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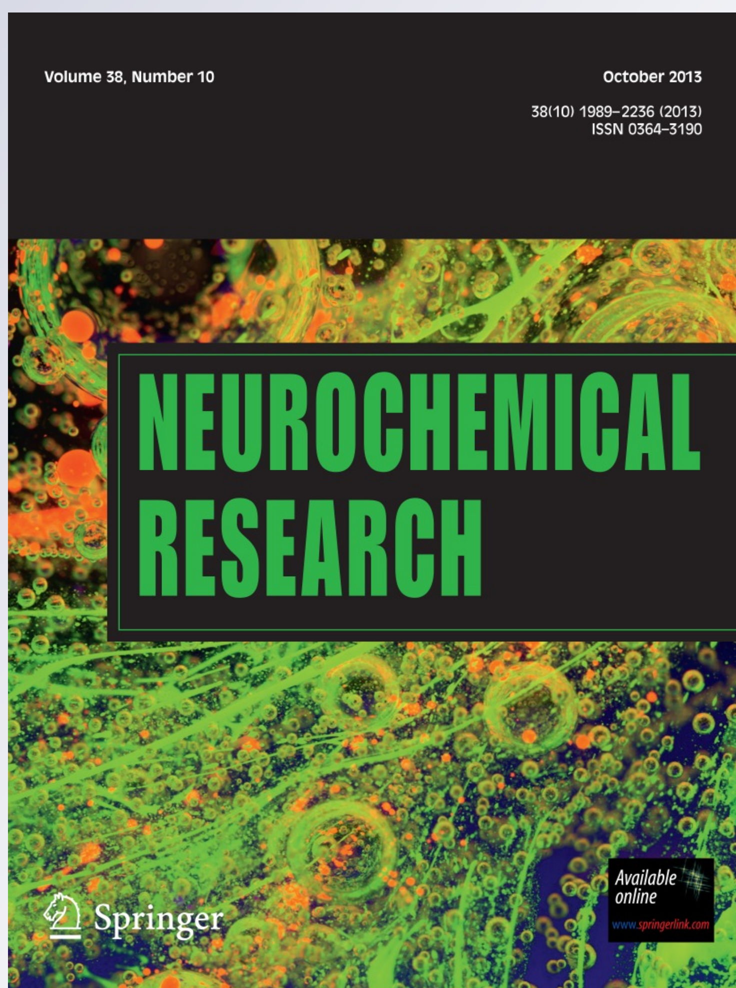

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ORIGINAL PAPER

Neuroprotective Effects of Hydroalcoholic Extract of *Ocimum sanctum* Against H_2O_2 Induced Neuronal Cell Damage in SH-SY5Y Cells via Its Antioxidative Defence Mechanism

M. P. Venuprasad · Kandikattu Hemanth Kumar · Farhath Khanum

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Abstract Oxidative stress mediates the cell damage in several ailments including neurodegenerative conditions. *Ocimum sanctum* is widely used in Indian ayurvedic medications to cure various ailments. The present study was carried out to investigate the antioxidant activity and neuroprotective effects of hydroalcoholic extract of *O. sanctum* (OSE) on hydrogen peroxide (H_2O_2)-induced oxidative challenge in SH-SY5Y human neuronal cells. The extract exhibited strong antioxidant activity against DPPH, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical and hydroxyl radicals with IC_{50} values of 395 ± 16.2, 241 ± 11.5 and 188.6 ± 12.2 μg/ml respectively, which could be due to high amount of polyphenols and flavonoids. The observed data demonstrates 41.5 % cell survival with 100 μM H_2O_2 challenge for 24 h, which was restored to 73 % by pre-treatment with OSE for 2 h. It also decreased the lactate dehydrogenase leakage and preserved the cellular morphology. Similarly OSE inhibited lipid peroxidation, DNA damage, reactive oxygen species generation and depolarization of mitochondrial membrane. The extract restored superoxide dismutase and catalase enzyme/protein levels and further downregulated HSP-70 over-expression. These findings suggest that OSE ameliorates H_2O_2 induced neuronal damage via its antioxidant defence mechanism and might be used to treat oxidative stress mediated neuronal disorders.

Keywords *Ocimum sanctum* · Oxidative stress · SH-SY5Y cells · Neuroprotection · DNA damage · HSP-70

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Introduction

Brain is a vital organ in human body which is more predisposed to oxidative stress than any other organ due to its high metabolic rate and high content of polyunsaturated fatty acids. The imbalance between oxidants and antioxidants leads to disruption of redox signaling leading to oxidative stress. The most common reactive oxygen species (ROS) include superoxide ($O_2^{·-}$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^·$) and reactive hydroxyl ($OH^·$) radicals [1]. Excessive generation of free radicals affects human health by macromolecular damage, such as DNA, cell membrane, proteins and cellular components which consequently induce degeneration, destruction and toxicity of various molecules that play an important role in metabolism [2]. The stress induced oxidative damage has been implicated in neurodegenerative diseases such as Alzheimers and Parkinsons disease [3].

Ocimum sanctum L. (*Lamiaceae*) is a well documented Indian traditional medicinal herb used for the treatment of various stress-related conditions and is distributed worldwide. The major bioactive phytochemical constituents of *O. sanctum* are ursolic acid, rosmarinic acid, flavonoids and tannins, eugenol, luteolin, apigenin, β-caryophyllene, methyl eugenol, β-pinene and Ocimumosides [4–6]. *O. sanctum* is an "elixir of life" and has been demonstrated to possess diverse pharmaceutical effects. It is used as diaphoretic and to cure gastric and hepatic disorders [7, 8]. *O. sanctum* has also been reported as radio protective, immuno modulatory and nootropic agent [9–12]. The terpenoid and fatty acid derivatives of *O. sanctum* have been reported to possess antimicrobial, anticancer and anti-HIV activities [13–15]. Recently we have evaluated the antifatigue activity of ethanolic extract of Ocimum [16].

