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Cytoprotective effect of methanolic extract of *Nardostachys jatamansi* against hydrogen peroxide induced oxidative damage in C6 glioma cells

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Oxidative stress has been implicated as an important factor in the process of neurodegeneration and hydrogen peroxide (H_2O_2) is one of the most important precursors of reactive oxygen species (ROS), responsible for many neurodegenerative diseases. This study used extracts from *Nardostachys jatamansi* rhizomes, known for nerve relaxing properties in Ayurvedic medicine, to ascertain their protective role in H_2O_2-induced oxidative stress in C6 glioma cells. The protective effect of methanolic, ethanolic and water extracts of *N. jatamansi* (NJ-MEx, NJ-EEx and NJ-WEx respectively) was determined by MTT assay. NJ-MEx significantly protected against H_2O_2 cytotoxicity when cells were pretreated for 24 h. The level of antioxidant enzymes, catalase, superoxide dismutase (Cu-ZnSOD), glutathione peroxidase (GPx), and a direct scavenger of free radicals, glutathione (GSH), significantly increased following pre-treatment with NJ-MEx. Lipid peroxidation (LPx) significantly decreased in NJ-MEx-pretreated cultures. The expression of a C6 differentiation marker, GFAP (glial fibrillary acidic protein), stress markers HSP70 (heat shock protein) and mortalin (also called glucose regulated protein 75, Grp75) significantly decreased when cells were pre-treated with NJ-MEx before being subjected to H_2O_2 treatment as shown by immunofluorescence, western blotting and RT-PCR results. The present study suggests that NJ-MEx could serve as a potential treatment and/or preventive measure against neurodegenerative diseases.

Key words: antioxidant enzymes, C6 glioma, hydrogen peroxide, *Nardostachys jatamansi*.

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INTRODUCTION

A large class of common diseases involving progressive loss of cognitive functions have been grouped as neurodegenerative disorders. No conclusive hypothesis has been proposed to date to explain the chemical and pathological events in a diseased neuronal cell but it is accepted that all neurodegenerative disorders, which include Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD) and cerebral ischemia (Chatterjee *et al.*, 2000; Liu *et al.*, 2002; Murphy, 1999; Simonian & Coyle, 1996) are upshots of oxidative stress and apoptosis. Brain cells are particularly vulnerable to oxidative damage due to their high energy expenditure and oxygen demand. Glial cells of the brain were earlier thought to provide little passive architectural and trophic support to neurons, but it is now clear that they actively participate in bidirectional communication (Hertz *et al.*, 1999). The CNS response to trauma, viral infection, inflammation, excitotoxicity, hypoxia/ischemia and degenerative diseases manifests reactive gliosis (Eng & Ghirnikar, 1994). Since C6 glioma, an N-nitrosomethylurea-induced rat glial cell line, shows normal glial cell properties, it has been extensively used as an *in vitro* glial model system (Benda *et al.*, 1968; Singh & Kaur, 2009). Upregulation of glial fibrillary acidic protein (GFAP) is a marker for reactive gliosis, trauma and degeneration in CNS, whereas HSP70 and mortalin (Heat shock protein 75/Hsp75/mtHsp70/Grp75/TRAP-1) are useful stress response markers. Experiments using both animal models and tissue culture systems have indicated the overexpression of HSP70 in neurons and glial cells under stress (Giffard *et al.*, 2004; Rajdev & Sharp, 2000). Mortalin is a mitochondrial member of the heat shock protein 70 (HSP70) family and is an essential mitochondrial chaperone. Mortalin is not heat-inducible, but like other HSP70 members, has been shown to be upregulated by various cellular insults including glucose deprivation, oxidative stress, thyroid hormone treatment, and ultraviolet A radiation (Carette *et al.*, 2002; Hadari *et al.*, 1997; Mitsumoto *et al.*, 2002). Mortalin induction was also found in focal cerebral ischemia (Massa *et al.*, 1995). Increased mortalin levels have been shown to be associated with cellular stress in smooth muscle and focal ischemia (Taurin *et al.*, 2002).

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Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; CNS, central nervous system; Cu-ZnSOD, copper-zinc superoxide dismutase; DDW, double distilled water; DEPC, diethylpyrocarbonate; DMEM, Dulbecco's modified eagle's medium; dNTP, deoxyribonucleoside triphosphate; Taq, *Thermus aquaticus*; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidase; Grp75, glucose-regulated protein 75; GSH, reduced glutathione; HD, Huntington's disease; HRP, horse raddish peroxidise; MTT, 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide; HSP70, heat shock protein 70 kDa; IC50, inhibitory concentration producing 50% cell death, IgG, Immunoglobulin G; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NBT, nitroblue tetrazolium; NGS, normal goat serum; PMSF, phenylmethylsulfonyl fluoride; NJ-EEx, *N. jatamansi* ethanolic extract; NJ-MEx, *N. jatamansi* methanolic extract; NJ-WEx, *N. jatamansi* water extract; PAGE, polyacrylamide gel eletrophoresis; PBS, phosphate buffered saline; PBST, phosphate buffered saline with tween 20; PCR, polymerase chain reaction; PD, Parkinson's disease; PVDF, polyvinylidene difluoride; ROD, Relative optical density; SDS, sodium dodecyl sulphate; TBA, thiobarbituric acid; TBS, Tris-buffered saline; TBST, Tris-buffered saline and Tween 20.

Brain has several antioxidant defence mechanisms to control mitochondrial decay-inducing oxidative stress molecules. The cytosol of brain cells has catalase which hydrolyzes H_2O_2 (Borniquel *et al.*, 2006) and glutathione peroxidases (GPx) which reduce organic hydroperoxides (Bjornstedt *et al.*, 1994). Mitochondria of neurons of the human CNS have superoxide dismutase which converts $^{\bullet}O_2^-$ to H_2O_2 and prevents $^{\bullet}ONOO^-$ formation from $^{\bullet}O_2^-$ and $^{\bullet}NO$ (Bayir *et al.*, 2007). Exogenous H_2O_2 can elevate oxidative stress beyond the protective capacity of endogenous antioxidant defences and induces apoptotic cell death by initiating mitochondrial dysfunction (Maroto and Perez-Polo, 1997).

So far, no effective drugs are available to successfully prevent neuronal cell death in neurodegenerative diseases, however, Ayurveda has numerous plants with amazing properties. Some of the actions of herbs that are described in Ayurveda are quite new to the conventional medicine. Polyphenolic compounds, found in vegetables, fruits, dry fruits, plant extracts, wine, and tea, are natural antioxidants having useful prophylactic properties for the treatment of excitotoxic and oxidative cell death (Zhang *et al.*, 2010). One such plant, *Nardostachys jatamansi* D.C. (Valerianaceae) is an Indian herb used in the Ayurvedic system of medicine for centuries to treat mental ailments. *N. jatamansi* was mentioned by Susruta in Sushruta samhita, an ancient book written centuries ago, as nerve tonic. Earlier studies on *N. jatamansi* rhizomes showed high phenolic content and antioxidant properties (Rasheed *et al.*, 2010; Sharma & Singh, 2012). However, no study has been published yet at the cellular level, using markers of cellular stress and antioxidant enzymes against H_2O_2-induced oxidative stress. The present study investigated the protective and antioxidant effect of *N. jatamansi*-methanolic extract (NJ-MEx), *N. jatamansi*-ethanolic extract (NJ-EEx) and *N. jatamansi*-water extract (NJ-WEx) against H_2O_2-induced oxidative stress in C6 glioma cells using antioxidant enzymes, GSH content, lipid peroxidation, GFAP, HSP70 and mortalin as markers.

