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Neuroprotective Effect of *Convolvulus pluricaulis* Methanol Extract on Hydrogen Peroxide Induced Oxidative Stress in Human IMR32 Neuroblastoma Cell Line

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Authors' contributions

This work was carried out in collaboration between all authors. Author KD designed the study, wrote the protocol, and performed the experiments. Authors VD, SSK and JS wrote the first draft of the manuscript managed the analyses of the study. Author GB managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The present study aimed to evaluate and ascertain the protective role of methanolic/ethanolic/water extracts of *Convolvulus pluricaulis* against H_2O_2 induced cytotoxicity in IMR32 Neuroblastoma cell line as model system and identify the factor responsible for the protective effect.
Study Design: Experimental study.
Place and Duration of Study: Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar & Department of Biotechnology, DAV College, Amritsar, PuCPab, between August 2010 and March 2012.
Methodology: Firstly, cytotoxic dose of H_2O_2 and non-toxic dose of methanolic, ethanolic and water extracts of *C. pluricaulis* (CP-MEx, CP-EEx and CP-WEx respectively) was determined by MTT assay. Protective effect of CP-MEx, CP-EEx and CP-WEx was determined using quercetin as a positive control. The expression of IMR32 cytoskeletal marker, Neurofilament (NF-200) and stress markers, Heat shock protein (HSP70) and

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(glucose regulated protein 75, Grp75) Mortalin studied by immunofluorescence and RT-PCR results. The level of antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase, direct scavenger of free radicals, Glutathione and lipid peroxidation were analysed by their standard procedures.

Results: The results showed that quercetin, CP-MEx, CP-EEx and CP-WEx displayed cytoprotective activity in IMR32 cells. Out of tested extracts CP-MEx significantly decreased hydrogen peroxide-induced cell death. Significant decrease in NF-200, HSP70 and Mortalin expression was observed in CP-MEx+H_2O_2 treated cultures as compared to H_2O_2 treated. Catalase, superoxide dismutase, glutathione peroxidase, Glutathione levels significantly increased in Quercetin and CP-MEx treated cultures. Lipid peroxidation was significantly decreased in both Quercetin and CP-MEx treated cultures.

Conclusions: The present work establishes the protective effect of CP-MEx on IMR 32 Human Neuroblastoma cell line which is as much as by quercetin. The cytoprotective effect of CP-MEx was due to induction of antioxidant machinery of the cell hence holds therapeutic value in the treatment and/or prevention of neurodegenerative disorders of oxidative stress.

Keywords: Convolvulus pluricaulis; IMR32 human neuroblastoma; hydrogen peroxide; antioxidnat enzymes; NF-200; HSP70; mortalin.

1. INTRODUCTION

Neurodegenerative disorders are chronically progressive group of diseases involving nerve cells death. Majority of these diseases including Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD) and cerebral ischemia are result of oxidative stress [1-3]. The Brain is a very active organ of human body weighing only 2% of the body weight but it consumes 20% of body oxygen and 25% of body glucose at rest [4]. Since reactive oxygen species (ROS) produced in any tissue is directly proportional to its oxygen consumption which further increases with intellectual process like thinking planning and reasoning [5], the brain is continuously under oxidation/antioxidation process which makes it prone to oxidative damage. Several antioxidant mechanisms are available within brain to combat ROS. Brain cells have catalase and glutathione peroxidase in their cytosol which hydrolyzes H_2O_2 and reduces organic hydroperoxides respectively. Neuronal mitochondria have superoxide dismutase for converting $^{\cdot}O_2^-$ to H_2O_2 which is further metabolized by catalase and thus preventing the formation of neurotoxic and inflammatory cytokine-inducing $^{\cdot}ONOO^-$ from $^{\cdot}O_2^-$ and $^{\cdot}NO$ [6]. Although, brain cells have defense mechanisms for dealing with ROS yet it has been practically found that when level of ROS goes unusually high or antioxidant defense goes low, cells bear oxidative damage ultimately leading to neurodegenerative disorders [7]. Exogenous H_2O_2 may also produce ROS beyond the capacity of cellular defence system leading to apoptotic cell death [8]. So far there are no effective drugs in conventional system which can effectively combat or check the onset or progression of neurodegenerative diseases. But, Ayurveda has used many herbs for centuries to successfully treat and prevent neurodegenerative diseases. There are very few scientific studies available for neuroprotective effect of these plants. Therefore search for novel therapies with little or no side effects is increasing day by day.

Medhya rasayana are Ayurvedic drugs known to improve physical and mental health and immunity of the body. *Convolvulus pluricaulis* Choisy is among frequently prescribed rasayanas for improvement of learning and memory and treat mental health problems. There

are hundreds of products in India where *C. pluricaulis* is used as a nerve tonic, stress reliever and recommended as a best memory enhancer. Evaluation of *C. pluricaulis* plants for their biological effect especially the memory enhancing and neuroprotection has recently attracted the attention of scientists. It is also reported that daily dose of 150 mg/kg *C. pluricaulis* for 3 months significantly alleviated aluminium induced neurodegeneration [9]. *C. pluricaulis* has also been shown to improve learning and memory in young and old mice [10]. The present study is aimed to investigate the neuroprotective and antioxidant effect of methanolic, ethanolic and water extracts of *C. pluricaulis* (CP-MEx, CP-EEx and CP-WEx) on human IMR32 Neuroblastoma cell line under oxidative stress induced by hydrogen peroxide (H_2O_2). We have investigated that CPMEx protected H_2O_2 induced oxidative stress and cell death in IMR32 cells by increasing the level of the catalase, superoxide dismutase, glutathione peroxidase and glutathione. We further revealed that level of NF-200 (Neurofilament) and stress markers HSP70 (Heat shock protein) and Mortalin (glucose regulated protein 75, Grp75) reduced in CPMEx pretreated cells with respect to H_2O_2 treated cell.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

For western blotting and immunocytofluorescence, primary antibodies were monoclonal anti-HSP70 (Clone BRM-22, Sigma-Aldrich) and anti-Grp 75 (Mortalin) (Abcam), and anti-α-tubulin (Clone AA13, Sigma-Aldrich). Anti-mouse IgG:HRP (Bangalore genei) and anti-mouse Alexa Fluor 568 (Invitrogen) were used as secondary antibodies. The 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT), Quercetin and 1'-1" Diphenyl-2'-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich. The PCR reagents including dNTP Mix, Random Hexamer Primer, 100bp ladder, Reverse Transcriptase and Taq DNA Polymerase were purchased from Fermentas, Thermo Fisher Scientific. Primers for synthesis of cDNA for α-tubulin, HSP and Mortalin were prepared from Biolink, India. All other chemicals and reagents were procured in their purest form available commercially from Indian companies.

