Comparative analysis of different dietary antioxidants on oxidative stress pathway genes in L6 myotubes under oxidative stress

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Abstract Enhanced oxidative stress plays an important role in the progression and onset of diabetes and its complications. Strategies or efforts meant to reduce the oxidative stress are needed which may mitigate these pathogenic processes. The present study aims to investigate the in vitro ameliorative potential of nine antioxidant molecules in L6 myotubes under oxidative stress condition induced by 4-hydroxy-2-nonenal and also to comprehend the gene expression patterns of oxidative stress genes upon the supplementation of different antioxidants in induced stress condition. The study results demonstrated a marked increase in the level of malondialdehyde and protein carbonyl content with a subsequent increase in the free radicals that was reversed by the pretreatment of different dietary

antioxidant. From the expression analysis of the oxidative stress genes, it is evident that the expression of these genes is modulated by the presence of antioxidants. The highest expression was found in the cells treated with Insulin in conjugation with an antioxidant. Resveratrol is the most potent modulator followed by Mangiferin, Estragole, and Capsaicin. This comparative analysis ascertains the potency of Resveratrol along with Insulin in scavenging the reactive oxygen species (ROS) generated under induced stress conditions through antioxidant defense mechanism against excessive ROS production, contributing to the prevention of oxidative damage in L6 myotubes.

Keywords T2D · Oxidative stress · ROS · HNE · Antioxidants · Myotubes

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Introduction

Diabetes mellitus is a chronic metabolic disease and its incidence is rapidly increasing, affecting about 366 million people in the world (Day 2001). The intimidating increase of diabetes is one of the major cause of morbidity and mortality of human beings worldwide and its highly increased annual rate indicates insufficiency of existing therapeutic options. The causal link between diabetes and its complications is recognized as hyperglycemia. The chronic hyperglycemia results

in increased oxidative stress due to overproduction of reactive oxygen species (ROS) as a result of glucose auto-oxidation and protein glycosylation (Laaksonen et al. 1998). ROS generation leads to oxidative damage of lipids, DNA and proteins of cells and creates diabetes-related complications (Robertson 2004). The impairment in the functioning of endogenous antioxidant enzymes also induce oxidative insult in cells (Wu et al. 2010) and prolong exposure to oxidative stress may cause insulin resistance by causing an alteration in the redox balance at the cellular level (Mc Garry 2002). The effect of ROS is balanced by the antioxidant action of non-enzymatic antioxidants as well as by antioxidant enzymes. In mammalian systems, the most efficient primary scavenger antioxidant enzymes involved in detoxifying ROS are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione s-transferase (GST) (MatEs et al. 1999) while, non-enzymatic antioxidants include non-protein thiol, vitamin C and vitamin E (Maritim et al. 2003; Halliwell and Gutteridge 1999; Wiernsperger 2003). These antioxidant defenses are extremely important because they provide maximum protection to biological sites by directly removing free radicals.

The multifaceted character of antioxidants in countering ROS and diabetes in animal models (Kowluru and Kenned 2001; Abiko et al. 2003) provides the impetus for identifying new and more efficient antioxidants, for treating diabetes and its complications. Antioxidants of plant origin (widely distributed in fruits, vegetables, beverages and herbal remedies) that are part of our regular diet can be important molecules to explore their therapeutic potential for countering issues related to ROS generated damages in diabetes. Of the different antioxidants of plant origin Curcumin, Mangiferin, Resveratrol, Hesperidin, Capsaicin, Baicalein, Carvacrol, Estragole, and Lycopene are some of the prominent once. Curcumin is the principal curcuminoid found in turmeric (Kochhar 2008) with many beneficial effects such as antioxidant, scavenging free radicals, anti-inflammatory and so on (Shao et al. 2012). Curcumin has been reported to inhibit insulin signaling pathway and glucose transport in 3T3-L1 adipocytes under normal culture condition (Zhang et al. 2015). Another important molecule mangiferin is reported to possess antioxidant effects (Sanchez et al. 2000). Mangiferin attenuates oxidative stress through activation of PI3K

induced Akt and Nrf-2 mediated signaling pathways in NKE cells (Saha et al. 2016). Resveratrol is a natural polyphenol whose hypoglycemic and hypolipidemic effects have been innumerate in streptozotocin-induced diabetic rats (Aderibigbe et al. 1999). In Swiss 3T3 fibroblasts, resveratrol showed the protective effect against 4-hydroxynonenal (4-HNE) induced oxidative stress and apoptotic death (Kutuk et al. 2004). Similarly, the hesperidin molecule has been shown to possesses hypoglycemic, antioxidant activities, anti-inflammatory, anti-carcinogenic and lipid-lowering properties (Choi et al. 2001). Recently, it has been reported that hesperidin has inhibitory effects in the production of reactive oxygen species and reactive nitrogen species in 3T3-L1 and RAW264 cells (Jeon et al. 2014). Capsaicin is the active component of capsicum which was found to inhibit 3T3-L1-preadipocytes adipogenesis in vitro and also prevented high-fat diet induced obesity (Zhang et al. 2007). Flavanoid, Baicalein is another potent antioxidant obtained from *Scutellariae radix* which possesses anti-inflammatory and antioxidant activities (Zhao et al. 2005). In HMEC-1 cells, Du et al. (2017) reported that baicalein abolishes oxidative stress. A phenolic monoterpene, Carvacrol, is another plant-based antioxidant produced by aromatic plants and spices, present in the genera *Origanum* and *Thymus* (Can Baser 2008). Carvacrol possesses several biological actions, including anti-inflammatory, antioxidative, and anti-apoptotic properties (Guimaraes et al. 2010). It was revealed that carvacrol has anti-proliferative properties on human non-small cell lung cancer cells, A549 (Koparal and Zeytinoglu 2003). Carvacrol was found to improve the cognitive function of diabetic rats, which was linked with its hypoglycemic, antioxidant, and anti-inflammatory properties (Deng et al. 2013). Estragole antioxidant is a component of various trees and plants, including turpentine (pine oil), anise, fennel, bay, tarragon, and basil. In human skeletal muscle cells, Wang et al. (2004) reported that alcoholic extract of *Artemisia dracunculus* improved glucose disposal in these insulin sensitive tissues. Another major fruit based antioxidant studied upon is Lycopene, found naturally in fruits and vegetables, belonging to the carotene family. Although found in many fruits and vegetables, Lycopene is mostly found in tomatoes and tomato products (Breinholt et al. 2000). It demonstrates antioxidant activity by destroying oxygen radicals,

