

Comprehensive Assessment of Antioxidant Activities of Apigenin Isomers: Vitexin and Isovitexin

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ABSTRACT

Introduction: Fenugreek is known to possess anti-diabetic as well as antioxidant properties known to increase with germination. Previous study from our laboratory has reported vitexin and isovitexin as major antioxidant compounds from germinated fenugreek seeds. The present study aimed to comprehensively assess and compare their antioxidant activity.

Methods: The antioxidant ability of these compounds against various radicals was checked using standard *in vitro* radical scavenging assays and pulse radiolysis. Protection conferred to mitochondria against oxidative damage was assessed by measuring the levels of lipid peroxides and protein sulphhydryls. Protection from hydrogen peroxide induced cytotoxicity in HepG2 cells was checked by MTT assay and by measuring intracellular ROS. Their ability to modulate intracellular antioxidant status was checked both at activity and RNA level. **Result:** Vitexin and isovitexin exhibited differential antioxidant activity against various radicals. Vitexin was a better nitric oxide scavenger while isovitexin scavenged superoxide radicals more efficiently. Pulse radiolysis studies revealed good antioxidant activity of both compounds against short-lived radicals and affected the formation and decay of the 2-dG transient species to the same extent. In oxidatively damaged mitochondria, lipid peroxidation was inhibited significantly by

isovitexin whereas vitexin prevented decrease in protein sulphhydryl content more effectively. These compounds protected HepG2 cells to the same extent against hydrogen peroxide induced oxidative insult by reducing intracellular ROS and modulating the levels of antioxidant enzymes.

Conclusion: The present study clearly demonstrates differential antioxidant potential of both vitexin and isovitexin and their role in mitigating oxidative stress induced damage and maintaining cellular redox homeostasis.

Key words: Antioxidant enzymes, Fenugreek, Flavonoids, Free radicals, Isoviteixin, Vitexin.

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INTRODUCTION

In the last few years there has been an exponential growth in the field of herbal medicines and these drugs are gaining popularity both in developing and developed country because of their natural origin and less side effects. India is the largest producer of medicinal herbs. Herbs like turmeric,¹ fenugreek, ginger, garlic and holy basil² are integral part of Ayurvedic formulation. There have been several studies on the antioxidant activities of various herbs³/plants⁴/extracts⁵ with medicinal values. However, the exact mechanism and mode of action of these formulations/extracts is not known. Therefore it is necessary to characterize the extracts in terms of their constituents and mechanism. Efforts are now being made to standardize the plant material/extracts and identify bioactive constituents with relevant biological activity.

Fenugreek seeds, commonly used in India and other countries as a condiment, are an excellent source of dietary fiber and are commonly used in many polyherbal formulations. Studies from our laboratory have shown that germinated fenugreek seeds exhibit high antioxidant activity.⁶ Bioactivity guided isolation of active antioxidant compounds from germinated fenugreek seeds revealed apigenin-8-C- β -glucopyranoside (vitexin) and apigenin-6-C- β -glucopyranoside (isovitexin) as major compounds.⁷ Both these compounds have received critical attention due to their antioxidant and anti-diabetic properties. Isoviteixin is known to inhibit xanthine oxidase activity and protect DNA against cellular ROS.⁸ It is also known to be an insulin secretagogue in non-diabetic rats.⁹ Vitexin on the other hand exhibits wide pleothera of biological activities such as anti-myeloperoxidase activity, anti-*Helicobacter pylori* activity as well as inhibition of H⁺, K⁺-ATPase activity.¹⁰ Vitexin has been shown to reduce H₂O₂ induced oxidative stress in polymorphonuclear cells.¹¹ The ability of vitexin to inhibit adipogenesis in 3T3-L1 cells has also been demonstrated.¹² Both vitexin and isovitexin possess

strong α -glucosidase inhibitory activity¹³ as well as anti-glycation ability.¹⁴ Though these different biological activities are demonstrated, their direct interaction with various known free radicals is still unclear. In the present study we have comprehensively assessed antioxidant property of these two C-glycosides of apigenin, namely Vitexin and Isoviteixin against various model stable and systemic free radicals using steady state kinetics and Pulse radiolysis. Their role in mitigating hydrogen peroxide induced oxidative stress in human hepatic epithelial cell line (HepG2) was also studied by assessing cellular redox status and expression of antioxidant defense enzymes in presence and absence of these phytochemicals.

MATERIAL AND METHODS

Chemicals

2, 2-Diphenyl 1-picrylhydrazyl (DPPH), L-Ascorbic acid, 2, 2-azino bis (3-ethylbenzothiazoline-6-sulfonate) ABTS, 2, 4, 6-2-thiobarbituric acid (TBA), 1, 1, 3, 3-tetramethoxypropane (TMP), vitexin, isovitexin standards (HPLC grade) were purchased from Sigma Chemical Co., U.S.A. HPLC grade acetonitrile, methanol and trifluoroacetic acid (TFA) and Hydrogen peroxide were purchased from Merck (Germany). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBCO life technologies. All other chemicals used in this study were of analytical grade and were procured from local suppliers.

Biologicals

Male or female Wistar rats weighing 250 $\pm$ 20 g were used to isolate liver mitochondria and were locally purchased from Institute of Veterinary and Biological Sciences, Pune. They were housed in polypropylene cages

maintained at $25 \pm 2^\circ\text{C}$ with 12:12 h light and dark cycle. They were given feed and water *ad libitum*. Prior approval from Savitribai Phule Pune University institutional animal ethical committee was obtained.

Preparation of standard solutions

Vitexin and Isovitexin were dissolved in methanol to obtain 10 mM stock which were used for all further experiments.

Interaction of Vitexin and Isovitexin with various commercially available and biologically important free radicals

Interaction of Vitexin and Isovitexin with 2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) radical was monitored using method described previously.¹⁵ Similarly interaction of these compounds with, 2'-azinobis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS) radical cation generated by interacting ABTS with ammonium persulfate for 16 h in dark was monitored following previously described method.¹⁶ Ability of the compounds to scavenge phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH) generated superoxide radical was monitored using previously reported method by Aruoma (1993).¹⁷ Nitric oxide radical was generated using 50 mM sodium nitroprusside in 1X PBS and quantified by using Griess reagent (1% sulphanilamide and 0.1% N-naphthyl ethylene diamine dihydrochloride (NEDD) in 2% H_3PO_4).¹⁸ An experimental control without test compounds but with equivalent amount of vehicle was also kept parallelly.

Pulse radiolysis

Vitexin and isovitexin were tested for their ability to interact with $\text{ABTS}^{\cdot-}$ and $\text{CO}_3^{\cdot-}$ radicals using pulse radiolysis. LINAC facility at the 'National Center for Free Radical Research', Pune was utilized for Pulse radiolysis studies. Dosimetry was performed with aerated 0.01 mol dm^{-3} KSCN solution with a G of $2.6 \times 10^{-4} \text{ m}^2 \text{ J}^{-1}$ per 100 eV at 480 nm.¹⁹ and the dose rate per pulse was determined to be 19 Gy. $\text{ABTS}^{\cdot-}$ was produced by interaction of radiolytically generated azide radicals with ABTS^{2-} (2 mM). $\text{CO}_3^{\cdot-}$ was generated using aqueous solutions of equimolar (0.05 M) NaHCO_3 and Na_2CO_3 . All samples exposed to high energy pulse were presaturated with N_2O . The decay traces of $\text{ABTS}^{\cdot-}$ or $\text{CO}_3^{\cdot-}$ were monitored in presence and absence of different concentrations of test compounds and were correlated with the concentration of ascorbic acid equivalents.⁶ $\text{ABTS}^{\cdot-}$ and $\text{CO}_3^{\cdot-}$ radicals were recorded at the corresponding λ_{max} 420 and 600 nm respectively.

DNA Fragmentation Assay

pBR322 plasmid DNA was exposed to hydroxyl radicals initiated by reaction between ferrous sulphate and hydrogen peroxide in presence or absence of different concentrations of test compounds.²⁰ The mixture was then loaded on 1% agarose gel and electrophoresed at 80 volts till BPB migrated ≈ 5 cm from the well. The gel was then stained with 2 $\mu\text{g}/\text{ml}$ of ethidium bromide solution for 20 min at room temperature, washed with water and observed under UV. The bands were recorded using gel documentation system (Alfa innotech, USA) and were analyzed by performing densitometry.