2.2 Preparation of CP-MEx, CP-EEx and CP-WEx

Dried whole plants of *C. pluricaulis* were procured from local Ayurvedic Merchants and got identified from The Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, India. These were then powdered and 10 g of dry rhizome powder each was suspended separately in 100 ml of methanol/ethanol/ distilled water and kept stirring for 48 hours at 30 ± 5ºC followed by filtration under sterile conditions. The filtrates thus obtained were concentrated with a vacuum rotary evaporator (Buchi, Switzerland) at temperature of 35ºC and pressures 280, 170 and 60 mbar for methanolic, ethanolic, water extracts respectively. The concentrated extracts thus obtained are air dried to make powder. These were further diluted in respective solvent to give final concentration of 50 µg/ml each for CP-MEx, CP-EEx and CP-WEx.

2.3 Cell Culture and Treatments

Human IMR32 Human Neuroblastoma cell line was obtained from NCCS, 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 37ºC and

humid environment containing 5% CO_2. The H_2O_2 dose (IC50) for neuroprotection studies was calculated by treating cells with H_2O_2 (7.5 µM to 1000 µM diluted in medium) at 50% confluency for 24 hours in serum free medium. To obtain the cytotoxicity profile and non-toxic dose of *C. pluricaulis* extracts were tested at higher doses from 25 to 2000 µg/ml. The IMR32 Human Neuroblastoma cells were treated with CP-MEx, CP-EEx, CP-WEx and quercetin at concentration from 1.5 µg/ml to 50 µg/ml diluted in medium for 24 hours at 30-40% confluency and then subjected to H_2O_2 (IC50 concentration i.e 250 µM) treatment for 24 hours in serum free medium. The medium of control culture without H_2O_2 and without extract was replaced with a fresh one.

2.4 Cell Viability Assay

MTT was used to assess cell integrity and potential cytotoxicity of the plant extract by monitoring the uptake of the vital mitochondrial dye, 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT) by cell mitochondria [11].

2.5 Chemical Standardization of CP-MEX and Nature of Active Components

CP-MEx was subjected to preliminary phytochemical screening for alkaloids, amino acids, anthroquinones, flavonoids, phytosterols, saponins, steroids, tannins, triterpenoids and reducing sugars following the methods of Harborne [12]. It was further subjected to thin-layer chromatography (TLC) using chloroform: methanol (19:1) as solvent front. TLC plate was subjected to iodine vapours for observation.

2.6 Estimation of Activities of Antioxidant Enzymes and Levels of Antioxidants

2.6.1 Catalase

Catalase activity was measured according to the method of Aebi [13]. 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 12mM 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.

2.6.2 Superoxide dismutase (SOD)

Superoxide dismutase was estimated according to the method of Kono [14]. 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 10.0, 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.

2.6.3 Reduced glutathione (GSH) and glutathione peroxidase (GPx)

Total glutathione was measured as described by Sedlak and Lindsay [15]. In brief, 100 μl sample was mixed with 4.4 ml of 10mM EDTA and 500 μl of trichloroacetic acid (50% w/v). Contents were centrifuged at 3000×*g* for 15 min. 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 [16]. The reaction mixture (1 ml) containing 100nM GSH, 15nM NADPH and 15nM H_2O_2 in potassium phosphate buffer (50 mM, pH 7.5) was mixed with sample (50μl) and the change in absorbance was monitored at 340 nm. Glutathione peroxidase activity is defined as 1μmol of NADPH oxidized per min at pH 7.5 at 25ºC using purified GPx enzyme.

2.6.4 Lipid peroxidation (LPx)

Method of Beuge and Aust [17] 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 melondialdehyde (MDA) upon decomposition. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) that gives a red colored product having absorption maximum at 532 nm. 100 μl sample was incubated with 100 μl each of FeSO4 (1 mM), ascorbic acid (1.5 mM) and Tris-HCl Buffer (150 mM, pH 7.1) in a final volume made of 1 ml, made up by DDW, for 15 minutes at 37ºC. 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 off and then centrifuged. The absorbance of supernatant so obtained was measured at 532 nm.

2.7 Immunocytochemistry

All cells, control and treated, were rinsed three times with ice cold 0.1M 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 hour at room temperature. Cells were incubated with mouse anti-NF-200, mouse anti-HSP70 and mouse anti-Mortalin and, diluted in 0.1% PBST, for 24h at 4ºC in humid chamber. Coverslips were then washed with 0.1% PBST thrice. Secondary antibody (anti-mouse Alexa Fluor 488, anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488) was applied diluted (1:200) in 0.32% PBST for 2h at room temperature. Coverslips were washed three times with 0.1% PBST and final washing was given with 0.1M PBS. These were on the slides with anti fading mounting media (Sigma) and were observed under fluorescent microscope Nikon E600. Images were captured using Cool Snap CCD camera and the pictures were analyzed using ImageJ 1.44p, NIH, USA.

