Final progress report (1/08/2012 to 31/12/2015):

Evaluation of mechanism of antioxidant and antidiabetic potential of vitexin

1. Introduction

Vitexin is an C-glycoside derivative of apigenin. Various biological activities of vitexin are reported . They include anti-myloperoxidase activity, anti-Helicobacter pylori activity as well as inhibition of H^+, K^+-ATPase activity (Quileza et al., 2010). Vitexin was shown to reduce H_2O_2 induced oxidative stress efficiently in polymorphonuclear cells (Jie et al., 2010). Its strong α-glucosidase inhibitory activity as well as its ability to inhibit adipogenesis in 3T3-L1 cells has been demonstrated (Li et al., 2009; Kim et al., 2010). In PC12 rat pheochromocytoma cells, vitexin inhibited HIF-1α and reduced mRNA level of hypoxia-inducible genes i.e., VEGF, smad3, aldolase A, enolase 1, and collagen type III possibly by activating c-jun N-terminal kinase (JNK) (Choi et al., 2006). Though these different biological activities are demonstrated for vitexin its precise mechanism of action is still to be elucidated. The present study aimed at studying its ability to scavenge free radicals as well as modulate cellular antioxidant defense at molecular level.

2. Material and Methods

In vitro antioxidant assays

For all the *in vitro* assays, vitexin at a concentration of 10,100 and 1000 μM was used. Interaction of vitexin with 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) radical was monitored using method described previously (Aquino et al., 2001). Similarly interaction of vitexin with, 2'-azinobis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS**)** radical cation generated by interacting ABTS with ammonium persulfate for 16h in dark was monitored following previously described method (Maurya et al., 2010). Ability of vitexin to scavenge phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH) generated superoxide radical was monitored using previously reported method by Aruoma et al., (1993). Nitric oxide radical was generated using 50 mM sodium nitroprusside in 1X PBS and the ability of vitexin to scavenge these radicals was quantified by using Griess reagent (1 % sulphanilamide and 0.1 % N-naphthyl

ethylene diamine dihydrochloride (NEDD) in 2 % H_3PO_4) (Hazra et al., 2008). An experimental control without test compound but with equivalent amount of vehicle was also kept in parallel.

Pulse radiolysis

Vitexin was tested for its ability to interact with short lived radicals using pulse radiolysis. LINAC facility at the 'National Center for Free Radical Research', SPPU, Pune was utilized for Pulse radiolysis studies. Dosimetry was performed with aerated 0.01 mol dm^{-3} KSCN solution with a G ε of 2.6×10^{-4} m^2 J^{-1} per 100 eV at 480 nm. (Buxton et al., 1995) and the dose rate per pulse was determined to be 19 Gy. $ABTS^{\bullet-}$ was produced by interaction of radiolytically generated azide radicals with $ABTS^{2-}$ (2mM). $CO_3^{\bullet-}$ were 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 $ABTS^{\bullet-}$ or $CO_3^{\bullet-}$ were monitored in presence and absence of different concentrations of test compound and was correlated with the concentration of ascorbic acid equivalents (Dixit et al., 2005). $ABTS^{\bullet-}$ and $CO_3^{\bullet-}$ 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 (Ernst et al., 1997). 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 μg/ml of ethidium bromide solution for 20 min at room temperature, washed with water, observed under UV and bands observed were recorded using gel documentation system (Alfa innotech, USA) and were quantitated by densitometry.

Interaction of vitexin 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 $^{\bullet}OH$ with 2-dG were recorded. 2 ml reaction mixture containing different concentrations of vitexin and 2-dG (2mM) 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 and decay plots were plotted (Phadatare et al., 2011).

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 (Devasagayam et al., 1983). 0.25 mg/ml rat liver mitochondria were exposed to Fenton radical initiated by ascorbate-Fe^{2+} induced oxidative damage 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 sulphydryl groups was measured using Ellmans reagent, (5,5'-dithiobis 2-nitrobenzoic acid- DTNB) (Santos et al., 1998) and was expressed as nmoles protein sulphydryl/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 μg/ml), supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/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 (1, 5 and 10 μM). After incubation for 24 h, 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) (Wang et al., 2006). Cells (2×10^4 cells) were seeded in a 96-well plate in medium supplemented with 10% FBS. On the next day cells were treated with various concentrations of vitexin (1, 5 and 10 μM) for 24 h. Media was then 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. Resulting intracellular purple colored formazan was quantified by measuring absorbance at 550 nm using ELISA plate reader (Multiskan Ex, microplate reader, Thermofischer Scientific U.K).

Estimation of intracellular reactive oxygen species (ROS)

Intracellular ROS was quantified using DCFH-DA (2′, 7′-Dichlorodihydrofluorescein diacetate) (Alia et al., 2005). 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 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 $[(Ft_{30}-F_{t0})/F_{t0} * 100]$, where Ft_{30} = Fluorescence at time 30 min. and Ft_0 = Fluorescence at time 0 min. The data is expressed in terms of % DCFH fluroscence (Arbitary 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 (0.5M) and 1% Triton X 100), 1mM 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 method (Lowry et al., 1951) and lysates were stored at -20°C until use. Quantification of antioxidant enzymes namely superoxide dismutase (EC – Number 1.15.1.1) (Beauchamp et al.,1971) catalase (EC – Number 1.11.1.6) (Aebi,1983), glutathione reductase (EC – Number 1.8.1.10) (Goldbery et al., 1983) and Glutathione peroxidase (EC – Number 1.11.1.9) (Lawrence et al., 1976) were carried out using standard protocol.

Estimation of total glutathione (GSH)

Total intracellular GSH was estimated according to the protocol of Teixeira and Meneghini, (1996). 5% sulfosalicyclic acid was added to the cellular lysates to precipitate the proteins. The supernatant (10 μl of 1:2 diluted) was added to reaction mixture (170 μl) 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.