MATERIALS AND METHODS

Chemicals and reagents. The primary antibodies used for the Western blot and/or immunocytofluorescent analysis were monoclonal rabbit anti-GFAP (Sigma-Aldrich), mouse anti-HSP70 (Clone BRM-22, Sigma-Aldrich), mouse anti-Grp75 (mortalin) (Abcam), and mouse anti-α-tubulin (Clone AA13, Sigma-Aldrich). The secondary antibodies used were goat anti-mouse IgG:HRP (Sigma), anti-rabbit IgG:HRP (Bangalore genei), anti-mouse Alexa Fluor 568 (Invitrogen) and anti-rabbit Alexa Fluor 488 (Invitrogen). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma-Aldrich. The PCR reagents which include dNTP Mix, Random Hexamer Primer, 100bp ladder, Reverse Transcriptase and Taq DNA Polymerase were from Fermentas Life Sciences. Primers for synthesis of cDNA for GFAP, α-tubulin, HSP70 and mortalin were from Biolink, India. All other chemicals and reagents including FC reagent, hydrogen peroxide, EDTA, L-ascorbic acid, sodium hydroxide and solvents were procured in their purest form available commercially from Qualigens, Himedia and Sisco Research Laboratories (Indian companies).

Preparation of NJ-MEx, NJ-EEx and NJ-WEx. Rhizomes of *N. jatamansi* were procured from local Ayurvedic merchants and identified at the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, India. The rhizomes were powdered and 10 g of dry rhizome powder was suspended in 100 ml of methanol or ethanol or distilled water and kept stirring for 48 hours at 30 ± 5°C followed by filtration with muslin cloth and centrifugation at 17 000 × *g* for 15 min. The supernatant thus obtained was concentrated with a vacuum rotatory evaporator (Buchi, Switzerland) under reduced pressure and air dried to make powder. These were further diluted in respective solvent to make final concentration of 200 μg/ml each for NJ-MEx, NJ-EEx and NJ-WEx.

Cell culture and treatments. Rat C6 glioma cell line was obtained from the National Centre for Cell Sciences, Pune, India and maintained on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with streptomycin (100 U/ml), gentamycin (100 μg/ml), 10% FCS (Life Technologies) at 37°C and humid environment containing 5% CO_2. The H_2O_2 dose (IC50) for cytoprotective studies was calculated by treating cells with H_2O_2 (7.8 μM to 1000 μM diluted in medium) at 50% confluency for 24 h in serum-free medium. The C6 glioma cells were treated with NJ-MEx, NJ-EEx or NJ-WEx at concentration from 1.5 μg/ml to 50 μg/ml diluted in medium for 24 h at 30–40% confluency and then subjected to H_2O_2 (IC50 concentration) treatment for 24 h in serum-free medium. The medium of control culture without H_2O_2 and without extract was replaced with a fresh one. For enzyme assays, immunofluorescence, western blotting and RT-PCR the following four groups were used: untreated cultures, control; NJ-MEx-treated cultures, NJ-MEx; NJ-MEx-pretreated cultures before H_2O_2 treatment, NJ-MEx+H_2O_2; and H_2O_2-treated cultures, H_2O_2.

Cell viability assay. MTT was used to assess cell integrity and cytotoxicity by monitoring the uptake of the vital mitochondrial dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) by cell mitochondria (Hansen *et al.*, 1989).

Chemical standardization of NJ-MEX and nature of active components. NJ-MEx was subjected to preliminary phytochemical screening for alkaloids, amino acids, anthraquinone, flavonoids, phytosterols, saponins, steroids, tannins, triterpenoids and reducing sugars following the methods of (Harborne, 1998)). It was further subjected to thin-layer chromatography (TLC) using chloroform: methanol (24:1) as solvent. TLC plate was subjected to iodine vapours for observation.

Estimation of activities of antioxidant enzymes and levels of antioxidants

Preparation of whole cell extract. Cells were washed twice with ice-cold PBS (pH 7.4), harvested with PBS–EDTA (1 mM), and centrifuged at 400 × *g* for 10 min. The pellet so obtained was homogenized in 10 volumes of chilled homogenizing buffer containing 250 mM Sucrose, 12 mM Tris-HCl, 0.1 mM DTT, at pH 7.4 by repeated vortex mixing at 4°C for 10–15 minutes. Homogenates were centrifuged at 12 000 × *g* for 10 min at 4°C. The supernatant was transferred to chilled eppendorf tubes and used for the following estimations.

Estimation of catalase and CuZnSOD. Catalase activity was measured according to the method of (Aebi, 1984). The rate of decomposition of H_2O_2 by catalase was measured spectrophotometrically at 240 nm. The reaction mixture (1 ml) contained 0.8 ml phosphate buffer (0.2 M, pH 7.0) containing 12 mM H_2O_2 as substrate, 100 μl enzyme sample and distilled water to make up the volume. The decrease in absorbance/minute at 240 nm was recorded against H_2O_2-phosphate buffer as blank.

Superoxide dismutase was estimated according to the method of Kono (1978). This method is based on the principle of the inhibitory effects of SOD on the reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are generated by the autoxidation of hydroxylamine hydrochloride. The reduction of NBT was followed by an absorbance increase at 540 nm. In the test cuvette, the reaction mixture contained the following: 1.3 ml sodium carbonate buffer (50 mM, pH 10), 500 µl NBT (96 µM) and 100 µl triton X-100 (0.6%). The reaction was initiated by addition of 100 µl of hydroxylamine hydrochloride (20 mM), pH 6.0. After 2 min, 50 µl enzyme sample was added and the percentage inhibition in the rate of NBT reduction was recorded.

Reduced glutathione (GSH) and glutathione peroxidase (GPx). Total glutathione was measured as described by Sedlak & Lindsay (1968). For GSH content, 100 µl cell homogenate was mixed with 4.4 ml of 10 mM EDTA and 500 µl of trichloroacetic acid (50% w/v). Contents were centrifuged at 3000×*g* for 15 min at 4°C. The supernatant so obtained was mixed with 50 µl of 5,5'-dithiobis(2-nitrobenzoic acid) (10 mM) and absorbance was measured at 540 nm. Standard curve was prepared using pure glutathione.

Glutathione peroxidase activity was measured indirectly by monitoring the oxidation of NADPH. The reaction mixture (1 ml) containing 100 nM GSH, 15 nM NADPH and 15 nM H_2O_2 in potassium phosphate buffer (50 mM, pH 7.5) was mixed with sample (50 µl) and the change in absorbance was monitored at 340 nm. One unit of glutathione peroxidase activity is defined as 1 µmol of NADPH oxidized per min at pH 7.5 at 25°C.

