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Effects of methanolic extract of *Bacopa monnieri* against hydrogen peroxide induced oxidative stress in C6 glioma cells

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ABSTRACT

Researchers have inclined their focus for better perception of mechanism and efficacy of medicinal plants in neurodegenerative disorders. Bacopa monnieri (BM) has been the fascinating candidate to treat the disorders of mental frailty. Cerebral disorders are the upshots of disparity between cellular oxidant and antioxidant levels. Hydrogen peroxide (H_2O_2), pioneer of various detrimental reactive oxygen species (ROS), induces oxidative damage ensuing brain infirmity. The current study was designed to investigate the neuroprotective potency of BM extracts against H_2O_2 induced oxidative damage in C6 glioma cells. The protective efficacy of methanolic, ethanolic and water extracts of BM (BM-MEx, BM-EEx and BM-WEx) was determined by MTT assay. The prior treatment of BM-MEx remarkably upregulated the expression levels of antioxidant enzymes, catalase, superoxide dismutase, glutathione peroxidase and glutathione in the H_2O_2 exposed cells. The lipid peroxidation level was found to be remarkably down-regulated with BM-MEx pre-treatment. Immunofluorescence and RT-PCR demonstrated that the prior treatment of BM-MEx significantly alleviated the expression of GFAP (cytoskeletal marker), HSP70 and Grp75 (stress markers) in H_2O_2 exposed cells. The current investigation suggests that BM-MEx can provide protective ability to combat against cerebral infirmity.

Key words: *Bacopa monnieri, C6 glioma, H_2O_2, HSP70, Grp75, GFAP, oxidative stress*

INTRODUCTION

Currently, the synthetic drugs are becoming irresponsive to meet the therapeutic needs of patients with mental infirmity and other health troubles. The herbal medication turns out to be the foremost hope for these health issues. Hence, the western and developed countries are budging over to herbal remedies (Husain *et al.*, 2007). In Indian traditional medicine system (Ayurveda), number of plants are reputed for their medicinal relevance and have been traditionally used as health tonics. *Bacopa monnieri* (BM), vastly used as neurotonic, is well acknowledged for its nerve relaxing and memory boosting properties. Earlier, the BM has been recognised as anxiolytic and anti-depressant in mice (Chatterjee *et al.*, 2010). BM is also well established for having protective potential against varied neurological crisis like dementia, epilepsy, ischemia and other neurodegenerative diseases (Hosamani, 2009; Mathew *et al.*, 2011; Saini *et al.*, 2012; Le *et al.*, 2015). The glory for this protective ability is owed to diverse bioactive constituents such as Bacosides, saponins and triterpenoids in the BM (Aguiar & Borowski, 2013; Le *et al.*, 2015). The underlying mechanism is its antioxidant prospective of bioactive components that help to detoxify the unnecessary free radical species generated. Number of medicinal plants like *Hypericum perforatum* and *Hippophae rhamnoides* have also been found to provide protection against oxidative stress mediated damages in PC12 neuronal and C6 glioma cells respectively (Narayanan *et al.*, 2005; López & Calvo, 2011).

Brain being a vital organ of body has to be hale and hearty. Any impairment in its functioning can cause deleterious outcomes like diverse neurological disorders; Alzheimer disease, Parkinson disease and many more. These neurodegenerative diseases are the consequence of imbalance between the production of oxidative stress and its nullification by the cellular defence mechanism (Simonian & Coyle, 1996; Murphy, 1999; Liu *et al.*, 2002; Kasture *et al.*, 2007; Zhao & Zhao, 2013). Nitric oxide, superoxide and hydroxyl radicals are the prime cellular free radical species, whereas hydrogen peroxide (H_2O_2) and peroxynitrite are not oxidative entities *per se* but assist significantly to the cellular redox status (Albers & Beal, 2000). These free radical species mediate cytotoxicity by oxidizing cellular components like lipids and nucleic acids which is confronted by inbuilt cellular defence machinery involving antioxidant enzymes and endogenous antioxidants (Björnstedt *et al.*, 1994; Maroto & Perez-Polo, 1997; Sun & Chen, 1998; Blokhina *et al.*, 2003; Warner *et al.*, 2004; Borniquel *et al.*, 2006). When the levels of oxidants outstands the antioxidant capacity of cells, some exogenous assistance must be provided to avert the consecutive damage of the

central nervous system. So by memorizing the defensive ability of BM, current study was designed to evaluate the probable antioxidant and neuroprotective potential of BM on cellular model of neurodegenerative diseases by artificial induction of oxidative stress using H_2O_2, which induces the production of hydroxyl radicals, and hence a potent oxidative stress generator (Maroto & Perez-Polo, 1997).

An N-nitrosomethyl urea induced rat glial cell culture was used as a cellular model to validate the protective ability of BM. C6 glioma cells have been enormously used as an *in vitro* system. Earlier, glial cells were thought to impart only architectural support to the neuronal cells but later its role in bidirectional communication has been recognised (Hertz *et al.*, 1999; Singh & Kaur, 2009). Any cellular insult like hypoxia, viral infection or excitotoxicity in the CNS leads to reactive gliosis (Eng & Ghirnikar, 1994). These evidences portrait C6 glioma cells as a suitable model to study the consequences of oxidative stress in the CNS. To validate the above hypothesis of neuroprotection, the expression level of selected stress markers including GFAP (Glial fibrillary acidic protein), HSP70 (Heat shock protein) and Grp75 (Mortalin) was demonstrated. The level of endogenous antioxidants and enzymatic machinery was also determined.

MATERIALS AND METHODS

Chemicals and reagents

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagles Medium and Hydrogen peroxide were procured from Sigma-Aldrich. Fetal bovine serum was procured from Invitrogen. The primary antibodies used for Immunofluorescence were monoclonal rabbit anti-GFAP (Sigma- Aldrich), mouse anti-HSP70 (Clone BRM-22, Sigma-Aldrich) and mouse anti-Mortalin (Abcam anti-mouse).Anti-mouse Alexa Fluor 568 (Invitrogen) and Alexa Fluor 488 (Invitrogen) were used as secondary antibodies. The PCR reagents including 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, β-actin, HSP70 and Grp75 were from Biolink. All other chemicals and reagents including FC reagent, EDTA, sodium hydroxide and solvents were procured in their purest form from Qualigens, Himedia and Sisco Research Laboratories.