Western Blotting

After pre-treatment with the vitexin followed by hydrogen peroxide, HepG2 (2×10^6) cells were collected in 1X PBS and proteins were isolated using lysis buffer. Equal amounts of protein (60 μg) was loaded and separated on 10% and 12% polyacrylamide resolving gels and were transferred on activated PVDF membrane (Millipore, U.S.A.). Protein binding sites were blocked with 2% blocker (Thermoscientific, Germany) in TBST for 2 hrs. Membrane was washed thrice with 1X TBST. PVDF membranes were incubated with primary antibodies (at 1:1000 dilution in TBST-2%blocker) for 90 minutes at 4°C. Membranes were washed thrice with TBST and incubated with secondary antibody (goat anti-rabbit horseradish peroxidase-conjugated; Banglore Genei, India) at 1:2000 dilution in TBST-2% blocker) and washed thrice with TBST. Antigen-antibody complex was detected using enhanced chemiluminescence kit (ECL, Roche, Germany).

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). cDNA products were amplified by PCR using gene specific primers.

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). G3PDH, a house keeping gene was used as a standard for normalization.

Statistical Analysis

All experiments were done in 3 to 5 replicates, and final values are expressed as average ± 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 analyses were carried out using SPSS software (Version 19) licensed to Dept. of Zoology, SPPU.

3. RESULTS

3.1. *In vitro* antioxidant assays

Fig. 1a shows DPPH radical scavenging activity of vitexin. It was observed that 1000 µM vitexin showed significantly high DPPH radical scavenging activity (0.171 ± 0.012 mM AEAC) ($p<0.05$) compared to rest of the fractions. In ABTS radical scavenging assay, concentration dependent scavenging activity of vitexin was seen (Fig. 1b). Superoxide and nitric oxide are biologically relevant radicals. Ability of vitexin to scavenge these radicals was also checked. 1000 µM of vitexin scavenged nitric oxide more efficiently (50.3 ± 0.5%) ($p<0.05$) (Fig. 1c) than superoxide radicals (20 ± 2.2%) (Fig. 1d).

Fig.1: In vitro radical scavenging activity of different concentrations of vitexin as assessed by a) DPPH radical scavenging b) ABTS radical scavenging c) Nitric oxide radical scavenging and d) Superoxide radical scavenging. Values are expressed as mean ± S.D of three independent experiments.

Fig. 1a

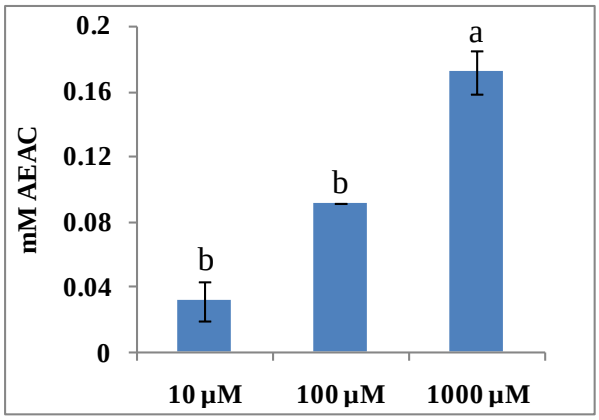


Fig. 1b

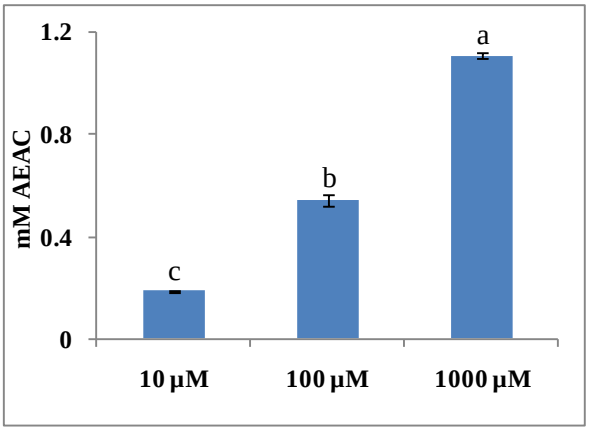


Fig. 1c

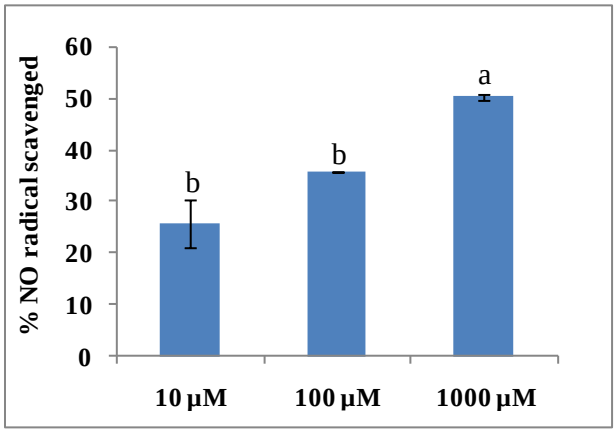
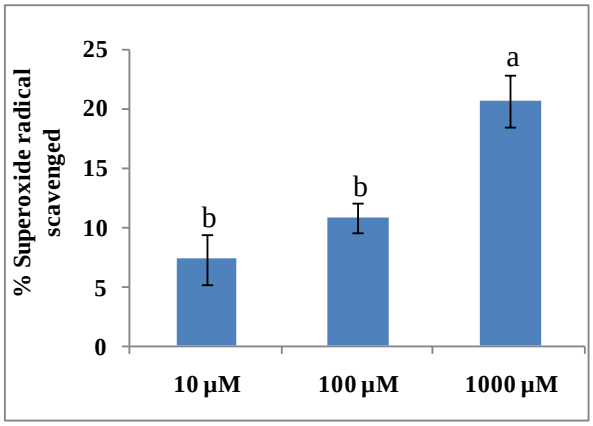


Fig. 1d



The radical scavenging ability of vitexin was quantitated using pulse radiolysis. In this study $ABTS^{\bullet+}$, $CO_3^{\bullet-}$ radicals were generated by pulse radiolysis and scavenging activity of these radicals by vitexin was checked. Ascorbic acid was used as standard antioxidant to measure the activity of these compounds. Fig. 2 shows the decay curves of $ABTS^{\bullet+}$ (Fig. 2a) and $CO_3^{\bullet-}$ (Fig. 2b) radicals in presence of different concentrations of vitexin.

