Vegetables' juice influences polyol pathway by multiple mechanisms in favour of reducing development of oxidative stress and resultant diabetic complications

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ABSTRACT

Objective: Hyperglycemia induced generation of free radicals and consequent development of oxidative stress by polyol pathway is one of the crucial mechanisms stirring up development of diabetic complications. We evaluated influence of ten vegetables' juice on polyol pathway along with their antioxidant and antioxidative stress potentials. **Materials and Methods:** Aldose reductase activity was determined utilising goat lens and human erythrocytes. In goat lens, utilization of nicotinamine adenine dinucleotide phosphate (NADPH) and aldose reductase inhibition was assayed. In human erythrocytes, sorbitol formation was measured as an index of aldose reductase activity under normoglycemic and hyperglycemic conditions. Ability of juices in inhibiting oxidative damage to deoxyribose sugar and calf thymus DNA and inhibitory activity against hydrogen peroxide induced hemolysis of erythrocytes was also analysed. Phytochemical contents like total polyphenol, total flavonoid and total protein were measured to find their influence on biological activities. **Results:** Vegetables' juice displayed varying degrees of inhibitory potentials in mitigating NADPH dependent catalytic activity of aldose reductase in goat lens, accumulation of sorbitol in human erythrocytes under different glucose concentrations; Fenton-reaction induced oxidative damage to deoxyribose sugar, and calf thymus DNA. Substantial variations in vegetables phytochemicals content were also noticed in this study. **Conclusions:** Vegetables' juice possesses potent activities in influencing polyol pathway by various mechanisms in favour of reducing development of oxidative stress independent of their inherent antioxidative properties. Juice of ivy gourd followed by green cucumber and ridge gourd were among the most potent for they displayed strong activities on various parameters analysed in this study. These vegetables' juice may become part of mechanism-based complementary antioxidant therapy to prevent development of diabetic complications.

Key words: Aldose reductase, antioxidant activity, diabetic complications, hyperglycemia, oxidative stress, polyol pathway, sorbitol, vegetables' juice

INTRODUCTION

Although, intensive blood glucose control is still the main objective to halt initiation or progression of diabetic complications, the impact of existing therapies have several limitations due to difficulties in maintaining blood glucose level close to normal range. Diabetic vascular complications are the major cause of retinopathy, nephropathy, neuropathy, cardiovascular complications, stroke and limb amputation. Therefore, management of diabetes and its complications is not only a serious public health issue but also a socioeconomic burden in most parts of the world.[1,2]

Hyperglycemia induced increased generation of free radicals and consequent development of oxidative stress has been recognized as one of the crucial pathway stirring up development of diabetic complications. Several mechanisms have been proposed by which hyperglycemia induces increased generation of free radicals resulting development of oxidative stress. One of the important mechanisms by which hyperglycemia induces oxidative stress is polyol pathway.[3] Under euglycemic condition

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only trace amount (~3%) of glucose enters polyol pathway; however, increased flux (>30%) of glucose has been noticed under hyperglycemic situation.[4,5] The rate limiting step of polyol pathway is reduction of glucose to sorbitol catalysed by enzyme aldose reductase (ALR) at the expense of reduced nicotinamide adenosine dinucleotide phosphate (NADPH).[5] Sorbitol is, in turn converted to fructose by sorbitol dehydrogenase (SDH) with the help of oxidized form of nicotinamide adenine dinucleotide (NAD^+) as a co-factor.[5]

Depletion of NADPH by ALR hampers regeneration of reduced glutathione (GSH), an important intracellular antioxidant [Figure 1] leading to ineffective scavenging of reactive oxygen species (ROS) and thereby exacerbates development of oxidative stress.[1] Furthermore, during conversion of sorbitol into fructose by SDH, the co-factor NAD^+ is converted into NADH.[1] NADH is substrate for NADH oxidase responsible for generation of superoxide anions [Figure 1].[6] Taken together, reduction in antioxidant enzyme GSH and increased generation of free radicals (ROS) through polyol pathway contributes to the development of oxidative stress [Figure 1]. Oxidative stress and free radicals induced damage to biomolecules results in the imbalance of normal physiological functions leading to the development of diabetic complications.