Interaction of vitexin and isovitexin with 2 deoxy guanosine (2-dG) base transient

2-dG which is the most favorable site for DNA damage was exposed to 7 Gy radiation dose of 50 ns pulse width using PULAF (Pune University Linear Accelerator Facility). The time resolved transient absorption spectra for the reaction of $\cdot\text{OH}$ with 2-dG were recorded. 2 ml reaction mixture containing different concentrations of vitexin/ isovitexin and 2-dG (2 mM) was exposed to high energy pulse to study the interaction

of these compounds with 2-dG transient. Decay of 2-deoxy guanosine transient species was monitored at λ_{max} , 310 nm.²¹

Isolation of rat liver mitochondria and exposure to oxidative stress

Three months old Wistar rats (weighing about 250 ± 20 g) were used for the preparation of mitochondria using standard protocol.²² 0.25 mg/ml rat liver mitochondria were exposed to Fenton radical initiated by ascorbate- Fe^{2+} and oxidative damage induced to the lipids was estimated by TBARS method. Malonaldehyde (MDA) one of the major end product of lipid peroxidation was estimated spectrophotometrically using TBA reagent. A standard graph was prepared by acid hydrolysis of known concentrations of tetramethoxypropane (TMP). The data was expressed as nmoles of TBARS/mg protein. Protection offered to protein sulphhydryl groups was measured using Ellmans reagent, (5,5'- dithiobis 2-nitrobenzoic acid- DTNB).²³ Results are expressed as nmoles protein sulphhydryl/mg protein.

Cell culture and treatment

HepG2 (human hepatocellular carcinoma) cells were purchased from National Centre for Cell Sciences, Pune. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C .

For treatment with the compounds, cells were exposed to different concentration of vitexin and isovitexin (1, 5 and 10 μM). After incubation for 24 hrs, medium was discarded and fresh medium containing 500 μM of hydrogen peroxide was added and incubation was continued for 6 hrs.

Cell viability

Cell viability was determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide).²⁴ Cells (2×10^4 cells) were seeded in a 96-well plate in medium supplemented with 10% FBS. On the next day the cells were treated with various concentrations of vitexin and isovitexin (1, 5 and 10 μM) for 24 h. The treatment media was removed and the cells were washed with phosphate-buffered saline (1X PBS) and exposed to 500 μM hydrogen peroxide for 6 h. 10 μl of 5 mg/ml of MTT was added to each well. Cells were incubated at 37°C for 3 hrs and solubilized overnight with 100 μl of solubilizing buffer. The resulting intracellular purple colored formazan was quantified by measuring absorbance at 550 nm using ELISA plate reader (Multiskan Ex, microplate reader, Thermo-fischer Scientific U.K).

Estimation of intracellular reactive oxygen species (ROS)

Intracellular ROS was quantified using DCFH-DA (2', 7'-Dichlorodihydrofluorescein diacetate).²⁵ For the assay, HepG2 cells with density of 1×10^4 per well were seeded in 96-well black plate in triplicates. On the next day, cells were treated by fresh culture medium containing different concentrations of vitexin and isovitexin for 24 h, and later washed with 1X phosphate buffered saline (PBS). 10 μM of DCFH-DA was added in each well. Cells were washed with PBS after 30 min to remove excess amount of dye and were immediately exposed to 500 μM H_2O_2 prepared in FBS free incomplete cell culture medium. Fluorescence was measured at 485 and 530 nm excitation and emission wavelengths respectively after 30 min using BIO-TEK FL600 fluorescence plate reader (U.S.A) at 37°C . The percentage increase in fluorescence was calculated by the formula $[(F_{t_{30}} - F_{t_0})/F_{t_0} \times 100]$, where $F_{t_{30}}$ =Fluorescence at time 30 min. and F_{t_0} =Fluorescence at time 0 min. The data is expressed in terms of % DCFH fluorescence (Arbitrary Units, AU).

Measurement of antioxidant enzyme activities

Treated cells were collected in 1X PBS, centrifuged and pellet was resuspended in thrice the volume of protein extraction buffer (Tris-Cl (pH 7.8), EDTA (0.5 M) and 1% Triton $\times$ 100), 1 mM PMSF and protease inhibitor cocktail (Pierce). Cells were kept on ice and vortexed vigorously with two minutes interval, repeating the cycle three times. The homogenates were then centrifuged at 10,000 rpm for 10 minutes. The supernatant cell lysate was used for further assessment of cellular antioxidant enzymes. Protein content in the lysates was estimated by using Lowry method²⁶ and lysates were stored at -20°C until use. Quantitation of antioxidant enzymes namely superoxide dismutase (EC-Number 1.15.1.1)²⁷ catalase (EC-Number 1.11.1.6),²⁸ glutathione reductase (EC-Number 1.8.1.10)²⁹ and Glutathione peroxidase (EC-Number 1.11.1.9)³⁰ was carried out using standard protocol.

Estimation of total glutathione (GSH)

Total intracellular GSH was estimated according to the protocol of Teixeira and Meneghini, (1996).³¹ 5% sulfosalicylic acid was added to the cellular lysates to precipitate the proteins. The supernatant (10 μ l of 1:2 diluted) was added to reaction mixture (170 μ l) containing 3.76 mM DTNB, 500 units/ml GR and 2.5 mM NADPH. Increase in absorbance at 412 nm was recorded within every two min interval continuously for 15 min. Amount of GSH in the sample was calculated by using a standard GSH plot. Values are expressed as nmol GSH/mg protein.

RNA extraction and quantitation of genetic expression of antioxidant enzymes using RT-PCR

Expression of antioxidant enzymes at transcriptional level was studied by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Cellular RNA from the treated and untreated cells was extracted using TRIZOL-chloroform method. One microgram of RNA was reverse transcribed using Verso cDNA synthesis kit (Thermoscientific, Germany). The cDNA products were amplified by PCR using the following primers:

Gene	Primer Sequence	PCR Product Size (bp)
Catalase	Forward: 5'-TGCTGAATGAGGAACAGAGG-3' Reverse: 5'-GTGTGAATCGCATTCTTAGG-3'	184
CuSOD	Forward: 5'-AAGGCCGTGTGCGTGCTGAA-3' Reverse: 5'-CAGGTCTCCAACATGCCTCT-3'	300
GPx	Forward: 5'-GCGGCGGCCAGTCGGTGTA-3' Reverse: 5'-GAGCTTGGGGTCGGTCATAA-3'	220
GR	Forward: 5'-CAGTGGGACTCACGGAAGAT-3' Reverse: 5'-TTCAGTCAACAGCAAAACC-3'	400
G3PDH	Forward: 5'-TGGGGAAGGTGAAGGTCGGA-3' Reverse: 5'-GGGATCTCGCTGCTGGAAGA-3'	220

Conditions used for PCR amplification were: Initial denaturation at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s and final extension at 72°C for 6 min. PCR products were electrophoresed on 1.8% agarose gel containing ethidium bromide and bands were documented and quantitated using the gel documentation system (Alfa innotech, U.S.A). Band intensity was normalized to values for G3PDH.

Statistical Analysis

All experiments were done in 3 to 5 replicates, and final values are expressed as average $\pm$ S.D of these replicates. One-way ANOVA paired with Tukey's test was performed to determine statistical significance of

difference between various experimental groups. Dissimilar alphabets a, b, c and d in the superscript indicate significant difference at $P \leq 0.05$. All statistical analysis was carried out using SPSS software (Version 19) licensed to Dept. of Zoology, SPPU.

RESULTS

Radical scavenging by Vitexin and Isovitexin

For all the antioxidant assays, 10, 100 and 1000 μ M concentration of both vitexin and isovitexin were used. Figure 1 shows different radicals scavenging activity of both the compounds. It was observed that 1000 μ M vitexin showed significantly high DPPH radical scavenging activity (Figure 1a) with a value of 0.171 ± 0.012 mM AEAC ($P \leq 0.05$) followed by isovitexin (0.014 ± 0.0006 mM AEAC). In ABTS radical scavenging assay, 1000 μ M concentration of vitexin and isovitexin showed equivalent activity (1.11 ± 0.011 and 1.15 ± 0.00075 mM AEAC respectively) (Figure 1b) while in case of biologically relevant radicals namely superoxide and nitric oxide, 1000 μ M of vitexin scavenged nitric oxide (Figure 1c) more efficiently ($50 \pm 0.5\%$) ($P \leq 0.05$) than isovitexin ($35 \pm 1.4\%$). On the other hand isovitexin scavenged ($25 \pm 2.6\%$) superoxide radicals (Figure 1d) significantly ($P \leq 0.05$) than vitexin ($20 \pm 2.2\%$). Direct interactions of vitexin and isovitexin with various radicals were recorded by fast reaction kinetic studies using pulse radiolysis. In this study $\text{ABTS}^{\cdot-}$ and $\text{CO}_3^{\cdot-}$ were generated by pulse radiolysis and scavenging activity of these radicals by vitexin and isovitexin was checked. Ascorbic acid was used as standard antioxidant to measure the activity of these compounds. Figure 2 shows the decay curves of $\text{ABTS}^{\cdot-}$ and $\text{CO}_3^{\cdot-}$ in presence and absence of different concentrations of vitexin and isovitexin. Both compounds effectively scavenged $\text{ABTS}^{\cdot-}$ (Figure 2a) as well as $\text{CO}_3^{\cdot-}$ (Figure 2b) radicals. Vitexin and isovitexin at a concentration of 10 μ M scavenged ABTS radicals with a value of 38 and 29 μ g/ml of AEAC respectively. Maximum $\text{CO}_3^{\cdot-}$ radical scavenging activity was exhibited by 10 μ M of vitexin and isovitexin with a value of 1.9 and 2.6 μ g/ml of AEAC respectively.