Fig. 2: Decay curve of (a) $ABTS^{\bullet+}$ radicals and (b) $CO_3^{\bullet-}$ radicals generated by pulse radiolysis by different concentration of vitexin.

Fig. 2a

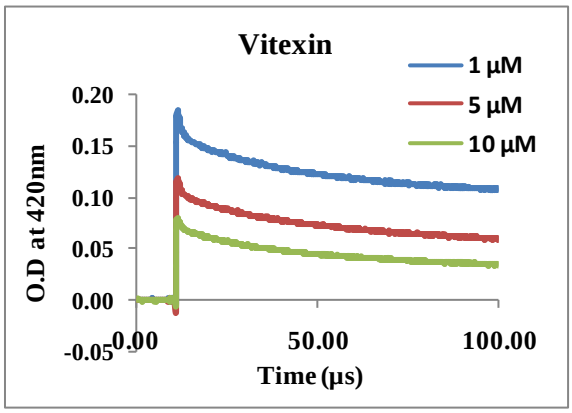
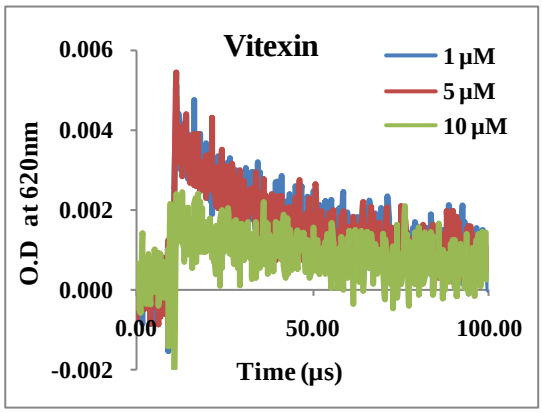


Fig. 2b



Vitexin effectively scavenged $ABTS^{\bullet+}$ as well as $CO^{\bullet-}_3$ radicals. At a concentration of 10 µM vitexin scavenged ABTS radicals with a value of 38 µg/ml of AEAC. Maximum $CO^{\bullet-}_3$ radical scavenging activity was exhibited by 10 µM of vitexin with a value of 1.9 µg/ml of AEAC respectively.

Protection to biomolecules of oxidatively damaged mitochondria

Rat liver mitochondria were subjected to oxidative stress by Ascorbate – Fe^{2+} and damage was assessed by measuring lipid peroxidation and protein sulphydryl as oxidative stress markers. Lipid peroxidation in case of control and oxidatively damaged mitochondria by ascorbate-Fe^{2+} was 1.1 ± 0.2 and 17.04 ± 0.28 nmoles of TBARs/mg protein, respectively (Fig.3a). Vitexin significantly reduced lipid peroxide formation (11.4 ± 0.26 nmoles TBARs / mg protein) and exhibited high protection (33%) ($p<0.05$). Exposure of rat liver mitochondria to Ascorbate – Fe^{2+} lead 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 prevented effectively in presence of 100 µM of vitexin (2.9 ± 0.09 nmoles protein sulphydryl/mg protein) (Fig.3b).

Fig. 3: Protection to biomolecules of rat liver mitochondria against ascorbate-Fe^{2+} by different concentrations of vitexin a) Inhibition of lipid peroxidation measured in terms of nmoles of TBARs formed/mg protein b) Protection to protein sulphydryl measured in terms of nmoles protein sulphydryl/mg protein. Values are expressed as mean ± S.D of three independent experiments.

Fig. 3a

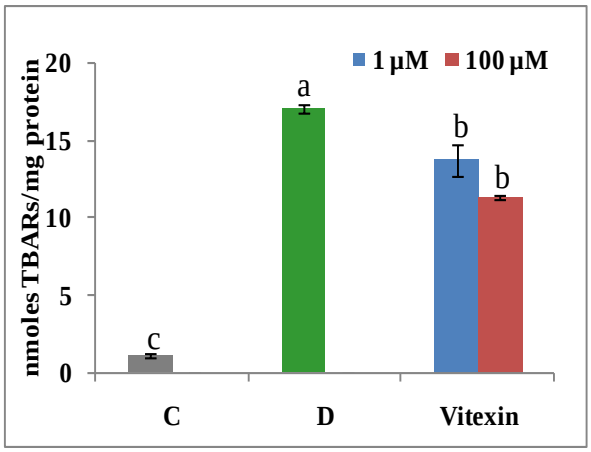
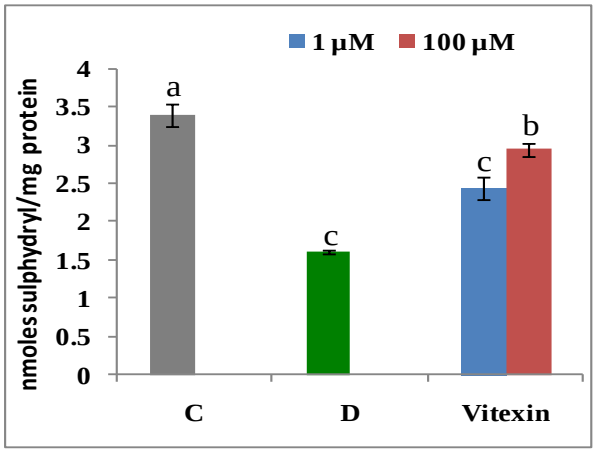


Fig. 3b



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

Agarose gel electrophoretic pattern of plasmid DNA exposed to OH radicals generated by Fenton reaction in the presence of compounds was monitored to evaluate the protective effect of compounds to oxidatively damaged plasmid DNA. Oxidative damage to plasmid was measured in terms of conversion of supercoiled (SC) to nicked circular (NC) form. Protection offered by the compounds was estimated by quantifying the amount of DNA in both nicked circular and supercoiled form in presence of these compounds. Additionally any damaging effect of compound alone on pBR322 was also assessed. Figure 4a shows electrophoresis pattern of oxidatively damaged pBR322 in presence and absence of different concentrations of vitexin. Significant reduction in the supercoiled form of DNA was observed in presence of the fenton reaction (Lane 2, Fig. 4a and b), whereas this reduction was prevented in a concentration dependent manner in presence of vitexin. (Lanes 3,5 and 8). Vitexin by itself did not cause any damage to the plasmid DNA.