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Oxidative stress mediated death of dopaminergic neurons has been observed particularly in Parkinsonism. Hence in the present investigation we mimicked the Parkinsonism symptoms in dopaminergic SH-SY5Y human neurons by H_2O_2 challenge [17, 18]. Although literature pertaining to neuroprotective activity of *O. sanctum* is reported, there is no data available on the modulation of the oxidative stress induced neuronal cell damage by the *Ocimum* extract. Therefore, we made an attempt to explore the neuroprotective and antioxidant potential of ethanolic extract of *O. sanctum* leaves against H_2O_2 induced neuronal cell damage.

Materials and Methods

Chemicals and Reagents

DMEM-F12 from HIMEDIA (Bangalore, India), MTT, 2′,7′-$DCFH_{2DA}$, rhodamine 123, RIPA buffer, protease and phosphate inhibitor cocktail were obtained from Sigma (St Louis, MO, USA) while H_2O_2 was procured from Merck (Bangalore, India) all the other chemicals were of analytical grade and procured from Rankem (Bangalore, India).

Preparation of *O. sanctum* Extract (OSE)

The *O. sanctum* L. leaf material was obtained from the local market of Mysore, India, and identified by Ex-Prof. Chandrashekharappa, Department of Botany, JSS College, Mysore, India. The leaves were shade dried, finely powdered and macerated at room temperature with 70 % ethanol by keeping the powder and solvent ratio of 1:10 (w/v). The extraction was continued till the samples were decolorized and the extracts were pooled and concentrated using a rotary vacuum evaporator. The concentrate was finally freeze dried and yield was 5.84 %. The final powder was dissolved in dimethyl sulfoxide, filtered through 0.2 μm membrane filter (Millipore, India) and used in the subsequent experiments.

Estimation of Polyphenols and Flavonoids

The total phenolic contents were determined according to the methods of Kujala et al. [19] using Folin-Ciocalteu reagent (FCR) where as gallic acid was used as a standard antioxidant. To 3 ml of appropriately diluted extract, 0.5 ml of FCR was added, followed by incubation at room temperature (10 min) and addition of 7 % Na_2CO_3 (2 ml) solution. The mixture was boiled for 1 min and color intensity was observed at 650 nm (Shimadzu, Kyoto, Japan). The results were expressed in μg gallic acid equivalent (GAE)/mg extract.

The flavonoid content was determined according to the method of Delcour and Varebeke [20]. Briefly, to 1 ml of appropriately diluted extracts, 5 ml of chromogen reagent (0.1 % cinnamaldehyde solution in a cooled mixture of 75 ml methanol and 25 ml concentrated HCl) was added and incubated for 10 min and the absorbance was recorded at 640 nm. The total flavonoid content was expressed in μg catechin equivalents (CE)/mg extract.

DPPH Radical Scavenging Activity

The hydrogen donating/radical scavenging activity of OSE was assessed by DPPH˙ scavenging activity according to the method of Blois [21]. The solution of DPPH (500 μM) was prepared with methanol and mixed with different concentration of OSE or butylated hydroxy toluene (BHT) standard. The absorbances were read spectrophotometrically at 517 nm after 30 min of incubation and then percentage of decolourisation was determined and the scavenging activity was determined by calculating IC_{50} values using the equation

$$\text{DPPH}^{\cdot}\,\text{scavenging effect}\ (\%) = (\text{OD}^{\text{control}} - \text{OD}^{\text{samples}})/\text{OD}^{\text{control}} \times 100)$$

Iron Chelating Activity

The chelating of ferrous ions by OSE was estimated according to Dinis et al. [22]. Briefly, the extract was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM Ferrozine (0.2 ml) and the mixture was incubated at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe2+ complex formation was calculated as:

$$[(A_0 - As)/As] \times 100$$

where A_0 was the absorbance of the control, and As was the absorbance of the extract or EDTA (positive control).

Hydroxyl Radical Scavenging Activity

This assay was employed according to Kunchandy and Rao [23]. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid (TBA). The Fenton reaction was initiated to generate hydroxyl radicals (OH˙) which degrade DNA deoxyribose, using Fe^{3+}-ascorbate-EDTA-H_2O_2.The reaction mixture composed of 2-deoxy-2-ribose (2.8 mM); KH_2PO_4–KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); EDTA (100 μM); H_2O_2 (1.0 mM); ascorbic acid (100 μM) and various concentrations (0–200 μg/ml) of OSE/standard (ascorbic acid). After incubation for 1 h at 37 °C, 0.5 ml of

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the reaction mixture was added to 1 ml 2.8 % trichloroacetic acid, then 1 ml 1 % aqueous TBA was added and the mixture was incubated at 90 °C for 15 min to develop the colored product which was measured at 532 nm and the results were expressed in terms of IC_{50} values.

ABTS Radical Scavenging Activity

ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical) was performed according to Re et al. [24]. The stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate solution was prepared. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml of methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm. Plant extracts (1 ml) and BHT-standard were allowed to react with 1 ml of the ABTS solution and the absorbance was measured at 734 nm. The percentage inhibition [%] was calculated as

$$I\,\% = [(\text{Ablank} - \text{Asample})/\ \text{Ablank}\ ] \times 100).$$

where, 'A' blank is the absorbance of ABTS radical + methanol; 'A' sample is the absorbance of ABTS radical + sample extract/standard.