Protection conferred by Vitexin and Isovitexin to oxidatively damaged mitochondria

Rat liver mitochondria were exposed to Ascorbate- Fe^{2+} and oxidative damage induced to lipids and proteins was assessed by estimating thiobarbituric acid reactive substances (TBARS) and proteins sulphydryls respectively. Lipid peroxidation in case of control and oxidatively damaged mitochondria was 1.1 ± 0.2 and 17.04 ± 0.28 nmoles of TBARS/mg protein, respectively (Figure 3a). Isoviteixin significantly ($P \leq 0.05$) reduced lipid peroxide formation (1.96 ± 0.04 nmoles TBARS/mg protein) and exhibited good protection. Vitexin on the other hand could not protect (11.4 ± 0.26 nmoles TBARS / mg protein) mitochondria as effectively as isovitexin even at 100 μ M concentration (Figure 3a). Exposure of rat liver mitochondria to Ascorbate- Fe^{2+} led to significant depletion in the protein sulphydryl formed (1.6 ± 0.01 nmoles protein sulphydryl/mg protein) compared to the control (3.3 ± 0.14 nmoles protein sulphydryl/mg protein). This depletion in protein sulphydryl was effectively attenuated in presence of 100 μ M vitexin (2.9 ± 0.09 nmoles protein sulphydryl/mg protein) and isovitexin (2.5 ± 0.03 nmoles protein sulphydryl/mg protein) (Figure 3b).

Protection to pBR322 DNA and the possible interaction with 2-deoxy guanosine base transient

pBR322 DNA was exposed to $\cdot\text{OH}$ generated by ammonium ferrous sulfate and hydrogen peroxide (H_2O_2). Oxidative damage to DNA was assessed in terms of conversion of supercoiled (SC) to nicked circular (NC) forms. Densitometry was performed to quantify the amount of DNA

in both NC and SC in presence and absence of different concentrations of vitexin and isovitexin alone and also when exposed to $\cdot\text{OH}$ radicals. Figure 4a and b shows oxidatively damaged pBR322 DNA in presence and absence of different concentrations (1, 5 and 10 μM) of vitexin and isovitexin respectively. Amount of supercoiled DNA was significantly ($p \leq 0.05$) reduced when it was exposed to $\cdot\text{OH}$ initiated by Fenton reaction (Lane 2, Figure 4a and b). This reduction in the amount of SC DNA was prevented in presence of vitexin and isovitexin (Lanes 3,5,7, Figure 4a and b) in a concentration dependent manner. Both the compounds alone did not induce any DNA damage (Lanes 4, 6, 8, Figure 4a and b).

The time resolved transient absorption spectra for the reaction of $\cdot\text{OH}$ with 2-deoxy guanosine base was recorded with a dose rate of 7 Gy/pulse. The transient showed absorption maxima at 310 nm, resembling to the spectral features reported earlier. The decay of 2-dG transient species was monitored in presence and absence of 1 μM vitexin and isovitexin. It was evident that both vitexin and isovitexin affected the 2-dG transient formation as the absorbance of the transient was decreased in presence of both compounds (Figure 5).

Protection by vitexin and isovitexin to HepG2 cells against hydrogen peroxide induced stress

Viability of HepG2 cells in presence of hydrogen peroxide at different concentration and time was checked using MTT assay (Figure 6a). Concentration and time dependent decrease in the cell viability was observed in the cells exposed to various concentrations of H_2O_2 . In presence of 500 μM H_2O_2 for 6 h, $48 \pm 2.5\%$ HepG2 cells were viable. This IC_{50} was used for further experiments. Similarly, cell-viability in presence of vitexin and isovitexin alone was also determined (Table 1). Cells were pre-treated with different concentrations of vitexin and isovitexin (1, 5 and 10 μM) for 24 h and exposed to 500 μM H_2O_2 for 6 h. Cell viability was calculated and compared with H_2O_2 treated group. All concentrations of vitexin significantly ($P \leq 0.05$) protected the cells against H_2O_2 induced cytotoxicity. Treatment of cells with 500 μM H_2O_2 alone decreased cell viability to $51 \pm 1.5\%$, which was significantly ($P \leq 0.05$) increased in 1 μM ($83 \pm 2.4\%$), 5 μM ($84.5 \pm 2.8\%$) and 10 μM ($80 \pm 1.9\%$) of vitexin. While in case of isovitexin the viable cell population at 1, 5 and 10 μM was 85 ± 3.15 , 83.3 ± 7.0 and $80 \pm 5.6\%$ respectively (Figure 6b).

Attenuation of H_2O_2 induced increase in ROS by vitexin and isovitexin

Estimation of ROS is highly important parameter to study cellular redox status. The increase in fluorescence intensity resulting from oxidation of H_2DCF to DCF in various experimental groups was estimated at 30 mins after exposure to 500 μM H_2O_2 . Rapid increase in the ROS levels was observed on H_2O_2 treatment ($306 \pm 26\%$) compared to control (100 ± 0.0) group ($P \leq 0.05$). This increase in ROS was significantly attenuated by 10 μM vitexin ($224 \pm 3\%$) and isovitexin ($232 \pm 8\%$) (Figure 6c).

Replenishment of H_2O_2 induced depletion in intracellular glutathione (GSH) by Vitexin and Isovitexin

Glutathione is an important systemic antioxidant molecule and plays key role in maintaining cellular redox homeostasis. Figure 7 shows H_2O_2 induced depletion and replenishment of cellular GSH by isovitexin and vitexin. Hydrogen peroxide treatment significantly reduced glutathione levels (454 ± 23 nmoles GSH/mg protein) compared to control (989 ± 183 nmoles GSH/mg protein) ($P \leq 0.05$). Isovitexin and vitexin both effectively prevented this depletion and restored the GSH almost equivalent to control. At 10 μM concentration, isovitexin and vitexin restored the glutathione levels significantly ($P \leq 0.05$) to 888 ± 105 nmoles/mg protein ($P \leq 0.05$) and 1012 ± 49 nmoles/mg protein, respectively. Cells treated

with vitexin and isovitexin alone did not show any depletion of cellular glutathione

Modulation of intracellular antioxidant enzyme activities and their expression by vitexin and isovitexin

Activities of different antioxidant enzymes from HepG2 cells treated with vitexin and isovitexin (1,5 and 10 μM) alone and in presence of 500 μM H_2O_2 are shown in Table 2. Untreated cells served as a control. In the control group, catalase activity was 6931 ± 463 units/mg protein. Exposure to 500 μM of H_2O_2 significantly ($P \leq 0.05$) increased this activity to 12634 ± 575 units/mg protein, which was concentration dependently reduced in the cells treated with vitexin and isovitexin. 10 μM isovitexin and vitexin reduced the catalase activity to 8561 ± 573 and 8767 ± 488 unit respectively. HepG2 cells treated with 10 μM isovitexin and vitexin alone (7588 ± 375 and 7768 ± 551 units/mg protein, respectively) did not show change in the catalase activity and was almost comparable to the control.

Exposure to H_2O_2 , also significantly ($P \leq 0.05$) increased superoxide dismutase (SOD) activity (5678 ± 120 units/mg protein) compared to control (3941 ± 412 units/mg protein). This increase was significantly ($P \leq 0.05$) reduced by 10 and 5 μM vitexin (4564 ± 400 and 4981 ± 482 units/ mg protein, respectively) and isovitexin (4358 ± 113 and 4594 ± 162 mg protein, respectively) 10 μM isovitexin treatment alone did not affect the SOD activity (3653 ± 382 units/mg protein) but treatment with 10 μM vitexin alone showed marginal increase in the SOD activity (4558 ± 199 units/mg protein).

In case of Glutathione peroxidase (GPx), Control cells showed 110 ± 7 units/mg protein GPx activity, and was found to be significantly ($P \leq 0.05$) increased (293 ± 19 units/mg protein) when exposed to H_2O_2 . Treatment with isovitexin and vitexin (10 and 5 μM) reduced the H_2O_2 induced increase in the GPx activity in a concentration dependent manner. 10 μM isovitexin significantly ($P \leq 0.05$) reduced the GPx activity to 120 ± 7 units/mg protein and was almost equivalent to the control, whereas in vitexin treated group, it was 189 ± 21 units/mg protein. Cells treated with 10 μM isovitexin and vitexin alone did not show any effect on the GPx activity (99 ± 4 and 133 ± 16 units/mg protein respectively).