These advances in understanding pathophysiology of diabetic complications have increased interest in determining beneficial effects of antioxidant therapy that can complement to intensive glucose control. Even though, the efficacy of classical antioxidants in preventing development of diabetic complications is still uncertain, it is being advocated that identification of mechanism-based antioxidant therapies may become more promising therapeutic strategy.[1] Vegetables have been identified as

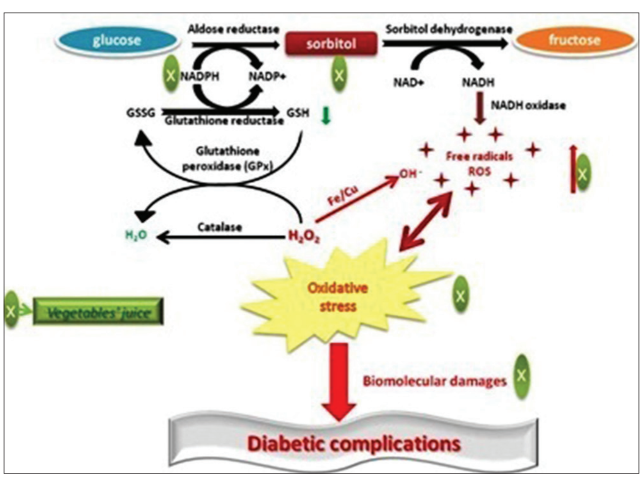

Figure 1: Mechanism of polyol pathway induced development of diabetic complications and effects of vegetables'juice on various stages

an economical natural source of potent antioxidants[7] and important in the maintenance of health and prevention of several diseases.[8] Furthermore, eating vegetables before carbohydrate rich meals have been found to improve postprandial glycemic excursion in clinical settings.[9] Recently, certain vegetables' juice has been identified to display antihyperglycemic activities[10,11] through various mechanisms.[11,12] Vegetables' juice has also been observed to reduce development of hyperglycemia induced oxidative stress and imbalance in physiological functions.[13]

This study explored effect of vegetables' juice on polyol pathway, and found that apart from their inherent antioxidant and antioxidative stress activities, they also possess potentials of influencing polyol pathway against development of oxidative stress and therefore, may serve as an economical complementary therapy in preventing development of diabetic complications.

MATERIALS AND METHODS

Chemicals

Aluminium chloride ($AlCl_3.6H_2O$), Ammonium sulfate ($(NH_4)_2SO_4$), Ascorbic acid, Bovine Serum Albumin (BSA), Bradford's reagent, Ethidium bromide, Ferric chloride ($FeCl_3$), Folin-Coicalteu reagent, Gallic acid, Glucose, Glycerol, DL-Glyceraldehyde, Glycine, Hydrogen peroxide (H_2O_2), Disodium ethylenediaminetetraacetate dihydrate (EDTA), Reduced nicotinamine adenine dinucleotide phosphate (NADPH), Nicotinamine adenine dinucleotide (NAD^+), Perchloric acid ($HClO_4$), Phenylmethanesulfonylfluoride (PMSF), Sodium chloride (NaCl), Sorbitol dehydrogenase (SDH), Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Tris-HCl were procured from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Other chemicals of analytical grade were purchased from Indian manufacturers.

Preparation of vegetables' juice

Vegetables namely, Ash gourd (AG) fruit (*Benincasa hispida* Thunb.Cogn.), Bottle gourd (BG) fruit (*Lagenaria siceraria* Molina standl), Banana stem (BS, *Musa paradisiaca* L.), Carrot (CT) root (*Daucus carota* L.), Green Cucumber (GC) fruit (*Cucumis sativus* L.), Ivy gourd (IG) fruit (*Coccinia grandis* L. J.Voigt), Radish (RD) root (*Raphanus sativus*), Ridge gourd (RG) fruit (*Luffa acutangula* L. Roxb.), Snake gourd (SG) fruit (*Trichosanthes cucumerina* L.) and Yellow Cucumber (YC) fruit (*Cucumis melo var. chito*) were procured daily afresh from local vegetable markets of Hyderabad (India).

Vegetables were thoroughly washed. As a precaution, any vegetable with bitter taste was discarded. A weighed amount of each finely chopped unpeeled vegetables were ground in a food-grade grinder and squeezed in a clean sterile muslin cloth to obtain juice. Percentage yield of the juice was calculated from the volume of obtained juice.[12] The juice was further centrifuged (Eppendorf centrifuge 5430R, Eppendorf AG, 22331 Hamburg, Germany) for 30 min at 5000 rpm (18°C) and supernatant was used for analysis.

Determination of ALR activity

ALR activity was determined utilising goat lens and human erythrocytes. In goat lens, utilization of NADPH and ALR inhibition was assayed. In human erythrocytes, sorbitol formation was measured as an index of ALR activity under normoglycemic and hyperglycemic conditions.

Isolation of lens

Goat eyes from freshly slaughtered animal were obtained from local slaughter house in ice-cold container. Each eye was cleaned in cold distilled water. Cornea, sclera, rhodopsin pigment, aqueous humour, muscle and fat tissue around the eye were carefully removed. Clear lens was washed in cold distilled water and stored at -80°C.