Fig.4a: Agarose gel electrophoretic pattern showing protection to oxidatively damaged pBR322 DNA by vitexin. Lane 1: Control pBR322 DNA; Lane 2: oxidatively damaged DNA; Lanes 3, 5 and 7 are oxidatively damaged DNA in presence of 1, 5 and 10 µM vitexin respectively, Lanes 4, 6 and 8 are DNA treated with 1, 5 and 10 µM vitexin alone

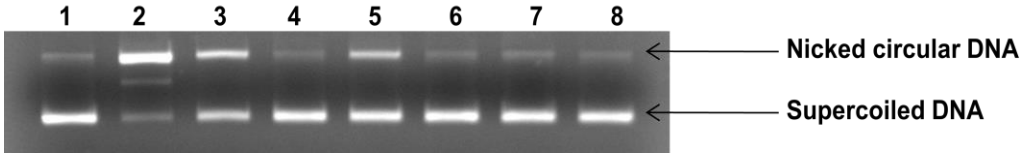
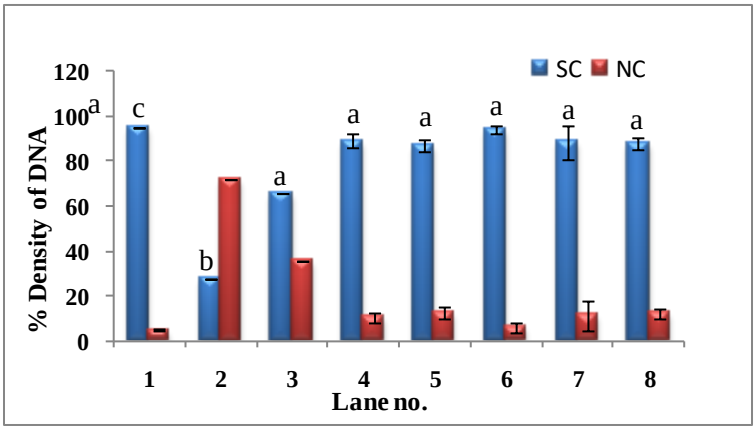
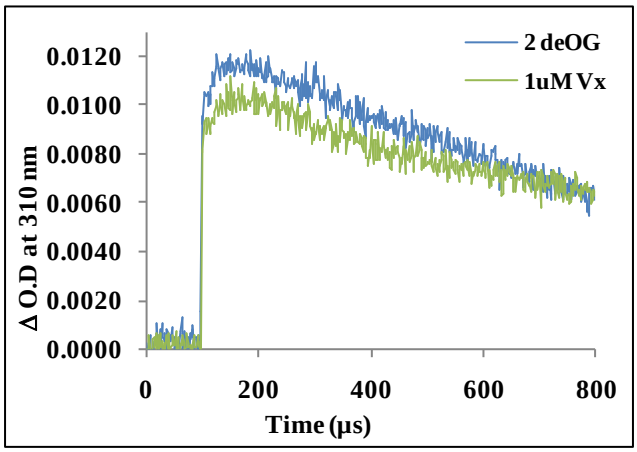


Fig. 4b: Amount of supercoiled and nicked circular DNA estimated from densitometric measurements of electrophoreogram. Amount of nicked circular DNA is substantially increased in oxidatively damaged DNA, while in the presence of the vitexin majority of the DNA remained in the supercoiled form.



The time resolved transient absorption spectra for the reaction of •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 1µM vitexin. It was evident that vitexin affected 2-dG transient formation as the absorbance of the transient was decreased in presence of vitexin (Figure 5).

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



Cytotoxicity measurement by MTT assay

For HepG2 cells, hydrogen peroxide was used as oxidative stress generator. Viability of cells in presence of different concentration and time of exposure of hydrogen peroxide was checked using MTT assay. Cell viability was determined as percent formazon formed compared to untreated cells. Fig.6 shows the effect of H_2O_2 on cell viability using MTT assay. Concentration and time dependent decrease in the cell viability was observed in H_2O_2 treated cells. Cells incubated with H_2O_2 at a concentration of 500 µM for 6 h exhibited 48 ± 2.5 % cell death. This IC_{50} concentration and time period was used for carrying out rest of the experiments.

The protective effect of vitexin on HepG2 cells was also checked by MTT assay. Different concentrations of vitexin were added to the cells and incubated for 24 hrs and cell viability was calculated. Fig. 7 shows the effect of vitexin on HepG2 cells. Vitexin at concentration of 5, 10 and 20 µM significantly prevented the cells from oxidative damage with a value of 100 ± 5.2, 93 ± 5.5 and 89 ± 2.2 % respectively. Therefore 1, 5 and 10 µM concentrations were used for further experiments.

Fig. 6: Percent cell viability of HepG2 cells treated with different concentrations of H_2O_2 and at different time points. Results are expressed as mean ± S.E of three independent experiments.