Preparation of plant extract

The aerial part of BM was utilized in this study. The whole plant was procured from the local market and got authenticated from the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar. The plant was shade dried, grinded and 10 g of plant powder was extracted with 100 ml of methanol, ethanol and water at 37 ºC using orbital shaker and hence labeled as BM-MEx, BM-EEx and BM-WEx respectively. The resultant mixture was centrifuged at 15,000x g. The supernatant was air dried to get concentrated solvent fractions. These fractions were further diluted with culture medium to obtain final working concentrations.

Total Flavonoid Content

Total flavonoid content was measured by the colorimetric aluminium chloride method using catechin as standard (Zhishen *et al.*, 1999). One ml of plant extract was mixed with 4 ml of double distilled water (DDW) and followed by 300 µl of 5% $NaNO_2$. The mixture was incubated at room temperature for 5 minutes and then 300 µl of 10 % $AlCl_3$ was added. After one min, 2 ml of 1M NaOH was added and finally reaction mixture was diluted with 2.4 ml of DDW. The resultant mixture was read spectrophotometrically at 510 nm. The total flavonoid content was calculated as mg Catechin Equivalents (CE) per 100mg of dried extract.

Total Phenolic Content

The amount of total Phenolic content was determined by Folin-Ciocalteu (FC) reagent method with some modifications (Yu *et al.*, 2002). Gallic acid monohydrate was used as a standard. Hundred µl of plant extract was mixed with 0.5 ml of FC reagent. Then 1.5 ml of 20% Sodium carbonate was added and the mixture volume was raised to 10ml by addition of DDW. The mixture was incubated at room temperature for two hours. Finally the absorbance was measured at 765 nm. The total phenolic content was calculated from standard curve. The total phenolic content was determined in terms of mg gallic acid equivalents (GAE) per 100 mg of dry weight of extract.

Free radical scavenging assay

The free radical scavenging potential of various extracts of *B. monnieri* was determined by the DPPH (2, 2'-diphenyl-1-picrylhydrazyl) assay (Blois, 1958). Caffeic acid, Catechin and Rutin were used as standards. Two ml of 0.1 mM freshly prepared DPPH solution in methanol was added to the test tubes and measured spectrophotometrically at 517 nm as a blank. Then 300µl of standard or plant extract was added. After incubation of 30 minutes, the discoloration of the DPPH solution was measured.

The percentage scavenging activity was determined by following formula:

$$\textbf{(\%) DPPH scavenging potential} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 = Absorbance of Blank A_1 = Absorbance of Extract/Standard

Cell culture and extract treatment

C6 glioma culture was obtained from NCCS, Pune, India. The cells were maintained on DMEM supplemented with 10% fetal bovine serum (Life Technologies), glutamine (20 mM), penicillin (120 µg/ml), streptomycin (100 U/ml) and gentamycin (100 µg/ml) at 37° C and humid atmosphere with 5% CO_2 in air. Primarily, the C6 glioma cells were exposed to different concentrations of all the BM extracts ranging from 1.5 µg/ml to 200 µg/ml to verify the probable cellular toxicity. To investigate the protective ability of BM, H_2O_2 was used as an artificial oxidative stress inducer. In order to determine IC_{50} value of H_2O_2, cells were treated with varied concentrations of H_2O_2 ranging from 7.8 µM to 1000 µM at 50% confluency. To elucidate the protective potential of BM-MEx, BM-EEx and BM-WEx, the cells were first treated with different concentrations of extracts in the range of 1.5 µg/ml to 50 µg/ml at 30% confluency for 24 hours and consequently incubated with IC_{50} dose of H_2O_2 for 24 hours. The control group medium was deprived of H_2O_2 and extracts. The cellular integrity and toxicity was determined by the MTT assay (Hansen *et al.*, 1989). This vital mitochondrial dye reacts with enzyme succinate dehydrogenase and gets converted to purple coloured formazan crystals. Immunofluorescence, RT-PCR and enzyme assays were used to validate the protective prospect of BM-MEx at molecular level. For advance experiments 4 individual groups were named as BM-MEx treated, H_2O_2 treated, BM-MEx + H_2O_2 treated and control cultures.

Expression analysis of endogenous antioxidant enzymes and antioxidants

Preparation of cell lysate

The above mentioned different cell groups were rinsed twice with 0.1M phosphate buffer and then extricated with 1mM PBS–EDTA. The cells were then pelleted down at 400 × *g* for 10 minutes. The pellet was resuspended in 5 volumes of chilled homogenizing buffer, pH 7.4 (250 mM Sucrose, 12 mM Tris-HCl, 0.1 mM DTT) by continual vortexing for 15 minutes. Cell lysate so obtained was centrifuged at 12,000 × *g* for 10 minutes. The supernatant was used for evaluating the expression levels of endogenous antioxidant enzymes and antioxidants.

Assessment of cellular antioxidant machinery

The expression level of catalase enzyme was determined by the standardized method based on the rate of decomposition of H_2O_2 (Aebi, 1984). The enzyme superoxide dismutase (SOD) expression was examined by the tetrazolium dye procedure (Kono, 1978). The amount of total glutathione content (GSH) in the cell lysate was determined by the Ellman's reagent method (Sedlak & Lindsay, 1968). The total GSH content was calculated by plotting a standard curve using pure glutathione. The activity of Glutathione peroxidase (GPx) was determined indirectly by measuring NADPH oxidation (Paglia & Valentine, 1967). The lipid peroxidation (LPx) was measured by thiobarbituric acid (TBA) method (Buege & Aust, 1978).

Validation of neuroprotection at molecular level

Immunofluorescence

The cells were grown on poly-lysine coated coverslips (Himedia) in 24 well tissue culture plate. After the respective treatment of mentioned groups, the cells were washed thrice with chilled PBS. The cells were then incubated with 4% paraformaldehyde solution for 30 minutes for fixation purpose. Further, the cells were permeabilized with 0.32% PBST for 20 minutes followed by blocking with 5% normal goat serum in 0.1% PBST for 1 hour at 37ºC. Afterwards, the cells were incubated with rabbit anti-GFAP (1:500), mouse anti-HSP70 (1:500) and anti-Grp75 (1:100) prepared in 0.1% PBST, overnight at 4ºC in moist compartment. The cells were then tagged with specific secondary antibody, anti-rabbit Alexa Fluor 488 (anti-GFAP) and anti-mouse Alexa Fluor 568 (anti-HSP70 and anti-Grp75) in 0.1M phosphate buffer for 4 hours at room temperature. The cells were finally washed with PBS to remove unbound antibody. The coverslips were mounted on glass slides with Fluoromount (Sigma) and visualized under Nikon A1R confocal system. The images were analyzed with software ImageJ 1.44p (NIH, USA).