2.8 Reverse Transcription-PCR

The IMR32 cells from 25cm^2 culture flask were homogenized in TRI Reagent (Sigma). Total RNA was extracted and reverse transcriptized 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 CCCACACTGTGCCCATCTACGA3', reverse primer

5'CAGCGGAACCGC TCATTGCCAATGG3'); NF200 (forward primer 5'-AAGTGAACACAGATGCTATGCG-3' NF200 reverse primer 5'-CTGTCACTCCTTCCGTCACC-3'; HSP70 (forward primer 5'GAGTTCAAGCGCAAACACAA3', reverse primer HSP70 5'CTCAGACTTGTCGCCAATGA3'); Mortalin (forward primer 5'CAGTCTTCTGGTGGATTAAG3', reverse primer 5'ATTAGCACCGTCACGTAACACCTC3'), 10× buffer (5.0 µl), cDNA (2.0 µl), 25 mmol/l MgCl2 (3.0 µl), 10 mmol/l dNTPs (1.0 µl), and Taq polymerase (2.5 U). PCR amplification cycles consisted of denaturation at 94ºC for 1min, primer annealing at 57ºC for 45 s and extension at 72ºC for 45 s, for a total of 30 cycles followed by final extension at 72ºC. The PCR product was separated by electrophoresis on 2% agarose gels.

2.9 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 SPSS statistics 17.0 SigmaStat.

3. RESULTS AND DISCUSSION

Oxidative stress can occur when there is imbalance between free radical production and their detoxification by cellular antioxidants. If this imbalance go uncorrected, injury can occur in many tissues [3, 18-20]. Antioxidant defense systems which involve cellular antioxidant molecules and enzymes, scavenge and alleviate the free radicals damage, but they may not be effective all the time and lead to widespread damage to macromolecules, such as DNA, RNA and proteins [21]. Therefore, augmenting the antioxidant defense mechanism, through diet-derived antioxidants, may be an effective strategy to prevent this deleterious oxidative damage.

C. pluricaulis extracts have been shown to display high antioxidant activity in chemical assays in earlier work [22]. However, there is a need to confirm their activity at the cellular level. Therefore, the work presented in this paper was designed to investigate the antioxidant activity of the extracts at a cellular level. Protection against oxidative damage caused by H_2O_2 in the human neuroblastoma cell line IMR32 was used as a cellular antioxidant assay for plant extracts, with quercetin as a positive control in cell viability and antioxidant enzyme studies. The possible mechanism of cytoprotection activity has also been determined, by studying the changes in expression of selected proteins believed to exert cytoprotective functions.

Present study revealed that the CP-MEx pacified H_2O_2-induced toxicity in a dose-dependent manner which was comparable to quercetin. Although *C. pluricaulis* has been reported to improve learning and memory in rats and act as protective agent in rats [9, 10], but the methanolic extracts of *C. pluricaulis* have been evaluated for the first time for its neuroprotective effect on human neuroblastoma cell line.

3.1 CP-MEX Protected IMR32 Human Neuroblastoma Cells against H_2O_2 Induced Cytotoxicity

IMR32 Human Neuroblastoma cells in the present study were cultured in the presence methanolic, ethanolic and water extracts of *C. pluricaulis* at various concentrations to find their neuroprotective effects against H_2O_2-induced cytotoxicity. To obtain appropriate concentration of H_2O_2 producing 50% cells death, IMR32 were treated with different concentrations of H_2O_2 for 24 h (Fig. 1A). Concentration dependent cell death was observed. The IC50 for H_2O_2 in IMR32 cells was found to be 250 µM. In toxicity profile assay, concentration of *C. pluricaulis* extracts below 100 µg/ml did not produce any cytotoxic effect (Fig. 1B). However at higher doses, cytotoxic effect was observed in a dose dependent manner. Similar trend has earlier been observed in methanolic extract of Jeju native plant [23].

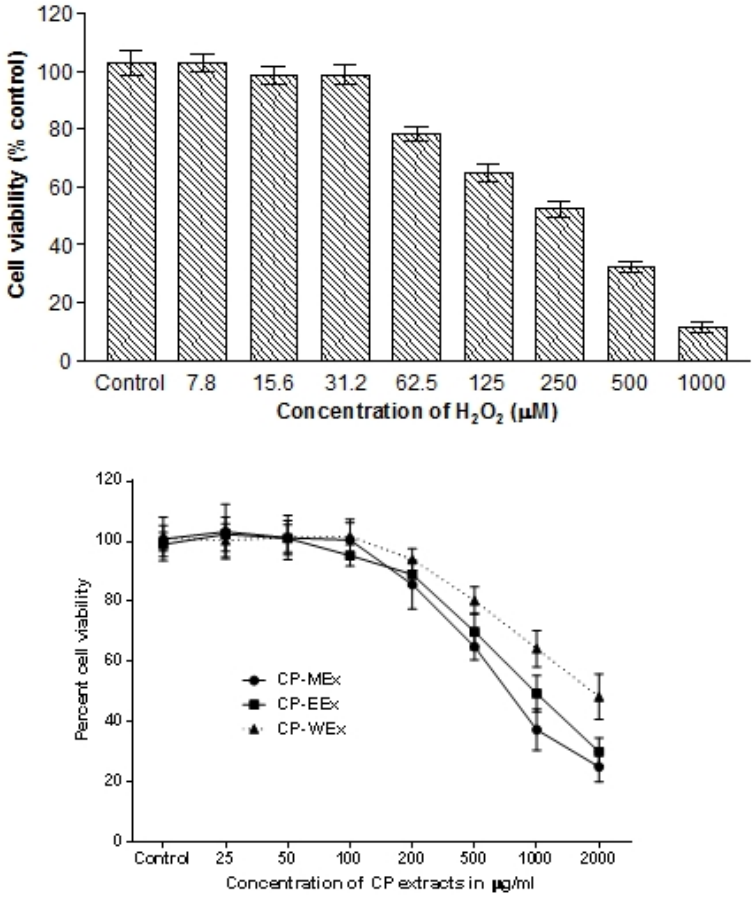

Fig. 1. (A) Dose-dependent cytotoxic effect of H_2O_2 on IMR 32 neuroblastoma cell viability. Incubation of H_2O_2 with IMR32 cells for 24 h produced cytotoxicity with IC50 at 250 µM. (B) Effect of *C. pluricaulis* extracts on IMR 32 cell viability. The extract dose below 100 µg/ml did not show any toxicity however the higher doses exhibited dose dependent toxic effect.