Lipid peroxidation (LPx). The method of Buege & Aust (1978) was followed to measure the lipid peroxidation level. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) upon decomposition. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) that gives a red product having absorption maximum at 532 nm. A 100 µl sample was incubated with 100 µl each of $FeSO_4$ (1 mM), ascorbic acid (1.5 mM) and Tris/HCl buffer (150 mM, pH 7.1) in a final volume of 1 ml, made up with DDW, for 15 minutes at 37°C. The reaction was stopped by adding 1 ml of trichloroacetic acid (10% w/v). This was followed by addition of 2 ml thiobarbituric acid (0.375% w/v). After keeping in boiling water-bath for 15 min, contents were cooled and then centrifuged at 3000×*g* for 10 min at 4°C. The absorbance of supernatant so obtained was measured at 532 nm.

Immunocytochemistry. All cells, control and treated, were rinsed three times with ice-cold 0.1 M PBS and fixed with paraformaldehyde (4%) for 30 minutes. Permeabilization was carried out with 0.32% PBST for 15 minutes. Coverslips were washed thrice with 0.1% PBST followed by blocking with 5% NGS (Normal Goat Serum) prepared in 0.1% PBST for 1 h at room temperature. Cells were incubated with rabbit anti-GFAP (1:500), mouse anti-HSP70 (1:500) and mouse anti-mortalin (1:100), diluted in 0.1% PBST, for 24 h at 4°C in humid chamber. Coverslips were then washed with 0.1% PBST thrice. Secondary antibody anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 568 was applied diluted (1:200) in 0.1% PBST for 2 h at room temperature. Coverslips were washed three times with 0.1% PBST and final washing was given with 0.1 M PBS. These coverslips were then mounted on slides with anti-fading mounting media, Fluoromount (Sigma), and were observed under a Nikon E600 fluorescence microscope. Images were captured using a Cool Snap CCD camera and the pictures were analyzed using ImageJ 1.44p, NIH, USA.

Protein assay and Western blotting. All cells, treated and untreated, were rinsed twice with ice-cold PBS and harvested with PBS-EDTA. The cells from 25 cm^2 culture flasks of same group were pooled together and centrifuged at 400×*g* for 5 min at 4°C. Cell pellet was homogenized in 5 volumes of chilled homogenizing buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 100 µM $NaVO_4$, 1 mM PMSF and 0.5 mM DTT) and centrifuged for 10 min at 12000×*g* at 4°C. Protein content in the supernatant was determined by the Bradford method. Each homogenate was then diluted in homogenizing buffer to equal protein content in all the samples. The samples were mixed 1:1 with sample buffer (0.25 M Tris/HCl, pH 6.8, 20% glycerol, 4% SDS (sodium dodecyl sulfate), 10% β-mercaptoethanol and 1 mg bromophenol blue] and stored at –20°C. Samples containing 30 µg of protein were electrophoresed on one-dimensional 11% SDS/PAGE under standard denaturing conditions. The separated proteins were then blot transferred onto a PVDF membrane using a western blotting system at 25 V for 3 hours. Subsequently, membranes were blocked for 24 h at room temperature with 5% skimmed milk solution in TBST buffer (13.3 mM Tris. 0.8%, w/v, NaCl; pH 7.6) containing 0.1% Tween-20 (Sigma) and immediately incubated with rabbit anti-GFAP (1:3000), mouse anti-HSP70 (1:1000), mouse anti-mortalin (1:3000) and mouse anti-tubulin (1:3000) monoclonal antibodies overnight. After three washes for 10 minutes each in TBST, horse radish peroxidase-conjugated anti-mouse IgG (1:3000) and anti-rabbit IgG (1:3000) secondary antibodies were added for 2 h for hybridization with primary antibodies followed by three washes in TBST for 10 minutes and finally washed with TBS. Immunoreactive bands were visualized using the EZ-ECL Chemiluminescence Kit for HRP detection (Biological Industries, Israel) according to the manufacturer's instructions and exposed to Super RX Fuji X-ray film. The films were then developed and the antibody-labeling intensity (relative optical density) was analyzed using AlphaEaseFC 4.0. In order to account for potential variations in protein estimation and sample loading, expression of each protein was compared to that of α-tubulin in each sample. Tubulins are abundant cytoskeletal proteins that are highly expressed in brain and α-tubulin in particular is known to show stabilized expression in the adult stage of life. Each blot was stripped in 62.5 mM Tris, 2% SDS and 100 mM 2-mercaptoethanol (pH 6.7) for 30 min at 50°C and reprobed with an anti-α-tubulin antibody and relative optical density (ROD) measured as described above. The values for each sample were then expressed as ROD obtained using α-tubulin.

Reverse transcription-PCR. The C6 cells from 25 cm^2 culture flask were homogenized in TRI Reagent (Sigma). Briefly, cells were collected by centrifugation at 400×*g* and then lysed in 1 ml of TRI Reagent by repeated pipetting. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 minutes at room temperature, then 0.2 ml of chloroform was added. Sample was tightly covered and shaken vigorously for 15 seconds, and allowed to stand for 15 minutes at room temperature. The resulting mixture was centrifuged at 10000×*g* for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added and mixed. The sample was allowed to stand for 10 minutes at room temperature and then centrifuged at 12000×g

for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed by adding 1 ml of 75% ethanol. The sample was vortexed and centrifuged at 7 500 × *g* for 5 minutes at 4°C. The RNA pellet was briefly dried for 5 minutes in air. An appropriate volume of DEPC water was added and the RNA pellet dissolved with repeated pipetting with a micropipette at 55–60°C for 10–15 minutes.

Total RNA was reverse transcribed according to the manufacturer's instruction. Briefly, the cDNA was amplified in a 50-µl reaction containing primer pairs (each 1.0 µl): β-actin (forward primer 5″TCA CCCACACT-GTGCCCATCTACGA3', reverse primer 5'CAGCG-GAACCGC TCATTGCCAATGG3′); GFAP (forward primer 5'GGCGCTCAATGCTGGCTTCA3', reverse primer 5″TCTGCCTCCAGCCTCAGGTT3′); HSP70 (forward primer 5'GAGTTCAAGCGCAAACACAA3', reverse primer 5'CTCAGACTTGTCGCCAATGA3′); mortalin (forward primer 5'CAGTCTTCTGGTGGAT-TAAG3', reverse primer 5'ATTAGCACCGTCACG-TAACACCTC3′), 10× buffer (5.0 µl), cDNA (2.0 µl), 25 mM $MgCl_2$ (3.0 µl), 10 mM dNTPs (1.0 µl), and Taq polymerase (2.5 U). PCR amplification cycles consisted of denaturation at 94°C for 1 min, primer annealing at 57°C for 45 s and extension at 72°C for 45 s, for a total of 30 cycles followed by final extension at 72°C for 5 min. The PCR product was separated by electrophoresis on 2% agarose gels.

Statistical analysis. Results were expressed as the mean ± S.E.M. from at least three independent experiments. Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Bonferroni test was used according to the statistical program SigmaStat (Jandel Scientific, Chicago, IL, USA).