Preparation of ALR enzyme from lens

Enzyme from the lens was isolated by method of Hayman *et al.*,[14] with suitable modifications. Lens were washed thouroughly and homogenised in three volumes of cold distilled water at 4°C. Homogenate was centrifuged for 30 min at 15000g (4°C) (Eppendorf centrifuge 5430R, Eppendorf AG, 22331 Hamburg, Germany) and pellet was discarded. The supernatant was saturated with solid ammonium sulfate to obtain 30% saturation. Mixture was allowed to stand for 20 min with occassional stirring and centrifuged again. Supernatant was then saturated (75%) with solid ammonium sulfate for 60 min. The saturated supernatant was centrifuged and pellet was reconstituted in equal volume of NaCl (0.05 M) as a source of partially purified ALR[15] and stored at -20°C.

Determination of ALR activity

Kinetics of NADPH utilisation as a function of ALR catalytic activity was determined spectrophotometrically (Perkin Elmer $_{\text{precisely}}$, Lambda 25, UV/Vis spectrometer, Massachusetts, USA) with suitable modifications.[16] Reaction mixture contained, 100µLs of vegetables' juice, 0.3 mM NADPH, 10mM DL-Glyceraldehyde, and 600 µL of 0.1M sodium phosphate buffer (pH 6.2) in a final volume of 1mL. Kinetics of NADPH utilisation was measured at 340 nm for 5 min at an interval of 30 sec with the addition of 100 µL enzyme.

Suitable blank was employed without enzyme in the reaction mixture to counter background absorbance.

Percentage of unutilized NADPH over time was calculated as follows: (100-ΔC) Vs Time for control and (100-ΔS) Vs time in presence of vegetables juice. ΔC represents percentage of NADPH utilised over time in control and was calculated as follows:

$$\Delta C = \frac{C_0 - C_t}{C_0} \times 100$$

ΔS represents percentage of NADPH utilised over time in presence of vegetables' juice and was calculated as follows:

$$\Delta S = \frac{S_0 - S_t}{S_0} \times 100$$

Percentage ALR inhibition (I_{Lens} at 't' 5th min) was calculated by applying following formula:

$$I_{Lens} = \frac{\Delta C - \Delta S}{\Delta C} \times 100$$

Where, C_0 = Absorbance of control at '0' time
C_t = Absorbance of control at time 't'
S_0 = Absorbance with vegetable juice at '0' time
S_t = Absorbance with vegetable juice at time 't'.

Preparation of erythrocyte suspension

Blood was withdrawn from healthy human volunteers after overnight fasting. 1 mL of whole blood was centrifuged (Eppendorf centrifuge 5430R, Eppendorf AG, 22331 Hamburg, Germany) at 1500 rpm for 10 min with 4 volumes of normal saline at 18°C. This procedure was repeated thrice in order to get packed cells. One volume of washed and packed erythrocytes was suspended in four volumes of Kreb's bicarbonate buffer.[17]

Determination of Sorbitol content in erythrocytes as an index of ALR activity

150 µL of erythrocytes suspension was incubated with 120 µL vegetable juice for 30 min at 37°C. Mixture was incubated for 3h in presence of 120 µL of 100 mg/dL and 300 mg/dL glucose respectively. Erythrocytes were homogenised (Heidolph silent crusher s) in 9 volumes of 0.8 M perchloric acid and centrifuged (Eppendorf centrifuge 5430R, Eppendorf AG, 22331 Hamburg, Germany) at 5000g for 10 min at room temperature. Supernatant was used for the determination of sorbitol concentration.[18]

Determination of Sorbitol content

Supernatant was incubated with equal volume of glycine buffer (0.05M glycine, 0.2mM NAD^+ and 2 U/mL SDH (20 U = 1 mg), pH 9.4) for 30 min at 37°C. Fluorescence was measured (BioTek $^{\text{synergy4}}$ multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA) at excitation and emission wavelengths of 366nm and 452nm respectively.[17] The percentage inhibition (I) of sorbitol formation by vegetables' juice was calculated using following formula:

$$I=\frac{F_{Control}-F_{Juice}}{F_{Control}}\times 100$$

where $F_{Control}$ and F_{Juice} represent fluorescence intensity recorded in control and samples incubated with vegetables' juice.