As shown in Fig. 2 A-D, *C. pluricaulis* extracts and Quercetin protected IMR32 cells against H_2O_2 induced-cell death in a concentration dependent manner, and was itself non-cytotoxic in the concentration range tested (Fig. 2A-D). As compared to other two extracts, CP-MEx maximally protected IMR32 cells at 25µg/ml, which was comparable to quercetin protection at12.5 µg/ml (Fig. 2A-D). Ethanolic and water extracts showed less protective effect than CP-MEx.

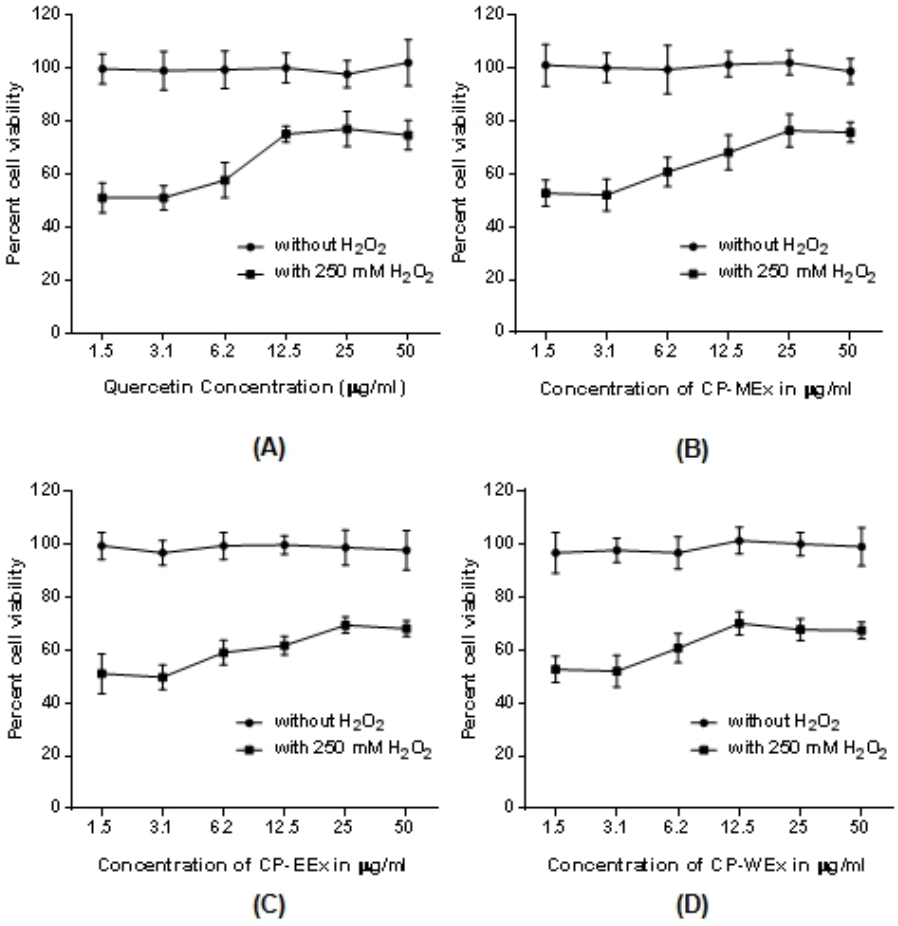

Fig. 2. The effects of quercetin on IMR32 neuroblastoma cell viability in the absence or presence of H_2O_2. (A) Incubation of quercetin with IMR32 cells for 24 h protected against the cytotoxicity elicited by H_2O_2 in a concentration-dependent manner. No cytotoxicity was observed in the cells after incubation with quercetin alone for 24 h in the concentration range 1.5 to 50 µg/ml. (B) The effects of CP-MEx on IMR32 cell viability in the absence or presence of H_2O_2. Incubation of the extract with HepG2 cells for 24 h protected against the cytotoxicity elicited by H_2O_2 in a concentration-dependent manner. No cytotoxicity was observed in the cells after incubation with the extract alone for 24 h in the concentration range 1.5 to 50 µg/ml. (C) The effects of CP-EEx on IMR32 cell viability in the absence or presence of H_2O_2. Incubation of the extract with HepG2 cells for 24 h protected against the cytotoxicity elicited by H_2O_2 in a concentration-dependent manner. No cytotoxicity was observed in the cells after incubation with the extract alone for 24 h in the concentration range 1.5 to 50 µg/ml. (D) C) The effects of CP-WEx on IMR32 cell viability in the absence or presence of H_2O_2. Incubation of the extract with HepG2 cells for 24 h protected against the cytotoxicity elicited by H_2O_2 in a concentration-dependent manner. No cytotoxicity was observed in the cells after incubation with the extract alone for 24 h in the concentration range 1.5 to 50 µg/ml.

3.2 Nature of Bioactive Components of CP-MEx

Preliminary screening tests for phytochemicals showed the presence of flavonoids, steroids, tannins, triterpenoids, saponins, and alkaloids in CP-MEx (Table 1). The TLC profile of CP-MEx generated by Chloroform:Methanol (19:1) solvent elucidated the presence of seven spots with Rf values 0. 08, 0.12, 0.38, 0.45, 0.61, 0.70 and 0.85 (Fig. 3). As there are no reports for bioactive components of *C. pluricaulis,* the presence of specific flavonoids, triterpenoids and saponins require further investigation.

Table 1. Analysis of phytochemicals in CP-MEx

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

"+": presence; "−": absence.