RESULTS

NJ-MEx protected C6 glioma cells against H_2O_2 cytotoxicity

In the present study, rat C6 glioma cells were cultured in the presence of NJ-MEx, NJ-EEx or NJ-WEx to determine their protective effect against H_2O_2-induced cytotoxicity. The IC50 of H_2O_2 for C6 cells was evaluated with MTT mitochondrial function assay (Fig. 1a). A dose dependent cell death was observed and an IC50 concentration of 125 µM was selected for further studies. Decrease in cell density with increasing doses of H_2O_2 in phase contrast micrographs also supported the MTT results (figure not shown). For cytoprotective effect, C6 cells were treated with NJ-MEx, NJ-EEx or NJ-WEx before subjecting to H_2O_2 treatment (IC50 dose). Out of the three extracts of *N. jatamansi* used in the present study, NJ-MEx significantly prevented the H_2O_2-induced cell death at 6.2 µg/ml which increased cell viability to 70.7 ± 6.4% (Fig. 1b) ($p < 0.05$). NJ-EEx and NJ-WEx also protected the cells at 12.5 µg/ml with cell viability of 58.7 ± 5.1% ($p < 0.05$) and 60.2 ± 5.4% ($p < 0.05$) (Fig. 1c, 1d). The higher concentrations of NJ-MEx, NJ-EEx and NJ-WEx exhibited cytotoxic effect (data not shown). Because of its highest protective effect, only NJ-MEx was selected for further investigation.

Nature of bioactive components of NJ-MEx

Preliminary screening for phytochemicals demonstrated the presence of flavonoids, steroids, tannins, triterpenoids, saponins and alkaloids in NJ-MEx (Table 1). In the TLC profile of NJ-MEx generated with the chloroform: methanol (24:1) solvent system, eight spots with

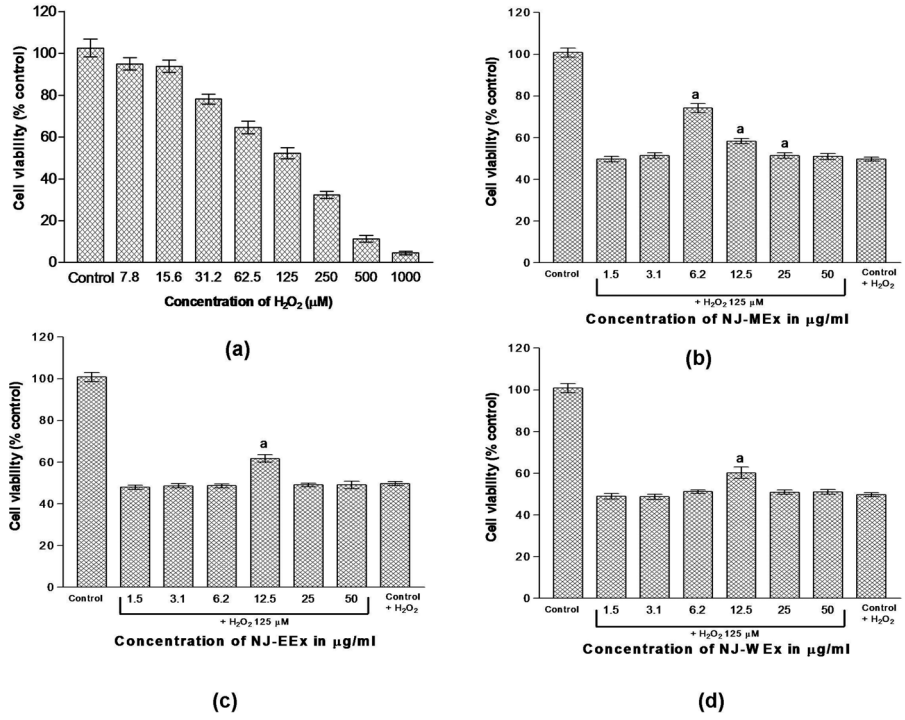

Figure 1. Cytotoxicity of H_2O_2 against C6 glioma cells
(**a**) Dose-dependent cytotoxic effect of H_2O_2 on C6 glioma cell viability. Neuroprotective assay of *N. jatamansi* extracts against H_2O_2 cytotoxicity (**b–d**). (**b**) Effect of pretreatment with NJ-MEx on H_2O_2-induced cytotoxicity (**c**) Effect of pretreatment with NJ-EEx on H_2O_2-induced cytotoxicity (**d**) Effect of pretreatment with NJ-WEx on H_2O_2-induced cytotoxicity. Glial cell viability was measured using MTT assay after 24 h of incubation with H_2O_2. The data represents mean ± S.E.M. from four independently experiments. a, Statistically significant difference between H_2O_2 cultures and NJ-MEx+H_2O_2 cultures.

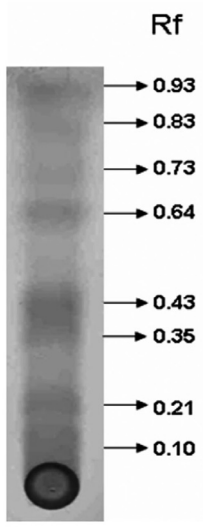

Figure 2. Analysis of NJ-MEx by thin layer chromatography. Chloroform: methanol (24:1) solvent system revealed eight different spots. These spots were visualized using iodine vapors.

R_f values of 0.10, 0.21, 0.35, 0.43, 0.64, 0.73, 0.83 and 0.93 were observed (Fig. 2).

Effect of NJ-MEx and H_2O_2 treatment on the activities of Cu-ZnSOD, Gpx and catalase and the content of GSH and LPx

The activities of Cu-ZnSOD, catalase, GPx, and the level of GSH and LPx observed in five independent experiments are shown in Table 2. The activity of catalase was significantly reduced in H_2O_2-treated cultures as compared to control ($p<0.05$). A significant increase of catalase activity was observed in NJ-MEx cultures in comparison to control ($p<0.05$). Further, the activity of catalase was significantly higher in NJ-MEx+H_2O_2 cultures with respect to H_2O_2 cultures ($p<0.05$). A similar trend was observed for Cu-ZnSOD, i.e., it was significantly decreased in H_2O_2 cultures compared to control ($p<0.05$). NJ-MEx treatment significantly increased the Cu-ZnSOD activity in comparison to control ($p<0.05$). The Cu-ZnSOD activity was higher in the NJ-MEx+H_2O_2 than in the H_2O_2 cultures.

The activity of GPx was found to decrease significantly ($p<0.05$) following H_2O_2 treatment. However, NJ-MEx pretreatment of H_2O_2 cultures significantly increased GPx activity as compared to control cultures ($p<0.05$).

The H_2O_2 cultures had a significantly lower level of GSH as compared to control ($p<0.05$). In NJ-MEx cultures, GSH level increased considerably ($p<0.05$) as compared to control. An increased GSH content was also observed in NJ-MEx+H_2O_2 cultures compared to H_2O_2 cultures ($p<0.05$), indicating a protective effect of NJ-MEx.

A considerable increase in lipid peroxidation was observed in H_2O_2 cultures as compared to control ($p<0.05$), but a pretreatment with NJ-MEx decreased lipid peroxidation significantly ($p<0.05$). The NJ-MEx treatment also decreased lipid peroxidation in cultures not subjected to H_2O_2 treatment, confirming its antioxidant effect.