Determination of erythrocytes hemolysis

The antioxidative stress potentials of vegetables juice was determined by measuring H_2O_2 induced hemolysis of erythrocytes.[19] 2.8% suspension of washed and packed erythrocytes was prepared in sodium phosphate buffer saline (150 mM NaCl, 8.1 mM Na_2HPO_4, 19 mM NaH_2PO_4) of pH 7.4. In 96 well micro plate 100 μL of vegetable juice was incubated with 50 μL of erythrocyte suspension for 10 min at room temperature with shaking (Eppendorf centrifuge 5430R, Eppendorf AG, 22331 Hamburg, Germany) at 1200rpm. 100 μL of 2.5mM H_2O_2 was added and shaking was continued for another 20 min at 37°C. The increase in absorbance (A) due to lysis of erythrocytes was measured spectrophotometrically (BioTek synergy4 multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA) at 660nm. Percentage inhibition of erythrocytes hemolysis ($I_{Hemolysis}$) by vegetables' juice was calculated using the following formula:

$$I_{Hemolysis}=\frac{A_{Control}-A_{Juice}}{A_{Control}}\times 100$$

where $A_{Control}$ and A_{Juice} represent absorbance recorded for control and samples incubated with vegetables' juice.

Deoxyribose degradation assay

Deoxyribose assay is a practical application of studying free radical reactions in biological systems induced by fenton reaction and has been utilized to determine antioxidant activity.[20] Reaction mixture contained 50 μL each of vegetable juice, 2-deoxy-D-ribose (2.8mM), $FeCl_3$ (25mM) premixed with EDTA (100 μM) in 10mM potassium phosphate buffer (KH_2PO_4/KOH) of pH 7.4, H_2O_2 (2.8mM). Ascorbic acid (100 μM) was added as promoter of the reaction reducing Fe (III) to Fe (II). Tubes containing samples were incubated in water bath at 37^0C for 1 h. 1 mL each of 2.8% (w/v) TCA and 1% (w/v) TBA in 50mM NaOH were added thereafter and reaction mixtures were heated in a water bath at 80ºC for 20 min. Pink colored TBA-MDA adduct generated due to oxidative degradation of 2-deoxy-D-ribose was measured spectrophotometrically (BioTek synergy4 multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA) at 532 nm.[20]

The percentage prevention (%P) of 2-deoxy-D-ribose degradation by test material was calculated from the absorbance (A) with respect to control absorbance as follows:

$$\%P=\frac{A_{Control}-A_{Juice}}{A_{Control}}\times 100$$

where $A_{Control}$ and A_{Juice} represent absorbance recorded for control and samples incubated with vegetables' juice.

Determination of calf thymus DNA damage induced by Fenton reagent under influence of vegetables' juice

Fenton reagent induced damage to genetic calf thymus DNA has been utilised to determine preventive effects of antioxidants.[21] 10μL of calf thymus DNA (293 ng/mL) was incubated with 10 μL vegetable juice in presence of Fenton reagent (80 μM $FeCl_3$, 50μM Ascorbic acid, 30 mM H_2O_2) for 60 min at 37°C. DNA incubated with Fenton reagent was taken as control. DNA samples were applied to agarose gel (0.8%) along with 6X DNA loading dye and run in 1X TAE (40 mM Tris-acetate, 1 mM EDTA) buffer at 100V for 30 min and stained with ethidiumbromide.[21] Gels were transilluminated using BioDoc-It™ Imaging System, UV Transilluminator UVP (Cambridge UK) and electrophoretic migration profile was captured.

Phytochemical analysis in vegetables juice

Total flavonoids

Total flavonoids content in vegetables' juice was measured by incubating equal volume of juice with 2% $AlCl_3.6H_2O$ in a 96 well micro plate. Absorbance was recorded spectrophotometrically (BioTek synergy4 multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA) at 430nm. Results were expressed as micrograms of rutin equivalent (RE) per milliliter of juice (μg RE/mL).[13]

Total polyphenols

Total polyphenol content was measured using Folin-Coicalteu reagent. In brief, fresh juice (25 μL) was reconstituted in 2.5mL distilled de-ionized water, followed by addition of Folin-Coicalteu reagent (1 N, 250 μL) and Sodium carbonate (20% w/v Na_2CO_3, 250 μL). Mixture was incubated at room temperature (60 min). Absorbance (765 nm) was recorded spectrophotometrically on microplate reader (BioTek synergy4 multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA). Total polyphenolic content was expressed as micrograms of Gallic Acid Equivalent per milliliter of the juice (μg GAE/mL).[12]

Total protein

Protein content in fresh juice was analysed using Bradford's reagent. Briefly, 10 μL of juice was incubated with 240 μL of Bradford's reagent for fifteen minutes and absorbance (595nm) was measured (BioTek synergy4 multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA) as above. Total protein content was expressed as micrograms of BSA Equivalent per milliliter of juice (μg BSAE/mL).[12]