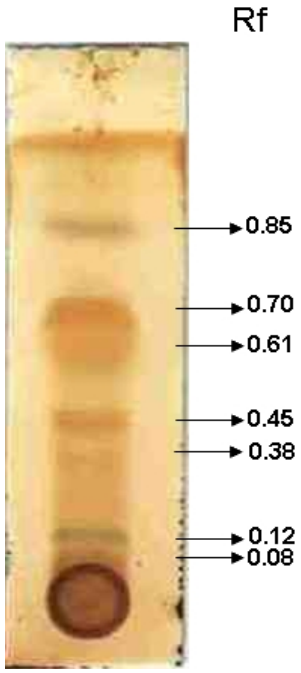

Fig. 3. Analysis of CP-MEx by thin layer chromatography. Chloroform: methanol (19:1) solvent system revealed eight different spots. These spots were visualized using iodine vapors

3.3 Effect of CP-MEx and H_2O_2 Treatment on Catalase, SOD, Gpx and Content of GSH and LPx

The total activities of SOD, catalase and GPx observed in the five independent groups are shown in Table 2. The activity of catalase was significantly reduced in H_2O_2 treated cultures compared to control cultures (P= .012). The activity of catalase increased significantly in CP-MEx treated cultures. The activity of catalase further increased significantly in CP-MEx+H_2O_2 treated cultures (P= .024). Similar types of results were observed for the activity of SOD, which was significantly decreased in H_2O_2 treated cultures when compared to control cultures (P= .008). CP-MEx treatment leads to a significant increase in SOD activity in comparison to control cultures (P= .035). SOD activity was restored close to control cultures in CP-MEx+H_2O_2 treated cultures (P = .026). The activity of GPx was found to diminish significantly (P = .007) in H_2O_2 treated cultures with respect to control cultures. Whereas, CP-MEx treated cultures with (P= .018) or without H_2O_2 (P= .031) treatment showed a significant increase in the activity of GPx when compared to control cultures. Exposure of IMR32 Human Neuroblastoma cells to CP-MEx before giving the dose of H_2O_2 revealed the protective effect of CP-MEx by the induction of antioxidant scavenger system of the cell. The activity of catalase, SOD and GPx were found to be restored close to control by quercetin pretreatment in H_2O_2 cultures. However, the increase in catalase, SOD and GPx activity in control cultures was non-significant in quercetin treated cultures. The increased SOD activity by aqueous extract of *C. pluricaulis* in scopolamine-induced cognitive impairment in Wistar rats have been reported [24]. The increase in SOD activity indicates possible de novo protein production which leads to enhanced antioxidant defense system [25]. The H_2O_2 produced by SOD is metabolized by Catalase and GPx. The level of Catalase and GPx are which are also increased by the pretreatment of CP-MEx which further substantiate the protective effect of CP-MEx through induction of endogenous antioxidant enzymes. The decrease in catalase and SOD activity in H_2O_2 treated cultures may be either due to increase in instability of catalase and SOD mRNAs as the post-transcriptional regulation of catalase and SOD gene have been reported to be the major factor regulating its expression under oxidative stress [26]. SOD and H_2O_2 metabolizing enzymes guard the cell against ROS formed during normal aerobic respiration, but under the stressed conditions they seem to be incapable of coping up with high production of ROS. The increase in the activities of SOD, catalase and GPx due to pretreatment with CP-MEx further reveal the protective role of CP-MEx in H_2O_2 toxicity and this observation is supported by a variety of studies [27-29].

The cultures treated with H_2O_2 had significantly low level of GSH content when compared to control cultures (P= .012). GSH content of the CP-MEx treated cultures increased significantly (P= .019) in comparison to control cells. However, restoration of GSH content of CP-MEx+H_2O_2 treated cultures (P= .027) close to control cultures indicates the protective effect of CP-MEx. Quercetin was also able to restore the GSH content of the cell when H_2O_2 cultures were pretreated with it (P= .002). However, control culture treated with quercetin, did not produce significant increase in GSH content. A significant increase in lipid peroxidation level was observed in H_2O_2 treated cultures when compared with control cultures (P= .005), where as treatment of cultures with CP-MEx decreased the lipid peroxidation significantly as compared to control cultures (P= .016). CP-MEx+H_2O_2 treated cultures exhibited decreased level of lipid peroxidation as compared to cultures treated with H_2O_2 (P= .011). Similar effect was produced by quercetin in lipid peroxidation when control cultures were treated and H_2O_2 treated cultures were pretreated. Increased level of GSH in CP-MEx treated cultures with H_2O_2 further endorse its protective effect as GSH plays a crucial role by scavenging the free radicals directly. Glutathione system has also been

reported to protect cultured IMR32 Human Neuroblastoma cultures [30]. Lipid peroxidation comprises of polyunsaturated fatty acids cleavage at their double bonds leading to membrane damage. A significant increase in lipid peroxidation due to H_2O_2 treatment with respect to control cells is due to oxidative stress indicative of peroxidative degradation of cell membrane as reported previously [31]. The decrease in the lipid peroxidation level in CP-MEx treated cultures can be related to increase in the anti-oxidant enzymes. Conserving the activity of antioxidant enzymes (SOD, Catalase and GPx) and level of GSH, and reducing the level of lipid peroxidation in CP-MEx pretreated cultures against H_2O_2 induced cytotoxicity provides evidence to design therapeutic strategy which could be of used in future for protection against variety of neurodegenerative disorders. The effect of CP-MEx matched with quercetin in restoration of antioxidant machinery when H_2O_2 cultures were pretreated with both however only CP-MEx was able to produce significant increase in cellular antioxidant machinery under normal circumstances. Such increase may be due to some other bioactive components present in the extract. Earlier reports also support this idea of increase in antioxidant enzymes [27].

3.4 Effect of CP-MEx on mRNA and Protein Expressions of NF200 in IMR32 Cells Exposed to H_2O_2 Induced Cytotoxicity

NF-200 are intermediate filament proteins specifically found in IMR32 Human Neuroblastoma cells. We examined the changes in expression of NF-200 by immunofluorescence (Fig. 4a,b,c,d). In order to corroborate the protective effect, we examined the expression of cytoskeletal marker, NF-200, and found that CP-MEx+H_2O_2 treated cultures had significantly less NF-200 as compared to H_2O_2 treated cells (P = .009) (Fig. 4e). The protective effect of CP-MEx was further confirmed at the transcription level by studying the expression of NF-200 in RT-PCR (Fig. 4f,g). The level of NF-200 level was reduced in CP-MEx+H_2O_2 treated cells when measured against H_2O_2 treated cells (P = .001). There was no significant difference between expression of NF-200 in control cultures and CP-MEx treated IMR32 Human Neuroblastoma cultures in immunofluorescence and RT-PCR. The IMR32 Human Neuroblastoma marker, NF-200, is involved in the intricate cellular functions which control the cell morphology, differentiation and proliferation [32]. The increase in NF-200 was observed in IMR32 Human Neuroblastoma cells after H_2O_2 treatment in immunofluorescence and RT-PCR results. The decrease of NF-200 level in CP-MEx+H_2O_2 treated cells in comparison to H_2O_2 treated cells indicate the low oxidative stress profile of the cells which has been provided by increase in anti-oxidant enzymes.

