysis of wine prepared from *tendu* fruits reveals that it contains total sugar of 3.78 g/100 mL, titratable acidity 1.32 g tartaric acid/ 100 mL; pH 3.12; total phenolics 0.95 g/100 mL; β-carotene 8 µg/100 mL; ascorbic acid 1.52 mg/ 100 mL; lactic acid 0.39 mg/100 mL; methanol 3.5 % (v/v), and ethanol 6.8 % (v/v). The tendu wine also possesses potent antioxidant activity[9].

Sustained postprandial hyperglycemia is a precursor for inducing a number of diabetic complications. The closest link between hyperglycemia and the development of diabetic complications is hyperglycemia-induced oxidative stress[10]. Protracted postprandial hyperglycemia exacerbates postprandial oxidative stress which is associated with a higher risk for development of diabetes, diabetic complication, obesity and atherosclerosis[11]. The postprandial glycemic peak and width of glycemic excursion are closely related with the intensity of postprandial hyperglycemia-induced oxidative stress[12]. Therefore, dietary material that displays relatively flattened postprandial glycemic response[13] and simultaneously possesses antioxidative stress potentials[11] may become important functional food material to tackle growing incidences of dysmetabolic disorders.

Tendu fruit being popular in tribal communities to quench heat and provide long-time energy, was selected to study antioxidant activities and antioxidative stress potentials applying various *in vitro* methods. Simultaneously, this study also compared and evaluated postprandial glycemic excursions due to consumption of tendu fruit and sucrose solution. To unravel tribal belief that consumption of tendu fruit provides energy for a longer duration; efforts were made to understand the effect of fruit on carbohydrate and lipid-digesting enzymes.

Materials and Methods

Materials

Collection and identification of plant material

Unripe *tendu* fruits were collected from Janampeta village of Bhadrachalam-Khammam district of Telangana state (India) in the month of March 2016. Fruits were identified as *Diospyros melanoxylon* Roxb. (Family: Ebenaceae) by Dr. Sateesh Suthari of Department of Plant Sciences, School of Life Sciences, University of Hyderabad (Telangana) and the sample was deposited in departmental herbarium (accession no.2172).

Processing and extraction of plant material

Whole fruits were washed properly in running tap water. Part of fruits was kept at room temperature in the closed cartoon to ripen. It took 24-48 hours for the fruit to ripen. Photographs of unripe and ripened fruits are presented in Fig. 1. The taste of unripe fruits was highly astringent, however, ripened fruits were mild sweet in taste. Peel, pulp and seeds of unripe as well as ripe fruits were separated. Finely chopped pieces of individual parts were dried at 37 °C in an incubator (Innova 4230 Refrigerated Incubator Shaker). Fine

Fig. 1 — Photographs of *D. melanoxylon* fruits. a) Unripe fruit and b) Ripened fruit.

powder of peel and pulp were prepared by grinding in food grade mixer-grinder.

Aqueous methanol extract (1:1 ratio) of peel and pulp was prepared as described earlier[14] for phytochemicals analysis and evaluation of *in-vitro* biological activities. Whole edible pulp powder of unripe and ripened fruit was used for animal experimentation.

Phytochemicals analysis and biological activities assays

Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid)], gallic acid, rutin, DCFDA (2′,7′-Dichlorodihydrofluorescein diacetate), intestinal acetone powders from rat, p-Nitrophenyl α-D-glucopyranoside, pancreatic α-amylase, pancreatic lipase type II, p-Nitrophenyl palmitate, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), tris-HCl, Aluminum chloride, DNS (3,5-dinitrosalicylic acid) were purchase from Sigma-Aldrich, St. Louis (USA). DMEM (Dulbecco modified eagle medium), FBS (fetal bovine serum), PNS (Penicillin-Streptomycin-Neomycin) were procured from Gibco (USA) and Folin–Ciocalteu reagent was obtained from Merck Specialties Pvt. Ltd Mumbai. Other fine chemicals of analytical grade were acquired from Indian manufacturers.

Total polyphenol

Aqueous methanol extract of *D. melanoxylon* was reacted with Folin-Ciocalteu reagent and Na_2CO_3 (20 %, w/v). After incubation of 60 min at room temperature, absorbance was read at 765 nm spectrophotometrically[15]. Gallic acid was used as the standard. Total phenol content was expressed in terms of Gallic acid equivalent (GAE).

Total flavonoids

Total flavonoids concentration in aqueous methanol extract was determined by reacting extract with 2 % aluminium chloride solution and recording absorbance at 430 nm spectrophotometrically. Rutin was taken as standard and results were expressed in terms of rutin equivalent[15].