as retinol, α -tocopherol, and carotenoids do (Erhardt et al. 2003). The effect of lycopene on oxidative stress in the H9c2 cell has been reported by Liu et al. (2015). Although, Curcumin, Mangiferin, Resveratrol, Hesperidin, Capsaicin, Baicalein, Carvacrol, Estragole, and Lycopene have been explored individually to establish their hypoglycemic, antioxidant, and anti-inflammatory properties etc., a comparative analysis of their potential in the oxidative setup is still not available. In view of this, it would be interesting to explore the most potent antioxidant molecule possessing ROS quenching properties to bridge this lacking information.

The present study was carried out hypothesizing that some of the well-known dietary antioxidants may be effective in reducing the oxidative stress-induced impairments in the skeletal muscle cell, impressed upon by HNE. The study aims to investigate the in vitro ameliorative potential of these nine antioxidant compounds in L6 myotubes in combating oxidative stress conditions and also to comprehend the gene expression patterns of oxidative stress genes upon supplementation of different antioxidant in induced stress condition. As insulin is a positive modulator of ROS, we were interested to study the effects of antioxidants treatment conditions by keeping the insulin treatment as a comparative reference. We also wanted to observe if the antioxidants were able to combat ROS individually or in combination with insulin. Additionally, we were interested in identifying the most potent antioxidant molecule having the potential to be used as an additive supplement to reduce the risk of oxidative stress-based diseases.

Materials and methods

Materials

Antioxidants were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum), and HS (Horse Serum) were purchased from Himedia Laboratories Pvt. Ltd., (Mumbai, India). DMSO (Dimethyl Sulfoxide) was purchased from Merck (Mumbai, India). HNE, Rosiglitazone, mannitol, insulin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), were purchased from Sigma-Aldrich Chemicals. Trizol, SYBR Green PCR master

mix, and Reverse Transcriptase III were purchased from Invitrogen, Life Technologies (Carlsbad, CA, USA). H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) and glucose uptake assay kit were purchased from Sigma-Aldrich.

Sample preparations

The antioxidants (cell culture grade) and rosiglitazone were dissolved in DMSO, as per the instructions were given in the manufacturer's product information to prepare the mother stocks. The final concentration of DMSO in the working stock solutions did not exceed 0.02%. Insulin was dissolved in DMEM for preparing the working stock concentration of 100 mM. Mannitol was dissolved in PBS and then diluted in DMEM to prepare a final working stock concentration of 100 mM. For preparing HNE stocks, it was dissolved in ethanol as per the manufacturer's product information and further working stocks were prepared in DMEM.

Cell culture and treatment

L6 myoblasts were purchased from National Centre for Cell Sciences (NCCS) (Pune, India). L6 cells were grown in DMEM containing 10% FBS, 100 $\mu\text{g}/\text{ml}$ / 100 units/ml antibiotics streptomycin and penicillin in an atmosphere of 5% CO_2 at 37 °C until they reached 80% confluence. For the experiment, cells were grown at a density of 3.5×10^4 cells/well on 6-well plates. To obtain fully differentiated myotubes, myoblasts were reseeded in DMEM having 2% HS for 8–10 days, with a medium change every 72 h.

Experimental design

Oxidative stress on L6 cells was induced by using HNE treatment at different concentrations of 50, 100 and 150 μM for 24 h. The cytotoxicity levels in the cells were measured and optimum dose for HNE, as an oxidant, was standardized. The effects of different antioxidants were tested for lower to higher concentrations (1–200 μM) for a period of 24 h. The concentration which generated 90% viability after 24 h of exposure was selected for treatment. The treatment pattern followed for the assays include pretreatment of cells with Antioxidants, Rosiglitazone and Mannitol for 24 h and then HNE treatment for

24 h, followed by 100 nm Insulin stimulation for 30 min.

Cell viability and cytotoxicity test

The viability of L6 cells was measured by means of MTT assay. Cytotoxicity of HNE and all antioxidants were standardized, based on concentration. Briefly, L6 cells after incubation with HNE and with antioxidant compounds were washed and MTT (50 µg/100 µL) was added to each well for the estimation of mitochondrial dehydrogenase activity (Mosmann 1983). After 4 h incubation, 100 µl of DMSO was added to each well and the absorbance at 570 nm of solubilized MTT formazan products was measured after 2 h using a microplate reader (BIOTEK Instruments, Winooski, VT, USA).

Measurement of intracellular reactive oxygen species

The intracellular ROS level in L6 myotubes was estimated by H₂DCFDA (Sigma-Aldrich) method. Cells grown in 6-well plates were administered different treatments and then treated with H₂DCFDA at 37 °C for 30 min in PBS, rinsed, and intracellular ROS containing cells were analyzed by cytoFLEX flow cytometer (Beckman Coulter, Brea CA, USA) with FlowJo (version 10) software to determine relative fluorescence intensity.

TBARS (2-thiobarbituric acid reactive substances) activity assay

Lipid peroxidation within cells occurs as a result of oxidative damage and is a direct marker for oxidative stress. TBARS are naturally present in the biological sample which measures malondialdehyde (MDA), a compound that generates from the decomposition of polyunsaturated fatty acid lipid peroxides. MDA is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products.