3.5 Study of CP-MEx on Level of HSP70 in IMR32 Cells Exposed to H_2O_2-Induced Cytotoxicity

In order to examine the changes in expression of HSP70 the cells were examined for the level of HSP70 at various levels of expression with immunofluorescence and RT-PCR (Fig. 5a,b,c,d). After a variety of nervous system insults, HSP70 is synthesized at especially high level and is present in cytosol, nucleus and endoplasmic reticulum. For the evaluation of protective effect of CP-MEx, we studies the expression of HSP70 in comparison to cells treated with CP-MEx+H_2O_2 treated with cells treated only with H_2O_2. The level of HSP70 was significantly less in CP-MEx+H_2O_2 treated cells (P = .004) (Fig. 5e). The protective effect of CP-MEx was further supported by the results of RT-PCR as the level HSP70 was significantly less in CP-MEx+H_2O_2 treated cells as compared to H_2O_2 treated cells (P = 0.002) (Fig. 5f,g). No significant variation was observed in expression of HSP70 when control cultures were compared to CP-MEx treated IMR32 cells.

Table 2. Effect of cytoprotective activity of CP-MEx on antioxidant scavenger system in IMR32 Neuroblastoma cells

Groups	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	14.57 ± 0.86	19.05 ± 1.10	3.05 ± 0.36	3.94 ± 0.28	16.53 ± 1.18
CP-MEx	16.76 ± 0.68 a	22.14 ± 1.23 a	3.88 ± 0.24 a	4.78 ± 0.17 a	10.71 ± 0.53 a
H_2O_2	8.55 ± 0.77 b	11.75 ± 0.96 b	1.56 ± 0.35 b	2.31 ± 0.35 b	25.27 ± 1.36 b
CP-MEx + H_2O_2	12.65 ± 0.48 c d	15.87 ± 0.81 c d	2.48 ± 0.45 c d	3.37 ± 0.21 c d	21.42 ± 0.38 c d
Quercetin	14.98 ± 0.55	19.89 ± 0.89	3.35 ± 0.49	4.24 ± 0.34	9.59 ± 0.67
Quercetin + H_2O_2	11.05 ± 0.69 e	13.93 ± 0.92 e	2.20 ± 0.31 e	3.16 ± 0.30 e	23.15 ± 0.84 e

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 < .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.

Heat shock proteins are fundamental response to a wide variety of toxic conditions and attracted a great interest as an essential mechanism required for cell survival. Synthesis of HSP70 in mammalian cells is activated not only under heat shock but also in the conditions of disturbed cellular homeostasis, heavy metal toxicity and drugs cytotoxicity [18, 33, 34]. Our results in the present study shows that oxidative stress due to H_2O_2 treatment lead to marked upregulation of HSP70 level. But, pretreatment of CP-MEx increased the cellular anti-oxidant enzymes, inhibited free radical induced lipid peroxidation markedly and attenuated the expression of HSP70. The present observation of pretreatment of IMR32 Human Neuroblastoma cells with CP-MEx increased the cell survival against H_2O_2 induced cytotoxicity.