Total anthocyanins

The pH differential method was used to determine monomeric anthocyanin contents in aqueous methanol extract. Equal volume of extract was mixed with 25 mM potassium chloride solution (pH 1.0) and 0.4M sodium acetate buffer (pH 4.5). Absorbance was recorded at 510 nm and 700 nm. Results were explained as described by Guisti *et al*[16].

***In-vitro* biological activities**

DPPH radical scavenging activity

Decolourization of DPPH by extract was determined at physiological pH (tris-HCl buffer pH 7.4) by recording absorbance at 517 nm spectrophotometrically[17]. Ascorbic served as the standard radical scavenger.

ABTS radical scavenging activity

Scavenging of $ABTS^{+\cdot}$ radical cation was measured in phosphate buffer medium (6.8 mM, pH 8.0)[17]. Absorbance 734 nm was recorded spectrophotometrically. Ascorbic acid served as the standard radical scavenger.

Free radicals induced DNA damage and protective effects of fruit parts

The method described by Chang *et al*.[18] was adopted with suitable modifications to assess DNA damage in pUC18 DNA. The reaction was carried out in tris-buffer (pH 7.4) at 37 ºC. $FeCl_3$ and H_2O_2 were used to generate hydroxyl radicals ($^{\cdot}OH$). In an Eppendorf tube, for control test, pUC18 DNA (2 µg) was incubated with 5 µLof tris-buffer. In another set, pUC18 DNA (2 µg) along with 5 µLof tris-buffer was enacted with $FeCl_3$ (5 µL) and 10 µL of 30 % H_2O_2. The next set was prepared with pUC18 DNA (2 µg), 5 µL of tris-buffer and 5-10 µL of extract (5 mg/mL prepared in tris-buffer) and incubated for 10 min at RT. $FeCl_3$ (5 µL) and 10 µL of 30 % H_2O_2 were added to induce free radical reaction. The volume of the reaction mixture was equalized with the addition of tris- buffer. Tubes were incubated at 37 ºC for 15 minutes. To this reaction mixture, 3 µL of 6X gel loading dye was added. Electrophoresis was performed on 0.8 % agarose gel containing 3 µL ethidium bromide (10 mg/mL), at 85 V for 35 min. The gel was viewed under transilluminating UV light and photographed (Bio-Red ChemiDoc™ XRS+ with Image Lab™ Software).

Antioxidative stress potential in cell lines

NIH 3T3 (mouse embryonic fibroblast, CRL-1658) cells were obtained from National Centre for Cell Sciences, Pune (India). Cells were grown in tissue culture flask in DMEM supplemented with 10 % FBS and 1× antibiotic solution (Sigma) in a CO_2 incubator at 37 ˚C (5 % CO_2 and 90 % relative humidity).

Hydrogen peroxide induced oxidative stress in cells and antioxidative stress activity by samples

The effect of aqueous methanol extract on H_2O_2 induced oxidative stress on cells was determined by MTT assay[19]. NIH 3T3 cells (1×10^6) were seeded in 96 well plates for 24 hours. After 24 hours of incubation, cells were treated with different concentration (1 µg, 5 µg and 10 µg) of extract for 48 h in presence and absence of 10 µM H_2O_2. After 48 hours of incubation, 10 µL MTT (5 mg/mL) was added to each well and plates were further incubated for 4 h at 37 °C in dark. Culture medium from each well was carefully removed out and 100 µL of DMSO was added. Reduction of MTT by metabolically viable cell was determined by measuring absorbance at 570 nm spectrophotometrically. Antioxidative stress potential (AoxP) of test extract was calculated as follows:

$$100-[\{(A_c-A_t)/A_c\}\ x100]$$

where, 'A_c' represent absorbance of cells without H_2O_2 treatment and 'A_t' represent absorbance of cells treated with H_2O_2 in presence or absence of extract.

Hyperglycemia induced oxidative stress in NIH 3T3 cells and antioxidative stress activity by samples

NIH 3T3 cells (1×10^6) were seeded in 96 well plates for 24 hours. After 24 hours of incubation, cells were treated with different concentration of extract for 48 hours in presence and absence of high glucose DMEM (9 gm/L). After 48 hours of incubation, 10 µL MTT was added to each well and plates were further incubated for 4 hr at 37 ºC in dark. Culture medium from each well was carefully removed out and 100 µL of DMSO was added in each well. Reduction of MTT by metabolically viable cell was determined by measuring absorbance at 570 nm spectrophotometrically.

Antioxidative stress potential (AoxP) of test extract was calculated as follows:

$$100-[\{(A_c-A_t)/A_c\}\ x100$$

Where 'A_c' represents absorbance of cells without high glucose medium treatment and 'A_t' represent absorbance of cells treated with high glucose medium in presence or absence of extract.