A significant increase in the amount of glutathione reductase (GR) was observed in cells exposed to H_2O_2 (845 ± 48 units/mg protein) ($P \leq 0.05$) while in case of control it was, 562 ± 30 units/mg protein. This increase in GR activity was considerably reduced by the pretreatment of 10 μM isovitexin and vitexin. The GR activity in 10 μM pretreated isovitexin was 666 ± 45 units/mg protein and in case of vitexin was, 671 ± 79 units/mg

Table 1: Effect of vitexin and isovitexin alone on cell viability of HepG2 cells. Data are mean $\pm$ S.E of three independent experiments and are expressed as percent cell viability compared to control which is considered as 100%

No.	Compound concentration	Cell viability (%)
1	5 μM isovitexin	93 ± 0.1
2	10 μM isovitexin	90 ± 0.6
3	20 μM isovitexin	80 ± 0.9
4	60 μM isovitexin	74 ± 3.8
5	100 μM isovitexin	67 ± 4.7
6	5 μM vitexin	100 ± 5.2
7	10 μM vitexin	93 ± 5.5
8	20 μM vitexin	89 ± 2.2
9	60 μM vitexin	75 ± 4.2
10	100 μM vitexin	66 ± 2.5

Table 2: Antioxidant enzymes activity in HepG2 cells under different conditions

Groups	Catalase (units/mg protein)	Superoxide Dismutase (units/mg protein)	Glutathione peroxidase (units/mg protein)	Glutathione reductase (units/mg protein)
Control	6931 ± 63 ^{ef}	3941 ± 412 ^{ef}	110 ± 7 ^{ef}	562 ± 30 ^{ef}
H ₂ O ₂ alone	12634 ± 575 ^a	5678 ± 120 ^a	293 ± 19 ^a	845 ± 48 ^a
H ₂ O ₂ +10 µM isovitexin	8561 ± 573 ^{cd}	4358 ± 113 ^{de}	120 ± 7 ^e	666 ± 45 ^d
H ₂ O ₂ +10 µM vitexin	8767 ± 488 ^c	4564 ± 400 ^{cd}	189 ± 21 ^{bc}	671 ± 79 ^{de}
H ₂ O ₂ +5 µM isovitexin	9377 ± 704 ^{bc}	4594 ± 162 ^{cd}	158 ± 25 ^{cd}	705 ± 42 ^{cd}
H ₂ O ₂ +5 µM vitexin	9540 ± 677 ^{bc}	4981 ± 482 ^{bc}	201 ± 21 ^b	766 ± 74 ^{abc}
H ₂ O ₂ +1 µM isovitexin	10068 ± 754 ^b	4990 ± 92 ^b	210 ± 29 ^b	755 ± 37 ^{bc}
H ₂ O ₂ +1 µM vitexin	10246 ± 798 ^b	5462 ± 201 ^a	224 ± 4 ^b	817 ± 41 ^{ab}
10 µM isovitexin alone	7558 ± 375 ^e	3653 ± 382 ^f	99 ± 4 ^f	546 ± 33 ^f
10µM vitexin alone	7768 ± 55 ^{de}	4558 ± 199 ^{cd}	133 ± 16 ^{cd}	554 ± 32 ^f

Values are expressed as mean ± S.E of five independent experiments. Different alphabets indicate significant difference at P≤0.05.

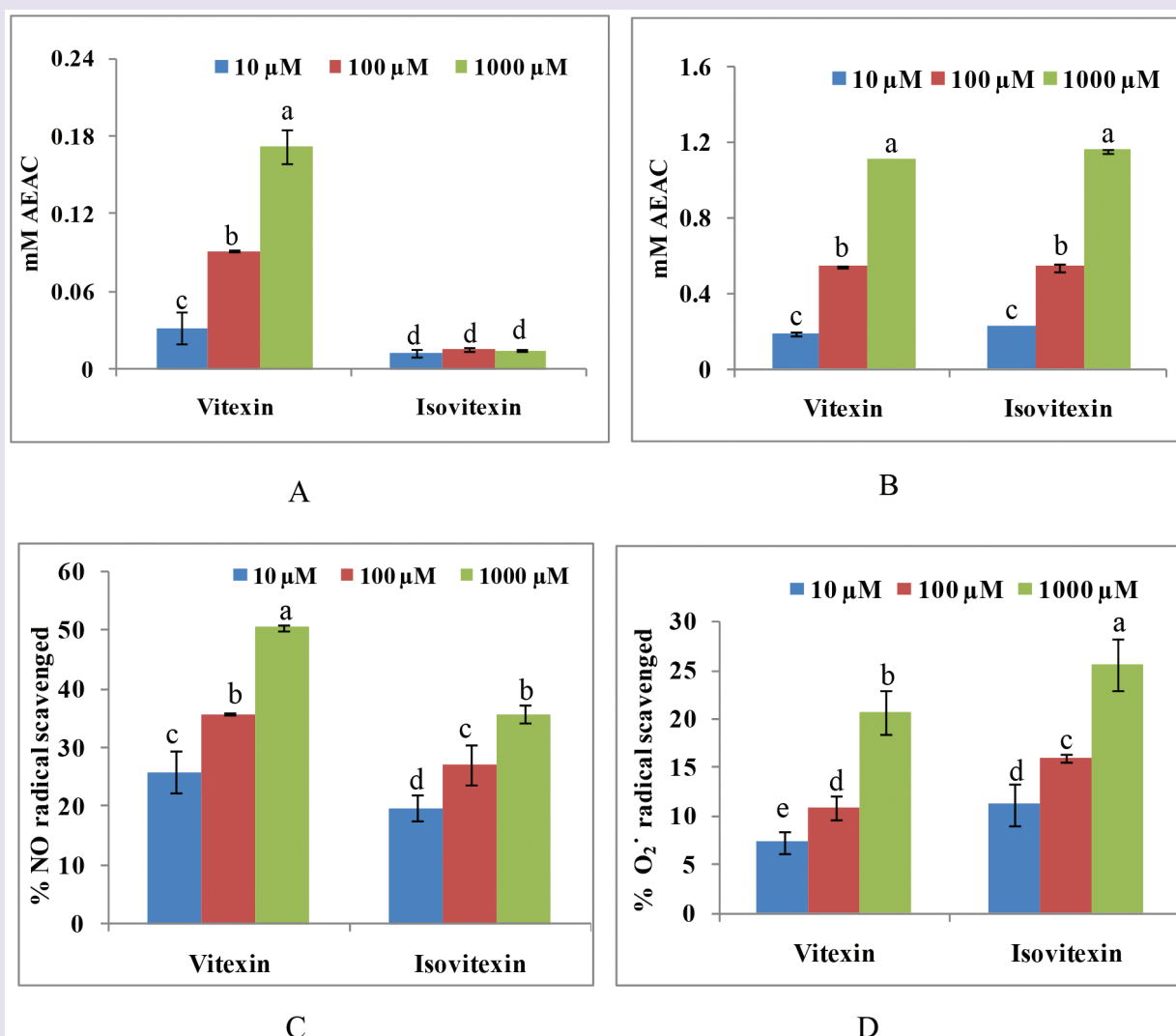


Figure 1: Radical scavenging ability of vitexin and isovitexin, as assessed by A) DPPH radical scavenging B) ABTS radical scavenging C) Nitric oxide radical scavenging and D) Superoxide radical scavenging. Values were expressed as mean ± S.D of three independent experiments. Different alphabets indicate significant difference at P≤0.05.