Cell Culture and Treatments

The SH-SY5Y human neuroblastoma cell line was obtained from National Centre for Cell Sciences, Pune, India. Cells were cultured into petri plates, flasks or dishes as required in DMEM/F-12 mixture supplemented with 10 % FBS, 2 mM L-glutamine, antibiotic and antimycotic solution Sigma (St Louis, MO, USA) in a humid atmosphere of 5 % CO_2 and 95 % air at 37 °C. The media was changed on alternative day and freshly prepared 100 µM H_2O_2 was added for 24 h to the cells with or without pretreatment with OSE for 2 h before any experiment. All the experiments were carried out in 0.5 % serum media.

Analysis of Cell Viability

The metabolic status of the mitochondria of SH-SY5Y cells was analyzed by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. The cells were cultured in 96-well plates at a density of 1×10^4 cell/ml (100 µl) and incubated for 24 h before treatments. The cells were then subjected to the treatments of interest. After 24 h of adherence, MTT (0.5 mg/ml) was added to each well. Further the cells were incubated for 2 h at 37 °C and the formed insoluble formazan crystals were dissolved by addition of 100 µl of DMSO. The resulting absorbance was measured at 540 nm using a VERSA max Hidex plate chameleon^{TM} V (Finland). Wells without cells were used as blanks and were subtracted as background from each sample and the results were expressed as percentage of control.

Lactate Dehydrogenase (LDH) Release Assay

Further, the cytotoxicity was confirmed by measuring the extracellular LDH released in the medium with a LDH-estimation kit (Agappe-11407002) according to the protocol provided by the manufacturer. LDH activity was measured through the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD^+) at a wavelength of 340 nm. The rate of increase in enzyme activity due to the formation of reduced nicotinamide adenine dinucleotide (NADH) is directly proportional to the LDH activity in the sample. The SH-SY5Y cells were plated in 24-well plates at a density of 4×10^5 cells/ml (500 µl) and cultured for 24 h followed by treatments of interest. After treatment period, 10 µl of 2 % Triton X-100 was added to lyse the untreated cells, which were selected as the total LDH activity. The cells were precipitated by centrifugation at 1,000*g* for 5 min at 4 °C and the supernatant (100 µl) was mixed with 900 µl of reaction mixture. The cell damage was evaluated by measuring the leakage of intracellular LDH into the medium.

Observations of Morphological Changes

The cells were seeded in 60 mm × 15 mm size petri dishes (3×10^5 cells/ml) (3 ml) and cultured till confluency and treated with 100 µM H_2O_2 for 24 h with or without pre-treatment of OSE for 2 h. The cellular morphology was observed and photographed using a phase contrast microscope (Olympus, Japan) equipped with Cool SNAP® Pro color digital camera.

Estimation of Superoxide Dismutase (SOD) and Catalase (CAT)

The cells (1×10^7 cells/ml) (15 ml) were cultured in 75 cm^2 flasks and treated as described earlier. After treatments the cells were collected by trypsinization, lysed (50 mM potassium phosphate buffer, pH 7.4, 2 mM EDTA and 0.1 % Triton X-100) by sonication and the cell debris was removed by centrifugation at 13,000*g* for 10 min at 4 °C. The protein content in the supernatant was measured by Bradford method [25] with BSA as the standard. The activity of the antioxidant enzyme SOD was estimated according to the kit supplier protocol (Randox, Cat no. SD. 125, Canada). While, CAT was estimated by measuring the decay of 6 mM H_2O_2 solution at 240 nm by the spectrophotometric degradation method [26].

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Estimation of Intracellular ROS

The intracellular ROS was measured spectrofluorimetrically by oxidation-sensitive dye $DCFH_{2DA}$ [28]. SH-SY5Y cells were seeded in 24-well plates (4.0×10^5 cells/ml) and treated as described earlier. After treatment, $DCFH_{2DA}$ (5 mg/ml) was added to the cells and incubated for 30 min. Further the cells were washed twice with PBS and the fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Hidex plate chameleon™ V (Finland).

Estimation of Lipid Peroxidation

The lipid peroxidation products were measured as the malondialdehyde content according to Ohkawa et al. [27]. SH-SY5Y cells were cultured in 75 cm^2 flasks at a concentration of 1.0×10^7 cells/ml and incubated at 37 °C. After confluence, the cells were treated as mentioned earlier, after treatments the cells were harvested and lysed (1.15 % KCl with 1 % Triton X-100) by sonication. A 100 µl of the cell lysate was mixed with 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid (pH 3.5), and 1.5 ml of 0.8 % thiobarbituric acid and the volume was made up to 4.0 ml with distilled water and boiled for 2 h. After cooling, the contents were centrifuged at 500*g* for 10 min, the supernatants were separated and the absorbance was measured at 532 nm.

Measurement of Mitochondrial Membrane Potential (MMP)

The mitochondrial health was estimated in terms of MMP using the fluorescent probe rhodamine 123. The cells were cultured in 24 well plates (4.0×10^5 cells/ml) and on cover slips in petri dishes (3×10^5 cells/ml) coated with poly L-lysine for fluorimetric measurement and imaging, respectively. The cells were treated as described earlier. After treatments rhodamine 123 (10 µg/ml) was added to the cells and incubated for 60 min at 37 °C. The cells were washed twice with PBS and the fluorescence was estimated at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Hidex plate chameleon™ V (Finland). The cells grown on cover slips were photographed using fluorescence microscope (Olympus microscope, Japan) equipped with Cool SNAP® Pro color digital camera.