Semi-quantitative reverse transcription-PCR

The above stated cell groups were extracted with 1 ml of Trizol Reagent (Invitrogen) and allowed to stand at room temperature for 10 minutes to ensure the total dissociation of nucleoprotein complexes. The cell suspension was transferred to eppendorf tubes followed by addition of 0.2 ml chloroform. The cell suspension was then shaken vigorously for 30 seconds and kept undisturbed at 37ºC for 15 minutes. The samples were centrifuged at 10,000× *g* for 10 minutes. The aqueous phase so obtained was incubated with 0.5 ml of isoamyl alcohol for 15 minutes at 37ºC followed by centrifugation at 12,000× g at 4°C for 10 minutes. The pellet so obtained was rinsed with 75% ethanol, vortexed and again centrifuged

at 7,500× *g* at 4°C for 5 minutes. The RNA pellet was air-dried for 10 minutes and dissolved in 50µl of Tris-EDTA buffer (10mM Tris-HCL, pH 7.5 and 1 mM EDTA, pH 8) with continuous pipetting. The RNA samples were then kept at 55–60°C for 10 minutes and read spectrophotometrically at 260 and 280 nm. The 260/280 nm ratio depicts the percentage purity of RNA samples.

Total RNA was reverse transcribed by following the manufacturer's protocol (Fermentas, Life sciences). Briefly, cDNA was amplified in a 20 µl reaction containing primer pairs (each 1.0 µl): β-actin (forward primer 5′TCACCCACACT-GTGCCCATCTACGA3′, reverse primer 5′CAGCGGAACCGCTCATTGCCAATGG3′); GFAP (forward primer 5′GGCGCTCAATGCTGGCTTCA3′, reverse primer 5′TCTGCCTCCAGCCTCAGGTT3′); HSP70 (forward primer 5′GAGTTCAAGCGCAAACACAA3′, reverse primer 5′CTCAGACTTGTCGCCAATGA3′); Grp75 (forward primer 5'CAGTCTTCTGGTGGATTAAG3', reverse primer 5′ATTAGCACCGTCACGTAACACCTC3′), 10X reaction 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). Amplification of cDNA comprised initial denaturation at 94°C for 1 minute, primer annealing at 55°C, 52°C and 45°C (GFAP, HSP70 and Grp75 respectively) for 45 sec and extension at 72°C for 45 sec, in total of 30 cycles followed by concluding extension at 72°C for 5 minutes. The PCR product so obtained was electrophoresed on 2% agarose gel.

Statistical analysis. The experimental data was expressed in terms of mean ± S.E.M. from at three independent experiments. One-way analysis of variance (ANOVA) was used to analyze multiple variable comparisons. For the evaluation of significance between groups, Bonferroni test was used according to the statistical program Sigma Stat (Jandel Scientific, Chicago, IL, USA).

RESULTS

Total Flavonoid Content

Total flavonoid content of various extracts of *B. monnieri* was calculated as mg Catechin Equivalents (CE)/100mg dry weight of extract. A calibration curve was plotted for various catechin concentrations against absorbance which was explained by linear regression equation $y = 0.002x + 0.003$, ($R^2 = 0.996$).

Out of different extracts of *B. monnieri* tested, BM-MEx was found to have maximum total flavonoid content of 19.17 ± 1.25 mg CE/100mg dry weight of extract followed by BM-

WEx with 16.83 ± 1.04 mg CE/100mg dry weight of extract. The lowest amount of 12.16 ± 0.76mg CE/100mg dry weight of extract was found in BM-EEx.

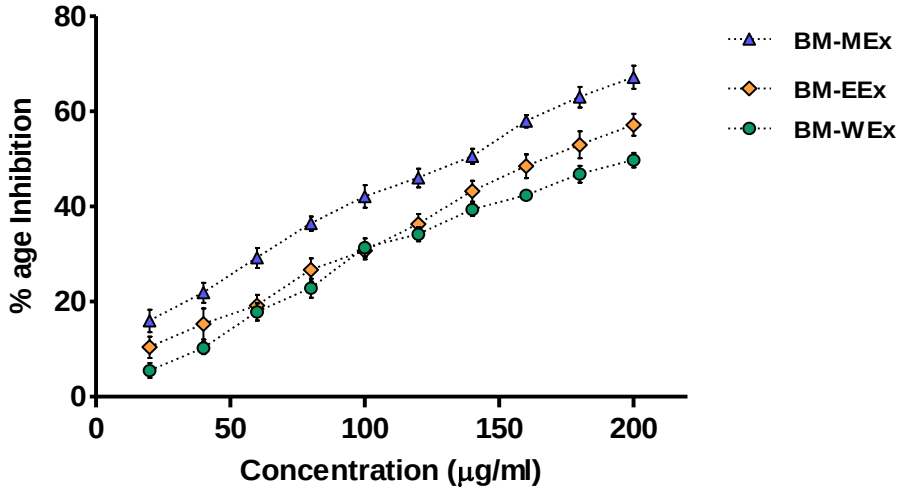
Total Phenolic Content

The amount of total phenolic content present in various *B. monnieri* extracts was determined in terms of mg gallic acid equivalents/100mg dry weight of different solvent extracts. A standard curve was plotted for gallic acid, explained by linear regression equation $y = 0.001x - 0.009$ ($R^2 = 0.996$). It was found that BM-MEx had maximum phenolic content of 23.67 ± 1.15 mg GAE/100mg dry weight of extract followed by BM-WEx which had 21.66 ± 1.15 mg GAE/100mg. The lowest phenolic content of 17.7 ± 0.57 mg GAE/100mg was obtained in case of BM-EEx.