Protein carbonyl content assay

The most commonly used stable oxidative damage marker to assess protein oxidation is the protein carbonyl content. The protein carbonyl content assay

Kit from Sigma-Aldrich was used as a direct method for measuring carbonyl content in our experiments. Carbonyl content was determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts, which were detected spectrophotometrically at 375 nm, the amount being proportional to the carbonyls present.

Glucose uptake assay

Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-glucose (2DG) using a commercial glucose uptake activity assay kit (Sigma-Aldrich). In this assay, 2-DG uptake was determined by a coupled enzymatic assay in which the 2-DG6P when oxidized, results in the generation of NADPH, which was then determined by a recycling amplification reaction in which the NADPH is utilized by glutathione reductase in a coupled enzymatic reaction produces glutathione. Glutathione reacts with DTNB to product TNB, which was detected at 412 nm.

Antioxidant enzyme activity assay

To determine the enzymatic activity of SOD and CAT, L6 myotubes were incubated in different treatment combinations for 24 h and assays were performed after lysing cells with 1X RIPA buffer.

SOD assay

Superoxide dismutase (SOD), is one of the most important antioxidative enzymes which catalyzes the dismutation (or partitioning) of the superoxide anion into either ordinary molecular oxygen or hydrogen peroxide. SOD is produced as a by product of oxygen metabolism and if not regulated, causes many types of cell damage. The Sigma SOD Assay Kit-WST produces a water-soluble formazan dye upon reduction with a superoxide anion. The rates of the reduction with O₂ are linearly related to the xanthine oxidase (XO) activity and are inhibited by SOD. The SOD activity was quantified colorimetrically by measuring the decrease in the color development at 440 nm.

CAT assay

Catalase (CAT) is an antioxidant enzyme present in mammalian and non-mammalian aerobic cells. This enzyme catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. The Sigma Catalase assay kit uses a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of H_2O_2 and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyrinyl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine) that absorbs at 520 nm to indicate the presence of H_2O_2 .

Extraction and analysis of RNA and quantification by real-time RT-PCR

Extraction and cDNA conversion of RNA

For RNA isolation, L6 myotubes were grown in DMEM medium. For inducing treatment conditions, the experiments were carried out in four groups, of which the first group comprised of HNE untreated cells, the second group comprised HNE treated, the third group was HNE untreated cell supplemented with insulin and the fourth group included HNE treated cell supplemented with insulin as depicted (Table 1). The reference gene GAPDH was amplified using forward primer 5' TGCACCACCAACTGCTTAGC 3' and reverse primer 5' GGATGCAGGGATGATGTTCT 3'.

Table 1 Treatment conditions used in the experiments

Group I	Group II
Control	HNE 100 μ M
Curcumin (1 μ M)	HNE (100 μ M) + Curcumin (1 μ M)
Mangiferin (20 μ M)	HNE (100 μ M) + Mangiferin (20 μ M)
Resveratrol (10 μ M)	HNE (100 μ M) + Resveratrol (10 μ M)
Hesperidin (10 μ M)	HNE (100 μ M) + Hesperidin (10 μ M)
Capsaicin (10 μ M)	HNE (100 μ M) + Capsaicin (10 μ M)
Baicalein (10 μ M)	HNE (100 μ M) + Baicalein (10 μ M)
Carvacrol (10 μ M)	HNE (100 μ M) + Carvacrol (10 μ M)
Estragole (20 μ M)	HNE (100 μ M) + Estragole (20 μ M)
Lycopene (10 μ M)	HNE (100 μ M) + Lycopene (10 μ M)
Rosiglitazone (1 μ M)	HNE (100 μ M) + Rosiglitazone (1 μ M)
Mannitol (100 mM)	HNE (100 μ M) + Mannitol (100 mM)
Group III	Group IV
Insulin (100 nmol)	Insulin (100 nmol) + HNE 100 μ M
Insulin (100 nmol) + Curcumin (1 μ M)	Insulin (100 nmol) + Curcumin (1 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Mangiferin (20 μ M)	Insulin (100 nmol) + Mangiferin (20 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Resveratrol (10 μ M)	Insulin (100 nmol) + Resveratrol (10 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Hesperidin 10 μ M	Insulin (100 nmol) + Hesperidin (10 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Capsaicin (10 μ M)	Insulin (100 nmol) + Capsaicin (10 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Baicalein (10 μ M)	Insulin (100 nmol) + Baicalein (10 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Carvacrol (10 μ M)	Insulin (100 nmol) + Carvacrol (10 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Estragole (20 μ M)	Insulin (100 nmol) + Estragole (20 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Lycopene (10 μ M)	Insulin (100 nmol) + Lycopene (10 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Rosiglitazone (1 μ M)	Insulin (100 nmol) + Rosiglitazone (1 μ M) + HNE (100 μ M)
Insulin (100 nmol) + Mannitol (100 mM)	Insulin (100 nmol) + Mannitol (100 mM) + HNE (100 μ M)

Table 2 The primers used in the qPCR study

Gene name	Accession no.	Primer	Sequence of primer	Amplicon size (bp)
SOD	M25157.1	Forward	GTCCACGAGAAACAAGATGA	98
		Reverse	ATCCCAATCACACCACAAG	
CAT	NM_012520.2	Forward	GAATGAGGAGGAGAGGAAAC	107
		Reverse	GGTGGACGTCAGTGAAAT	
GPx1	NM_030826.3	Forward	GGGACTACACCGAAATGAA	93
		Reverse	CCTGATGTCCGAACTGATT	
GST	M28241.1	Forward	CCCAATGGAGTAACAAGTAGG	117
		Reverse	GAGCAGGAAGGAGAGAGAA	

Total RNA was isolated from each treatment condition using Trizol reagent according to the manufacturer's protocol (Invitrogen, Life Technologies). One microgram of total RNA was reverse transcribed using the Reverse Transcriptase III (Invitrogen, Life Technologies) according to the manufacturer's protocols.