Fluorescence-activated cell sorting (FACS) analysis of H_2O_2 induced oxidative stress in NIH3T3 cells and effect of aqueous methanol extract

Quantification of antioxidative stress potentials of extracts was evaluated by flow cytometric analysis in NIH 3T3 cells. NIH 3T3cells were treated same as above and incubated for 48 h in presence or absence of 10 µM H_2O_2. Then cells were treated with fluorogenic dye DCFDA (2 µM) at 37 ˚C for 30 minutes and flow cytometric analysis was carried out[20]. Numerical values generated in triplicate for viable cells to quantify antioxidative stress potential of extract.

Intestinal α-glucosidase inhibition

Crude rat intestinal acetone powder was taken as a source of intestinal α-glucosidase enzyme. The acetone powder was sonicated in normal saline (100:1, w/v). The supernatant was collected as crude intestinal α-glucosidase enzyme after centrifugation at 3000 rpm at 4°C for 30 minutes. The enzyme was pre-incubated with extract in phosphate buffer saline (pH 6.8) for 5 minutes and then reacted with substrate *p*-nitrophenyl-α-D-glucopyranoside prepared in the same buffer for 5 minutes. The release of *p*-nitrophenol due to the action of the α-glucosidase enzyme was recorded spectrophotometrically at 405 nm[21]. Effect of extract on enzyme activity was compared with control where the enzyme was reacted with the substrate in absence of extract.

Pancreatic α-amylase inhibition

Extracts were primarily incubated with pancreatic α-amylase and then were reacted with soluble potato starch solution (0.5 % w/v in 20 mM phosphate buffer, pH 6.9) for 3 min. DNS colour reagent (1.0 g of 3, 5-dinitrosalicylic acid, 30 g of sodium potassium tartrate and 20 mL of 2 N NaOH to a final volume of 100 mL in distilled water) was added. Closed tubes were placed in a water bath (85–90 °C) for 10 minutes to develop colour and cooled thereafter. About 50 µL of reaction mixtures was diluted with 175 µL of distilled water in a 96-well microplate. α- Amylase activity was determined by measuring the absorbance of the mixture at 540 nm spectrophotometrically[22].

Pancreatic lipase inhibition

Pancreatic lipase inhibition was determined according to the procedure described by Mc Dougall *et al* [23]. Porcine pancreatic lipase type II dissolved in MilliQ water was pre-incubated with extracts and mixture was reacted with substrate *p*-nitrophenyl palmitate. Enzyme activity was recorded measuring the release of *p*-nitrophenol spectrophotometrically at 405 nm. Effect of extract on enzyme was compared with control where enzyme and substrate were incubated in absence of extract.

Animal experiment

Postprandial glycemic excursion

All the experimental animals were procured from Palamuru Biosciences Pvt. Ltd and were housed in Bio-Safe animal house facility (Reg. No. 97/GO/RBi/S/ 1999/CPCSEA) of CSIR-IICT. The animal experiment was performed using adult Wistar rats (200±10 g body weight). Institutional Animal Ethical Committee (Protocol No. IICT/07/2017, Gov. of India) approval for the experiment was obtained. Animal experimentation was performed as per the procedure and dosage described earlier[24]. Experiments with live animals were conducted in compliance with the relevant laws and institutional guidelines. Rats were kept for overnight fasting. Next day forenoon, blood was collected from retro orbital plexus in EDTA containing tubes. Fasting plasma glucose levels ("0" h) levels were measured by Hexokinase method using auto-blood analyzer instrument (Siemens, Germany). Rats were divided into three groups (five rats in each group) as follows:

1. Sucrose treated (2 g/kg body weight)

2. Ripe fruit (RF) pulp powder treatment (4 g/kg body weight)

3. Unripe fruit (URF) pulp powder treatment (4 g/kg body weight)

Thereafter, blood sample was collected at intervals of 0, 30, 60, 90 and 120 minute(s) post feeding. Plasma was separated out for glucose measurement as described above. At 2 hours, postprandial Glycemic load ($AUC_{0\text{-}120\ min.}$ mg/dL) was calculated following trapezoidal rules[24].

Statistical analysis

One-way ANOVA followed by Tukey's multiple comparison tests was applied to compare differences within the groups. Unpaired t-test (two tailed) with Welch's correction was applied to compare differences between the groups. The criterion for statistical significance was $p < 0.05$. Statistical analyses were performed using GraphPad PRISM® Version 5.01 (GraphPad Software. Inc., CA, USA).