Table 1. Analysis of phytochemicals in NJ-MEx

Phytochemicals	NJ-MEx
Flavonoids	+
Steroids	+
Tannins	+
Anthroquinones	-
Triterpenoids	+
Amino acids	-
Saponins	+
Phytosterols	-
Alkaloids	+

"+", presence; "–", absence.

Effect of NJ-MEx on mRNA and protein expressions of GFAP in C6 cells exposed to H_2O_2

GFAP is an intermediate filament protein specific to glial cells in the CNS. We examined expression of GFAP by immunofluorescence (Fig. 3a–d). The increased expression of GFAP in H_2O_2 cultures was attenuated be pretreatment with NJ-MEx ($p<0.05$) (Fig. 3g). Expression of GFAP remained unchanged in NJ-MEx cultures, indicating no stress. GFAP and α-tubulin labeling in C6 glioma cell cultures are shown in Fig. 3e. The expression of GFAP normalized against α-tubulin is illustrated in Fig. 3h. The cytoprotection was confirmed at the protein level by western blotting. The increase in GFAP due to H_2O_2, indicating oxidative stress, was alleviated considerably with NJ-MEx pretreatment ($p<0.05$). The protection due to NJ-MEx was further confirmed at the transcript level using RT-PCR (Fig. 3f, i). The GFAP mRNA level was significantly lower in NJ-MEx+H_2O_2 cultures than in H_2O_2 cultures ($p<0.05$). No significant difference in GFAP expression was observed between control and NJ-MEx cultures using immunofluorescence, western blotting or RT-PCR.

Table 2. Effect of cytoprotective activity of NJ-MEx on antioxidant scavenger system in C6 glioma cell cultures.

Groups	Cu-Zn-SOD (U g tissue^{-1})	GPx (U g tissue^{-1})	Catalase U g tissue^{-1}	GSH (mg g tissue^{-1})	LPx (mg dl^{-1})
Control	13.29 ± 0.79	14.23 ± 0.86	2.18 ± 0.39	2.95 ± 0.19	10.26 ± 0.57
N. jatamansi	14.54 ± 1.56[a]	15.86 ± 1.05[a]	2.38 ± 0.45[a]	3.16 ± 0.35[a]	8.09 ± 0.31[a]
H_2O_2	7.63 ± 0.96[b]	8.59 ± 0.72[b]	1.30 ± 0.34[b]	1.89 ± 0.18[b]	18.49 ± 0.68[b]
N. jatamansi + H_2O_2	10.56 ± 0.84[c d]	12.93 ± 0.95[c d]	1.89 ± 0.28[c d]	2.76 ± 0.15[c d]	14.82 ± 0.43[c d]

The data represents mean ± S.E.M. of activities of enzymes, and reduced glutathione and lipid peroxidation content measured in homogenates obtained from cells of culture dishes (n = 5) derived from three independently prepared cultures. The values having $P<0.05$ are considered significant. [a], Statistically significant change in CP-MEx treated cultures with respect to control cultures; [b], statistically significant change in H_2O_2 treated cultures with respect to the control cultures; [c], statistically significant change in CP-MEx + H_2O_2 treated cultures with respect to the CP-MEx treated cultures; [d], statistically significant change in H_2O_2 treated cultures with respect to the CP-MEx + H_2O_2 treated cultures; [e], statistically significant change in Quercetin + H_2O_2 treated cultures with respect to the H_2O_2 treated cultures.