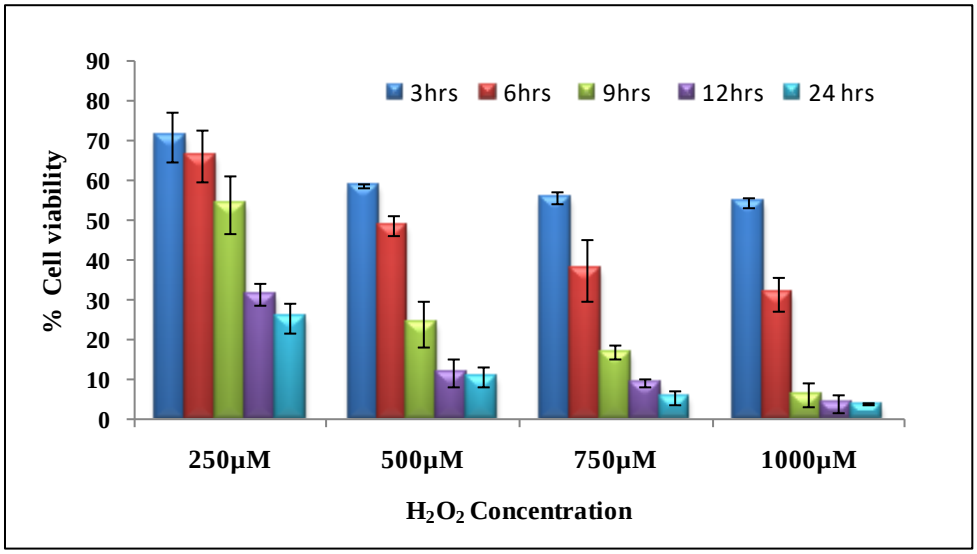
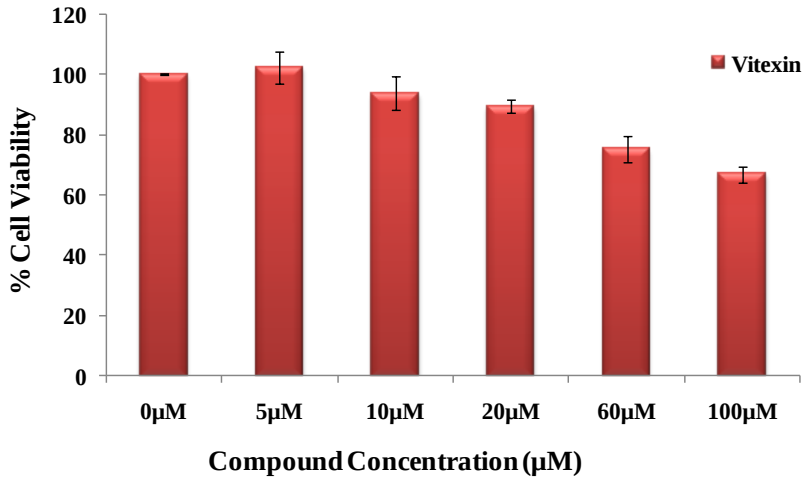


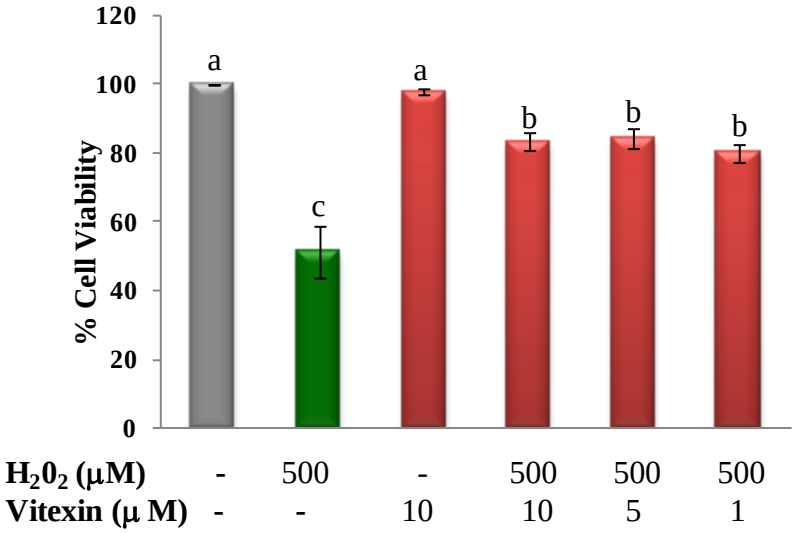
Fig. 7: Effect of vitexin alone on cell viability of HepG2 cells. Data expressed as % cell viability. Results expressed as mean ± S.E of three independent experiments.



To check of protective effect of vitexin against hydrogen peroxide induced cell death in HepG2 cells, MTT assay was used. Cells were pre-treated with different concentrations of both compounds for 24 hrs and incubated with 500 µM of H_2O_2 for 6hrs. After the incubation, cells

were treated with MTT and % viability was measured. 500 μM H_2O_2 induced significant damage in treated cells (51 ± 1.5%) ($p<0.05$) (Fig.8). This damage was significantly prevented ($p<0.05$) by pre-treatment of cells with 10 and 1μM of vitexin (83 ± 2, and 80 ± 1.9 % respectively) (Fig.3).

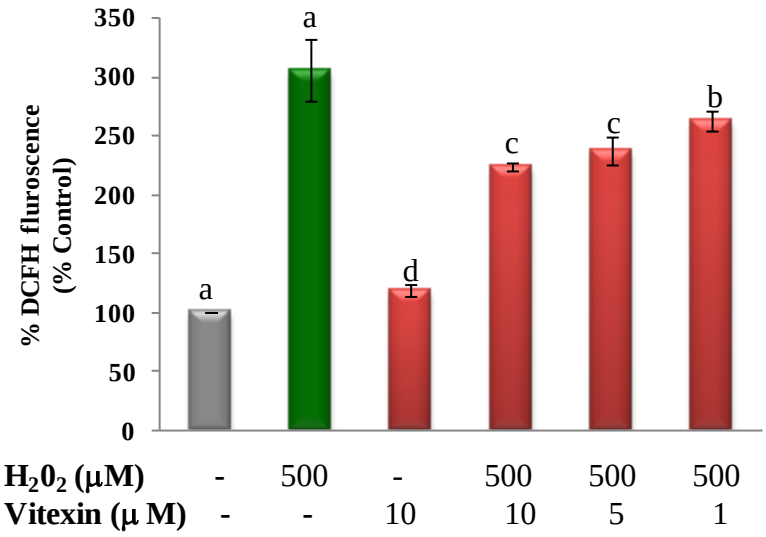
Fig. 8: Vitexin mediated cytoprotection against H_2O_2 induced oxidative stress. Data are represented as percent cell viability and mean of ± S.E of three independent experiments.