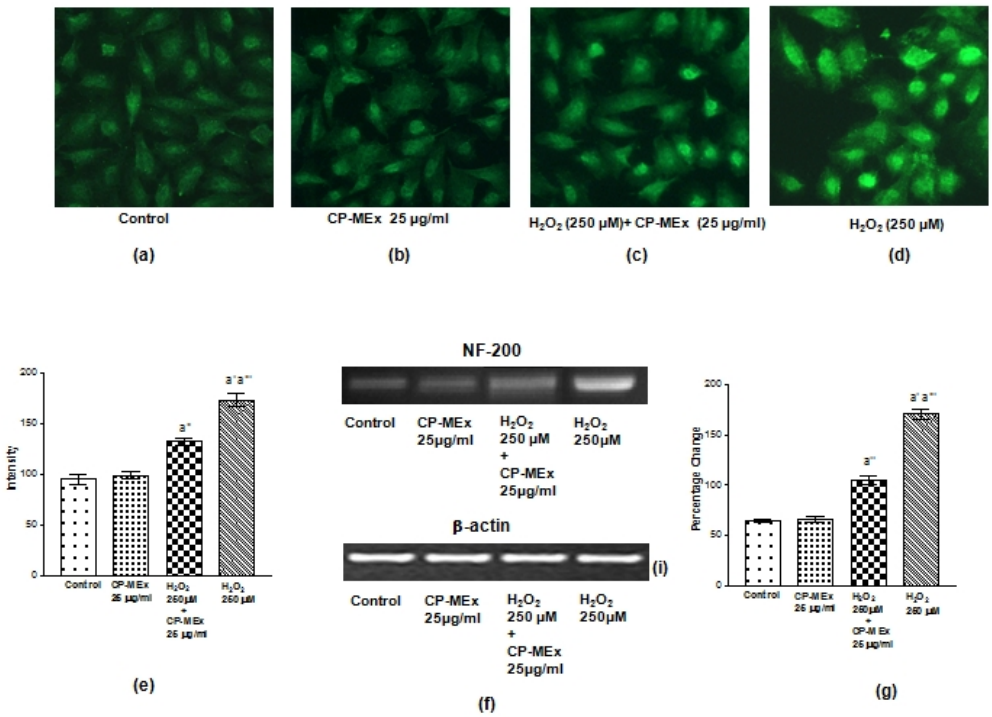

Fig. 4. Localization of NF-200 in IMR 32 neuroblastoma cells (a), Untreated control (b), CP-MEx treated (c) CP-MEx+H2O2 treated (d) H2O2 treated. Cells grown on coverslips (n = 5) for 4 days were fixed and stained for NF-200 (Alexa Fluor 488) immunoreactivity. (e) Relative intensity measurement of NF-200 immunofluorescence performed by ImageJ 1.44p. (f) Representative reverse transcription-polymerase chain reaction (RT-PCR) showing NF-200 and β-actin expression in untreated control, CP-MEx treated, CP-MEx+H_2O_2 treated, H_2O_2 treated IMR 32 neuroblastoma cells. (g) Relative optical density measurement of mean values of NF-200 expression in RT-PCR for each group expressed as percentage of β-actin. The values having P < .05 are considered significant. a′, statistically significant change in H_2O_2 treated cultures with respect to the control cultures; a″, statistically significant change in CP-MEx + H_2O_2 treated cultures with respect to the CP-MEx treated cultures; a‴, statistically significant change in H_2O_2 treated cultures with respect to the CP-MEx + H_2O_2 treated cultures.

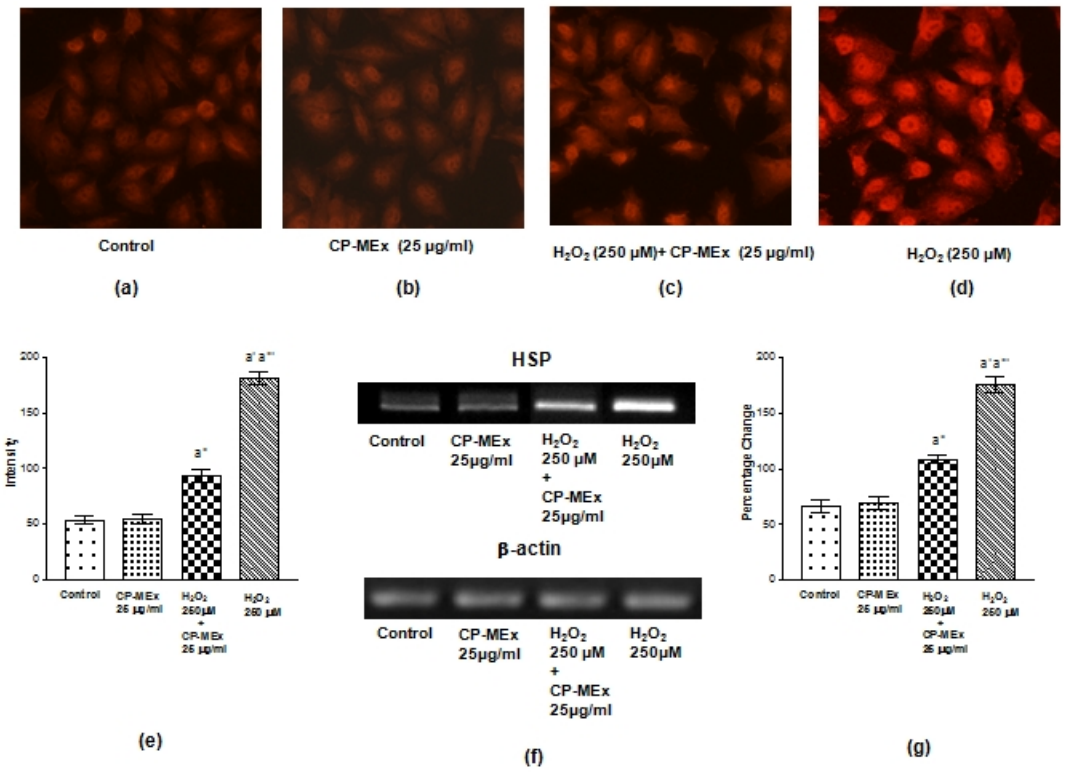

Fig. 5. Distribution of HSP70 in IMR 32 neuroblastoma cells (a), Untreated control (b), CP-MEx treated (c) CP-MEx+H2O2 treated (d) H2O2 treated. Cells grown on coverslips (n = 5) for 4 days were fixed and stained for HSP70 (Alexa Fluor 568) immunoreactivity. (e) Relative intensity measurement of HSP70 immunofluorescence performed by ImageJ 1.44p. (f) Representative reverse transcription-polymerase chain reaction (RT-PCR) showing HSP70 and β-actin expression in untreated control, CP-MEx treated, CP-MEx+H_2O_2 treated, H_2O_2 treated IMR 32 neuroblastoma cells. (g) Relative optical density measurement of mean values of HSP70 expression in RT-PCR for each group expressed as percentage of β-actin. The values having P < .05 are considered significant. a′, statistically significant (P < .05) change in H_2O_2 treated cultures with respect to the control cultures; a′′, statistically significant change in CP-MEx + H_2O_2 treated cultures with respect to the CP-MEx treated cultures; a′′′, statistically significant change in H_2O_2 treated cultures with respect to the CP-MEx + H_2O_2 treated cultures.