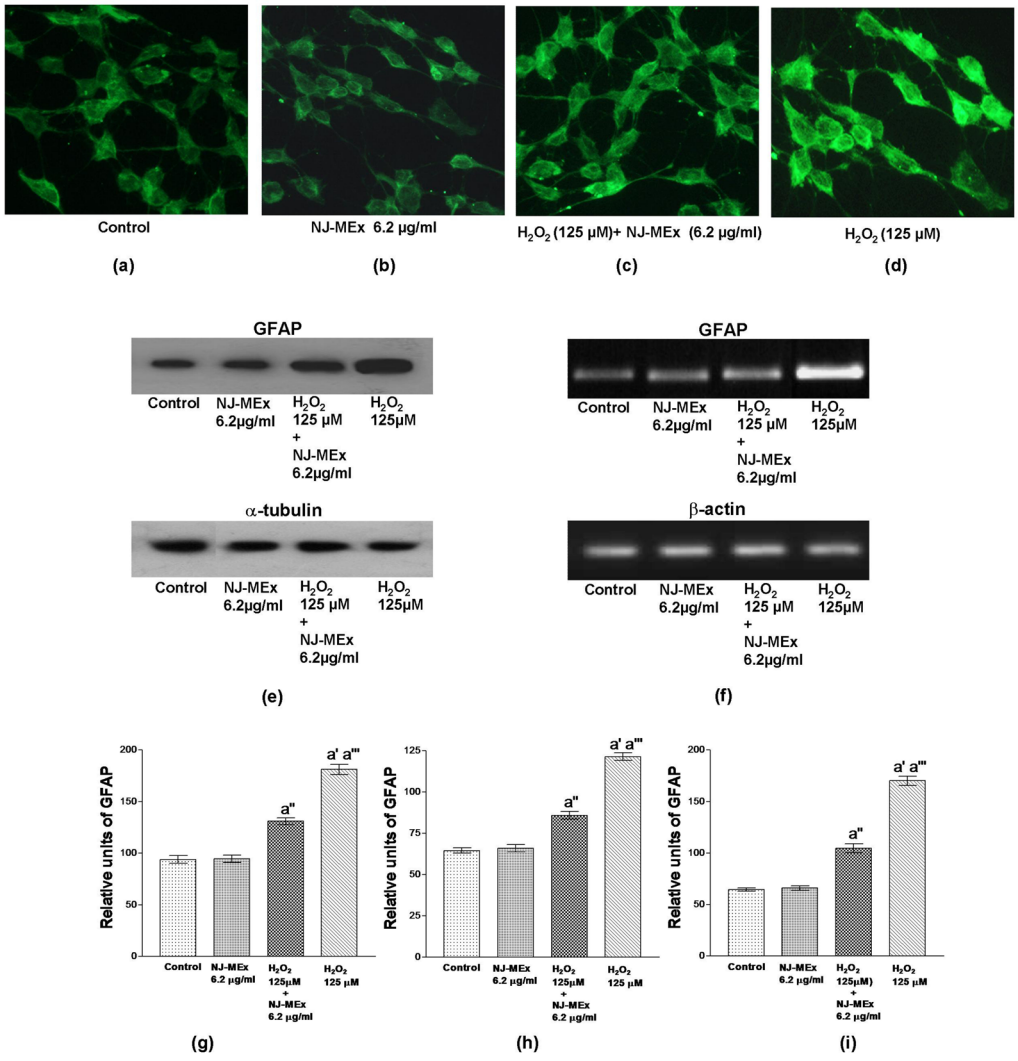

Figure 3. Localization of GFAP in C6 Glioma cells by immunofluorescence (a–d).
(**a**) Untreated control (**b**) NJ-MEx treated (**c**) NJ-MEx+H_2O_2 treated (**d**) H_2O_2 treated. Cells grown on coverslips (n = 4) for 4 days were fixed and stained for GFAP (Alexa Fluor 488) immunoreactivity.
Western blotting and Representative reverse transcription-polymerase chain reaction (RT-PCR) of GFAP (e–f).
(**e**) Representative western blot hybridization signals using antibodies specific for GFAP and α-tubulin from untreated control, NJ-MEx treated, NJ-MEx+H_2O_2 treated, H_2O_2 treated cultures. (**f**) Representative reverse transcription-polymerase chain reaction (RT-PCR) showing GFAP and β-actin expression in untreated control, NJ-MEx treated, NJ-MEx+H_2O_2 treated, H_2O_2 treated C6 glioma cells.
Relative intensity analyses of GFAP in immunofluorescence, western blot hybridization and RT-PCR (g–i)
(**g**) Relative intensity of GFAP immunofluorescence performed by ImageJ 1.44p. (**h**) Relative optical density of GFAP in western blot hybridization for each group expressed as percentage of α-tubulin (**i**) Relative optical density of GFAP expression in RT-PCR for each group expressed as percentage of β-actin. Relative units of GFAP were calculated for immunofluorescence by ImageJ 1.44p and for western blotting and RT-PCR by AlphaEaseFC 4.0. The values having $p < 0.05$ are considered significant. a', statistically significant difference H_2O_2 treated cultures and control cultures; a'', statistically significant difference between NJ-MEx + H_2O_2 treated cultures and NJ-MEx treated cultures; a''', statistically significant difference between H_2O_2-treated cultures and NJ-MEx + H_2O_2 treated cultures.

Effect of NJ-MEx on HSP70 expression in C6 cells exposed to H_2O_2

After a variety of nervous system insults, HSP70 is synthesized at especially high levels and is present in the cytosol, nucleus and endoplasmic reticulum (Giffard *et al.*, 2004). We examined C6 cells for HSP70 expression following exposure to H_2O_2 in the presence or absence of NJ-MEx using immunofluorescence, western blotting and RT-PCR (Fig. 4a–f). The Protective effect of NJ-MEx was confirmed by all three parameters as the increased level of HSP70 in H_2O_2 cultures was significantly reduced with pretreatment of NJ-MEx ($p < 0.05$) (Fig. 4 g–i).

Effect of NJ-MEx on mRNA and protein level of mortalin in C6 cells treated with H_2O_2

Mortalin is a heat-shock cognate protein whose level has been reported to increase under the stress (Taurin *et al.*, 2002). To study the perinuclear expression of mortalin, C6 glioma cells were subjected to immunofluorescence, western blotting and RT-PCR (Fig. 5a–f). The level of mortalin was significantly higher in H_2O_2 cultures as compared to control (Fig. 5g) ($p < 0.05$). We observed a reduction of the level of mortalin in NJ-MEx pretreated H_2O_2 cultures by immunofluorescence, clearly indicating protection (Fig. 5g) ($p < 0.05$). There was no significant difference in mortalin expression between control and