Designing of primers

The primers were designed using the IDT (Integrated DNA Technologies) Oligoanalyzer 3.1 software and the mRNA sequence was extracted from NCBI database. The primers were checked for their T_m values, hairpin loops, dimers, cross-dimers and number of repeats using the Oligoanalyzer 3.1. The gene names, GenBank accession numbers, amplicon sizes, and the sequences of the primers are listed in Table 2.

Real-time PCR analysis

Real-time PCR (RT-PCR) was performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using Power SYBR Green PCR master mix (Invitrogen, Life Technologies) according to the manufacturer's protocols. Briefly, PCR was performed in a final volume of 20 μ l comprising of 5 μ l of 80 ng sample cDNA, 0.2 μ l of 100 nM forward and reverse primers, and 10 μ l Power SYBR green PCR Master Mix. The following thermal cycling profile was used (40 cycles): 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Standard curves for each gene were generated by 1:5 dilutions of cDNA to determine the PCR efficiency of target and reference gene. Standard curves were established by plotting the threshold cycle

Ct versus Log10 (cDNA). The PCR efficiency was calculated by the following equation: PCR efficiency (E) = $(10^{-1/\text{slope}} - 1) \times 100$. Using the Ct comparative method, the relative abundance of the target transcript was calculated from triplicate samples after normalization against a housekeeping gene. The mRNA levels (arbitrary units) are expressed in relation to those of the control cells. Relative quantification was calculated using the $2^{-(\Delta\Delta C_t)}$ method, where $\Delta\Delta C_t = (C_t, \text{target} - C_t, \text{GAPDH}) \text{ treated sample} - (C_t, \text{target} - C_t, \text{GAPDH}) \text{ control sample}$. RT-PCR results are presented as the mean and SD of the relative fold changes to $2^{-(\Delta\Delta C_t)}$.

Statistical analyses

Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ($n = 3$) of three biological replicates. Data were subjected to one-way ANOVA and the significance of differences between means was calculated by Tukey's Multiple Comparison Test using GraphPad Prism Software and significance was accepted at $P \leq 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus HNE treated.

Results

Cell viability

L6 myotubes were cultured and seeded in 96 well dish (10,000 cells per well) in triplicates and cytotoxicity of HNE, Curcumin, Mangiferin, Resveratrol, Hesperidin, Capsaicin, Baicalein, Carvacrol, Estragole, and

Fig. 1 Cytotoxicity in cultured L6 muscle cells. **a** Effect of HNE on cell viability was evaluated based on concentration for a 24 h period of incubation. **b–e** Cytotoxicity of antioxidants

Lycopene was studied by MTT assay. The concentration of HNE and all antioxidants was standardized, based on cytotoxic levels determined by MTT assay (Fig. 1a). A concentration of 100 μ M for HNE was found to be less than 50% toxic for a period of 24 h. Antioxidants, Curcumin (1 μ M), Mangiferin (20 μ M), Resveratrol (10 μ M), Hesperidin (10 μ M), Capsaicin (10 μ M), Baicalein (10 μ M), Carvacrol (10 μ M), Estragole (20 μ M) and Lycopene (10 μ M) showed more than 90% survivability for an incubation period of 24 h (Fig. 1b–e). Results were expressed as a percentage of survival.

HNE induces intracellular ROS production

Intracellular ROS in L6 myotubes was determined by H₂DCFDA. Our results demonstrated that tested concentration of HNE (100 μ M) produced 121% increase in ROS activity (Fig. 2), when compared to control cells which produced a negligible amount of ROS. Treatment with insulin and antioxidants led to ROS production almost similar to control cells. Addition of antioxidant Resveratrol yielded ROS

determined after 24 h preincubation in L6 myoblast. Each value represents mean $\pm$ SD (standard deviation) from triplicate measurements ($n = 3$) of three different experiments

production similar to untreated controls. Production of total ROS decreased by 44 and 27% on treatment with insulin combined with antioxidants Resveratrol and Mangiferin ($P < 0.001$), respectively, when compared to control cells, in the presence of HNE. Also, ROS production was significantly ($P < 0.001$) lowered by 49% when compared to control cells, on treatment with Estragole combined with Insulin, in the presence of HNE.

TBARS (2-thiobarbituric acid reactive substances) activity assay

Lipid peroxidation, determined by the thiobarbituric acid method, indicated that cells exposed to HNE for 24 h showed a significant increase in malonaldehyde, the metabolic product of lipid peroxidation, over that of control L6 myotubes (Fig. 3). Pretreatment of antioxidants significantly ($P < 0.001$) reduced the malonaldehyde level to that of control. The lipid peroxidation level was observed to be maximum in HNE treated cells 0.022 nmol/mL and lowered in antioxidants and insulin treated cells (0.015 nmol/mL).

Fig. 2 HNE stimulated intracellular production of ROS in L6 muscle cells. ROS level in L6 myotubes was estimated by H₂DCFDA by FACS caliber flow cytometer. Production of ROS was measured by addition of 100 μ M HNE and was compared to control, antioxidants, insulin and combination treated cells. **a–f** Represents the relative fluorescence intensity analysis of L6 myotubes. **g–h** Bars correspond to relative fluorescence intensity analysis of cells; each value represents mean $\pm$ SD

(standard deviation) from triplicate measurements ($n = 3$) of three different experiments. Results were subjected to one-way ANOVA and the significance of differences between means was calculated by Tukey's Multiple Comparison Test using GraphPad Prism Software and significance was accepted at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus HNE treated

Protein carbonyl content assay

Protein carbonyl groups are an important and immediate biomarker of oxidative stress. DNPH tagging of protein carbonyls has been one of the most common measures of oxidative stress. There was a significant increase in protein carbonyl content in L6 myotubes on induction of oxidative stress (0.335 ± 0.009 nmole/ μ g protein) than that of untreated control (0.0968 ± 0.001 nmole/ μ g protein). Pretreatment with different dietary antioxidants significantly ($P < 0.001$) restricted the production of protein carbonyl (Fig. 4). The insulin treated cells with less oxidative damage generated lower carbonyl content (0.0146 ± 0.006 nanomole carbonyl/ μ g protein).