Results and Discussion

Phytochemical analysis

Total polyphenolic, flavonoid and anthocyanin contents

Dietary polyphenols have been identified to provide protection against oxidative stress and affect postprandial glycemic and lipidemic excursions[11]. Total polyphenols content in the pulp, as well as the peel of *D. melanoxylon* fruit, decreased sharply when the fruit ripened. In unripe fruit, total polyphenolic content was higher than the polyphenols content in ripe fruit (Table 1). More than 30 times ($p < 0.0003$) decrease was observed in the polyphenol content of pulp and 17 times ($p < 0.0005$) in peel when the fruit ripens (Table 1). Similar was the case observed in total flavonoids content. More than 10 times ($p < 0.0007$) decrease in total flavonoid content in pulp and 16 times ($p < 0.0001$) in the peel of ripe fruit was observed by in our study (Table 1). Decrease in polyphenols after fruits ripening has also been observed other investigators[25,26]. In addition to these observations, our analysis report decline in total flavonoids content also during the ripening process of fruit. The anthocyanin content in unripe fruit pulp was 35 % ($p < 0.001$) more than the anthocyanin content in the peel of the unripe fruit (Table 1). These anthocyanins may be considered as monomeric proanthocyanins. Applying the pH differential analytical method, only monomeric proanthocyanins can be detected due to observed absorbance differences at pH 4.5 and pH 1.0. However, when anthocyanins are polymerized and are present in the oligomerized state, the differences in absorbance at two pH levels are not observed[27]. Therefore, the presence of polymeric oligomerized anthocyanins cannot be ruled out in ripe fruits pulp and peel.

Table 1 — Phytochemical analysis in aqueous methanol extracts of *D. melanoxylon* fruit

	Total Polyphenol (µg/mL, GAE)		Total Flavonoids (µg/mL, RE)		Total Anthocyanin (% w/W)	
	Unripe	Ripe	Unripe	Ripe	#Unripe	Ripe
Pulp	340±5.77	10.28±1.47[a]	11.58±0.11	1.04±0.25[c]	1.33±0.02	#
Peel	290±5.77	17.5±1.92[b]	14.36±0.00	0.89±0.04[d]	0.98±0.01	#

Values represent mean±SEM, n ≥3, GAE; gallic acid equivalent, RE; rutin equivalent.

[#]Differences in absorbance between pH 1.0 and pH 4.5 as a measure of structural changes in anthocyanins chromophores were noticed in unripe fruit's pulp and peel. Therefore, anthocyanins present in these fruits represent monomeric proanthocyanins.

[# #] These differences could not be detected, however, in ripened fruit extracts. Hence, the anthocyanins present in ripened fruit extracts may be considered as polymerized oligomeric anthocyanins[27].

Unpaired t-test (two tailed) with Welch's correction was applied to compare differences between the groups. [a]$p < 0.0003$, [b]$p < 0.0005$, [c]$p < 0.0007$, [d]$p < 0.0001$ when compared with their respective counter parts.

***In-vitro* biological activities**

ABTS^{+} and DPPH radicals scavenging activity

ABTS+ is an amphiphilic cation radical applied to identify both the hydrophilic antioxidants as well as lipophilic antioxidants[28] and DPPH is organic nitrogen centred radical often applied to assess reducing the power of an antioxidant[29]. The ABTS+ cation is a planar radical hence an antioxidant even with low redox potentials may be identified using this test model, however, they may react slowly or even not when tested on DPPH radical due to the steric hindrance of N radical[28]. The ABTS+ radical cation and DPPH radical scavenging potentials of aqueous methanol extracts of *D. melanoxylon* fruit are presented in Table 2. The ABTS+ radical scavenging potential in unripe fruit peel was significantly higher (p <0.003) than that present in the pulp. However, differences between unripe pulp and peel's DPPH radical scavenging activity were not significantly different (p= 0.128, Table 2). The ABTS+ radical cation and DPPH radical scavenging activity could not be observed in the extract of ripe fruit' pulp and peel in our analysis. Our observations are in accordance with the earlier report of Kim *et al*[25], who observed a drastic decrease in free radicals scavenging activities in ripe mango.

Pancreatic α-amylase, intestinal α-glucosidase and pancreatic lipase activities

Clinically, use of intestinal α-glucosidase and pancreatic α-amylase inhibitors has shown promising strategies in mitigating diet-induced postprandial hyperglycemic burden in diabetic people[30]. However, several disadvantages are associated with the use of these synthetic drug molecules. The reason being these drugs are competitive inhibitors of carbohydrate digestion enzymes. Due to their competitive nature with substrate carbohydrate, digestion of food material remains partial in the intestine which results in flatulence, bloating and diarrhoea. Therefore, identification of dietary food materials containing inherent inhibitory activities for these enzymes may prove advantageous over existing drugs in controlling postprandial hyperglycemic spikes in diabetics.