Single Cell Gel Electrophoresis (SCGE) Assay

The DNA damage was measured by alkaline comet assay as described by Singh et al. [29]. SH-SY5Y cells were (4×10^5 cells/ml) treated with 100 µM H_2O_2 with or without pretreatment of OSE (75 µg/assay). Later the cell were collected and the cell suspensions were mixed with 100 µl of 0.7 % (w/v) low melting agarose (LMA) and pipetted on to the frosted slides pre-coated with 1.0 % (w/v) normal melting agarose. After agarose solidification, the slides were covered with another 100 µl of 0.7 % (w/v) LMA and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, 0.1 % SDS and 1 % Triton X-100 and 10 % DMSO; pH 10.0) for 90 min. Then, the slides were transferred to unwinding buffer (3 M NaOH, 10 mM EDTA; pH 13.0) in an electrophoresis tank for DNA unwinding. Later the slides were run with an electric current of 25 V/300 mA for 20 min and the slides were washed twice with neutralizing buffer (0.4 M Tris–HCl; pH 7.5). Further the DNA damage was photographed from the slides stained with ethidium bromide (20 µg/ml) with fluorescence microscope (Olympus microscope, Japan) equipped with Cool SNAP® Pro color digital camera. The tail length was determined by Image Pro® plus software and per cent inhibition of tail length was estimated with OSE treatment.

Immunoblotting

The cells (1×10^7 cells/ml) were seeded in 75 cm^2 flasks and treated as mentioned earlier. After treatment, the cells were washed twice with PBS and then lysed using ice-cold RIPA buffer with protease and phosphatase inhibitor cocktail. Cell lysates were centrifuged at 12,000*g* for 10 min at 4 °C, and the protein concentrations were determined as mentioned earlier. The proteins were separated by 8–12 % SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked overnight at 4 °C with 5 % (v/v) non-fat dry milk in Tris-buffered saline with Tween-20 (TBS-T) (10 mM Tris–HCl, 150 mM NaCl, and 0.1 % Tween-20, pH 7.5) and incubated with primary antibodies namely α-Tubulin (sc-5286), HSP-70 (sc-66048), SOD (sc-8637), CAT (sc-34280), (Santa Cruz Biotechnology, CA, USA) at 1:1,000 dilution for 3 h with shaking. The membranes were then washed in TBS-T followed by incubation for 2 h at room temperature in dark with horseradish peroxidase (HRP) conjugated rabbit anti-goat, goat anti-mouse and goat anti-rabbit secondary antibodies (DAKO, Denmark) at 1:10000 dilutions. The membranes were washed again and the immunoreactivity was detected by using the enhanced chemiluminescence peroxidase substrate kit (CPS-160, Sigma, St Louis, MO, USA).

Statistical Analysis

The results were represented as the mean ± SD. Statistical significance was analyzed with one-way analysis of

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variance followed by a Tukey's HSD-post hoc test. Differences with *P* value less than 0.05 were considered statistically significant.

Results and Discussion

Polyphenol and Flavonoid Content

Polyphenols and flavonoids are the principle bioactive constituents of herbal extracts and several studies have illustrated the neuroprotective and anti-stress activity of these compounds [30, 31]. *O. sanctum* possess a broad spectrum of polyphenols such as apigenin, luteolin, apigenin-7-O-ß-D-rutinopyranoside, luteolin-7-O-ß-D-glucopyranoside, vicenin-2, vitexin, isovitexin, orientin, isoorientin, aesculetin, aesculin, chlorogenic acid and caffeic acid [32]. In the present study we found 148.9 mg/g gallic acid equivalents of polyphenols and 14.16 mg/g catechin equivalents of flavonoids contents which may account for the bioactivity of the present investigation. Previous study by Kaul et al., [33] had demonstrated the atherogenic effect of polyphenols extracted from *O. sanctum*.

Antioxidant and Free Radical Scavenging Activity of OSE

The antioxidant activities of herbal extracts were estimated by an array of free radical scavenging methods such as DPPH˙, iron chelating, ABTS and hydroxyl radical assays. Here, we observed that OSE scavenges DPPH˙, Fe^{2+}, ABTS and HO˙ radicals. The maximum inhibition was observed with increasing concentration of extract and the IC_{50} values were represented in Table 1. Our results are in line with several studies which demonstrate the protective ability of herbal extracts against radical mediated stress [34, 35].

Protective Effect of OSE Against H_2O_2 Induced Cytotoxicity

Several herbal extracts have been shown to inhibit the H_2O_2 induced neuronal cell damage [36, 37]. In the present study we have evaluated the protective effect of OSE against H_2O_2 challenge by MTT reduction assay in cultured SH-SY5Y cells. The assay is based on the principle that mitochondrial dehydrogenase reduces the MTT dye to formazan. The H_2O_2 (25–200 µM) treatment decreased the cell proliferation in a dose-dependent manner with 41.5 % viability at 100 µM H_2O_2 challenge which was used for further assays (Fig. 1a). However, the cells pretreated with different concentrations of OSE (10–75 µg) for 2 h before 100 µM H_2O_2 treatment (24 h) showed significant improvement in cell survival up to 73.2 % with 75 µg of OSE (Fig. 1b).