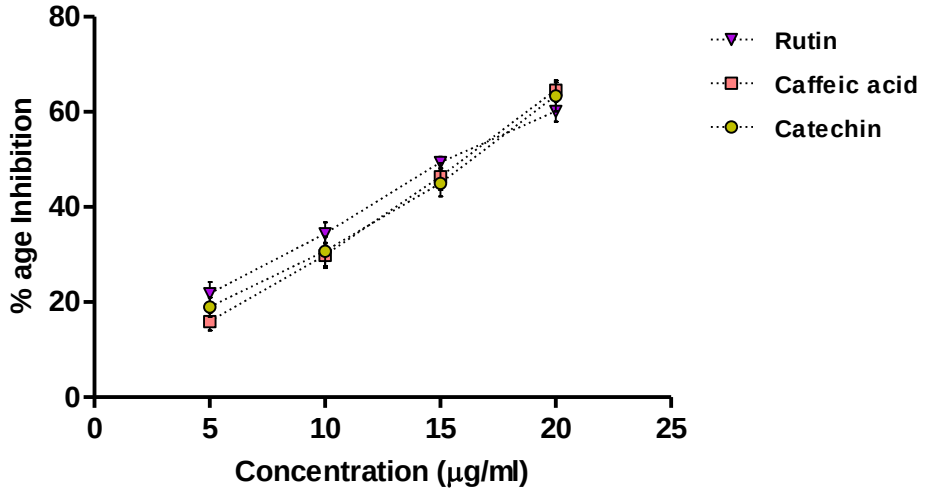
Free radical scavenging activity

The percentage scavenging activity of different *B. monnieri* extracts is directly dependent on the amount of antioxidants present. The DPPH (2, 2'-diphenyl-1-picrylhydrazyl) contains an odd electron, which on gaining another electron gets stabilized, leading to discoloration from deep purple color to pale yellow. This change can be measured spectrophotometrically at 517 nm. The percent scavenging activity of *B. monnieri* was evaluated with the caffeic acid (a phenol), rutin (a flavonoid) and catechin (a flavonoid).

The graph plotted between concentration and percentage inhibition, elucidated the dose dependent behavior (Fig. 1a-b). The IC_{50} value of individual sample was calculated from its respective linear regression equations. The linear regression equations for DPPH radical scavenging activity of BM-MEx, BM-EEx and BM-WEx are $y = 0.284x+11.7$ ($R^2 = 0.994$), $y = 0.268x + 4.487$ ($R^2 = 0.997$) and $y = 0.251x + 2.388$ ($R^2 = 0.981$) respectively. The linear regression equation for the Caffeic acid, Catechin and Rutin are $y = 3.253x - 1.498$ ($R^2 = 0.996$), $y = 2.952x + 2.582$ ($R^2 = 0.989$) and $y = 2.600x + 8.880$ ($R^2 = 0.996$) respectively. Out of the different extracts tested the BM-MEx was found to have maximum percentage scavenging activity with an IC_{50} value of 129.7 ± 9.65 μg/ml. The BM-EEx had also shown the percentage scavenging potential with IC_{50} value of 169.6 ± 15.2 μg/ml followed by BM-WEx of 190.3 ± 13.3 μg/ml. The three different polyphenols caffeic acid, catechin and rutin, well known for their antioxidant activities has been employed as reference samples. Caffeic acid, catechin and rutin were found to have highest percentage inhibition even at very low concentrations with IC_{50} values of 15.82 ± 1.14, 16.1 ± 1.69 and 15.84 ± 0.58 μg/ml respectively.



(a)



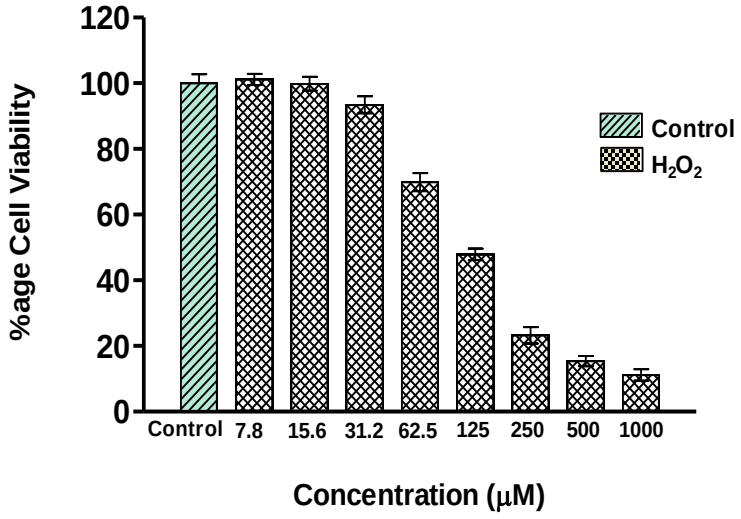
(b)

Fig.1. (a) Standard curve for the determination of total antioxidant capacity of extracts by DPPH assays. **(b)** Standard curve for the determination of total antioxidant capacity of standards by DPPH assay.

BM-MEx protection against H_2O_2 induced cytotoxicity

In the present study, the C6 glioma cells were first analyzed to find out the non-toxic doses of various BM extracts, to be used for further experimentation. It was found that the doses beyond 50µg/ml of each extract exhibited potential toxicity on C6 glioma cells (Data not shown). Further, the cells were exposed to different concentrations of H_2O_2 to calculate the prospective IC_{50} value (Fig. 2a). A dosage dependent cell damage was observed and respective IC_{50} value was 126.58 µM/ml, calculated by the regression equation y= -22.0 ln(x)

+ 156.5 (R^2 = 0.951). The computed IC_{50} value was further rounded off to 125 μM/ml for future experimentations. BM-MEx turned out to be a most potent candidate to protect against H_2O_2 induced damage followed by BM-EEx and BM-WEx. BM-MEx and BM-EEx delivered maximum protection at 25 μg/ml with a cellular viability of 81.65 ± 3.79% and 71.41 ± 4.02% respectively ($p < 0.05$). BM-WEx also improved the cellular viability to 65.40 ± 2.98% ($p < 0.05$) with a dose of 50 μg/ml (Fig. 2b-d). As BM-MEx offered maximum protection to C6 glioma cells against H_2O_2 generated damage, so the same was selected for downstream studies.