Presences of pancreatic α-amylase, as well as intestinal α-glucosidase inhibitory activities, were observed in both, the unripe as well as ripe fruit of *D. melanoxylon* (Table 2). The α-amylase inhibitory activity was significantly (p <0.03) higher in ripe fruit pulp when compared with activity present in unripe fruits pulp, however, the differences between unripe and ripe peel were not statistically significant (Table 2). Extract of unripe fruit's pulp and peel displayed significantly higher intestinal α-glucosidase inhibitory activity than the ripe fruit extracts (p <0.002 and p <0.001 respectively, Table 2). Presence of these inhibitory activities in *D. melanoxylon* fruit shows that digestion of carbohydrates after consumption of this fruit may be slow and therefore, the presence of these activities may slow down sudden increase in blood glucose level after fruit consumption.

Hyperlipidemia is considered as disorders of lipid metabolism. One of the recent approaches of reducing diet-induced postprandial hyperlipidemic excursion is through the slowing down the activity of pancreatic lipase which is involved in digestion of dietary lipids[31]. Our study recorded the presence of pancreatic lipase inhibitory activity in the extract of unripe fruits pulp and peel. The lipase inhibitory activity was significantly (p <0.002) higher in the extract of unripe fruit pulp than present in the peel (Table 2). However, in ripe fruits pulp or the peel, this activity could not be detected (Table 2).

Concentration-dependent percentage of α-glucosidase inhibitory activity plot is presented in Fig. 2. From Fig. 2, it is evident that in contrast to the linear (as displayed by standard drug acarbose)

Table 2 — Free radicals scavenging, carbohydrate and lipid digestion enzymes inhibitory activities in aqueous methanol extract of *D. melanoxylon* fruit

	$ABTS^{+\cdot}$ (SC_{50}, µg/mL)		DPPH (SC_{50}, µg/mL)		Pancreatic α-amylase (% inhibition at 1 mg/mL)		Intestinal α-glucosidase (% inhibition at 1 mg/mL)		Pancreatic lipase (% inhibition at 1 mg/mL)	
	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe
Pulp	58.3±0.33	ND	146.9±1.00[b]	ND	12.82±0.77	15.98±0.40[c]	61.17±1.69	38.45±1.52[d]	79.06±2.12[f]	ND
Peel	89.00±1.73[a]	ND	101.5±18.05	ND	16.45±0.96	14.52±0.13	87.25±1.66	42.77±0.30[e]	47.52±2.57	ND
Ascorbic acid	77.67±0.33		661±2							
Acarbose					22.36±1.69		46.69±1.31			
Orlistat									45.80±1.44	

or hyperbolic slope as is usually observed in case of reversible enzyme inhibition (competitive and non-competitive inhibitors)[32], the slope of curve with *D. melanoxylon* aqueous methanol extract intersects horizontal to the percentage of inhibition axis (Y). In such a case, enzyme inhibition is considered to be irreversible-type[33]. Therefore, α-glucosidase inhibitory activity present in the extract of *D. melanoxylon* fruit is of irreversible in nature. Such type of Inhibitory activities have been reported in food materials also reported beneficial to control diet-induced hyperglycemia[34,35].

Antioxidative stress capacity

Oxidative stress is the result of normal metabolic activities. Aerobic organisms are equipped with an inbuilt antioxidant defence mechanism. However, under consistent and overstressed circumstances such as hyperglycemia and hyperlipidemia, this defence mechanism fails to prevent bodily damages caused by a variety of free radicals arising under oxidative stress conditions. Therefore, easing out of oxidative stress nowadays has become a core subject to maintain better health and also therapeutics development. A diet that encompasses antioxidative stress potentials together with antihyperglycemic and antihyperlipidemic properties hence, offers a better choice for health maintenance and settle oxidative stress arising due to a disturbance in metabolic activities[11].