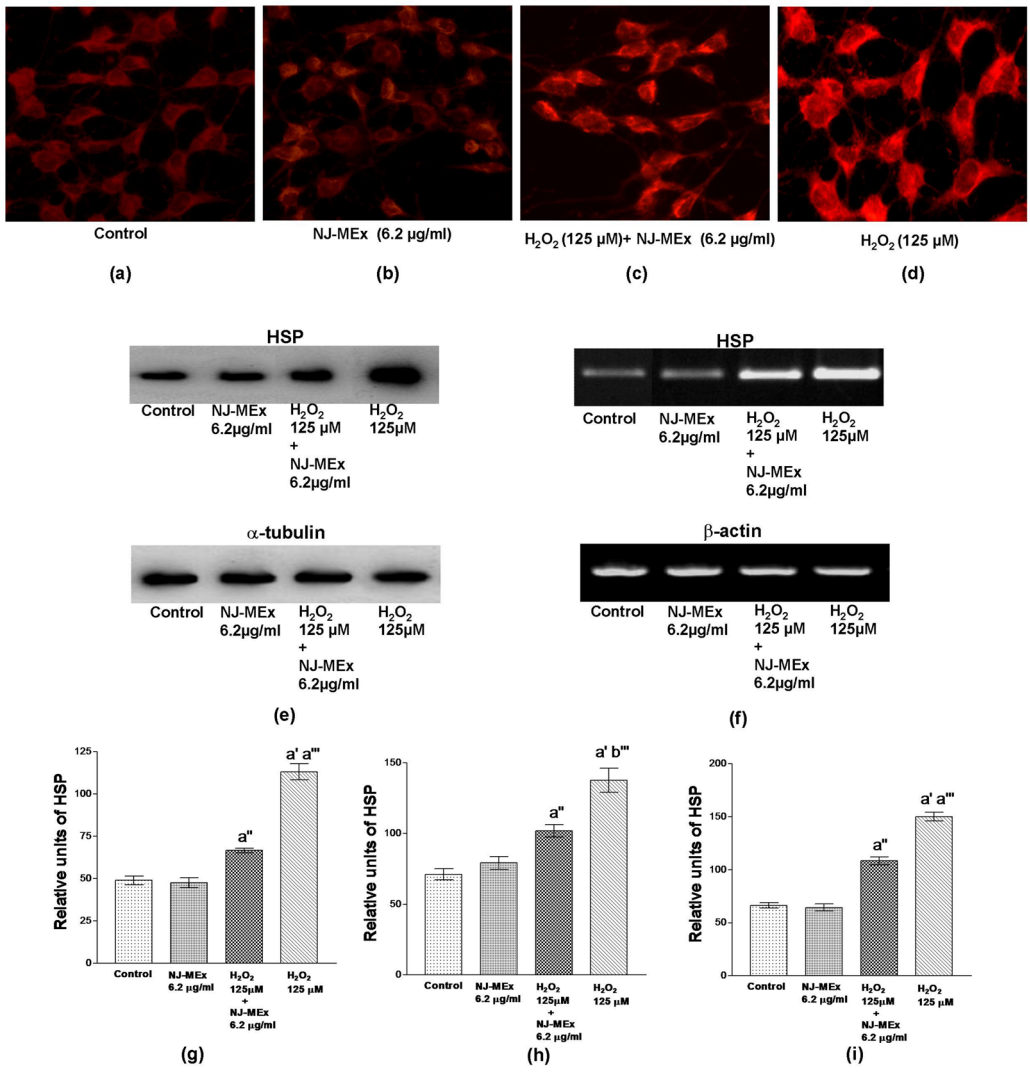

Figure 4. Distribution of HSP70 in C6 Glioma cells by immunofluorescence (a–d).
(**a**) Untreated control (**b**) NJ-MEx treated (**c**) NJ-MEx+H_2O_2 treated (**d**) H_2O_2 treated. Cells grown on coverslips (n=4) for 4 days were fixed and stained for HSP70 (Alexa Fluor 488) immunoreactivity.
Western blotting and Representative reverse transcription-polymerase chain reaction (RT-PCR) of HSP70 (e–f)
(**e**) Representative western blot hybridization signals using antibodies specific for HSP70 and α-tubulin from untreated control, NJ-MEx treated, NJ-MEx+H_2O_2 treated, H_2O_2 treated cultures. (**f**) Representative reverse transcription-polymerase chain reaction (RT-PCR) showing HSP70 and β-actin expression in untreated control, NJ-MEx treated, NJ-MEx+H_2O_2 treated, H_2O_2 treated C6 glioma cells.
Relative intensity analyses of HSP70 in immunofluorescence, western blot hybridization and RT-PCR (g–i).
(**g**) Relative intensity of HSP70 immunofluorescence performed by ImageJ 1.44p. (**h**) Relative optical density of HSP70 in western blot hybridization for each group expressed as percentage of α-tubulin (**i**) Relative optical density of HSP70 expression in RT-PCR for each group expressed as percentage of β-actin. Relative units of HSP70 were calculated for immunofluorescence by ImageJ 1.44p and for western blotting and RT-PCR by AlphaEaseFC 4.0. The values having $p<0.05$ are considered significant. a', statistically significant difference H_2O_2 treated cultures and control cultures; a", statistically significant difference between NJ-MEx + H_2O_2 treated cultures and NJ-MEx treated cultures; a"', statistically significant difference between H_2O_2-treated cultures and NJ-MEx + H_2O_2 treated cultures.

NJ-MEx treated cells (Fig. 5g). The data from western blotting and RT-PCR further supported the protective effect of NJ-MEx (Fig. 5h, i) ($p<0.05$).

DISCUSSION

Oxidative stress occurs when the cellular homeostasis, normally involving a fine balance between free radical generation and their detoxification by cellular antioxidants, is disturbed. Earlier studies have pointed to oxidative stress as a major reason for neuronal cell death leading to neurodegenerative disorders like ischemia, PD, AD and HD (Axelsen *et al.*, 2011; Caviness *et al.*, 2011; Chen, 2011; Doeppner and Hermann, 2010). The antioxidant machinery of the cell (natural antioxidant molecules and enzymes) scavenges and reduces free radicals production, but sometimes it may not be sufficient to manage this stress, initiating extensive damage to biological macromolecules, proteins, nucleic acids and lipids, ultimately leading to tissue damage (Halliwell, 2012; Halliwell & Gutteridge, 1999). Therefore, inducing antioxidant defense machinery through herbal means would be an effective strategy to prevent this harmful oxidative injury.

Nardostachys jatamansi extracts have earlier been shown to have antioxidant properties with chemical antioxidant assays (Rasheed *et al.*, 2010; Sharma & Singh, 2012). However, there is a need to authenticate this activity at

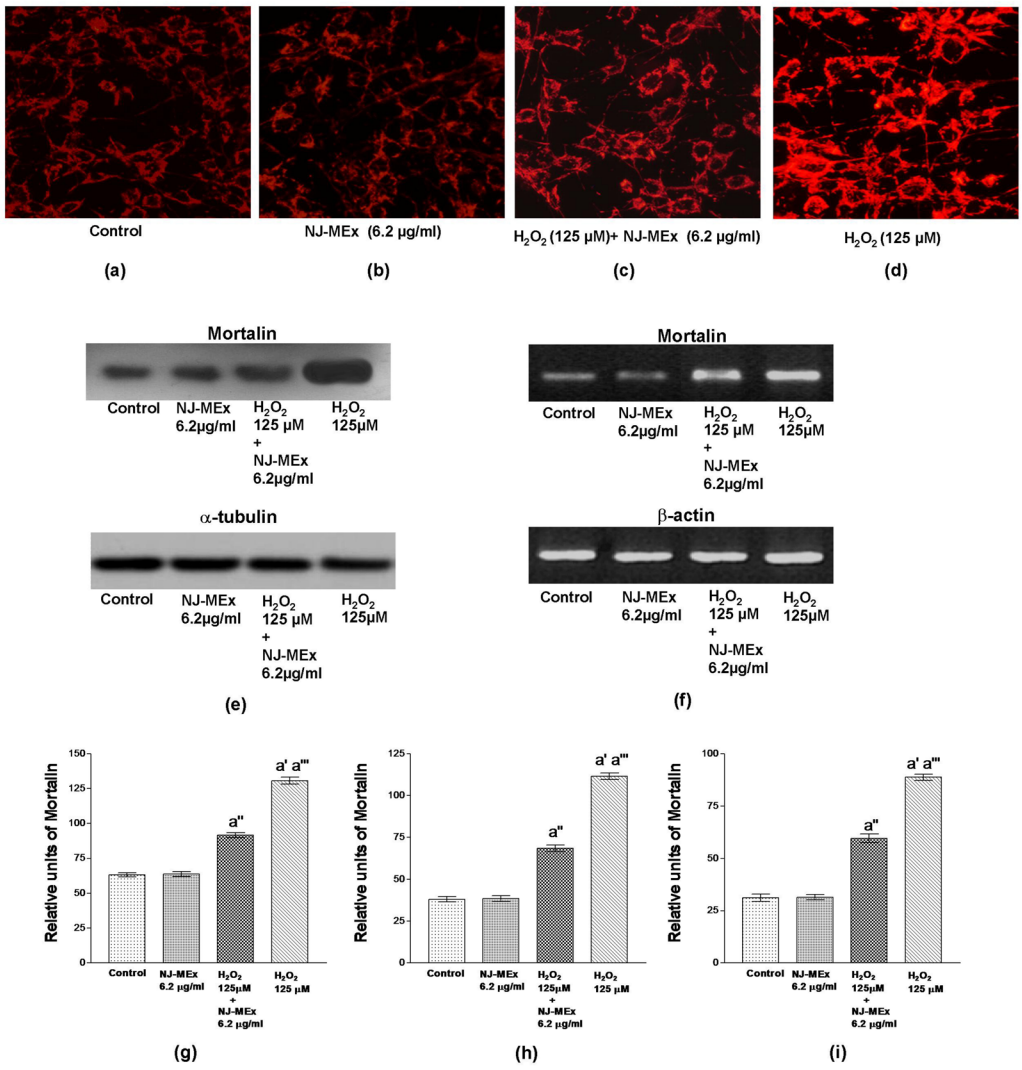

Figure 5. Localization of mortalin in C6 Glioma cells by immunofluorescence (a–d).
(**a**) Untreated control (**b**) NJ-MEx treated (c) NJ-MEx+H_2O_2 treated (**d**) H_2O_2 treated. Cells grown on coverslips (n = 4) for 4 days were fixed and stained for mortalin (Alexa Fluor 488) immunoreactivity.
Western blotting and Representative reverse transcription-polymerase chain reaction (RT-PCR) of mortalin (e–f).
(**e**) Representative western blot hybridization signals using antibodies specific for mortalin and α-tubulin from untreated control, NJ-MEx treated, NJ-MEx+H_2O_2 treated, H_2O_2 treated cultures. (**f**) Representative reverse transcription-polymerase chain reaction (RT-PCR) showing mortalin and β-actin expression in untreated control, NJ-MEx treated, NJ-MEx+H_2O_2 treated, H_2O_2 treated C6 glioma cells.
Relative intensity analyses of mortalin in immunofluorescence, western blot hybridization and RT-PCR (g–i).
(**g**) Relative intensity of mortalin immunofluorescence performed by ImageJ 1.44p. (**h**) Relative optical density of mortalin in western blot hybridization for each group expressed as percentage of α-tubulin (**i**) Relative optical density of mortalin expression in RT-PCR for each group expressed as percentage of β-actin. Relative units of mortalin were calculated for immunofluorescence by ImageJ 1.44p and for western blotting and RT-PCR by AlphaEaseFC 4.0. The values having $p < 0.05$ are considered significant. a', statistically significant difference H_2O_2 treated cultures and control cultures; a", statistically significant difference between NJ-MEx + H_2O_2 treated cultures and NJ-MEx treated cultures; a'", statistically significant difference between H_2O_2-treated cultures and NJ-MEx + H_2O_2 treated cultures.