RESULTS AND DISCUSSIONS

In diabetic eye, increased accumulation of sorbitol induces retinopathy resulting irreversible damage to eye. Sorbitol is generated due to NADPH-dependent reduction of glucose by ALR.[22] Therefore, mitigation of NADPH-dependent catalytic activty of ALR has been one of the favorite drug development targets. Figure 2 presents kinetics of NADPH utilization by ALR in goat lens under influence of various vegetables' juice. It was observed that close to 22% NADPH was utilised by the lens ALR in control experiment (in the absence of vegetables' juice) by fifth minute [Figure 2a]. However, pre-incubation of lens ALR with vegetables' juice inhibited (85 to 99%) utilization of NADPH [Figure 2a]. Juice of IG and BG were most potent in inhibiting NADPH utilization by lens ALR [Figure 2a]. AG and RD juice could not display consistent kinetics over time in our experiment (data not presented). Utilization of NADPH by ALR represents its catalytic activity. Figure 2b presents inhibition of ALR catalytic activity under the influence of various vegetables' juice. It was observed that juice of IG and BG inhibited ALR activity more than 90% whereas, AG and RD juice were mild (20%) inhibitors of lens ALR [Figure 2b].

Research over the years on ALR has revealed remarkable diversity in the reactivity with soluble NADPH oxidoreductases.[23] Similarly, difference in its susceptibility and kinetics to inhibitors under various experimental conditions has also been observed.[22] Therefore, it becomes interesting in studying polyol pathway in different tissue sites under various influencing conditions to identify variations in activity profile of inhibitors. Erythrocyte sorbitol content has been found to correlate with sorbitol content in the lens and sciatic nerve, increased in diabetic patients when compared with non-diabetics after an 8 h fast[24] and has been identified as an important indicator of diabetes control.[18]

We studied accumulation of sorbitol in human erythrocytes under different glycemic conditions [Figure 3]. It was found that sorbitol content in erythrocytes increased (17%) with increase in glucose (from 100 mg/dL to 300 mg/dL) concentration [Figure 3a and b]. Furthermore, the inhibitory potential of vegetables' juice also varried under these two experimental conditions. Reduction in sorbitol content in erthrocytes incubated with 100 mg/dL glucose solution was not significant under the influence of YC and SG juice when compared with sorbitol content in control [Figure 3a] however, it was significantly reduced (87 and 42% respectively) when incubated with 300 mg/dL glucose solution [Figure 3b]. RD juice was identified most potent inhibitor (93%) of sorbitol accumulation in erythrocytes when incubated with 300 mg/dL glucose solution [Figure 3b]. Its inhibitory activity was three times less when erythrocytes were incubated with 100 mg/dL glucose solution [Figure 3a]. Under hyperglycemic condition (300 mg/dL glucose) the inhibitory activity of vegetables' juice varried between 32 (AG) to 93% (RD) [Figure 3b]. These observations show variation in activity levels of vegetables' juice incubated under different conditions and also their susceptibility to the source of enzyme.

Increased ALR activity leads to imbalance in NADPH/NAD^+ ratio which adversely affects other NADPH-dependent enzymes, such as glutathione

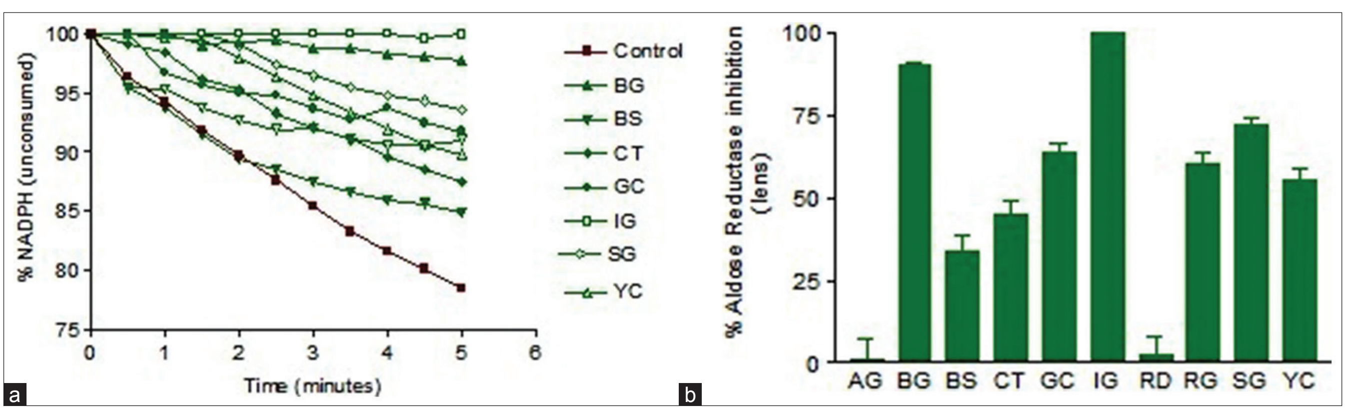

Figure 2: Prevention of utilization of NADPH by aldose reductase (goat lens) under influence of vegetables juice over time (a), Inhibition of aldose reductase (goat lens) by vegetables fresh juice (b). Data represents mean of triplicates