The free radicals scavenging antioxidant activity in aqueous methanol extract of *D. melanoxylon* pulp and peel presented in table 2 showed that unripe fruit has potentials of scavenging free radicals. In order to verify the antioxidative stress potential of unripe *D. melanoxylon* fruit, we challenged 3T3 cells with biological radical generator H_2O_2 and found that cells viability was significantly (p <0.001) compromised under the hyper physiological concentration of H_2O_2 (Fig. 3). However, when cells were pre-incubated with different concentrations of pulp extract and then challenged with a hyper physiological concentration of H_2O_2, the cells viability was retained (Fig. 3). Interestingly, however, although at lower pulp extract concentrations (1µg and 5 µg) of pulp extract, the cells viability was improved significantly (p <0.001 and p <0.01 respectively), the higher concentration (10 µg) of pulp extract could not protect cells against H_2O_2, induced oxidative stress. Beneficial antioxidative stress effect could not be observed with the peel of the fruit (Fig. 3). These observations disclose the fact that over-consumption of antioxidant enriched-diet or dietary formulations may not always be beneficial. Such cautionary reports have started emerging from a number of studies wherein antioxidantenrichednatural formulations have displayed undesired effects[36].

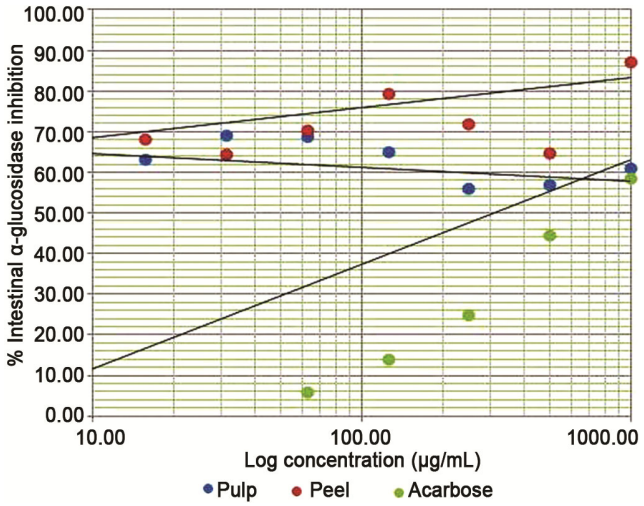


Fig. 2 — Concentration dependent α-glucosidase inhibition by aqueous methanol extract of *D. melanoxylon* fruit. In contrast to linear or the hyperbolic[32], the slope of the curve in presence of different dilutions of inhibitors intersects horizontal to the percentage of inhibition axis (Y) in case of irreversible enzyme inhibition[32,33]. Our experiment finds that inhibitor dilution line intersects 'Y' axis horizontally and hence the inhibition is irreversible.

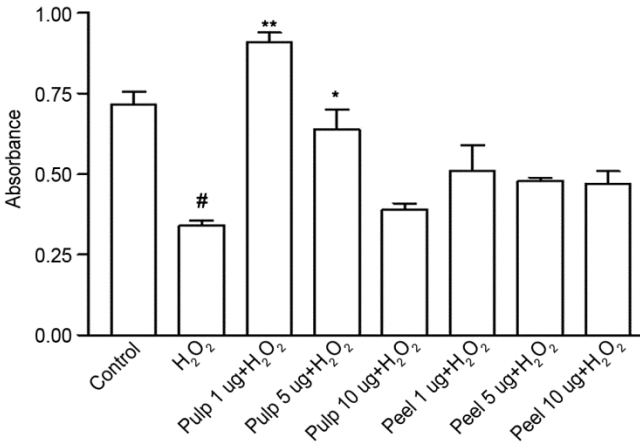


Fig. 3 — Hydrogen peroxide (H_2O_2) induced oxidative stress in NIH 3T3 cells and effect of aqueous methanol extract of unripe *D. melanoxylon* fruit.Values represent mean±SEM, n= 3. Tukey's multiple comparison test followed by ANOVA was applied to find differences within the groups. #p <0.001 (control vs H_2O_2), *p <0.01, (H_2O_2 vs pulp 5µg), **p <0.001 (H_2O_2 vs pulp 1 µg) .

Influence of aqueous methanol extract of unripe *D. melanoxylon* fruit on high glucose-induced oxidative stress and detection of total ROS by DCFDA in NIH 3T3 cells are presented in Fig. 3 and 4, respectively. Hyperglycemia is recorded factor inducing oxidative stress and supplementation of dietary antioxidants has been shown to alleviate sucrose-induced oxidative stress in animal models[37]. Incubation of NIH 3T3 cells under hyperglycemic environment significantly (p <0.001) reduced cells viability when compared with the normal control cells (Fig. 4). However, when cells were incubated along with pulp extract of unripe fruit at various concentrations (1µg, 5 µg and 10 µg), the viability of cells was retained close to normal level and high glucose-induced detrimental effect was significantly (p <0.05 to p <0.001) abolished (Fig. 4). Antioxidative stress potential of unripe *D. melanoxylon* fruit extracts was validated by FACS analysis. In this analysis, DCFDA dye was used to detect the generation of reactive oxygen species (ROS) generated due to H_2O_2. Representative FACS diagrams and histogram of triplicate analysis are presented in Fig. 5. The ROS generation (green right shift in FACS diagrams, Fig.5) was significantly (p <0.001) high when the cell was treated with H_2O_2 in comparison to normal control cells (Fig. 5). However, ROS were significantly (p <0.001) reduced when NIH 3T3 cells were pre-incubated with different concentrations (1, 5 and 10 µg) of pulp and peel extracts before challenging them with H_2O_2 (Fig. 5).