the cellular level by quantitating stress markers and the activity of antioxidant machinery. Therefore, the present study was planned to examine the antioxidant activity of *N. jatamansi* extracts at a cellular level. Cytoprotection against oxidative stress produced by H_2O_2 in C6 glioma cell line was used as a cellular antioxidant assay for plant extracts. The possible mechanism of cytoprotection was also addressed by evaluating expression of selected proteins known to be induced by oxidative stress.

H_2O_2 is well known to act as a potent inducer of reactive oxygen species (ROS) and capable of inducing cell injury both *in vitro* and *in vivo* (Kim *et al.*, 2012; Terashvili *et al.*, 2012). Particularly, in C6 glioma cells, H_2O_2 is known to induce oxidative damage, leading to lipid peroxidation, ROS generation, GSH depletion and reduction in antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) activity preceding cell death (Brenner *et al.*, 2010; Gulden *et al.*, 2010). Hence, H_2O_2 has been extensively used in studying the effects of antioxidant phytochemicals in C6 glioma cells (Brenner *et al.*, 2010; Gulden *et al.*, 2010). In the present work, C6 glioma cells were treated with H_2O_2 at a concentration of 125 µM (IC50 concentration, Fig. 1a). This concentration was further used in antioxidant assays and expression analysis of GFAP, HSP and mortalin. To ensure that there is no induction of stress proteins, only a non-toxic concentration of the plant extract was chosen for key experiments. The results demonstrated

that none of the *N. jatamansi* extracts assayed produced a toxic effect (after 24 h of treatment) at concentrations of 50 μg/ml or less (Fig. 1b–d).

NJ-EEx and NJ-WEx had very low cytoprotective effect, whereas NJ-MEx exhibited a potent cytoprotective activity against H_2O_2-induced oxidative injury. Such cytoprotection is in accordance with earlier studies on the antioxidant potential of *N. jatamansi* (Rasheed *et al.*, 2010; Sharma & Singh, 2012; Subashini *et al.*, 2007) Beside *N. jatamansi* extracts, *Ginkgo biloba* and Ashwagandha leaf extracts have also been reported to have cytoprotective effect against H_2O_2-induced oxidative damage in glial cells (Altiok *et al.*, 2006; Konar *et al.*, 2011).

The majority of ROS are normally produced during oxidative processes happening in live cells. To combat this stress, cells have a battery of antioxidant enzymes and antioxidant molecules such as glutathione to scavenge superoxide radical and hydrogen peroxide. If ROS go un-scavenged, impairment of physiological functions occurs, ultimately leading to cell death and tissue damage (Halliwell, 2012; Halliwell & Gutteridge, 1999). Catalase, SOD and GPx, along with other antioxidants of enzymatic and non-enzymatic nature, play crucial function in saving the cell from oxidative damage. In this study, a clear increase in lipid peroxidation was linked with H_2O_2 dose. Further, considerable reduction in catalase, SOD, GSH and GPx level was observed following treatment with H_2O_2, signifying damage in internal antioxidant defense system of the cell. However, pretreatment of C6 cells with NJ-MEx greatly mitigated the H_2O_2 effects. These findings apparently indicate protection of the endogenous antioxidant defense system.

To reveal the possible cytoprotective mechanism of NJ-MEx, immunofluorescence, western blotting and RT-PCR were used to examine the expression of three stress indicator proteins of the cell, GFAP, HSP70 and mortalin.

GFAP is a type-III intermediate filament, first identified in astrocytes (Eng, 1985). It is highly conserved throughout vertebrate evolution, suggesting that it plays a critical function in the central nervous system. In the current study, elevated expression of GFAP upon H_2O_2 exposure could be attributed to reactive gliosis and its stimulation. Augmented levels of intermediate filament proteins, particularly GFAP by reactive astrocytes are the best known characteristics of reactive gliosis. Such upregulation in intermediary filaments is known to occur in response to most brain injuries (Pekny & Nilsson, 2005). Such variation in protein levels is primarily controlled by transcriptional regulation (Landry *et al.*, 1990). Our observations illustrated that NJ-MEx directly down-regulated GFAP expression at both protein and mRNA levels in C6 glioma cells, indicating a possible mechanism of the cytoprotective effect of the extract in C6 cells.

Heat shock proteins are essential components of the response to a wide variety of toxic conditions and have attracted a great interest being essential for cell survival. The synthesis of HSP in mammalian cells is activated not only under heat shock, but also in the conditions of disturbed cellular homeostasis, heavy metal toxicity and drug cytotoxicity (Calabrese *et al.*, 2001; Colombrita *et al.*, 2003; Scapagnini *et al.*, 2002). HSP70 is known to play a protective role in animal and cellular models of neurotoxicity such as ischemia (Sauer *et al.*, 2001), trauma (Schipke *et al.*, 2001) and Alzheimer's disease (Chow & Brown, 2007). Increased HSP70 expression of H_2O_2-treated cultures was significantly attenuated by NJ-MEx pretreatment, substantiating its protective role.

In normal cell mortalin is scattered throughout the cytoplasm but its localization changes in immortal cells to the perinuclear region (Wadhwa *et al.*, 1993). An enhancement of mortalin expression is a type of stress response or an adaptive response to H_2O_2 treatment (Osorio *et al.*, 2007). Results in the present study show a significant increase in the mortalin expression, following H_2O_2 exposure. The pre-treatment of H_2O_2-treated cultures with NJ-MEx significantly reduced mortalin expression.

Glioblastoma is the most common of brain tumours and is very difficult to treat. Despite the use of different treatment regimens, which include surgery, radiotherapy, and chemotherapy, most patients do not live longer than a year after diagnosis. Based on our present data, we suggest the use of NJ-MEx and its constituents as effective glioma therapy.

CONCLUSION

Of the extracts of *N. jatamansi*, only NJ-MEx exhibited significant cytoprotective activity against H_2O_2-induced oxidative damage by induction of endogenous antioxidant enzymes, increase in glutathione level and prevention of direct membrane damage due to lipid peroxidation. These observations were further substantiated by the reduction in the level of GFAP, HSP70 and mortalin with pretreatment of C6 cells with NJ-MEx before subjecting to H_2O_2. Further study is needed to investigate other cytoprotective proteins which could be involved in the cytoprotection mechanism.

Conflict of interest

The authors have declared no conflicts of interest.

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