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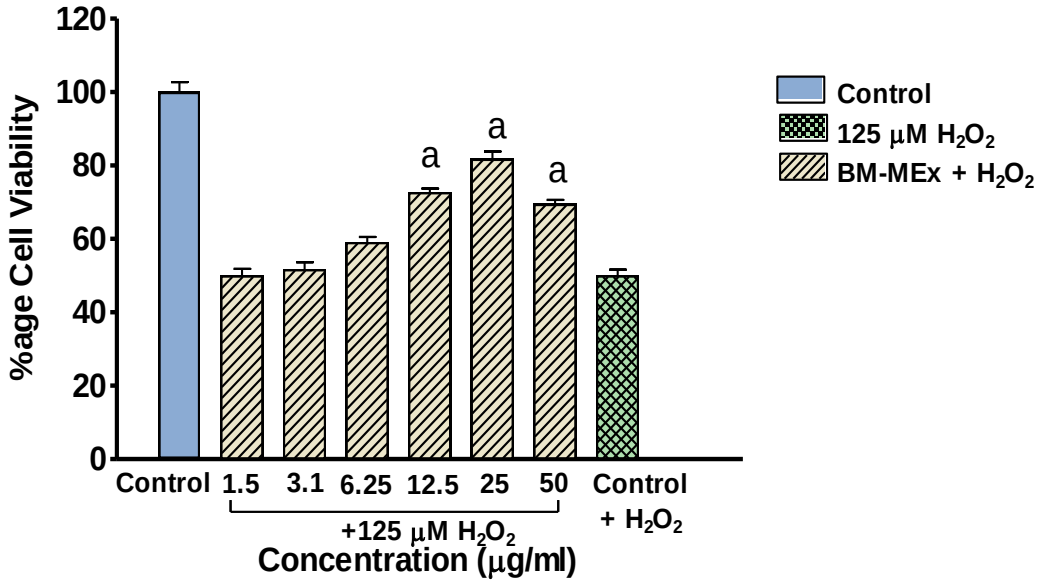
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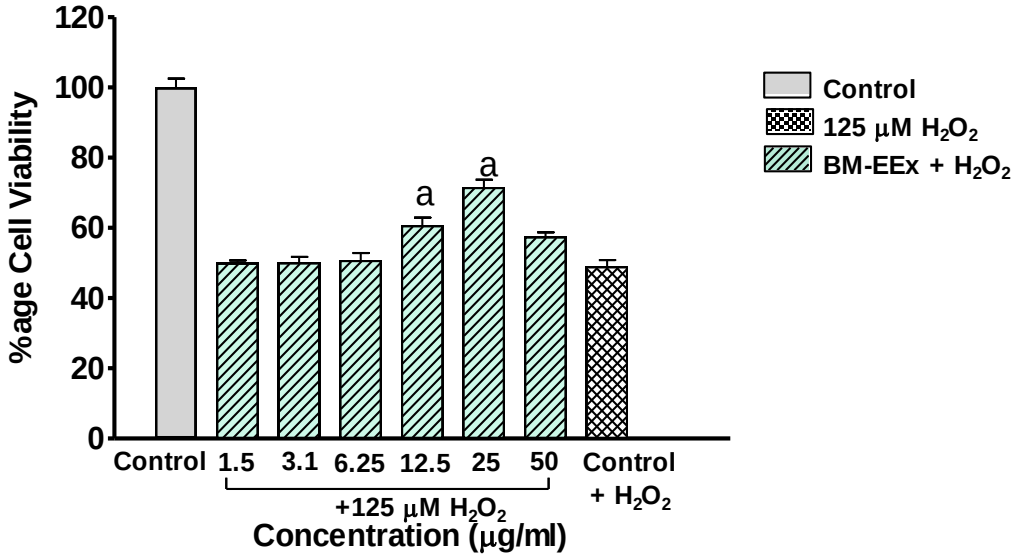
(b)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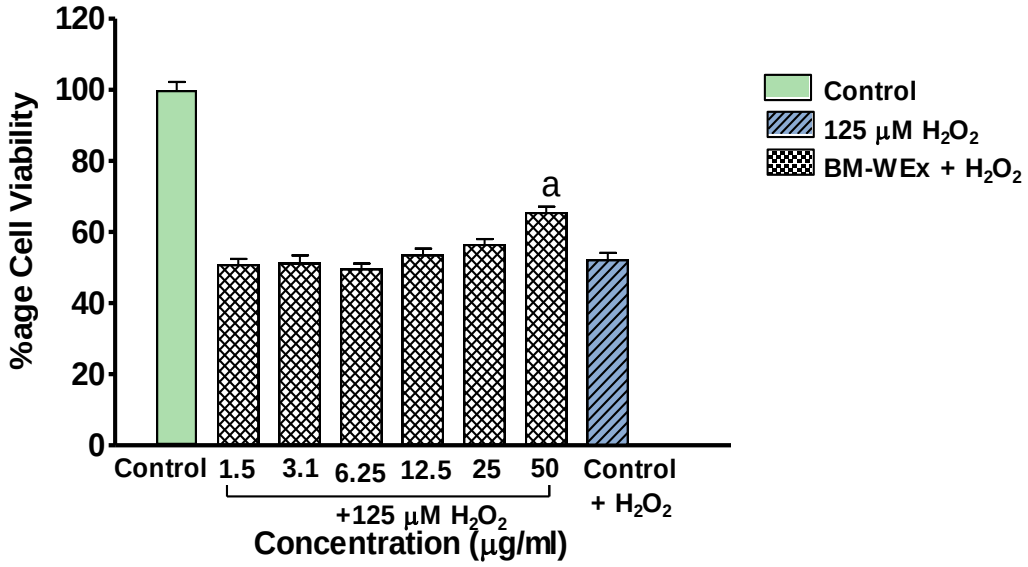
Concentration (μg/ml)

(c)



(d)

Fig.2. Neuroprotective effect of *B. monnieri* extracts on C6 glioma cell line.

(a) Dose-dependent cytotoxic effect of H_2O_2 (b-d) Effect of pretreatment with different extracts of *B. monnieri* on H_2O_2-induced cytotoxicity. The data represents mean ± S.E.M. from three independent experiments. a, Statistically significant difference between H_2O_2 cultures and various extracts + H_2O_2 cultures.

Expression of endogenous defense system

The expression analysis of cellular antioxidant enzymes and antioxidants was examined in the mentioned cell groups. Prior treatment with BM-MEx assisted the C6 glial cells to conquer H_2O_2 induced oxidative stress. The expression level of catalase, Cu-ZnSOD, GPx and GSH was found to be decreased significantly ($p < 0.05$) when exposed to H_2O_2 in comparison of control culture (Table 1). The prior treatment with BM-MEx facilitated the cells to overcome the deleterious effects of H_2O_2 and the expression of respective enzymes was increased considerably in contrast to H_2O_2 treated cells. Enhancement in lipid peroxidation was observed with H_2O_2 treatment in comparison to control ($p<0.05$), whereas the pre-treatment of BM-MEx to H_2O_2 exposed culture revealed a reduction in lipid peroxidation in comparison to H_2O_2 treated cells ($p < 0.05$). The investigation indicated the protective ability of BM-MEx which was further validated by downstream experimentations.