Glucose uptake measurements

Glucose uptake in L6 myotubes was analyzed by glucose uptake calorimetric assay kit (Sigma), which provides a simple and direct procedure for measuring glucose uptake in cells. There was no significant effect on glucose uptake in the cells treated with HNE as compared to control (Fig. 5). Preincubation with antioxidants significantly increased glucose uptake in L6 myotubes ($P < 0.001$) in the presence of oxidative stress induced by HNE.

Antioxidant enzyme activity of SOD and CAT

SOD enzyme activity was performed (in triplicates) with the L6 cell lysate by the Sigma SOD Assay Kit-WST (Supplementary Fig. 1a). SOD enzyme

Fig. 3 Levels of TBARS in control and treatment groups of L6 myotubes. Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ($n = 3$) of three biological replicates. Data were subjected to one-way ANOVA and the significance of

differences between means was calculated by Tukey's Multiple Comparison Test using GraphPad Prism Software and significance was accepted at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus HNE treated

activities were measured in whole groups, and it was observed that with only antioxidant treatment, the maximum SOD activity was generated with Estragole (34.14 ± 1.5) and Carvacrol (33.39 ± 0.71) and the positive control treatment mannitol (41.25 ± 1.7). A significant reduction in activities of enzymatic antioxidant upon HNE (15.97 ± 1.48) treatment was observed. However, treatment with antioxidants significantly ($P < 0.001$) increased the level of enzymatic antioxidant in the L6 cells. Highest SOD activity was observed in cells with insulin treatment (45.24 ± 1.65). Similarly, CAT activity was assayed using the same lysate that was used in SOD assay. A standard curve was plotted using the different concentration of hydrogen peroxide. With HNE treatment; the average CAT activity (micromoles/min/mol) was as low as 3.85 ± 0.264 . The presence of insulin enhanced the CAT activity to 38.35 ± 2.53 . Maximum CAT activity using antioxidants was generated with Estragole (30.65 ± 2.69) and Carvacrol (28.94 ± 2.13) and with the positive control treatment

Rosiglitazone (26.93 ± 2.13 and mannitol (29.68 ± 3.43) (Supplementary Fig. 1b).

Expression analysis of genes associated with oxidative stress pathway

The expression analysis of oxidative stress genes, including *SOD*, *CAT*, *GPx*, and *GST* decreased in cells treated with HNE as compared to the control. Pretreatment of cells with different dietary antioxidants (Curcumin, Mangiferin, Resveratrol, Hesperidin, Capsaicin, Baicalein, Carvacrol, Estragole, and Lycopene) upregulated the expression of oxidative stress genes, which was deleteriously suppressed due to HNE stress.

Expression analysis of SOD gene across various treatments set

The relative fold change of *SOD* gene was observed to be significantly ($P < 0.001$) downregulated in HNE

Fig. 4 Level of protein carbonyl content in control and treatment groups of L6 myotubes. Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ($n = 3$) of three biological replicates. Data were subjected to one-way ANOVA and the

significance of differences between means was calculated by Tukey's Multiple Comparison Test using GraphPad Prism Software and significance was accepted at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus HNE treated

stress as compared to control L6 myotubes (Fig. 6a). The pretreatment of antioxidants on HNE treated L6 myotubes significantly ($P < 0.001$) upregulated the expression of *SOD* gene. It was observed that only antioxidant treatment, the maximum *SOD* gene expression was with antioxidant Resveratrol then antioxidant Estragole and in the positive control mannitol treatment. In the case of all combination treatments, the maximum fold change was observed with mannitol + insulin treatment.

Expression analysis of CAT gene across various treatments set

In case of regulation of *CAT* gene, HNE treated cells were found to be significantly downregulated as compared to control L6 myotubes (Fig. 6b). However, with pretreatment of antioxidants to HNE treated L6 myotubes significantly ($P < 0.001$) increased the regulation of *CAT* gene throughout all treatment combinations. Among the antioxidant treatments, maximum fold change was observed in cells treated with Resveratrol followed by Mangiferin. Thus, for

CAT gene the overall maximum fold change was observed in mannitol + insulin treatment.

Expression analysis of GPx gene across various treatments set

The relative fold change of *GPx* genes across various treatments is shown in Fig. 6c. The *GPx* gene observed to be significantly downregulated in L6 myotubes treated with HNE when compared with control L6 myotubes. The pretreatment of antioxidants to HNE treated L6 myotubes significantly ($P < 0.001$) upregulated the expression of *GPx* gene. Among all antioxidant treatments, maximum upregulation was observed in Resveratrol treatment followed by Mangiferin treatment. *GPx* gene showed upregulation with Rosiglitazone (positive control). In the case of combination treatment, the maximum fold change was observed with the combination of mannitol + insulin (Fig. 6c).

Fig. 5 Glucose uptake activity assay level in control and treatment groups of L6 myotubes. Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ($n = 3$) of three biological replicates. Data were subjected to one-way ANOVA and the

significance of differences between means was calculated by Tukey's Multiple Comparison Test using GraphPad Prism Software and significance was accepted at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus HNE treated

Expression analysis of GST gene across various treatments set

The relative fold change of *GST* gene, across various treatments are shown in Fig. 6d. The *GST* gene observed to be significantly downregulated in L6 myotubes treated with HNE when compared with control L6 myotubes. The pretreatments of antioxidants on HNE treated L6 myotubes, significantly ($P < 0.001$) enhanced the regulation of *GST* gene among all treatment combination sets. Among all antioxidants, the maximum fold change was observed in Resveratrol followed by Mangiferin and in the positive control mannitol. In combination treatment, the maximum fold change was observed with the combination of mannitol + insulin (Fig. 6d).