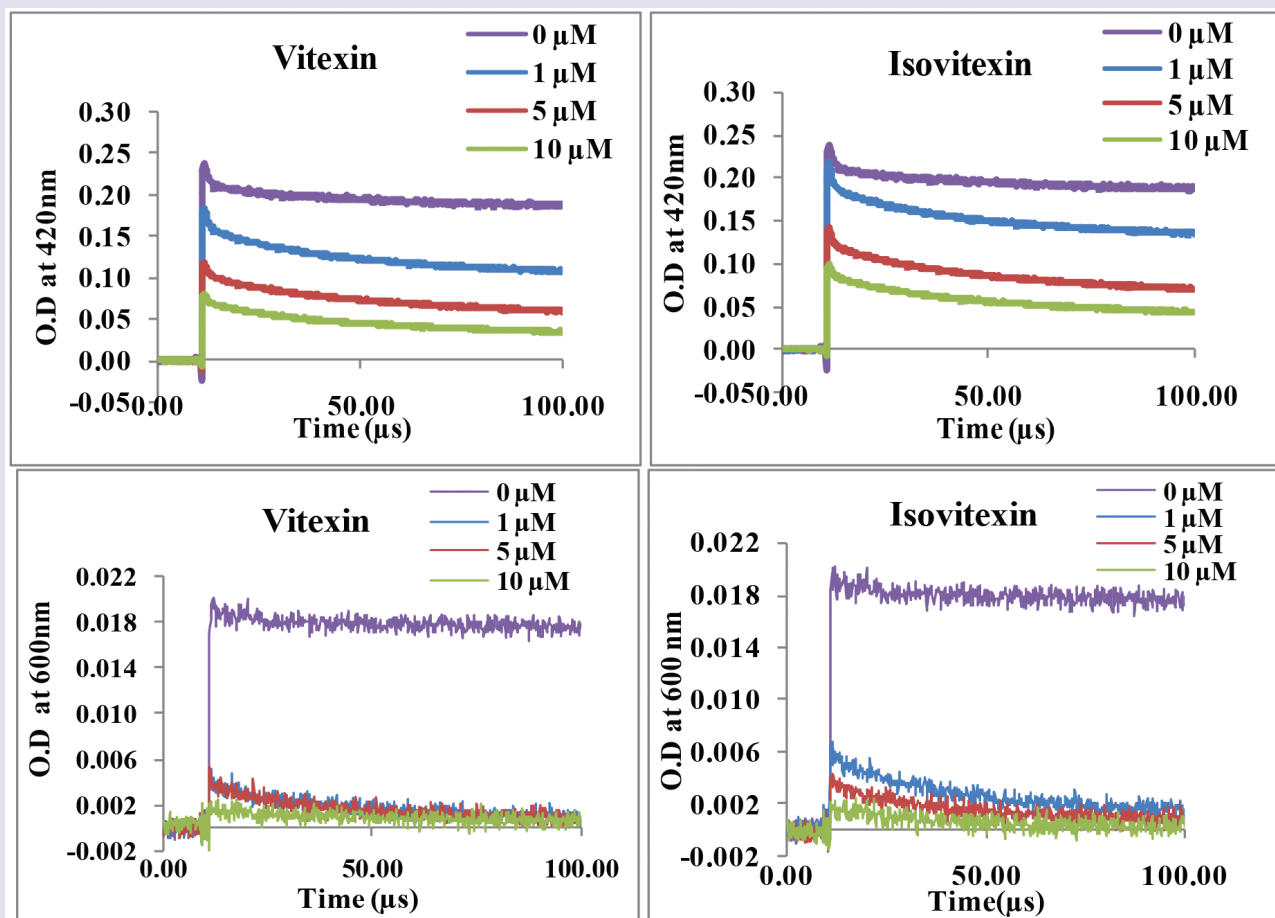


Figure 2: Decay curve of (A) ABTS•⁺ radicals (B) CO₃•⁻ radicals by pulse radiolysis in presence of different concentrations of vitexin and isovitexin.

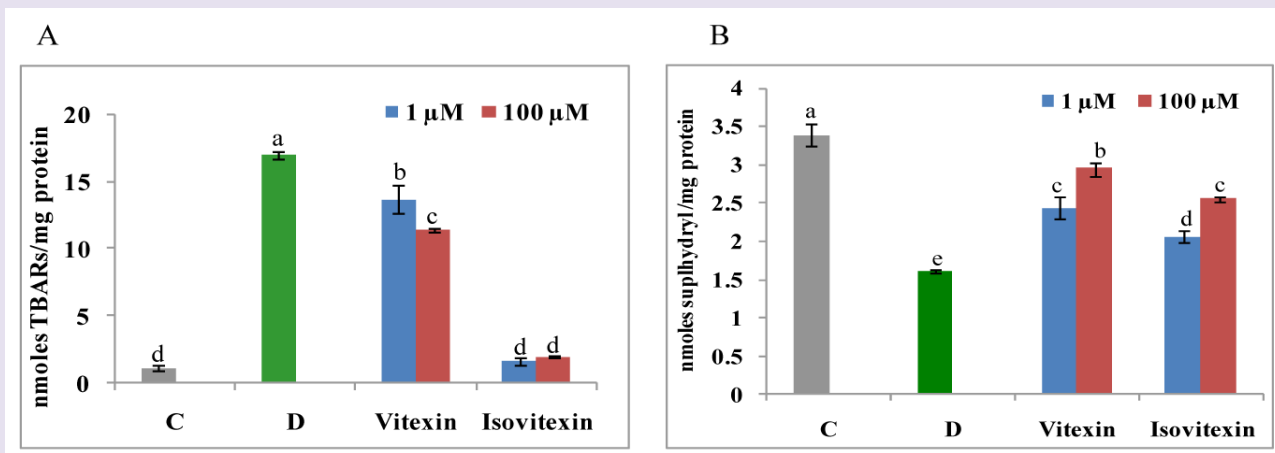


Figure 3: Protection to biomolecules of rat liver mitochondria against ascorbate-Fe²⁺ by different concentrations of vitexin and isovitexin A) Inhibition of lipid peroxidation was measured in terms of nmoles of TBARs formed/mg protein B) Protection to protein sulphhydryl was measured in terms of nmoles protein sulphhydryl/mg protein. Values are expressed as mean ± S.D of three independent experiments. C and D represent values in control and damaged mitochondria respectively. Different alphabets indicate significant difference at P≤0.05.

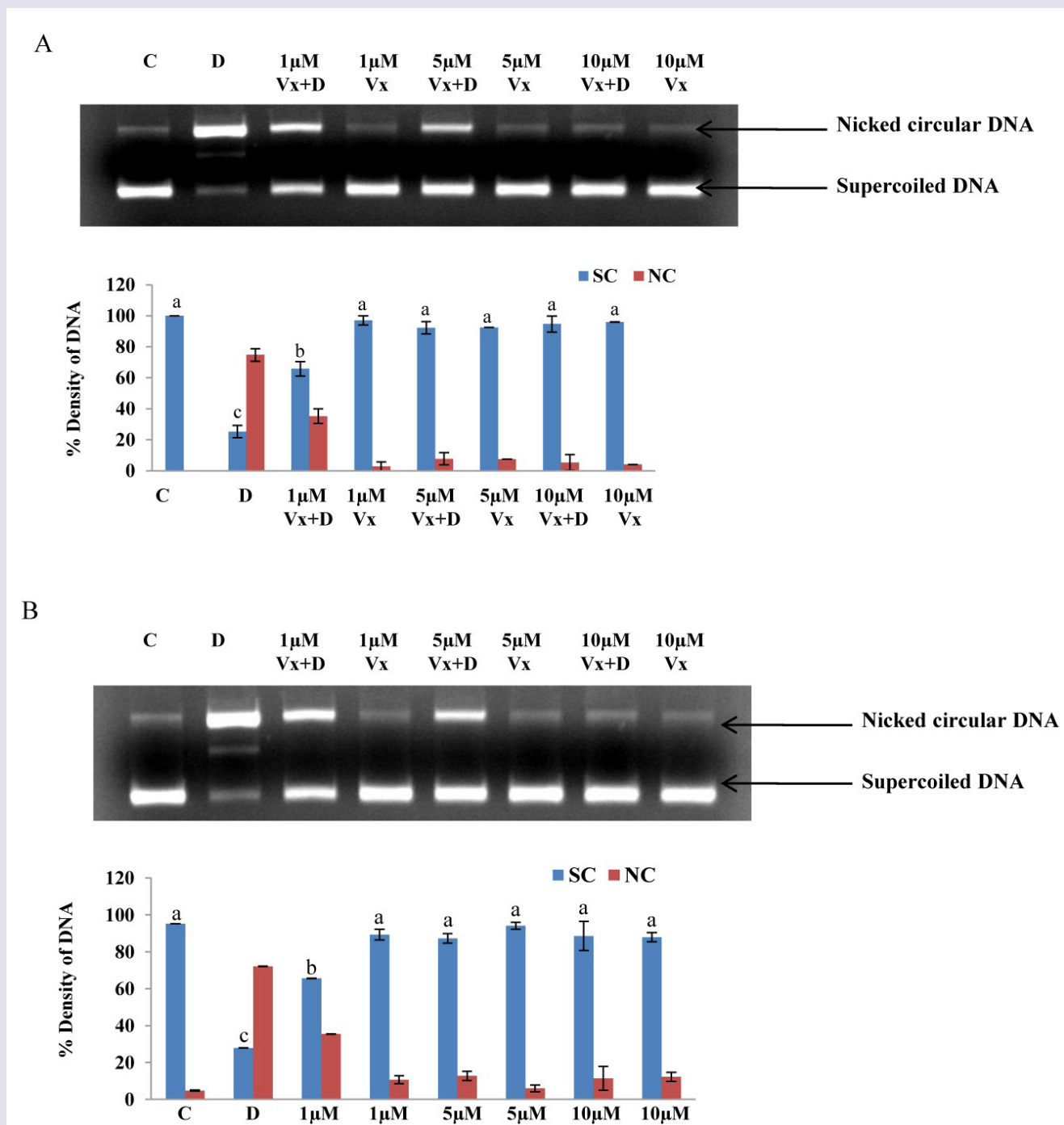


Figure 4: Protection to oxidatively damaged pBR322 DNA by A) Vitexin and B) Isovitexin. For quantification of supercoiled and nicked circular forms of DNA, densitometry was performed. Values were expressed as mean $\pm$ SE of three independent experiments. Different alphabets indicate significant difference at $P \leq 0.05$.