Determination of intracellular ROS production

Evaluation of ROS is a good indicator of the oxidative damage to living cells. Hydrogen peroxide within 30 minutes induced rapid and significant increase in the amount of intracellular ROS in a concentration dependent manner. 500 μM of H_2O_2 induced significant increase in the basal ROS levels. This increase was effectively decreased on pre-treatment with vitexin in a concentration dependent manner (Fig. 9).

Fig. 9: Inhibition of intracellular ROS formation by vitexin in response to H_2O_2. Values are expressed as percent of control and are mean of ± S.E of three independent experiments.



Quantitation of intracellular antioxidant enzymes

Table 1 shows the different antioxidant enzymes levels in HepG2 cells after the treatment with H_2O_2 in presence or absence of different concentrations of vitexin. In control untreated cells, catalase activity was 6931 ± 463 units/mg protein. Treatment of cells with 500 μM of H_2O_2 significantly increased this activity to 12634 ± 575 units/mg protein ($p<0.05$). This increase in catalase activity was prevented in presence vitexin in a concentration dependent manner. 10 μm vitexin along with H_2O_2 decreased the catalase activity to 8767 ± 488 followed by 5 μM which decreased the activity to 9540 ± 704 units/mg protein ($p<0.05$). HepG2 cells incubated with 10 μM vitexin alone showed no change in the activity and was comparable to the control (7768 ± 551 units/mg protein).

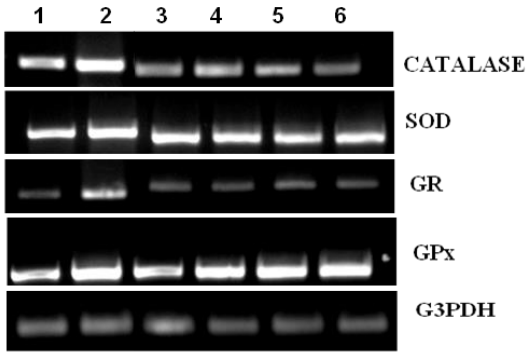
Table 1: Antioxidant enzymes activity in HepG2 cells under different conditions.

Groups	**Catalase** (units/mg protein)	**Superoxide Dismutase** (units/mg protein)	**Glutathione peroxidase** (units/mg protein)	**Glutathione reductase** (units/mg protein)
Control	6931 ± 463	3941 ± 412	110 ± 7	562± 30
H_2O_2 alone	12634 ± 575	5678 ± 120	293 ± 19	845 ± 48
10μM vitexin alone	7768±551	4558 ± 199	133 ± 16	554 ± 32
H_2O_2 + 10μM vitexin	8767± 488	4564 ± 400	189± 21	671 ± 79
H_2O_2 + 5μM vitexin	9540± 677	4981± 482	201± 21	766 ± 74
H_2O_2 + 1μM vitexin	10246±798	5462± 201	224 ± 4	817 ± 41

Treatment of HepG2 cells with H_2O_2 increased superoxide dismutase activity (5678 ± 120 units/mg protein) compared to untreated control (3941 ± 412 units/mg protein) ($p<0.05$). In presence of H_2O_2 and 10μM of vitexin the activity decreased to 4564 ± 400 units/ mg protein, ($p<0.05$). Treatment of HepG2 cells with 10 μM vitexin alone had no effect on the activity (4558 ± 199 units/mg protein) (Table 1). Glutathione peroxidase activity in control HepG2 cells was 110 ± 7 units/mg protein, and it increased to 293 ± 19 units/mg protein ($p<0.05$) in H_2O_2 treated cells. In presence of increasing concentration of vitexin along with H_2O_2, GPx activity decreased. 10 μM vitexin decreased the activity to 189 ± 21 units/mg protein ($p<0.05$). Incubation of cells in presence of 10 μM vitexin alone did not cause any increase in the activity compared with untreated control cells (133 ± 16 units/mg protein respectively). Similarly a significant increase in the amount of glutathione reductase was observed in cells treated with H_2O_2 (845 ± 48 units/mg protein) ($p<0.05$) compared with untreated control cells (562 ± 30 units/mg protein). This increase GR activity was reduced by vitexin effectively. The activity decreased to 671 ± 79

units/mg protein ($p<0.05$) in presence of 10 µM vitexin along with H_2O_2 (Table 1). The effect of 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 10, Lane 2) At 10 µM concentration of vitexin (Lane 4) this H_2O_2 induced increase in genetic expression was significantly ($P\leq0.05$) restored to normal.. Vitexin (Lane 3) alone, did not alter expression of these antioxidant enzymes.