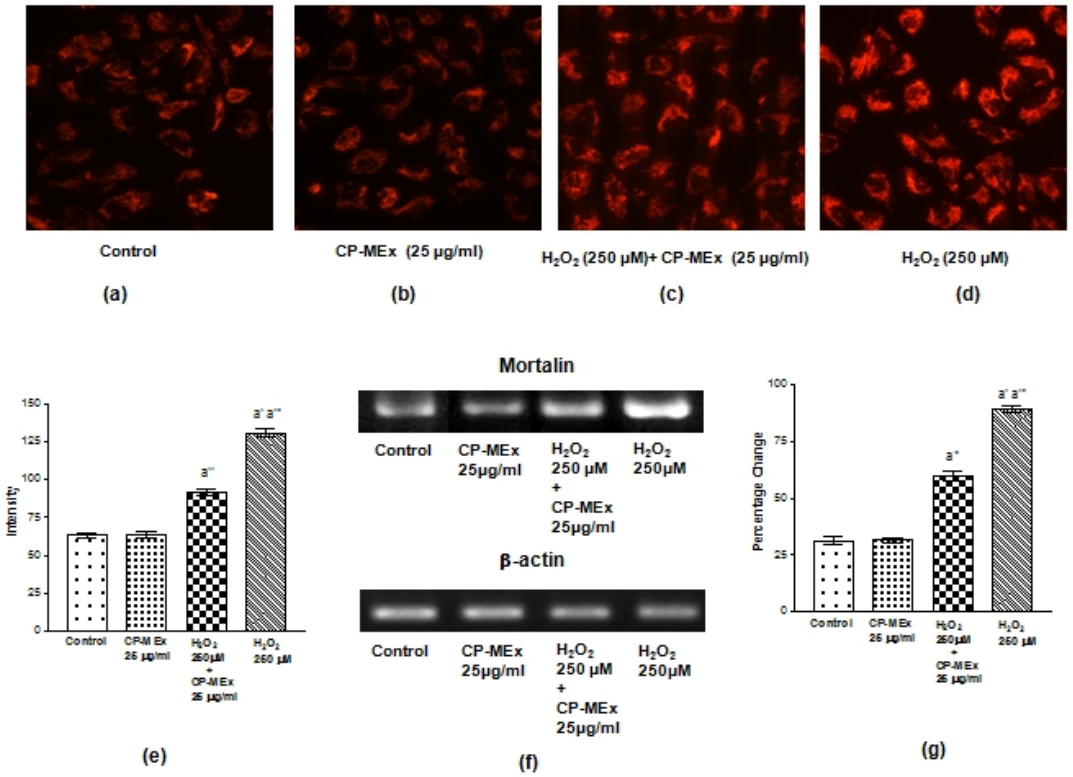

Fig. 6. Localization of Mortalin in IMR 32 neuroblastoma cells (a), Untreated control (b), CP-MEx treated (c) CP-MEx+H2O2 treated (d) H2O2 treated. Cells grown on coverslips (n = 5) for 4 days were fixed and stained for Mortalin (Alexa Fluor 568) immunoreactivity. (e) Relative intensity measurement of Mortalin immunofluorescence performed by ImageJ 1.44p. (f) Representative reverse transcription-polymerase chain reaction (RT-PCR) showing Mortalin and β-actin expression in untreated control, CP-MEx treated, CP-MEx+H_2O_2 treated, H_2O_2 treated IMR 32 neuroblastoma cells. (g) Relative optical density measurement of mean values of Mortalin expression in RT-PCR for each group expressed as percentage of β-actin. The values having P < .05 are considered significant. a′, statistically significant (P < .05) change in H_2O_2 treated cultures with respect to the control cultures; a′′, statistically significant change in CP-MEx + H_2O_2 treated cultures with respect to the CP-MEx treated cultures; a′′′, statistically significant change in H_2O_2 treated cultures with respect to the CP-MEx + H_2O_2 treated cultures.

3.6 Effect of CP-MEx on mRNA and Protein Level of Mortalin in IMR32 Cells Treated with H_2O_2

To study the perinuclear expression of mortalin in IMR32 Human Neuroblastoma cells after treatment of CP-MEx and H_2O_2, the cells were subjected to immunofluorescence. Mortalin is a heat-shock cognate protein and it has been reported that under the stressed conditions the level of mortalin increases. In this experiment we examined the level and staining of mortalin protein (Fig. 6a,b,c,d). There was no significant difference in the level of expression of mortalin in control and CP-MEx treated cells (Fig. 6e). The level of mortalin was significantly

high in H_2O_2 treated cells as compares to control cultures (Fig. 6e) (P = .006) whereas CP-MEx+H_2O_2 treated cells expressed less level of mortalin as compared to H_2O_2 treated cells (Fig. 6e) (P = .003). The reduction in level of mortalin suggests the protective effect of CP-MEx. The data from RT-PCR further confirmed the protective effect of CP-MEx (Fig. 6f). The expression of mortalin was significantly less in CP-MEx+H_2O_2 treated cells in contrast to H_2O_2 treated cells (Fig. 6g) (P = .004). No significant change was observed in level of mortalin on comparing control cultures with CP-MEx treated IMR32 cells in immunofluorescence, and RT-PCR. The expression of mortalin is scattered in a pancytoplasmatic form in normal cells, however its localization change in immortal cells to the perinuclear region [35, 36]. The elevation in the level of mortalin expression is a type of stress response or an adaptive response to the H_2O_2 treatment [37]. Results in the present study show a significant increase in the mortalin expression with the treatment of H_2O_2. The phenomenon behind such increase in mortalin in H_2O_2-treated in mortalin remains unknown. The pretreatment of cultures with CP-MEx before H_2O_2 significantly reduces the level of mortalin which indirectly shows that CP-MEx is reducing the oxidative stress of the cell. No significant difference was observed in the level of mortalin in control cultures but the level of anti-oxidant enzymes was increased in the only CP-MEx treated cultures which support the protective role of CP-MEx under normal conditions.

4. CONCLUSIONS

We conclude that prior treatment of IMR32 Human Neuroblastoma cells with CP-MEx increases the cell viability via stimulation of endogenous anti-oxidant enzymes, increase in glutathione level and prevention of direct membrane damage due to lipid peroxidation. The effect of CP-MEx was comparable to quercetin in restoration of cellular antioxidants close to control when H2O2 cultures were pretreated however CP-MEx also enhanced cellular antioxidant under normal condition which was absent in quercetin. The observations were corroborated by the decrease in the level of stress markers mortalin and HSP70 and cytoskeletal marker NF-200 with pretreatment of CP-MEx before H_2O_2 dose. Current study provides information about functional relationship of antioxidant machinery of the cell with various stress response markers of the cell. Since, present study was restricted to study of antioxidant enzyme levels and cell marker therefore further investigation is required to elucidate the signaling pathway involved in such type of protective effect of CP-MEx on cellular and animal models. However, the protective effect of CP-MEx against H_2O_2 induced cytotoxicity, increased level of endogenous antioxidant machinery suggest that CP-MEx could become a potential therapeutic strategy in the treatment of neurodegenerative disorders of oxidative stress.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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