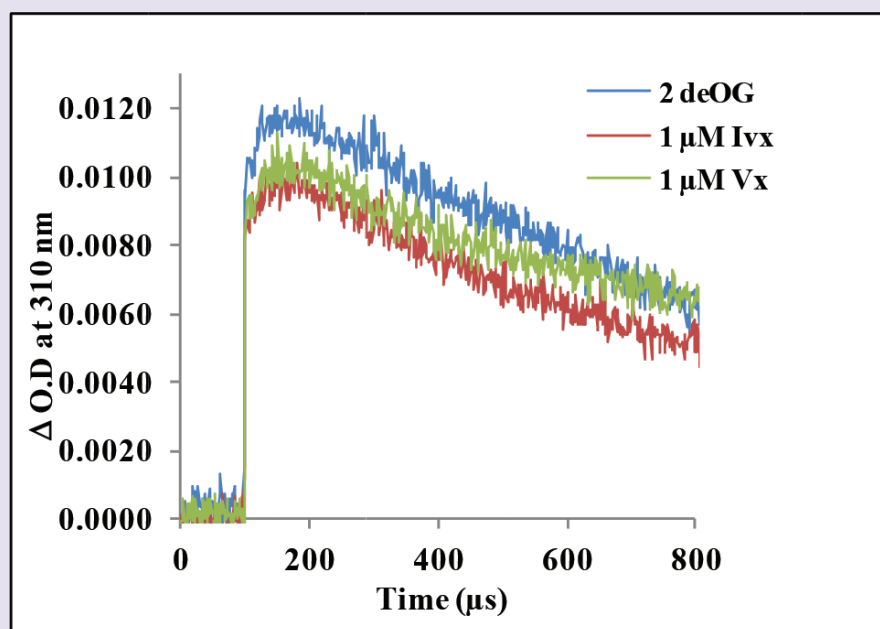


Figure 5: Interaction of vitexin (Vx) and Isovitexin (IVx) with 2 deOG transient base.

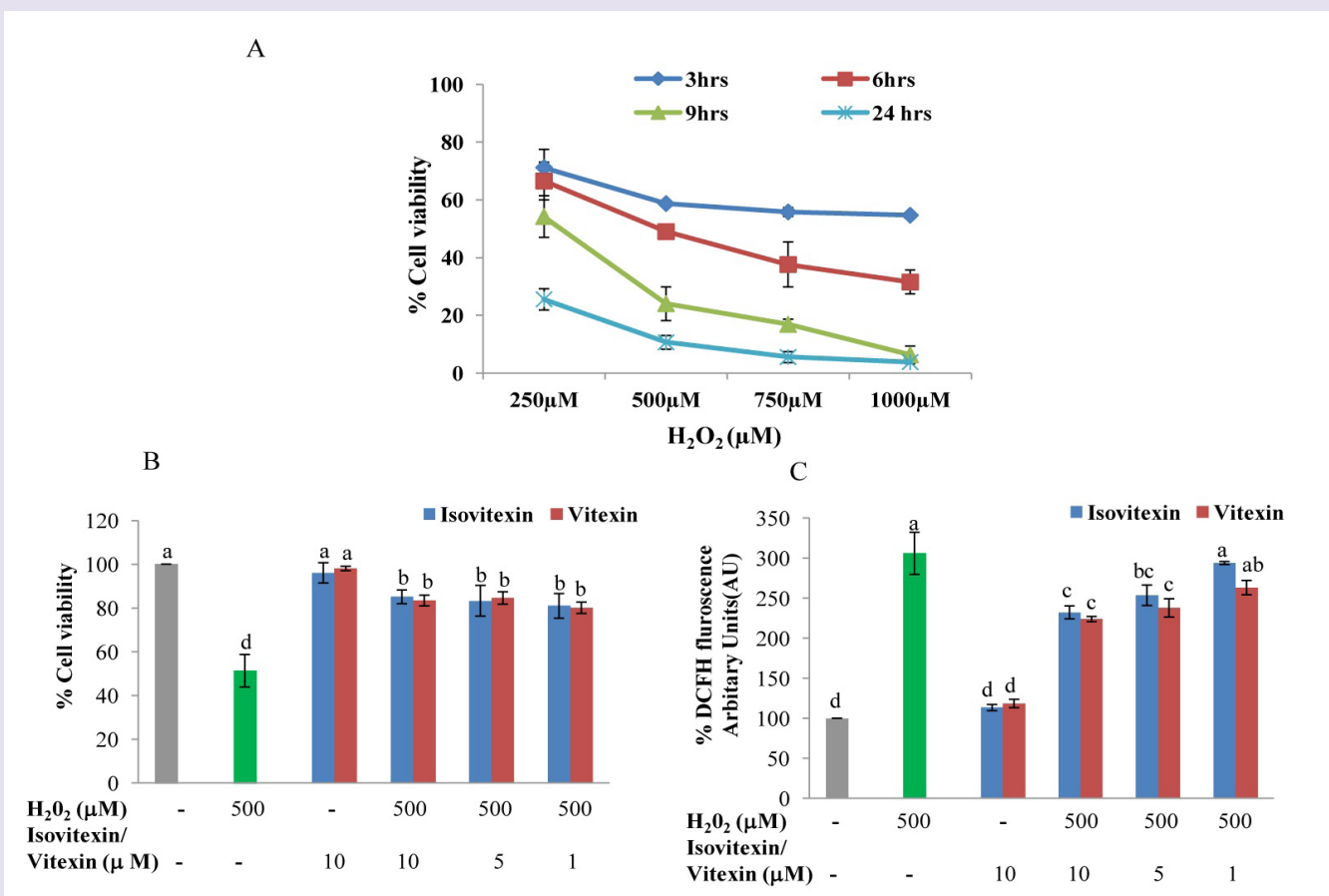


Figure 6: Protection conferred by vitexin and isovitexin against H₂O₂ induced oxidative stress. A) Percent cell viability of HepG2 cells treated with different concentrations of H₂O₂. B) Percent cell viability of HepG2 cells treated with vitexin and isovitexin in presence of H₂O₂. C) Inhibition of H₂O₂ induced ROS by vitexin and isovitexin. Results are expressed as mean ± S.E of three independent experiments. Different alphabets indicate significant difference at P≤0.05.

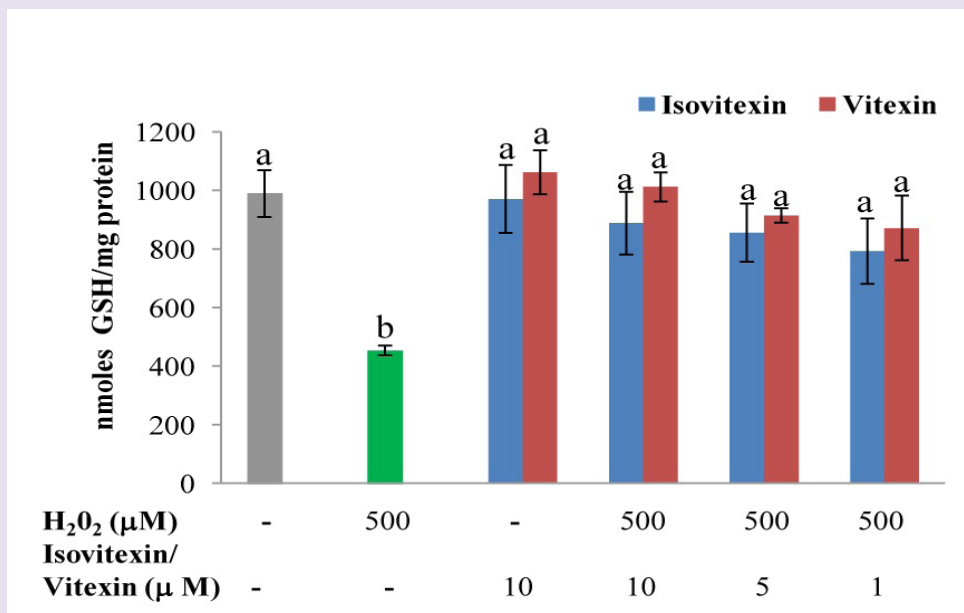


Figure 7: Vitexin and isovitexin replenish H_2O_2 induced depletion in intracellular glutathione (GSH). Results are expressed as nmoles GSH / mg protein and are mean $\pm$ S.E of three independent experiments. Different alphabets indicate significant difference at $P \leq 0.05$.