Table 1 Antioxidant and free radical scavenging activities of OSE

Assay	Activity IC_{50} (µg/ml)
DPPH radical scavenging	395 ± 16.2
Metal chelating	241 ± 11.5
Hydroxyl radical scavenging	188.6 ± 12.2
ABTS radical scavenging	97.6 ± 5.1

Each value represents the mean ± SD of three determinations

Protective Effect of OSE Against Plasma Membrane Damage

The cytotoxicity of H_2O_2 and the protective activity of OSE were further evaluated by LDH assay, which is based on the principle that the leakage of cytosolic LDH increases as the number of dead cells increases. SH-SY5Y cells were pretreated with 75 µg/ml of OSE for 2 h, before treatment with 100 µM H_2O_2 for 24 h (Fig. 1c). The results show that the release of LDH of 45.7 % of total enzyme with 100 µM H_2O_2 challenge which indicates that H_2O_2 induces cytotoxicity in the SH-SY5Y cells. In contrast, OSE pretreatment lowered the LDH release up to 15 % as compared with 100 µM H_2O_2-treated cells. The observed results demonstrate the protective effect of OSE against 100 µM H_2O_2-induced neurotoxicity. The protective effect of OSE was further more confirmed morphologically by bright field microscope. The 100 µM H_2O_2-challenged neurons exhibited cell shrinkage and disappearance of the cellular processes which was partially protected with pretreatment of OSE (Fig. 1d). The OSE extract was dissolved in DMSO and the final concentration added in the culture medium did not exceeded 0.01 % and the same concentration of DMSO has been used for control cells as well as 100 µM H_2O_2 treated group. The DMSO did not showed any effect to the control cells as well as the toxicity elicited by H_2O_2.

Effect of OSE on Antioxidant Enzymes

Antioxidant enzymes play a vital role in detoxification of free radicals generated due to oxidative damage of the cell thus maintains the redox status. The antioxidant enzyme SOD dismutates the $O_2^{\cdot -}$ radicals whereas H_2O_2 is converted to H_2O and O_2 by the catalytic activity of catalase and glutathione system. Compelling evidence demonstrates the decrease in antioxidant enzyme levels in Parkinson's disease [38]. Supplementation of diet rich of antioxidant principles enhances the defence system to detoxify the oxidative molecules [39]. In the present study we measured the SOD and CAT activity by spectrophotometric

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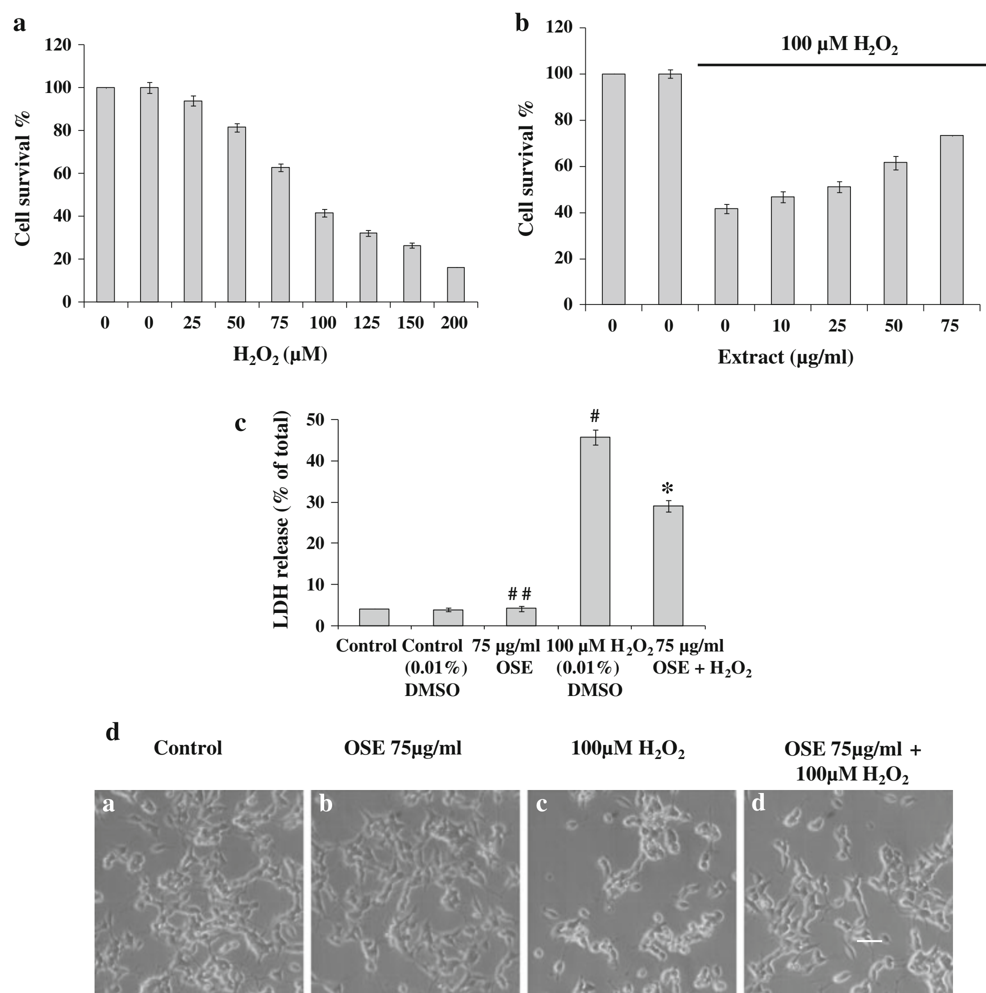

Fig. 1 **a** Cytotoxic effects of H_2O_2 on SH-SY5Y neuronal cell. **b** Dose dependent protective effect of pre-treatment of OSE for 2 h on 24 h treatment of 100 µM H_2O_2-induced cytotoxicity in SH-SY5Y cells, the cell viability was determined by MTT assay. **c** Effect of OSE for 2 h on 24 h treatment of 100 µM H_2O_2, the plasma membrane damage was analysed by LDH leakage and **d** Effects of OSE pretreatment for 2 h on 24 h treatment of 100 µM H_2O_2 induced morphological alterations in SH-SY5Y neurons by phase-contrast microscopy. All the groups were treated with 0.01 % DMSO. The data are represented as mean ± SD of three independent experiments. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus 100 µM H_2O_2 treated group, ## Non significant versus control group. *Scale bar* = 50 µm

degradation method (Fig. 2a, b), which shows that the oxidative damage generated by H_2O_2 leads to decreased enzyme activity of SOD and CAT. The pretreatment of OSE significantly restored the antioxidant enzyme levels which indicate the antioxidant defence of the active ingredients of OSE. Our observed results are in accordance with recent reports on anti-stress activity of OS via its antioxidant potential [40, 41].