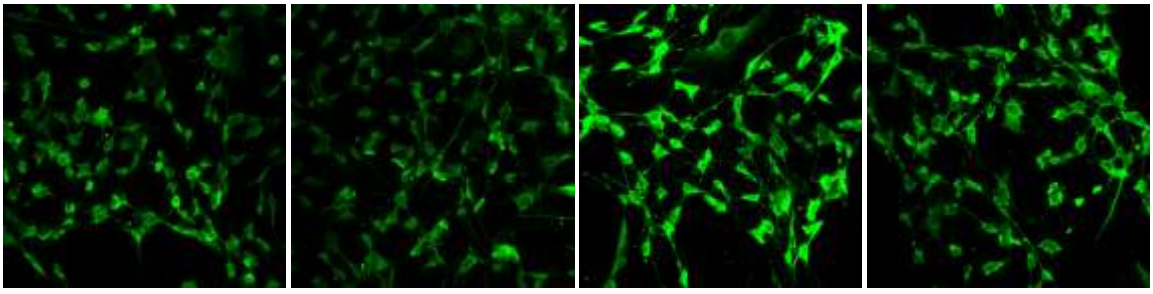
Table 1. Effect of protective potential of BM-MEx on oxidant scavenging machinery in C6 glial Cells.

Groups	Control	BM-MEx	H_2O_2	BM-MEx + H_2O_2
Catalase (U g tissue^{-1})	3.00 ± 0.31	3.43 ± 0.42 **a**	1.40 ± 0.29 **b**	2.51 ± 0.21 **c**
SOD (U g tissue^{-1})	14.29 ± 0.64	15.32 ± 0.96 **a**	7.76 ± 0.57 **b**	10.63 ± 1.02 **c**
GPx (U g tissue^{-1})	15.37 ± 1.13	15.71 ± 0.94 **a**	10.32 ± 0.71 **b**	13.32 ± 1.06 **c**
GSH (mg g tissue^{-1})	3.31 ± 0.39	3.67 ± 0.34 **a**	2.04 ± 0.19 **b**	2.63 ± 0.28 **c**
LPx (mg dl^{-1})	13.55 ± 0.78	9.58 ± 0.53 **a**	20.88 ± 1.32 **b**	16.26 ± 1.08 **c**

The data represents mean ± S.E.M. of protective potential of BM-MEx on oxidant scavenging machinery measured in homogenates obtained from cells of culture dishes (n = 3) derived from three independent cultures. The values having $P < 0.05$ are considered significant. **a**, Statistically significant change in BM-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 BM-MEx + H_2O_2 treated cultures with respect to the H_2O_2 treated cultures.

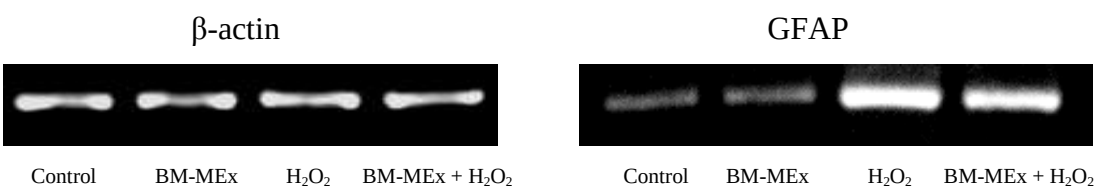
Effects of BM-MEx on GFAP expression in C6 glioma cell culture

GFAP is a major cytoskeletal protein specific to glial cells in the CNS. The expression level of GFAP was observed by immunofluorescence (Fig. 3a-d). The prior treatment with BM-MEx of H_2O_2 subjected cells assisted significantly to alleviate GFAP expression ($p < 0.05$) while the expression of GFAP remained stable in control cultures indicating no stress. The validation of protection by BM-MEx was also confirmed by studying the mRNA expression level of GFAP. The analysis revealed similar trend of expression as in immunofluorescence (Fig. 3e-f). The GFAP mRNA level was found to be reduced with the pre-treatment of BM-MEx in H_2O_2 exposed culture ($p < 0.05$). Control and BM-MEx cultures showed stabilized GFAP expression in immunofluorescence and RT-PCR.

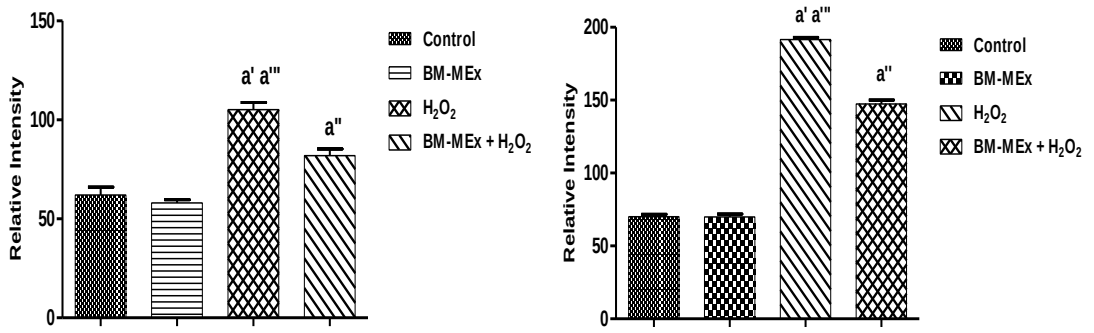


Control BM-MEx H_2O_2 BM-MEx + H_2O_2

(a) (b) (c) (d)



(e) (f)



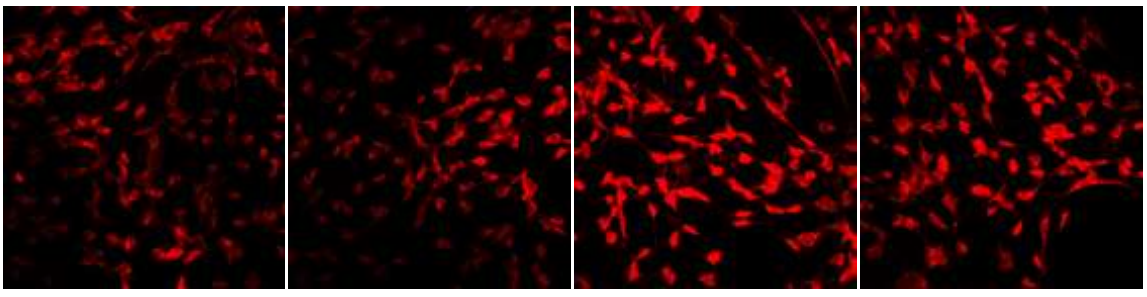
(g) (h)

Fig. 3. Expression analysis of GFAP in C6 cells by immunofluorescence and RT-PCR.