Fig. 10A



10 B

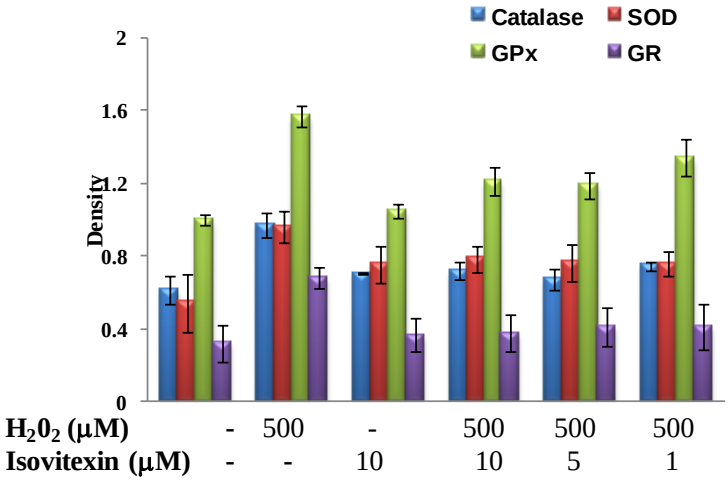


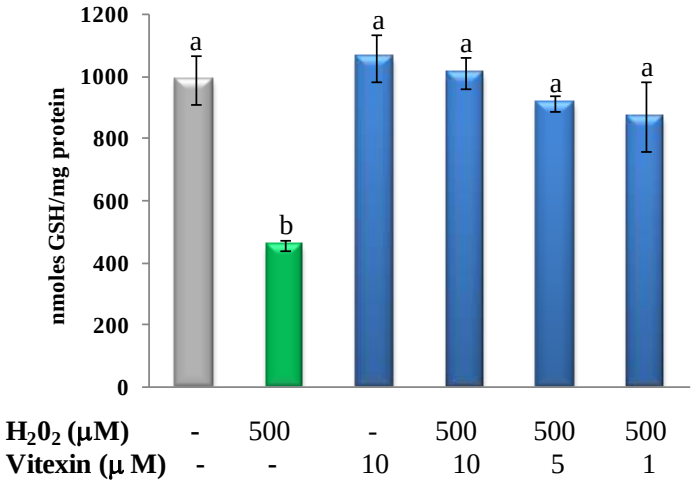
Fig. 10A: Representative RT-PCR image of different antioxidant enzymes B**)** Densitometry analysis of mRNA levels of catalase, SOD, GPx and GR. H_2O_2 treatment led to significant increase ($P\leq0.05$) (lane 2) in mRNA levels of all the enzymes compared to control. Treatment

with vitexin at 10 µM concentration (Lanes 4) prevented H_2O_2 induced increase in genetic expression significantly ($P \leq 0.05$)

Quantitation of intracellular glutathione

Glutathione is an important antioxidant molecule. Fig. 11 shows the nmoles of GSH formed after treatment with H_2O_2 in presence/absence of vitexin. Hydrogen peroxide treatment significantly reduced glutathione levels (454 ± 23 nmoles GSH/mg protein) compared with control untreated cells (989 ± 183 nmoles GSH/mg protein) ($p<0.05$). Pretreatment of cells with vitexin prevented this damage. 10 µM vitexin increased the glutathione levels to 1012 ± 49 nmoles/mg protein ($p<0.05$). Vitexin alone on the other hand did not cause any significant change in the glutathione levels (1062 ± 105 and 971 ± 115 nmoles/mg protein respectively) ($p<0.05$).

Fig. 11: Effect of different concentration of vitexin against H_2O_2 induced glutathione levels. Results are expressed as nmoles glutathione / mg protein.



DISCUSSION

Vitexin is a C-glycosides of apigenin possessing glucose at C8 position of A ring. It has been shown to inhibit adipogenesis in 3T3-L1 cells (Kim et al., 2010) and is known to possess strong α-glucosidase inhibitory activity (Li et al., 2009) as well as anti-glycation ability (Peng et al., 2008). In the present study, the antioxidant activity of vitexin against primary, secondary free

radicals was monitored by standard *in vitro* antioxidant assays and compared. The ability of the compound to scavenge radicals such as DPPH and $ABTS^{\bullet+}$ gives preliminary information about electron or hydrogen donating capacity of the antioxidant molecule. The data obtained suggest that vitexin scavenged DPPH as well as $ABTS^{\bullet+}$ radical efficiently. 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 pathological conditions (Tylor et al., 1997). Nitric oxide also reacts with superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$) and thereby causes more damage. Vitexin efficiently scavenged nitric oxide as well as superoxide radicals.

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}, $_H\bullet$ and $\bullet OH$. Presaturation with N_2O leads to the formation of $\bullet OH$, as a single major oxidizing radical. In the present study $ABTS^{\bullet-}$, and $CO_3^{\bullet-}$ were generated using the reaction of $\bullet OH$ in aqueous solutions of radical precursors in presence and absence of different concentrations of vitexin. Decay constants of vitexin suggest that they efficiently scavenge these radicals with almost equal rates indicating that this compound interferes with $ABTS^{\bullet-}$ and $CO_3^{\bullet-}$ radical formation probably by directly scavenging with $\bullet OH$.

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 $\bullet OH$ generated by Fenton reaction and was quantified in terms of amount of supercoiled DNA converted to nicked circular form. Vitexin effectively protected the DNA against free radical induced strand breaks probably by directly interacting with fenton radical. 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 prevents this, formation of 8-OH-2'-deoxyguanosine was subjected to pulse radiolysis studies in presence and absence of 1µM vitexin and the decay traces of 2-dG transient species were recorded at 310 nm. Vitexin effectively affected the formation of 2dG transient species, indicating its 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 cellular death. Vitexin inhibited lipid peroxidation (33%) significantly ($P \leq 0.05$), as well as attenuated ascorbate–Fe^{2+} induced depletion in mitochondrial protein sulphydryls indicating its ability to differentially protect cellular biomolecules. Thus, the observed antioxidant activity of vitexin indicates different reactivity of vitexin towards biological and non-biological free radicals and can be co-related to its structure. Antioxidant activity of vitexin 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.

To check whether the observed antioxidant activity also reflected their ability to modulate cellular antioxidant defense, HepG2 cells were used. H_2O_2 was used as an oxidizing agent which induces cellular damage (Nohl et al., 2007) by initiating sequential chain reactions involving formation of Fenton and Haberweiss radicals and secondary peroxyl radicals. Pre-treatment with vitexin for 24 h significantly ($P \leq 0.05$) decreased H_2O_2 induced cytotoxicity and inhibited intracellular ROS. Kang et al., (2010) 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 peroxyl 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 (Kim et al., 2005). In HepG2 cells, 10 μM vitexin significantly attenuated ROS in hydrogen peroxide exposed 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 H_2O_2 alone. This increased enzyme activities and their expression was normalized in presence of varying concentrations of vitexin 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 H_2O_2 induced ROS. This increase was significantly inhibited by vitexin in a 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 H_2O_2 alone, intracellular GSH levels in HepG2 cells were significantly reduced which were effectively replenished in the vitexin pretreated cells.