Free radical-induced damage to DNA has been linked to the etiology of numerous disease conditions[38]. Protection against oxidative DNA damage has been identified in dietary materials[39]. We induced hydroxyl radical-mediated damage to pUC18 DNA and found that *D. melanoxylon* fruit extract prevented damage to DNA (Fig. 6).

***In-vivo* postprandial glycemic excursion**

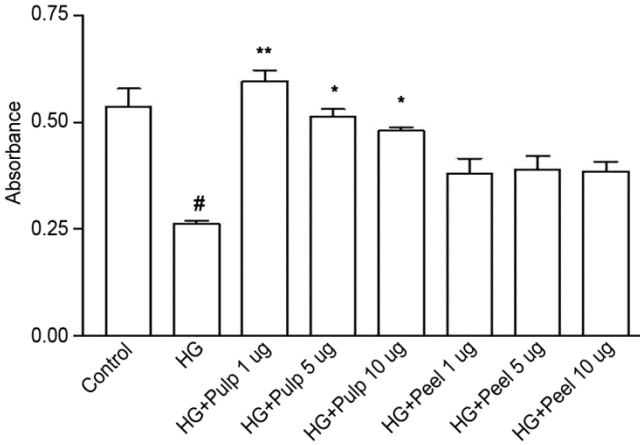
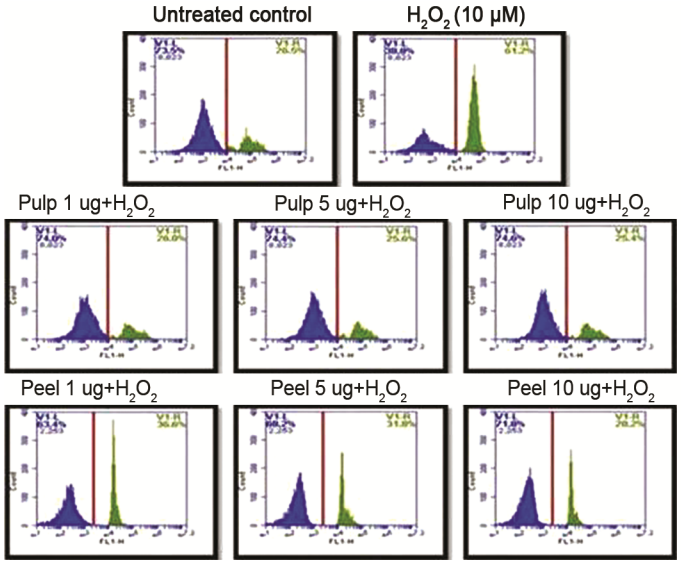


Fig. 4 — High-glucose (HG) induced oxidative stress in NIH 3T3 cells and effect of aqueous methanol extract of *D. melanoxylon* fruit.Values represent mean±SEM, n= 3. Tukey's multiple comparison test followed by ANOVA was applied to find differences within the groups. #p <0.001(control vs HG); *p <0.05 (H_2O_2 vs 5µg and 10 µg pulp concentration) and **p <0.001 (H_2O_2 vs 1 µg pulp concentration).



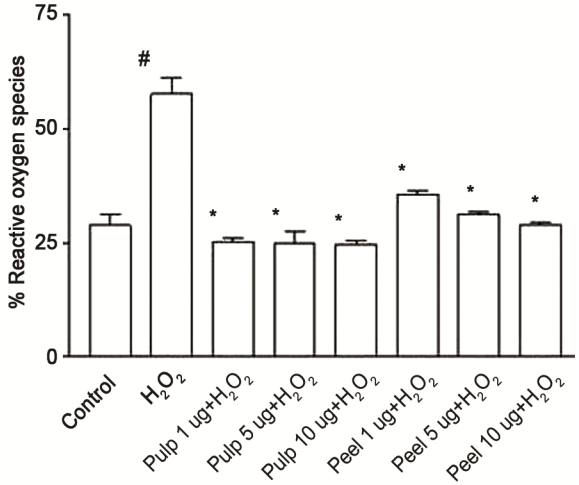


Fig. 5 — Detection of reactive oxygen species generated due to H_2O_2 in NIH 3T3 cells by FACS analysis and effect of aqueous methanol extract of unripe *D. melanoxylon* fruit.Values represent mean±SEM, n= 3. Tukey's multiple comparison test followed by ANOVA was applied to find differences within the groups. #p <0.001 (control vs H_2O_2), * p <0.001 (H_2O_2 vs pulp or the peels).