(**a**) Untreated control (**b**) BM-MEx treated **(c)** H_2O_2 treated **(d)** BM-MEx+H_2O_2 treated. Cells were cultured on coverslips (n = 3) for 3 days and then were fixed and stained for GFAP (Alexa Fluor 488) immunoreactivity **(e, f)** Representative reverse transcription-polymerase chain reaction (RT-PCR) of β-actin and GFAP **(g, h)** Relative intensity analyses of GFAP in immunofluorescence and RT-PCR. Relative optical density of GFAP in RT-PCR for each group expressed as percentage of β-actin. a', statistically significant difference H_2O_2 treated cultures and control cultures; a", statistically significant difference between BM-MEx + H_2O_2 treated cultures and BM-MEx treated cultures; a'", statistically significant difference between H_2O_2-treated cultures and BM -MEx + H_2O_2 treated cultures.

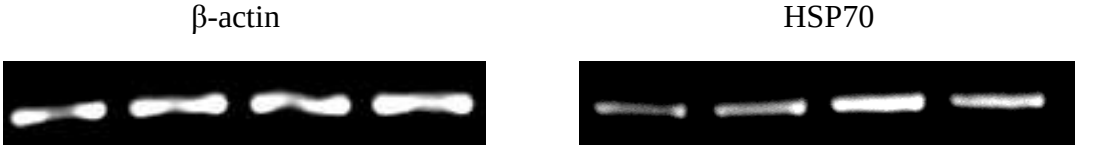
Effect of BM-MEx on transcript and protein levels of HSP70 in C6 cells exposed to H_2O_2

The cellular expression of HSP70 with H_2O_2 generated stress and BM-MEx treatment was observed in the C6 cells. There was reduction in HSP70 expression with prior treatment with BM-MEx in H_2O_2 subjected cultures ($p < 0.05$). The protective potential of BM-MEx was examined by using immunofluorescence and RT-PCR (Fig. 4).

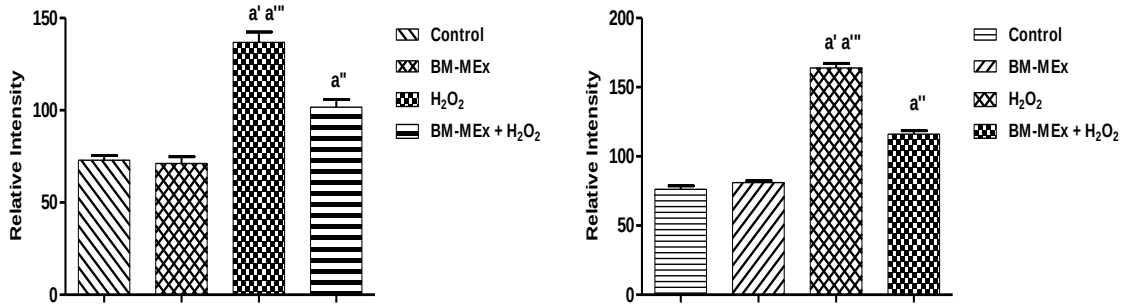


Control BM-MEx H_2O_2 BM-MEx + H_2O_2

(a) (b) (c) (d)



(e) (f)



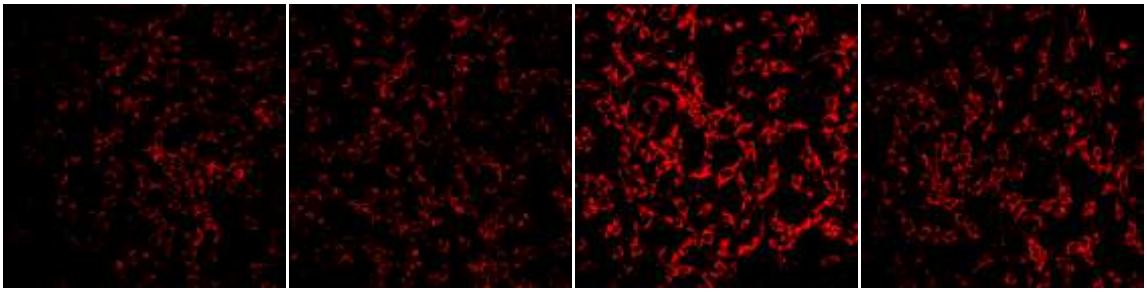
(g) (h)

Fig. 4. Expression analysis of HSP70 in C6 cells by immunofluorescence and RT-PCR.

(**a**) Untreated control (**b**) BM-MEx treated **(c)** H_2O_2 treated **(d)** BM-MEx+H_2O_2 treated. Cells were cultured on coverslips (n = 3) for 3 days and then were fixed and stained for HSP70 (Alexa Fluor 568) immunoreactivity **(e, f)** Representative reverse transcription-polymerase chain reaction (RT-PCR) of β-actin and HSP70 **(g, h)** Relative intensity analyses of HSP70 in immunofluorescence and RT-PCR. Relative optical density of HSP70 in RT-PCR for each group expressed as percentage of β-actin. a', statistically significant difference H_2O_2 treated cultures and control cultures; a", statistically significant difference between BM-MEx + H_2O_2 treated cultures and BM-MEx treated cultures; a'", statistically significant difference between H_2O_2-treated cultures and BM -MEx + H_2O_2 treated cultures.

Grp75 expression in H_2O_2 and BM-MEx + H_2O_2 C6 cell

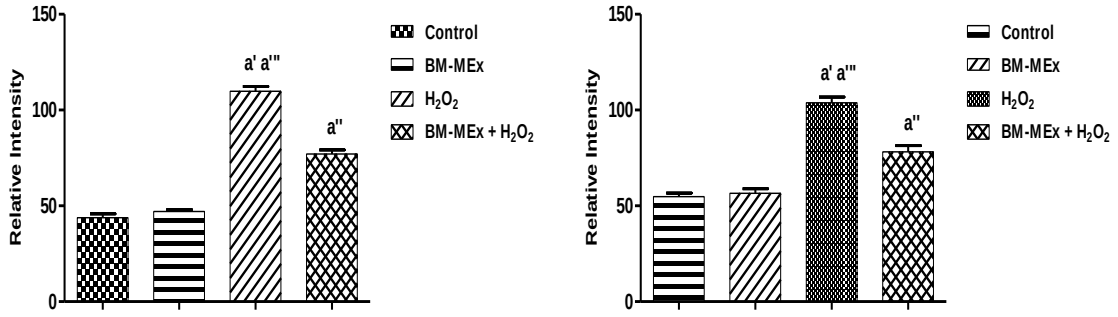
The expression levels of Grp75 was examined by immunofluorescence and RT-PCR (Fig. 5). The analysis showed enhancement of Grp75 expression in C6 cells exposed to H_2O_2 and the same was found to decreased with prior treatment of BM-MEx ($p < 0.05$). The analysis summarizes the protective prospective of BM-MEx against detrimental effects of H_2O_2.