Discussion

Oxidative stress plays a key role in the pathophysiology of T2D and its complications (Ramakrishna and

Jailkhani 2008). Oxidative insult in cells is mainly caused by the impairment in the functioning of endogenous antioxidant enzymes (Tiwari et al. 2013) that can be prevented by reduced lipid peroxidation and improved antioxidant status. Antioxidant in T2D patients neutralizes the activity of free radicals through several pathways, acting as an enzyme that destroys free radicals, binds to metals which stimulate the production of free radicals and thereby inhibits the formation of free radicals, as well as acting as scavengers of free radicals (Zatalia and Sanusi 2013).

In the present study, the significant increase of ROS in HNE treated cells was observed which validate that HNE contributes to the production of ROS. Our study results are in agreement with previous report by Usatyuk et al. 2006, who reported HNE treatment induced an increase in intracellular ROS production in L6 muscle cells and hence can be used as a medium to generate ROS induced oxidative stress condition in vitro models.

Defects in the antioxidant defense system are in close association with induction of lipid peroxidation

Fig. 6 a–d The expression level of *SOD*, *CAT*, *GPx* and *GST* gene. Each real-time PCR examination was carried out at least in triplicate. Data are shown as fold change in relative expression compared with *GAPDH* on the basis of Comparative Ct ($2^{-\Delta\Delta C_t}$) method. Values are shown as mean $\pm$ SD. Significance test

between various groups were determined by using one way ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism Software and significance was accepted at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus HNE treated

(Jagetiya and Baliga 2003). The level of TBARS has been shown to significantly increase in T2D with the duration of disease and development of complications (Hernandez-Munoz et al. 2013). It has been observed that levels of malondialdehyde, a byproduct of lipid peroxidation increases on induction of oxidative stress

with HNE. In the present study, the significant increase in lipid peroxidation in HNE treated cells by 0.0222 nmol/mL indicated significant induction of oxidative stress. The generation of HNE induced ROS was significantly reduced in cells that were pretreated with antioxidants, as compared to HNE exposed cells.

Fig. 6 continued

The present study shows that administration of different dietary antioxidants significantly normalized the TBARS levels in HNE treated cells (Fig. 3). Similar observation has been reported in in vivo models; previously Arulselvan and Subramanian (2007) reported that the increased level of lipid peroxidation in diabetic rats is due to increase in levels of TBARS and hydroperoxides in the liver and kidney. The present findings of lipid peroxidation come in accordance to Kakkar et al. (1995) and Limaye et al. (2003), who reported a significant increase of lipid peroxidation at 6 weeks of

streptozotocin-induced diabetes. Murugan and Pari (2006) also reported an increase of TBARS in STZ induced diabetic rats.

During stress conditions, elevated levels of oxidized protein are present in animals and cells (Berlett and Stadtman 1997). The oxidative stress conditions result in the inactivation of protein functions through protein oxidation content. Carbonylation is one of protein oxidation processes that can occur directly on proteins, often in response to the metal-catalyzed formation of hydroxyl radicals (Levine 2002; Stadtman and Levine 2000). In the present study, we have

observed an increased level of protein carbonyl in HNE treated cell as compared to control. The pretreatment of dietary antioxidant significantly decreased the protein carbonyl content, and such results have also been presented by Nakhaee et al. (2009), who reported a decrease in protein carbonyl content upon eucalyptus treatment in diabetic rats. Diabetes mellitus is associated with decreased glucose uptake in skeletal muscles due to the phenomenon of insulin resistance (Shirwaikar et al. 2006). The present study shows that antioxidants treatment reduced the elevation of ROS and enhanced glucose uptake in L6 myotubes. Our findings are in agreement with the results reported by Dachani et al. (2012) who demonstrated that *Trichodesma Indicum* extract can enhance glucose uptake under in vitro conditions. Oxidative stress markers which are enzymes SOD, CAT, GPx and GST change drastically under stress conditions as also evidenced in our study. Previous studies in regards to the effect of oxidative stress on antioxidant enzyme activities have been mostly reported from in vivo models where changes significantly vary from no changes (Hussein and Abu-Zinadah 2010), to decrease (Limaye et al. 2003) and to increase (Cho et al. 2002) depending on the duration of the experiments. Srinivas et al. (2003) reported that the antioxidant drug, bis-o-hydroxycinnamoyl methane, imparts protection to cells against ROS-mediated damage by enhancing antioxidants and reduces hyperglycemia in streptozotocin (STZ)-induced diabetes. It has also been reported that some antioxidants such as vitamins C and E, lipoic acid reduced hyperglycemia-induced biological changes, including cytokine expression and matrix synthesis (Hussein and Abu-Zinadah 2010; Abdulkadir and Thanoon 2012). Recently Kolsi et al. (2017) reported that the SOD, CAT and GPx levels enhanced significantly upon treatment of pancreas, liver, and kidney with *Cymodocea nodosa* extract when compared to the untreated diabetic rats.