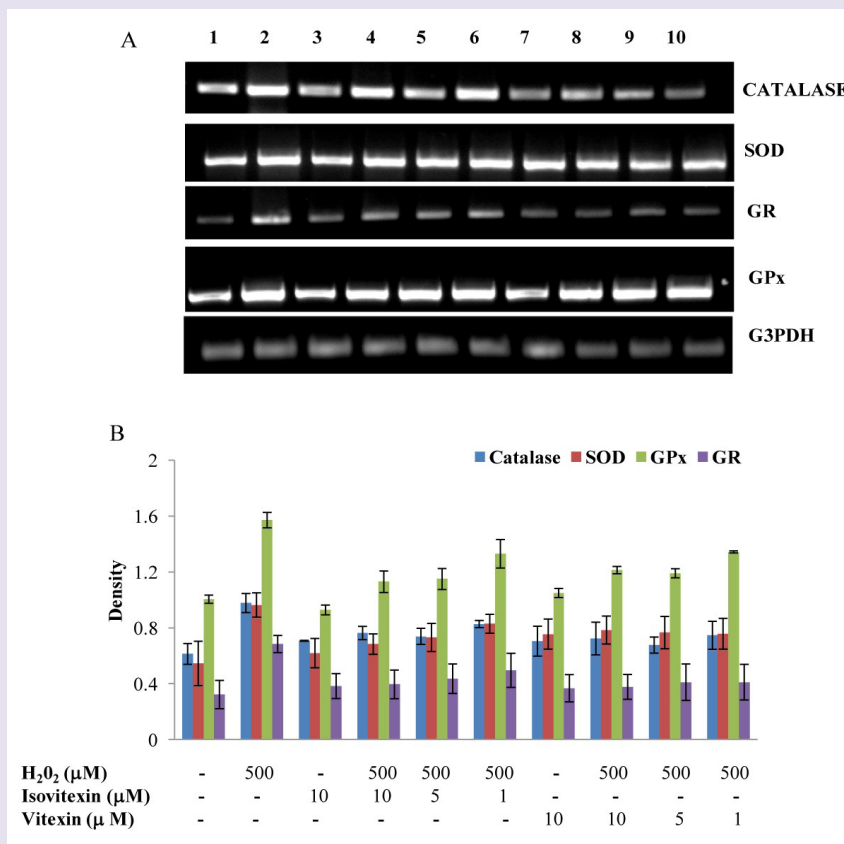
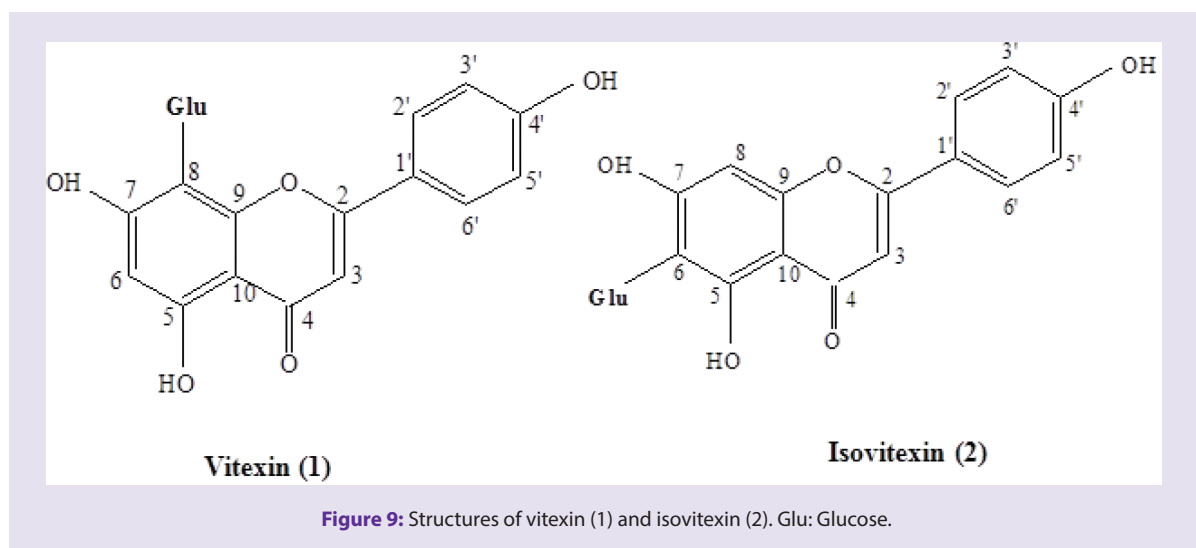


Figure 8A: Representative RT-PCR image of different antioxidant enzymes B) Densitometry analysis of mRNA levels of catalase, SOD, GPx and GR. H_2O_2 treatment led to significant increase ($P \leq 0.05$) (lane 2) in mRNA levels of all the enzymes compared to control. Treatment with isovitexin and vitexin at 10 μM concentration (Lanes 4 and 6 respectively) prevented H_2O_2 induced increase in genetic expression significantly ($P \leq 0.05$).



protein (Table 2). The effect of isovitexin and vitexin on antioxidant enzyme gene expression was studied by semi-quantitative RT-PCR. Exposure to 500 μM Hydrogen peroxide elevated the expression of all intracellular antioxidant enzymes (Figure 8, Lane 2) At 10 μM concentration of both isovitexin (Lane 4) and vitexin (Lane 8) this H_2O_2 induced increase in genetic expression was significantly ($P \leq 0.05$) restored to normal. Isoviteixin (Lane 3) and vitexin (Lane 7) alone, did not alter expression of these antioxidant enzymes.

DISCUSSION

Vitexin and isovitexin are C-glycosides of apigenin. Vitexin possesses glucose at C8 position of A ring whereas in isovitexin glucose is present at C6 position of A ring (Figure 9). Isoviteixin is known to protect DNA against cellular ROS and also acts an insulin secretagogue in non-diabetic rats.⁹ Vitexin has been shown to inhibit adipogenesis in 3T3-L1 cells.¹² Both compounds possess strong α -glucosidase inhibitory activity¹⁶ as well as anti-glycation ability.¹⁴ In the present study, the antioxidant activity of vitexin and isovitexin against primary, secondary free radicals was monitored by standard *in vitro* antioxidant assays and compared. The ability of these compounds to scavenge radicals such as DPPH and $\text{ABTS}^{\bullet+}$ gives preliminary information about electron or hydrogen donating capacity of the antioxidant molecule. The data obtained suggest that vitexin scavenged DPPH more efficiently than isovitexin, while for $\text{ABTS}^{\bullet+}$ radical both showed similar rate of scavenging. Lower activity of isovitexin against DPPH radical could be due to steric hindrance associated with the 6th positioned C-glycosylation. Earlier observation by Gerhauser (2009)³² suggests that apigenin and its derivatives are poor DPPH radical scavengers. Our data clearly demonstrates that C-8 glycosylation of apigenin (vitexin) enhanced its DPPH radical scavenging ability than its isomer C-6 glycosylated apigenin (isovitexin).

Superoxide anion is one of the major cellular damage causing radical, while nitric oxide is known to be an important mediator of various inflammatory processes. Sustained levels of nitric oxide are associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.³³ Nitric oxide also reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO^-) and thereby causes more damage. Between vitexin and isovitexin, vitexin efficiently scavenged nitric oxide radical while isovitexin was a better superoxide radical scavenger in a concentration dependent manner. This finding is in agreement with that reported by Zelinski and Zelinski, (2011),³⁴ who showed that the apigenin derivative

with glucose at C-6 position of A ring scavenged superoxide radical better than the apigenin derivative with glucose at C-8 position.

Most of the free radicals are short-lived, and hence require sensitive quantitative techniques such as pulse radiolysis for their detection at microsecond or picoseconds scale. In this technique, radiolysis of water by high energy electrons (e^-), produce e_{aq}^- , $\cdot\text{H}$ and $\cdot\text{OH}$. Presaturation with N_2O leads to the formation of $\cdot\text{OH}$, as a single major oxidizing radical. In the present study $\text{ABTS}^{\bullet+}$, and $\text{CO}_3^{\bullet-}$ were generated using the reaction of $\cdot\text{OH}$ in aqueous solutions of radical precursors in presence and absence of different concentrations of vitexin and isovitexin. Decay constants of vitexin and isovitexin suggest that they efficiently scavenge these radicals with almost equal rates indicating that these compounds interfere with $\text{ABTS}^{\bullet+}$ and $\text{CO}_3^{\bullet-}$ radical formation probably by directly scavenging with $\cdot\text{OH}$.