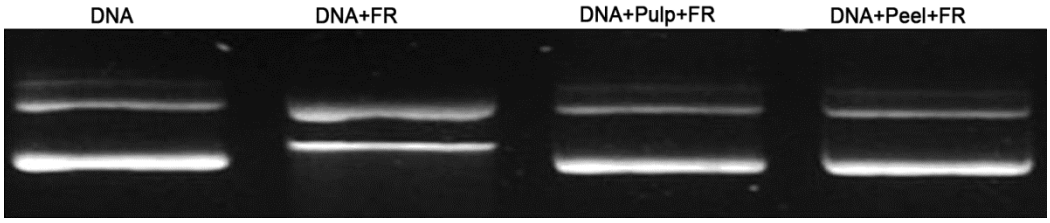


Fig. 6 — Influence of aqueous methanol extract of *D. melanoxylon* fruit on free radicals induced DNA_{pUC} damage. FR, free radical.

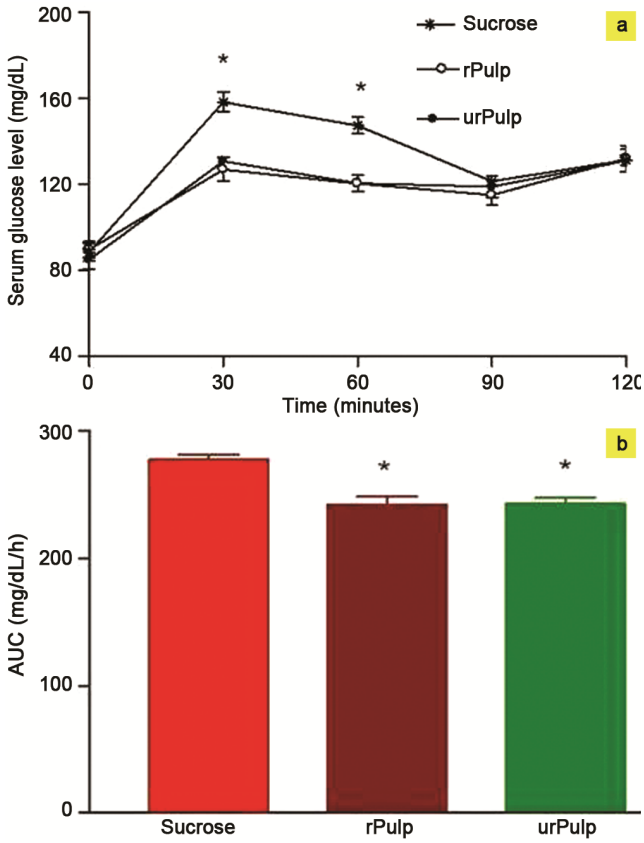


Fig. 7 — Glycemic response in rats following oral administration of sucrose and pulps (r-ripe,ur-unripe) of *D. melanozylon*. (a) Two hours postprandial glycemic curve following oral administration of sucrose and fruit pulp. Data represent mean±SEM, n= 6. *p <0.05 compared with rPulp & urPulp. (b) Total glycemic load (2 h) following oral administration of sucrose and fruit pulp. Data represent mean±SEM,n= 6. *p <0.05 compared with sucrose.

The post prandial glycemic response due to consumption of *D. melanoxylon* fruit was studied in Wistar rats and results are presented in Fig. 7. The glycemic response in rats was compared with the glycemic response induced due to consumption of sucrose. Plasma glucose levels of rats after administration of sucrose, unripe and ripe fruit pulp at different time points are presented in Fig. 7a. It is evident from plasma glucose response curve that even with 4 g/kg body weight the glycemic excursion induced by fruits pulp was significantly (p <0.05) less at 30th and 60th min when compared with plasma glucose spikes induced by 2 g/kg body weight oral sucrose feeding (Fig. 7a). The overall two-hour glycemic load due to *D. melanoxylon* fruit pulp consumption was significantly (p <0.05) less than that compared with sucrose control (Fig. 7b).

Conclusion

This research finds for the first time that fruit of tendu is a low glycemic fruit and can become a suitable alternative replacement for sucrose enriched beverages. Furthermore, identification of antioxidative stress properties in this wild fruit provides additional value for its commercialization as healthy fruit beverage.

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