Quantitation of mRNA was carried out by real-time PCR, which is a very sensitive and reliable method for the expression analysis (detection of a single mRNA molecule) of transcripts under induced stress to correlate to their enzymatic counterpart to decipher the role of the genes. In the study, Real-time PCR analysis was performed using comparative Ct method for *SOD*, *CAT*, *GPx* and *GST* genes after being normalized against housekeeping gene, *GAPDH* to

provide significant evidence on the effect of efficient antioxidant upon these tested oxidative stress genes. The different treatment combinations with and without the usage of antioxidant (refer to Table 1 for combinations) showed that insulin and mannitol combination possesses the highest combating power to scavenge ROS generated as evident from Fig. 6a–d for all studied genes. Treatment with HNE was found to alter the expression of *SOD*, *CAT*, *GPx*, and *GST*. The relative fold change of all genes was observed to be significantly downregulated in presence of HNE when compared with control L6 myotubes. Pretreatment with antioxidants on HNE treated L6 myotubes significantly upregulated the expression of tested genes. The increasing gene expression of *SOD*, *CAT*, *GPx*, and *GST* in our study seem to be a natural response of the cell to cope up with oxidative stress upon dietary antioxidants pretreatment. In our comparative analysis, it was observed that no antioxidant singly altered the expression of all oxidative stress genes in a similar fashion, however, of all target molecules Resveratrol and Mangiferin showed maximum alteration in the expression of the oxidative stress genes tested. Of all the tested molecules, Lycopene though till now has been described to be a very effective and potent antioxidant (Srinivas et al. 2003; Di Mascio et al. 1989), however in our study Lycopene seemed to alter the expression of most of the genes least. Insulin + mannitol effects were the same across all oxidative stress genes *SOD*, *CAT*, *GPx* and *GST* tested. Insulin remains the best modulator under the stress condition as reported in various studies.

Conclusions

Strategies to mitigate oxidative stress have currently become a significant pharmacotherapy in the treatment of diabetic complications. The results of this study indicate that antioxidant Resveratrol shows positive effects on oxidative stress genes under stress conditions. Resveratrol is the most potent followed by Mangiferin, Estragole, and Capsaicin amongst antioxidants. Resveratrol along with insulin performed the best to scavenge ROS generated during stress. The analysis of *SOD*, *CAT*, *GPx*, and *GST* gene with different treatment combinations along with different antioxidants indicates that the highest expression was found in cells treated with insulin and antioxidant

treatment combination, from which we can draw a conclusion that the antioxidants along with insulin work best to scavenge ROS generated during stress. Although previous studies have confirmed the potential of Insulin to combat diabetes solely, we observed that insulin in combination with the dietary and natural antioxidant was mostly more effective. Further experiments have to be performed in the in vivo models to provide more affirmative results regarding the exact potency of the antioxidants.

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Authors' contributions PS and AB performed the experimental work, and compilation of data. PS drafted the manuscript. SB designed the study, facilitated infrastructural and financial support to carry out the experiments. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

References

- Abdulkadir AA, Thanoon IA (2012) Comparative effects of glibenclamide and metformin on C-reactive protein and oxidant/antioxidant status in patients with Type II diabetes mellitus. *Sultan Qaboos Univ Med J* 12:55–61
- Abiko T, Abiko A, Clermont AC et al (2003) Characterization of retinal leukostasis and hemodynamics in insulin resistance and diabetes: role of oxidants and protein kinase C activation. *Diabetes* 352:829–837
- Aderibigbe AO, Emudianughe TS, Lawal BA (1999) Antihyperglycemic effect of *Mangifera indica* in rat. *Phytother Res* 13:504–507
- Arulselvan P, Subramanian S (2007) Effect of *Murraya koenigii* leaf extract on carbohydrate metabolism studied in streptozotocin induced diabetic rats. *Int J Biol Chem* 1:21–28
- Berlett BS, Stadtman ER (1997) Protein oxidation in aging. *Dis Oxid Stress* 272:20313–20319
- Breinholt V, Lauridsen ST, Daneshvar B et al (2000) Dose-response effects of lycopene on selected drug-metabolizing and antioxidant enzymes in the rat. *Cancer Lett* 154:201–210
- Can Baser KH (2008) Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr Pharm Des* 14:3106–3119
- Cho SY, Park JY, Park EM et al (2002) Alteration of hepatic antioxidant enzyme activities and lipid profile in streptozotocin-induced diabetic rats by supplementation of dandelion water extract. *Clin Chim Acta* 317:109–117
- Choi MS, Do KM, Park YS et al (2001) Effect of naringin supplementation on cholesterol metabolism and antioxidant status in rats fed high cholesterol with different levels of vitamin E. *Ann Nutr Metab* 45:193–201
- Dachani SR, Avanapu SR, Ananth PH (2012) In vitro antioxidant and glucose uptake effect of *Trichodesma indicum* in L-6 cell lines. *J Pharm Bio Sci* 3:810–819
- Day C (2001) The rising tide of type 2 diabetes. *Br J Diabetes Vasc Dis* 1:37–432
- Deng W, Lu H, Teng J (2013) Carvacrol attenuates diabetes-associated cognitive deficits in rats. *J Mol Neurosci* 51:813–819
- Di Mascio P, Kaiser S, Sies H (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys* 274:532–538
- Du C, Cao H, Sun H et al (2017) Protective effect of baicalein on oxLDL-induced oxidative stress and inflammation injury in endothelial cell. *Int J Pharmacol* 13:280–285
- Erhardt JG, Meisner C, Bode JC et al (2003) Lycopene, Beta-carotene and colorectal adenomas. *Am J Clin Nutr* 78:1219–1224
- Guimaraes AG, Oliveira GF, Melo MS et al (2010) Bioassay-guided evaluation of antioxidant and antinociceptive activities of Carvacrol. *Basic Clin Pharmacol Toxicol* 107:949–957
- Halliwell B, Gutteridge JM (1999) Free radicals in biology and medicine. Oxford University Press, Oxford
- Hernandez-Munoz R, Olguin-Martinez M, Aguilar-Delfin I et al (2013) Oxidant status and lipid composition of erythrocyte membranes in patients with type 2 diabetes, chronic liver damage, and a combination of both pathologies. *Oxid Med Cell Longev* 2013:9
- Hussein HK, Abu-Zinadah OA (2010) Antioxidant effect of curcumin extracts in induced diabetic wister rats. *Int J Zool Res* 6:266–276
- Jagetiya GC, Baliga MS (2003) Evaluation of the radioprotective effect of the leaf extract of *Syzygium cumini* (Jamun) in mice exposed to a lethal dose of gamma-irradiation. *Mol Nutr Food Res* 47:181–185
- Jeon HJ, Seo MJ, Choi HS et al (2014) Gelidium elegans, an edible red seaweed, and hesperidin inhibit lipid accumulation and production of reactive oxygen species and reactive nitrogen species in 3T3-L1 and RAW264. 7 cells. *Phytother Res* 28:1701–1709
- Kakkar R, Kalra J, Mantha SV et al (1995) Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. *Mol Cell Biochem* 151:113–119
- Kochhar KP (2008) Dietary spices in health and diseases (II). *Indian J Physiol Pharmacol* 52:327–354
- Kolsi RB, Salah HB, Jardak N et al (2017) Effects of *Cymodocea nodosa* extract on metabolic disorders and oxidative stress in alloxan-diabetic rats. *Biomed Pharmacother* 89:257–267