Inhibitory effect of OSE on H_2O_2 induced ROS generation and lipid peroxidation

Further we investigated the effect of OSE on H_2O_2 induced ROS generation using fluorescent probe $DCFH_{2DA}$. The non-ionic, non-polar fluorescent probe, $DCFH_{2DA}$, crosses the cell membranes and is hydrolyzed by intracellular esterases to nonfluorescent DCFH. Whereas, the ROS oxidizes DCFH to highly fluorescent dichlorofluorescein (DCF) [42]. The DCF fluorescence intensity was observed maximum of 153.5 ± 5.2 % of control in the cells treated with 100 µM H_2O_2, whereas, the pretreatment of OSE significantly decreased the DCF fluorescence up to 123.92 ± 3.8 % of control against 100 µM H_2O_2 stress (Fig. 3a). These results are in concomitant with our recent findings which demonstrates the ROS scavenging activity of *Cyperus* extract against 100 µM H_2O_2 induced neuronal stress [37]. Of late Zhao et al. [43] had also demonstrated the neuroprotective effect of apigenin one of the major constituent of *Ocimum*, which ameliorates Alzheimer's symptoms via regulation of ROS and mitochondrial damage.

Oxidative stress leads to generation of lipid peroxidation products which generates DNA reactive aldehydes such as 4-hydroxynoneal and malondialdehyde which are reported to play a role in carcinogenesis [44]. In the present investigation we observed the increase in lipid peroxidation products with 100 µM H_2O_2 treatment, which was decreased with pretreatment of OSE (Fig. 3b). Our results are in line with a recent report that *Ocimum* extract inhibits the lipid peroxidation against chronic unpredictable stress and swimming induced lipid peroxidation [16, 41].

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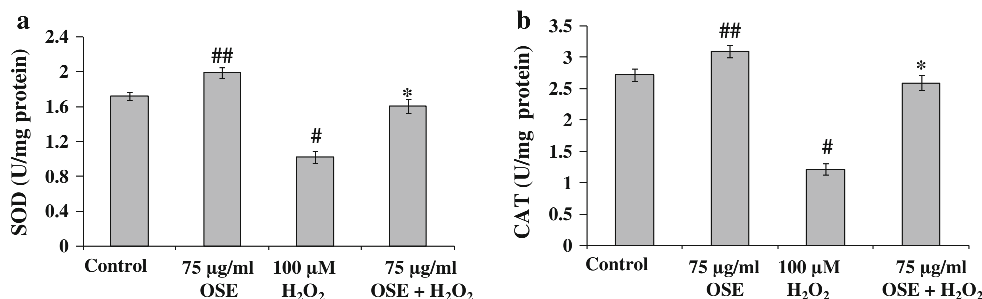

Fig. 2 Pre-treatment of OSE for 2 h on restoration of SOD and catalase enzyme activities in SH-SY5Y cells challenged with 100 µM H_2O_2 for 24 h. The data are represented as mean ± SD of three independent experiments. $^{\#\#}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus 100 µM H_2O_2 treated group

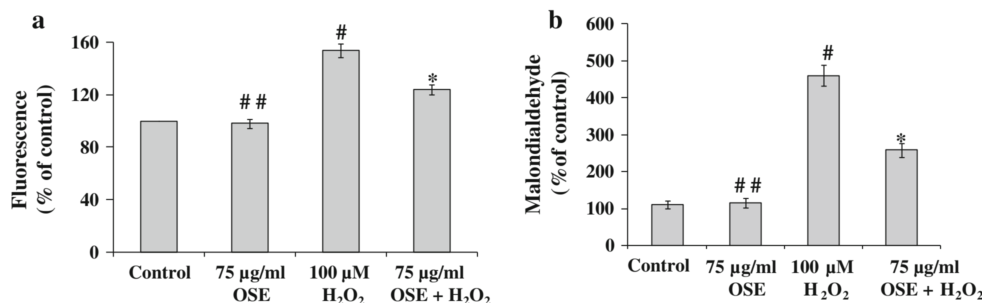

Fig. 3 **a** Estimation of intracellular ROS production using 2′,7′,DCFH$_{2DA}$ in SHSY5Y cells with pre-treatment of OSE for 2 h followed by 100 µM H_2O_2 for 24 h by spectrofluorimeter. **b** Estimation of lipid peroxidation products by TBARS assay in SHSY5Y cells with pre-treatment of OSE for 2 h followed by 100 µM H_2O_2 challenge for 24 h. The data are represented as mean ± SD of three independent experiments. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus 100 µM H_2O_2 treated group, ## Non significant versus control group

Effect of OSE on H_2O_2 Induced Inhibition of MMP

Mitochondrial damage has been observed in Parkinson's disease by inhibition of complex I activity which results in mitochondrial impairment [45]. In the present study mitochondrial health was estimated by measuring, the accumulation of fluorescent probe, rhodamine 123 by MMP assay. The lipophilic cationic dye partitions into mitochondria and interacts with the negative charges on the inner membrane of mitochondria. Whereas the radical induced damage of mitochondria partitions the dye to the cytosol. Hence, mitochondrial accumulation of the dye is proportional to mitochondrial health and membrane potential. In the present study we observed the dissipation of MMP with 100 µM H_2O_2 treatment to 69.96 ± 2.5 % of control. These results demonstrate that H_2O_2 induces the mitochondrial damage by depolarization of mitochondria. Whereas pretreatment of OSE restored the MMP to 88.25 ± 2.8 % of control which indicates the protective effect of OSE against 100 µM H_2O_2 induced mitochondrial damage (Fig. 4a, b). In an earlier study Halder et al. [46] has demonstrated the ameliorative effect of *Ocimum* extract against H_2O_2 induced ultra structural changes of human lens epithelial cells.