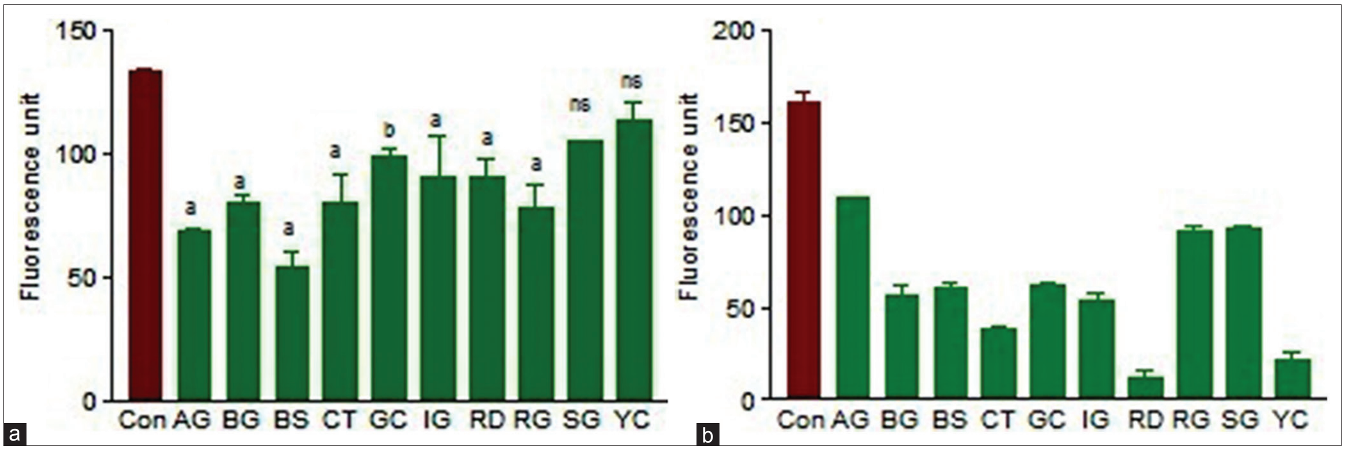

Figure 3: Influence of vegetables juice on sorbitol formation (polyol pathway) in human erythrocytes incubated with different concentrations of Glucose [a] 100 mg/dL glucose, [b] 300 mg/ dL glucose. Data represent mean ± SE of triplicates. ANOVA followed by Dunnett's Multiple Comparison Test was applied to compare differences between control and different vegetables group. [a]P<0.01, [b]P<0.05, ns = not significant when compared with control (Con) values in Figure (a), all the values in Figure (b) were significant (P<0.01) when compared with control (Con)

reductase.[1,25] The decreased activity of antioxidant enzyme glutathione reductase reduces free radicals scavenging potential of a system leading to the development of oxidative stress [Figure 1]. Furthermore, increased flux of sorbitol through polyol pathway leads to increase in NADH/NAD$^+$ ratio and accentuates activity of NADH oxidase resulting increased generation of ROS and subsequent development of oxidative stress [Figure 1]. Reduction in NADPH-dependent catalytic activity of ALR [Figure 2] and resultant flux of sorbitol [Figure 3] in multiple model systems in our study by vegetables' juice demonstrate that they can offer economical complementary therapy to diabetics in mitigating development of diabetic complications induced through polyol pathway [Figure 1].

Oxidative stress is defined as excessive production of reactive oxygen species (ROS) in the presence of diminished antioxidant defence. Increased oxidative stress could be one of the common pathogenic factors ensuing development of diabetic complications.[2] In order to evaluate antioxidative-stress capacity of vegetables' juice independent of polyol pathway, we utilised hemolysis of erythrocytes as a test- model. Several hypotheses have been proposed to explain mechanism of erythrocytes hemolysis following oxidative stress *in vivo* and *in vitro*.[26,27] Moreover, this test-model has been extensively utilised to evaluate antioxidative potential of natural products.[28] Figure 4 presents effect of vegetables' juice on H_2O_2 induced hemolysis of erythrocytes. It was observed that AG juice (94%) was most potent in inhibiting H_2O_2 induced hemolysis followed by RD (92%), YC (88%), IG (80%), RG (76%) and CT (55%). Juice of SG and BG could not prevent H_2O_2 induced hemolysis. Juice of BS (14%) and GC (24%) offered moderate protection against H_2O_2 induced erythrocytes hemolysis [Figure 4].