- Koparal AT, Zeytinoglu M (2003) Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line A549. *Cytotechnol* 43(1–3):149–154
- Kowluru RA, Kenned A (2001) Therapeutic potential of antioxidants and diabetic retinopathy. *Expert Opin Invest Drugs* 10:1665–1676
- Kutuk O, Adli M, Poli G et al (2004) Resveratrol protects against 4-HNE induced oxidative stress and apoptosis in Swiss 3T3 fibroblasts. *BioFactors* 20:1–10
- Laaksonen DE, Atalay M, Niskanen L et al (1998) Exercise and oxidative stress in diabetes mellitus. *Pathophysiology* 5(Suppl 1):112
- Levine RL (2002) Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic Biol Med* 32:790–796
- Limaye PV, Raghuram N, Sivakami S (2003) Oxidative stress and gene expression of antioxidant enzymes in the renal cortex of streptozotocin-induced diabetic rats. *Mol Cell Biochem* 243:147–152
- Liu Y, Tang Q, Hu Z et al (2015) Lycopene attenuates angiotensin II induced oxidative stress in H9c2 cells. *Zhonghua xin xue guan bing za zhi* 43:341–346
- Maritim AC, Sanders A, Watkins JB (2003) Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol* 17:24–38
- MatEs JM, Perez-Gomez C, De Castro IN (1999) Antioxidant enzymes and human diseases. *Clin Biochem* 32:595–603
- Mc Garry JD (2002) Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 51:7–18
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Murugan P, Pari L (2006) Effect of tetrahydrocurcumin on lipid peroxidation and lipids in streptozotocin–nicotinamide-induced diabetic rats. *Basic Clin Pharmacol Toxicol* 99:122–127
- Nakhaee A, Bokaeian M, Saravani M et al (2009) Attenuation of oxidative stress in streptozotocin-induced diabetic rats by *Eucalyptus globulus*. *Indian J Clin Biochem* 24:419–425
- Ramakrishna V, Jaikhan R (2008) Oxidative stress in non-insulin-dependent diabetes mellitus (NIDDM) patients. *Acta Diabetol* 45:41–46
- Robertson RP (2004) Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J Biol Chem* 279:42351–42354
- Saha S, Sadhukhan P, Sinha K et al (2016) Mangiferin attenuates oxidative stress induced renal cell damage through activation of PI3K induced Akt and Nrf-2 mediated signaling pathways. *Biochem Biophys Rep* 5:313–327
- Sanchez GM, Re L, Giuliani A et al (2000) OS Protective effects of *Mangifera indica* L. extract, mangiferin and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. *Pharmacol Res* 42:565–573
- Shao W, Yu Z, Chiang Y et al (2012) Curcumin prevents high-fat diet-induced insulin resistance and obesity via attenuating lipogenesis in liver and inflammatory pathway in adipocytes. *PLoS ONE* 7:28784
- Shirwaikar A, Prabhu KS, Punitha IS (2006) In vitro antioxidant studies of *Sphaeranthus indicus* (Linn). *J Exp Biol* 44:993–996
- Srinivas A, Menon VP, Periaswamy V et al (2003) Protection of pancreatic beta cell by the potential antioxidant bis-o-hydroxycinnamoyl methane, analogue of natural curcuminoid in experimental diabetes. *J Pharm Pharm Sci* 6:327–333
- Stadtman ER, Levine RL (2000) Protein oxidation. *Ann NY Acad Sci* 899:191–208
- Tiwari BK, Pandey KB, Abidi AB et al (2013) Markers of oxidative stress during diabetes mellitus. *J Biomark* 2013:378790
- Usatyuk PV, Parinandi NL, Natarajan V (2006) Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. *J Biol Chem* 281:35554–35566
- Wang Z, Zhang XM, Ribnicky DM et al (2004) Effect of an alcoholic extract of *Artemisia dracuncululus* (TarralinTM) on glucose uptake in human skeletal muscle culture. *Diabetes* 53:A406–A407
- Wiernsperger NF (2003) Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. *Diabet Med* 29:579–585
- Wu CH, Yeh CT, Yen GC (2010) Epigallocatechin gallate (EGCG) binds to low-density lipoproteins (LDL) and protects them from oxidation and glycation under high glucose conditions mimicking diabetes. *Food Chem* 121:639–644
- Zatalia SR, Sanusi H (2013) The role of antioxidants in the pathophysiology, complications, and management of diabetes mellitus. *Acta Med Indones* 45:141–147
- Zhang LL, Liu DY, Ma LQ et al (2007) Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity. *Circ Res* 100:1063–1070
- Zhang Y, Ye M, Chen L et al (2015) Role of the ubiquitin-proteasome system and autophagy in regulation of insulin sensitivity in serum-starved 3T3-L1 adipocytes. *Endocr J* 62:673–686
- Zhao Y, Li H, Gao Z et al (2005) Effects of dietary baicalin supplementation on iron overload-induced mouse liver oxidative injury. *Eur J Pharmacol* 509:195–200