Thus, from all the data obtained its evident that vitexin scavenged radicals both in cells as well as in cell free system and protects cell from oxidative damage. This observed antioxidant ability of vitexin could be attributed both to the free hydroxyl group in the B ring of the parent molecule apigenin and substitution of glucose at the C8 position of the A ring.

Reference

1. Aebi HE. Catalase. In: Bermeyer HU, editor. Methods of Enzymatic Analysis. Weinhein: Verlag chemie GmbH;1983. p. 277-282.
2. Alía M, Ramos S, Mateos R, Bravo L, Goya L. Response of the antioxidant defense system to t-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). J Biochem Mol Toxicol 2005;19(2):119–28.
3. Aquino R, Morelli S, Lauro MR, Abdo S, Saija A, Tomaino A (2001). Phenolic constituents and antioxidant activity of an extract of *Anthuriumversicolor* leaves. *J. of Nat. Prod.* 64: 1019-1023.
4. Aruoma OI, Murcia A, Butler J, Halliwell B. (1993). Evaluation of antioxidant and prooxidant actions of gallic acids and its derivatives. *J. Agric. Food. Chem.* 41: 1880-1885.
5. Beauchamp C, Fridovich I. Superoxide dismutase improved assay and an assay applicable to acrylamide gel. Anal Biochem. 1971;44(1):276-287.
6. Buxton GV, Stuart CR.Re-evaluation of the thiocyanate dosimeter for pulse radiolysis. J Chem Soc, Faraday Trans. 1995;91:279-281.
7. Choi HJ, Eun JS, Kim BG, Kim SY, Jeon H, Soh Y (2006). Vitexin, an HIF-1alpha inhibitor, has anti-metastatic potential in PC12 cells. *Molecules and cells,* 22(3): 291-9.
8. Devasagayam TPA, Pushpendra CK, Eapen J (1983). Differences in lipid peroxidation in rat liver rough and smooth microsomes. *Biochemica Biophysica Acta.*, 750: 91-97.
9. Dixit PP, Ghaskadbi SS, Mohan H, Devasagayam TPA. Antioxidant properties of germinated fenugreek seeds. Phytother Res. 2005;19(11):977-983.

10. Ernst S and Stuart L (1995). Formation, prevention and repair of DNA damage by ion/hydrogen peroxide. *J. of Biol. Chem.* 272: 19085-19089.
11. Ernst S, Stuart L. Formation, prevention and repair of DNA damage by ion/hydrogen peroxide. J Biol Chem. 1997;272(31):19085-19089.
12. Goldberg DM, Spooner. Glutathione reductase In: Bermeyer HU, editor. Methods of Enzymatic Analysis. Weinhein: Verlag chemie GmbH;1983.p. 258-265.
13. Hazra B, Biswas S, Mandal S. Antioxidant and free radical scavenging activity of Spondias pinnata. BMC Complement Altern Med. 2008;8:63
14. Kang J, Li Z, Wu T, Jensen GS, Schauss AG, Wu X. Antioxidant capacities of flavonoid compounds isolated from acai pulp. (*Euterpe oleracea* Mart.) Food Chem. 2010;122:610-617.
15. Kim J, I.Lee, J.Seo, M. Jung,Y.Kim, N.Yim , K.Bae (2010). Vitexin, Orientin and Other Flavonoids from *Spirodelapolyrhiza* inhibit Adipogenesis in3T3-L1 Cells. *Phyto. Res.* 24: 1543–1548.
16. Kim JH, Lee CB, Kim JH, Sim GS, Lee DH, Lee KE, Yun YP, Pyo HB. The isolation and antioxidative effects of vitexin from *Acer palmatum*. Arch Pharm Res. 2005;28(2):195-202.
17. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium deficient rat liver. Biochem Biophys Res Commun. 1976;71(4):952-958.
18. Li Huilin, S. Fengrui X, T.Rong, L.Zhiqiang, L.Shuying (2009). Screening and Structural Characterizationof α-Glucosidase Inhibitors from Hawthorn Leaf Flavonoids Extract by UltrafiltrationLC-DAD-$MS^n$and SORI-CID FTICR MS. *J. of American Society of Mass Spectro.* 20:1496–1503.
19. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951;193(1):265-275.
20. Maurya DK, Devasagayam TPA. Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. Food Chem Toxicol. 2010;48(12):3369-73.
21. Nohl H, Kozlov AV, Gille L, Staniek K. Cell respiration and formation of reactive oxygen species: facts and artefacts. Biochem Soc Trans. 2003;31(6):1308–1311.

22. Peng X, Zheng Z, Cheng KW, Shan F, Ren GX, Chen F, Wang M. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. Food Chem 2008;106(2):475–481.
23. Phadatare SD, Sharma KK, Rao BB, Naumov S, Sharma GK. Spectral characterization of guanine C4-OH adduct: a radiation and quantum chemical study. J Phys Chem B. 2011;115(46):13650-8.
24. Santos AC, Uyemura SA, Lopes JLC, Bazon JN, Mingatto FE, Curti C (1998). Effect of naturally occuring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria. *Free Radical in Biol. Med.* 24: 1455-1461.
25. Teixeira HD, Meneghini R. Chinese hamster fibroblast over expressing Cu-Zn superoxide dismutase undergoes global reduction in antioxidants and an increasing sensitivity to DNA by oxidative damage. Biochem J. 1996;315(3):821-825.
26. Tylor BS, Kion YM, Wang QI, Sharpio RA, Billiar TR, Geller DA. Nitric oxide down regulates hepatocyte-inducible nitric oxide synthase gene expression. Arch Surg. 1997;132(11):1177–1183.
27. Wang X, Ge J, Wang K, Qian J, Zou Y. Evaluation of MTT assay for measurement of emodin-induced cytotoxicity. Assay Drug Dev Technol. 2006;4(2):203-7