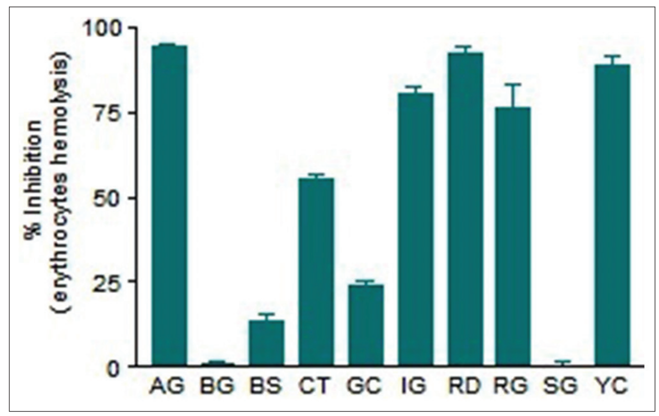

Figure 4: Influence of vegetables juice on H_2O_2 induced erythrocytes hemolysis. Data represents mean ± SD of triplicates

Although, damage to genomic DNA caused by oxidative mechanisms has been well-studied for its potential role in development of human diseases,[29] the oxidation of 2-deoxy-D-ribose in DNA has emerged only recently as a critical determinant of the cellular toxicity of oxidative damage to DNA.[29,30] Therefore, evaluation of antioxidative property of antioxidants on both, genomic DNA as well as its ribose sugar may reveal crucial information on genetic toxicology of oxidative stress and preventive role offered by an antioxidant.

Fenton reaction (FR) is applied to generate hydroxyl radicals (OH•) by ascorbic acid dependent iron salt-mediated degradation of H_2O_2. OH$^•$ attack and degrade deoxyribose, which forms pink chromogen when heated with TBA.[20] This test represents a unique complex-experimental model in determining antioxidant activity of compounds against free-radicals induced damage to biological molecules. Concentration dependent antioxidative property of vegetables' juice in inhibiting FR induced oxidation of 2-deoxy-D-ribose is presented in figure 5. Results show

Table 1: Analysis of phytochemical components, antioxidant activity in vegetables' juice and their ranking based on activity potentials

Vegetables	Yield (mL/100gm)	Total polyphenols (μgGAE/mL)	Total flavonoids (μgRE/mL)	Total protein (μgBSAE/mL)	Antioxidant activity (IC_{50}, % conc. of juice)	Rank
AG	48.9±2.0	130.7±11.0	4.7±0.2	235.3±24.3	2.8	4
BG	46.5±1.5	282.1±53.5	20.2±0.6	426.5±4.9	7.8	7
BS	55.4±1.2	85.8±19.4	10.9±0.3	ND	4.5	6
CT	37.0±1.3	305.7±47.7	30.8±0.5	778.3±37.8	5.9	9
GC	39.4±2.7	157.2±30	1.5±0.4	475.3±3.7	6.9	2
IG	36.7±2.8	177.3±13	2.4±0.3	445.5±8.3	5.8	1
RD	42.0±4.3	343.1±49.4	5.4±0.7	642.9±34.6	15.2	10
RG	37.5±7.2	175±19.6	6.1±0.1	832.4±49.8	2.3	3
SG	44.3±2.2	91.0±4.5	6.6±0.4	672.4±30.7	23.6	5
YC	38.7±5.4	371.3±34.4	4.6±0.2	451.8±34.7	24.1	8

GAE: Gallic acid equivalent; RE: Rutin equivalent; BSAE: Bovine serum albumin equivalent; ND: Not detected. Values represent mean±SD (*N*=3)

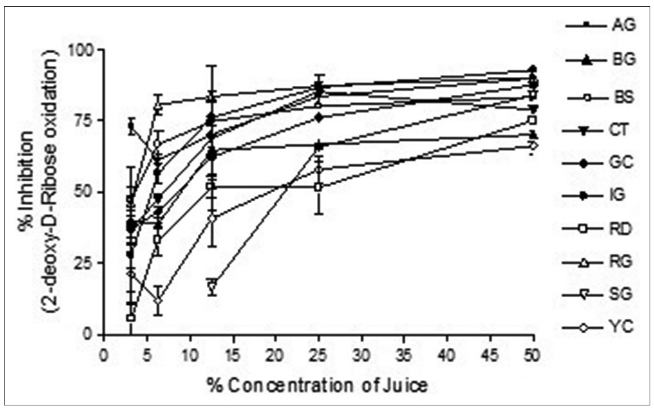

Figure 5: Concentration dependent inhibition of 2-deoxy-D-ribose oxidation induced by Fenton reagent (FR) under influence of vegetables juice. Data represents mean ± SD of triplicates