Protective Effect of OSE on H_2O_2 Induced DNA Damage

Various reports also demonstrate the damage of macromolecules such as proteins, lipids and DNA by oxidative modification in Parkinson's disease [45, 47]. The lipid products generated due to oxidative stress are highly reactive and have been implicated to induced DNA damage [48]. The comet assay is a sensitive, rapid and widely used screening test for evaluating ROS induced DNA damage. In the present study we evaluated the H_2O_2 induced DNA damage and its protective effect by OSE. The cells treated with 100 µM H_2O_2, showed increase in tail length up to ~65 µm, which indicates the DNA damage. (Fig. 5a, b) Pretreatment of the cells with OSE remarkably decreased the tail length to ~40 µm which shows significant inhibitory effects against 100 µM H_2O_2-mediated DNA damage. Our results are in line with Ovesna et al. [15] and Khanna et al. [49] on protective effects of *Ocimum* against chlorpyrifos induced DNA damage.

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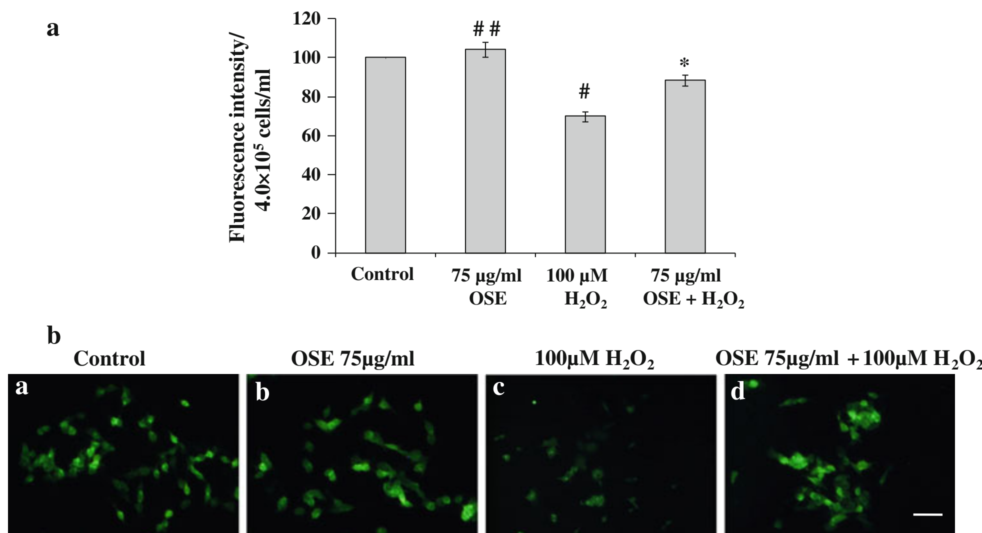
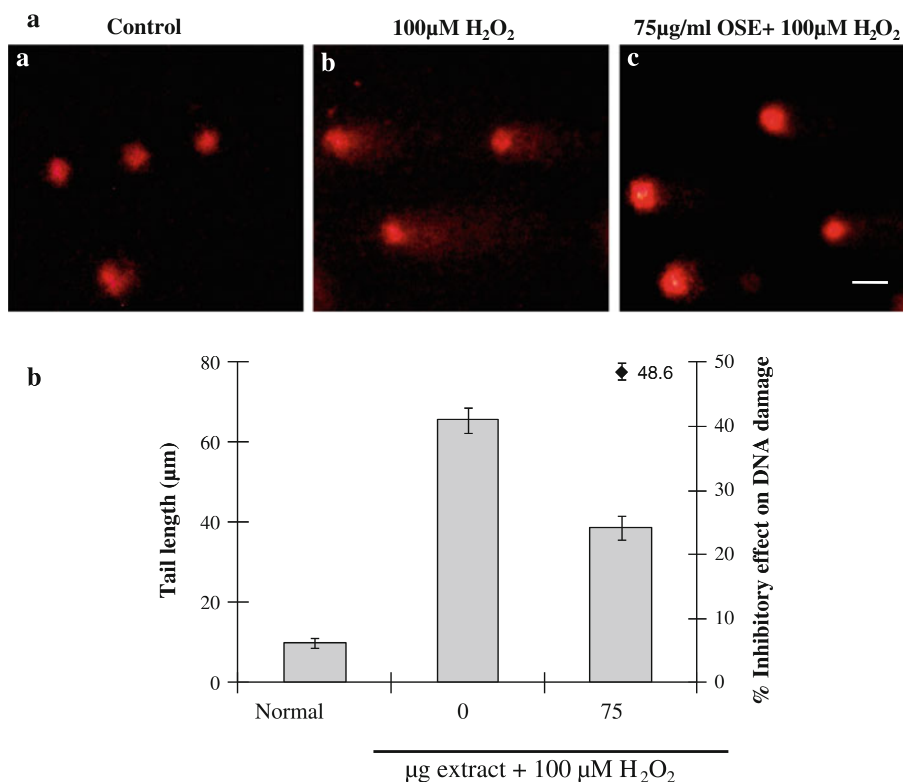