Control BM-MEx H_2O_2 BM-MEx + H_2O_2

(a) (b) (c) (d)

(e) (f)



(g) (h)

Fig. 5. Expression level of Grp75 in C6 cells by immunofluorescence and RT-PCR.

(**a**) Untreated control (**b**) BM-MEx treated **(c)** H_2O_2 treated **(d)** BM-MEx+H_2O_2 treated. Cells were cultured on coverslips (n = 3) for 3 days and then were fixed and stained for Grp75 (Alexa Fluor 568) immunoreactivity **(e, f)** Representative reverse transcription-polymerase chain reaction (RT-PCR) of β-actin and Grp75 **(g, h)** Relative intensity analyses of HSP70 in immunofluorescence and RT-PCR. Relative optical density of Grp75 in RT-PCR for each group expressed as percentage of β-actin. a', statistically significant difference H_2O_2 treated cultures and control cultures; a", statistically significant difference between BM-MEx + H_2O_2 treated cultures and BM-MEx treated cultures; a'", statistically significant difference between H_2O_2-treated cultures and BM -MEx + H_2O_2 treated cultures.

DISCUSSION

Nowadays, the traditional medicine system called Ayurveda is emerging as the center stage because of its efficacy to meet the speculated goals of the human welfare. A number of medicinal plants are acquiring authentication as remedy for deleterious illnesses because of the irresponsiveness and side effects of existing drugs. *Bacopa monnieri* is one of the well acknowledged medicinal plants universally accepted for the nervous frailty. It has been vastly used as nerve tonic for the enhancement of memory and intellectual ability (Calabrese *et al.*, 2008; Chatterjee *et al.*, 2010). BM has been found to possess the protective potential to combat the ongoing events in neurodegenerative disorders (Hosamani, 2009; Uabundit *et al.*, 2010; Shinomol *et al.*, 2012). The protective ability of BM is attributed to naturally occurring intrinsic bioactive constituents (Kasture *et al.*, 2007; Mathew *et al.*, 2011). Till date, the study of BM protection using *in vitro* culture is not well studied. So, the current study was designed to explore the protective efficacy of BM under the influence of oxidative damage using C6 glioma culture. The potency of protection by BM was determined by studying the expression levels of antioxidant machinery and selected stress markers.

Brain cells are extremely vulnerable to any damage because of nullified capacity of regeneration. Neurodegenerative diseases like AD, PD, HD and ALS are the upshots of oxidative stress (Uttara *et al.*, 2009; Gandhi & Abramov, 2012; Zhao & Zhao, 2013).

Reactive oxygen or nitrogen species mediated oxidative damage plays a main character in the deterioration of the nervous system. The intrinsic antioxidant cellular defense forces are capable enough to overcome the detrimental effects of oxidative stress only upto a certain limit, resulting into the irreversible cellular injury (Wei & Lee, 2002; Sies, 2013).

H_2O_2 *per se* is not an ROS but upon reduction with one electron it yield hydroxyl radicals which are one of the most potent ROS. These radicals indiscriminately reacts with all the vital cellular biomolecules including DNA, lipids or proteins resulting in cellular damage (Sies, 2013). In the present study, C6 cells were exposed to the varied concentrations of H_2O_2 to determine the IC_{50} value. The cells were also scanned to explore the toxicity profile of BM extracts (Figure not shown). The neuroprotective survey depicted BM-MEx to possess maximum protective ability against the H_2O_2 induced oxidative stress. So, BM-MEx being a superior contender in delivering protection, was selected for the downstream investigation. Earlier studies have also supported the protective efficacy of BM (Limpeanchob *et al.*, 2008; Shinomol *et al.*, 2012). Likewise BM, *Camellia sinensis* and *Hypericum perforatum* extracts have also been reported to protect against H_2O_2 induced cytotoxicity (Jang *et al.*, 2002; López & Calvo, 2011).

The neuroprotective efficacy of BM was explored by investigating the antioxidant enzyme levels, immunofluorescence and RT-PCR. GFAP, HSP70 and Grp75 proteins were elected as stress markers for the present study. The cellular defense machinery is potent enough to overcome the deleterious consequences of oxidative stress. Catalase, SOD, GPx and LPx are the components of defense system which can easily alter the detrimental ROS and defend the cell from any possible injury (Mates, 2000). In the current study, a significant reduction in the expression of these antioxidant enzymes (except LPx) following H_2O_2 exposure and controlled expression of enzymes with prior treatment of BM-MEx was observed. The expression of LPx was found to be reversed of rest of the studied enzymes.

GFAP is the intermediate cytoskeletal protein specific to glial cells of CNS. The expression of GFAP has been reported to be upregulated in reactive gliosis and under the influence of oxidative stress (Eng & Ghirnikar, 1994; Kataria *et al.*, 2012). Correspondingly, the expression level of HSP70 and Grp75have also been detected to elevate enormously in response to any cellular insult (Rajdev & Sharp, 2000; Carette *et al.*, 2002; Mitsumoto *et al.*, 2002). The present investigation demonstrated the increment in expression level of all the selected protein markers (GFAP, HSP70 and Grp75) following H_2O_2 treatment and down-regulation of the respective markers consequently following pretreatment with BM-MEx.

The present investigation clearly shows that the expression levels of selected stress markers and antioxidant machinery following prior treatment with BM-MEx restores to normal level. The pretreatment of BM-MEx significantly improves the cellular viability. These results authenticate the neuroprotective efficacy of BM and also proposes that BM-MEx can be employed as herbal remedy against nervous infirmity.

Conclusion

BM-MEx in contrary of BM-EEx and BM-WEx, has delivered potent protection against H_2O_2 mediated stress by enhancing the intrinsic antioxidant enzyme levels. The analysis was further confirmed by the alleviation of stress as revealed by the expression of GFAP, HSP70 and Grp75 following prior treatment with BM-MEx in H_2O_2 subjected cultures. Further investigations are required to explore the role of other cellular components in protection or severity.

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