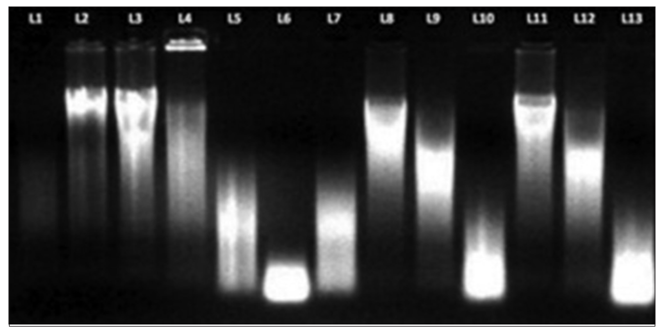

Figure 6: Electrophoretic migration profile of calf thymus DNA damage induced by Fenton reaction (FR) and influence of vegetables' juice. Experimental conditions are described in materials and methods section. L1 (DNA+FR); L2 (DNA, 6 μL); L3 (DNA, 10 μL); L4 (DNA+YC+FR); L5 (DNA+SG+FR); L6 (DNA+RG+FR); L7 (DNA+RD+FR); L8 (DNA+IG+FR); L9 (DNA+GC+FR); L10 (DNA+CT+FR); L11 (DNA+BS+FR); L12 (DNA+BG+FR); L13 (DNA+AG+FR

that vegetables' juice offered varying degree of protection against $OH^{\bullet}$ induced oxidative damage to ribose sugar. Based on IC_{50} values [Table 1], it was found that juice of RG and AG (with IC_{50} of 2.3 and 2.8% juice concentration respectively) were most potent inhibitors and juice of SG and YC offered least protection [Table 1].

In order to evaluate oxidative damage to DNA by FR and protective effect of vegetables' juice, we used calf thymus DNA as target. Movement of calf thymus DNA on gel electrophoresis has been identified as marker of oxidative DNA damage and used to assess antioxidant potential of antioxidants in preventing oxidative damage to DNA.[21] Slow is the migration, better is the protection offered by an antioxidant. Figure 6 displays gel electrophoretic migration of calf thymus DNA damage induced by FR. FR caused maximum oxidative damage to DNA as it is least visible on the gel [Figure 6, L1]. Migration of DNA on gel electrophoresis reveals varying degrees of protection when DNA was pre-incubated with vegetables' juice [Figure 6, L4-L13]. Results indicate that juice of IG and BS were most potent in preventing FR induced damage to DNA followed by BG, GC, YC, SG, RD, CT, AG, and RG [Figure 6]. Further experiments are required to delineate and clarify mechanism of action of vegetables' juice offering varying degrees of protection to oxidative DNA damage induced by FR.

Observations made in this study show that whether influence on polyol pathway or the antioxidant activity, potentials of vegetables' juice varies under different experimental conditions. Therefore, battery of tests and different experimental conditions are required to identify and evaluate optimal beneficial effects of vegetables' juice and no single test can reveal the true picture.

The antioxidants as well as the antihyperglycemic activities have been found affected by polyphenols, flavonoids and proteins content in vegetables' juice.[10] Total polyphenols, total flavonoids and protein content in vegetables' juice is presented in Table 1. YC, RD and CT juice were richer with polyphenols when compared to other vegetables' juice. Flavonoid content was more in juice of CT and BG. Juice of RG, CT, SG and RD were more proteinaceous than other vegetables' juice [Table 1]. Interestingly however, we could not find any correlation with the activities studied in this research with any of these phytochemicals present in vegetables' juice. Therefore, biological activities identified in this research may be ascribed to the holistic contents present in vegetables' juice.

In order to rank vegetables' juice for their holistic beneficial effects in mitigating polyol pathway induced development of oxidative stress; we ranked them by summing-up their overall activities. The juice of IG was identified to be more potent followed by GC and RG juice in imparting beneficial effects against polyol pathway induced development of oxidative stress and their inherent antioxidant activities than other vegetables [Table 1].

In conclusion, our study finds that vegetables' juice possess properties of influencing polyol pathway in favour of preserving native antioxidant potentials, preventing development of oxidative stress and hence mitigating development of diabetic complications by multiple mechanisms [Figure 1]. Amongst the ten vegetables' juice analysed in this research, juice of IG followed by GC and RG may be categorized as preferred ones, for they displayed potent activities on various parameters analysed. These vegetables' juice may become part of highly sought mechanism-based complementary antioxidant therapy to prevent development of diabetic complications.

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