Fig. 4 **a** Estimation of mitochondrial membrane potential in SH-SY5Y cells with 2 h pre-treatment of OSE on 24 h challenge of 100 µM H_2O_2. The fluorescence intensity was determined using spectrofluorimeter. **b** The membrane potential was monitored by fluorescent microscope (Olympus, Japan) Control cells without any treatment (*a*), 75 µg/ml OSE treatment for 2 h (*b*), 100 µMH_2O_2 treatment for 24 h (*c*), and cells pre-treated with 75 µg/ml OSE for 2 h then treated with 100 µM H_2O_2 for 24 h (*d*). The data are represented as mean ± SD of three independent experiments. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus 100 µM H_2O_2 treated group, ## Non significant versus control group. *Scale bar* = 50 µm

Fig. 5 **a** and **b** Protective effect of OSE on DNA damage induced by 100 µM H_2O_2 for 24 h in SH-SY5Y cells. Control cells without any treatment (*a*), Cells with 100 µM H_2O_2 treatment for 24 h (*b*), SH-SY5Y cells were pre-treated with OSE for 2 h at 75 µg/assay and induced with 100 µM H_2O_2 (*c*). Tail length (µm): *Bars*. Inhibitory effect of OSE on DNA damage *diamond*. *Scale bar* = 50 µm



Protective Effect of OSE on H_2O_2 Induced Expression of SOD, CAT and HSP-70

The effect of OSE against H_2O_2 stress was further evaluated by immunoblotting key enzymes of stress such as SOD, CAT and HSP-70 (Fig. 6) which play a vital role in cellular defence by detoxifying superoxide and H_2O_2 [2]. The antioxidant biomarkers SOD and CAT were down-regulated with 100 µM H_2O_2 treatment whereas OSE pre-treatment significantly restored the same. These immunoblot results

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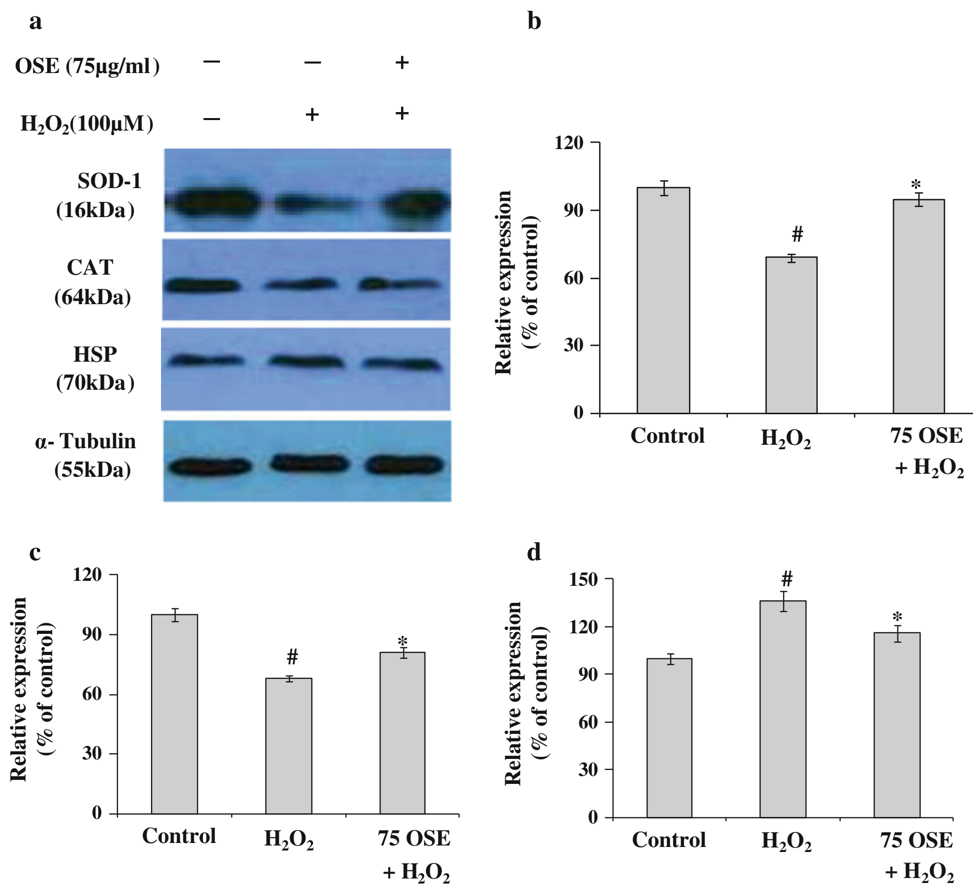

Fig. 6 **a** The protective effect of pre-treatment of OSE for 2 h on 24 h treatment of 100 µM H_2O_2 induced expression of oxidative stress marker proteins SOD, CAT and HSP-70 analyzed by immunobloting. **(b–d)** The band intensity is calculated by Image-J software. The data are represented as mean ± SD of three independent experiments. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus 100 µM H_2O_2 treated group

corroborate with earlier estimations of SOD and CAT enzyme activities. HSP-70, the molecular chaperone, gets over-expressed during stress conditions to combat proteolytic damage of cells [50]. In the present study we observed that OSE pretreatment down-regulated HSP-70 expression which is over-expressed with 100 µM H_2O_2 challenge which suggests the anti-stress activity mediated by OSE. In a recent study our group has demonstrated the protective activity of *Cyperus rotundus* against peroxynitrite induced neuronal damage by regulation of antioxidant marker proteins [51].

Conclusion

In the present study we observed the antioxidant and neuroprotective effects of OSE. The results demonstrate that OSE inhibits H_2O_2 induced neuronal death, ROS generation, lipid peroxidation and DNA damage. The extract restored MMP and SOD, CAT enzyme/protein levels and also inhibited HSP-70 over-expression. These data suggests that OSE may be employed to treat stress induced neurodegeneration. However, further in vitro studies are necessary to better clarify its neuroprotective mechanism of action.

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

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