Free radicals interact with DNA and induce deformation in the bases and/or strand breaks. In the present study oxidative damage to plasmid pBR322 DNA was induced by $\cdot\text{OH}$ generated by Fenton reaction and was quantified in terms of amount of supercoiled DNA converted to nicked circular form. Both compounds effectively protected the DNA against free radical induced strand breaks probably by directly interacting with fenton radical. Lin *et al.*, (2002)⁸ have reported the DNA protection conferred by isovitexin against $\cdot\text{OH}$ induced DNA damage using pUC19 plasmid DNA, with a IC_{50} , 9.52 μM . In the present study, at 10 μM isovitexin completely protected pBR322 DNA from $\cdot\text{OH}$ induced oxidative damage. Among the DNA bases guanosine is more susceptible to damage by free radicals and 8-hydroxy-2'-deoxyguanosine is the major adduct formed. To check whether Vitexin and isovitexin prevent formation of 8-OH 2'-deoxyguanosine was subjected to pulse radiolysis studies in presence and absence of 1 μM vitexin or isovitexin and the decay traces of 2-dG transient species were recorded at 310 nm. Both compounds equally affected the formation of 2-dG transient species, indicating their ability in protecting DNA against free radical induced oxidative damage. Mitochondria are the major targets of oxidative damage which leads to reduced cellular functions and ultimately to the cellular death. Vitexin and isovitexin prevented ascorbate- Fe^{2+} induced mitochondrial oxidative damage by inhibiting lipid peroxidation and preventing depletion in protein sulphhydryl levels. Ability of isovitexin to inhibit lipid peroxidation (88%) was significantly ($P \leq 0.05$) higher than that of vitexin (33%). Masuoka *et al.*, (2003)³⁵ reported strong inhibition of lipid peroxidation by isovitexin than α -tocopherol suggesting a potential role of isovitexin in conferring protection against lipid peroxidation. On the other hand

ascorbate-Fe²⁺ induced depletion in mitochondrial protein sulphhydryls was significantly ($P \leq 0.05$) attenuated by vitexin than isovitexin indicating the ability of both the compounds to differentially protect cellular biomolecules.

Thus, the observed antioxidant activity of both vitexin and isovitexin indicates different reactivity of these compounds towards biological and non-biological free radicals and can be co-related to different structural difference. It is well known that the antioxidant activity of the flavonoids depends largely on the differences in the structural features and nature of substitutions on rings B and C. One of the important structural features responsible for antioxidant properties of flavonoids involves presence of hydroxyl groups at the 3', 4' and 5' positions of the B ring. Another feature that is partly responsible for enhancing radical scavenging ability is the presence of a double bond between C-2 and C-3 and its conjugation with the 4-oxo group in the C-ring. In addition the presence of functional groups of ring B and 5 hydroxy group of ring A, contribute to the metal chelating ability of flavonoids.³⁶ The antioxidant activity of vitexin and isovitexin could be attributed to a single hydroxyl group in the B ring, a double bond between C-2 and C-3, conjugated with the 4-oxo group in the C ring and the resorcinol group in the A ring (Figure 9). Moreover Zielinska and Zielinski, (2011)³⁴ have reported that substitution of glucose at C6 position of the A ring of flavonoids increased the antioxidant activity when compared with substitution at C8 glucose.

To check whether the observed antioxidant activity also reflected their ability to modulate cellular antioxidant defense, HepG2 cells were used. H₂O₂ was used as an oxidizing agent which induces cellular damage³⁷ by initiating sequential chain reactions involving formation of Fenton and Haberweiss radicals and secondary peroxy radicals. Pre-treatment with vitexin and isovitexin for 24 h significantly ($P \leq 0.05$) decreased H₂O₂ induced cytotoxicity and inhibited intracellular ROS. Kang *et al.*, (2010)¹¹ reported that vitexin at very less concentration of 8×10^{-7} mg/ml reduced the hydrogen peroxide induced ROS in polymorphonuclear cells as well as exhibited high ORAC value indicating efficient peroxy radical scavenging. In yet another study, vitexin at a concentration of 100 µg/ml reduced UVB induced increase in DCF fluorescence to the baseline value.³⁸ In HepG2 cells, 10 µM vitexin significantly attenuated ROS in hydrogen peroxide exposed cells. Huang *et al.*, (2005)³⁹ have previously reported the ability of isovitexin to scavenge lipopolysaccharide induced hydrogen peroxide in mouse macrophage RAW264.7 cells. Similarly Lin *et al.*, (2002)⁸ showed reduction in cadmium induced hydrogen peroxide content in isovitexin treated HL-60 cells. Our observations of isovitexin are also in consistence with these earlier studies and at 10 µM concentration it significantly attenuated H₂O₂ induced ROS in HepG2 cells. Flavonoids can exhibit their antioxidant activity either by directly scavenging intracellular ROS or they may offer indirect protection by enhancing activities of a number of endogenous antioxidants. We monitored changes in intracellular antioxidant enzyme activities as a biomarker of the cellular response to oxidative insult by hydrogen peroxide. Significant increase in the enzymatic activity and expression of catalase and superoxide dismutase was observed in cells treated with H₂O₂ alone. This increased enzyme activities and their expression was normalized in presence of varying concentrations of vitexin and isovitexin in a dose-dependent manner. GPx catalyses the reduction of hydrogen peroxide and related peroxides using reduced glutathione and converts it to oxidized glutathione while GR recycles this glutathione back to reduced glutathione. We observed that exposure of cells to hydrogen peroxide significantly increased activity and expression of both GPx and GR, possibly to eliminate H₂O₂ induced ROS. This increase was significantly inhibited in dose dependent manner. Cellular redox balance is largely indexed by GSH content which is the most important redox buffer of the cell. When exposed to H₂O₂ alone, intracellular GSH levels in HepG2

cells were significantly reduced which were effectively replenished in the vitexin and isovitexin pretreated cells.

Thus, from all the obtained data it's evident that besides the free hydroxyl group in the B ring of apigenin, substitution of glucose at the C6 and C8 position of the A rings also plays a role in antioxidant activity. Both these compounds form a major component of germinated fenugreek seeds (16.84 and 17.25 mg/kg of germinated fenugreek seeds of vitexin and isovitexin respectively)⁷ and hence could together contribute effectively to the antioxidant activity of fenugreek seeds.

CONCLUSION

Vitexin and isovitexin both possess potent antioxidant abilities and exhibit differential interaction with free radicals as evident from steady state interactions and pulse radiolysis studies with several systemic and non systemic radicals. Additionally, these compounds can protect lipids, proteins and DNA from oxidative damage. Both these compounds affect the decay and formation of dG transient species and protect the DNA. Pre-treatment of HepG2 cells with vitexin and isovitexin prevented H₂O₂ induced cytotoxicity by attenuating intracellular ROS, replenishing GSH and modulating activities and expression of antioxidant enzymes implying their potential role in mitigating oxidative stress and maintaining cellular redox homeostasis. The data also points out the beneficial use of the structural disparity among the two compounds, indicating a combination of these compounds would be more effective antioxidant.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

ABBREVIATION USED

ROS: Reactive oxygen species; **DPPH:** 2,2-Diphenyl 1-picrylhydrazyl; **ABTS:** L-Ascorbic acid, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate); **TBA:** 2,4,6-2-thiobarbituric acid; **TMP:** 1,1,3,3-tetramethoxypropane; **TFA:** Trifluoroacetic acid; **DMEM:** Dulbecco's Modified Eagle's medium; **FBS:** Foetal bovine serum; **PMS/NADH:** Phenazine methosulfate/nicotinamide adenine dinucleotide; **NEDD:** Naphthyl ethylene diamine dihydrochloride; **MDA:** Malonaldehyde; **TMP:** Tetramethoxypropane; **DTNB:** 5,5'-dithiobis 2-nitrobenzoic acid; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **DCFH-DA:** 2', 7'-Dichlorodihydrofluorescein diacetate; **H₂O₂:** Hydrogen Peroxide; **SOD:** Superoxide dismutase; **GPx:** Glutathione Peroxidase; **GR:** Glutathione reductase.

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SUMMARY

- Vitexin and isovitexin, both glycoside derivatives of apigenin, showed antioxidant activity against various free radicals as well as protected mitochondrial lipids and proteins against oxidative damage.
- Both compounds inhibited oxidative damage induced single strand breaks in plasmid DNA probably by interacting with 2-deoxy guanosine base transient.
- Both compounds equally reduced H_2O_2 induced cytotoxicity as well as ROS generation in HepG2 cells, suggesting cytoprotective activity.
- An increase in the antioxidant enzymes namely catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase in presence of H_2O_2 in HepG2 cells was significantly reduced on pre-treatment of vitexin and isovitexin. This reduction was also observed at RNA level.
- Both vitexin and isovitexin inhibited decrease in GSH levels, indicating their ability to maintain cellular redox status.

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