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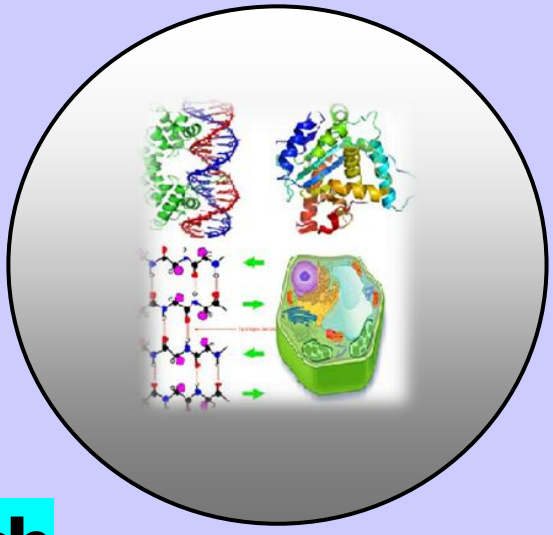

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RESEARCH PAPER

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Bacopa monnieri Suppresses Rotenone-Induced Microglial Activation: *In vitro* Study

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ABSTRACT

Neuroinflammation is involved in the pathogenesis of neurodegenerative diseases including Parkinson's disease (PD), a major neurodegenerative disorder characterized by dopaminergic neuronal death in the substantia nigra. We examined here, medicinal plant Bacopa monnieri (BM) for its anti-inflammatory properties towards an approach for development of a PD therapy. Rotenone, the active ingredient in various pesticides, has been identified as an inducer of PD. It has been revealed that rotenone induces activation of microglia and generation of pro-inflammatory factors in PD. In this study, our aim was to study the effect of BM on the inflammatory biomarkers induced by rotenone in neuroblastoma SH-SY5Y cell line. The levels of TNF-α, IL-1β, IL-6 were measured using ELISA kits. Furthermore, we performed the cell viability assay using MTT assay for evaluating the neuroprotective property of BM (Pre and post treatment) in Human neuroblastoma SH-SY5Y cells treated with rotenone. In rotenone-treated neuroblastoma SH-SY5Y cells, BM dose-dependently suppressed the up-regulation in the expression of iNOS in the cells, as well as the production of TNF-α, IL-6 and IL-1β in the cultured media. BM significantly inhibited rotenone-induced ROS production, in neuroblastoma SH-SY5Y cells. BM was also found to restore the viability of rotenone-treated SH-SY5Y cells. However, this rotenone-activated microglia-mediated death of SH-SY5Y cells was more significantly attenuated when the neuroblastoma SH-SY5Y cells were pre-treated with BM. Results demonstrated that BM pretreatment significantly reduces rotenone-induced cytotoxicity.BM can directly suppress rotenone-induced neuroinflammation in vitro by repressing ROS production and our results demonstrated that BM protects the neurons from the NO injury thereby BM proving itself to be a potent compound to combat PD.
Keywords: Neuroinflammation; Rotenone; Substantia nigra; MTT assay and Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by preferential loss of dopaminergic (DA) neurons in the substantia nigra pars compacta. Neuro-inflammation is commonly observed in several neurodegenerative disorders including PD and is mainly caused by microglial activation. Growing evidences implicate microglia in the loss of dopaminergic neurons in PD. However, factors mediating microglial activation in PD are poorly understood. Although, neuro-inflammation is a mechanism that protects the organism from infection and injury, if not turned off in a timely manner, it can become chronic and contribute to neurodegeneration [Tansey, M.G. and Goldberg, 2012, Blandini, 2013]. Rotenone, a widely used pesticides can selectively inhibit complex I of the mitochondrial electron transport chain, thereby increasing the production of free hydroxy radicals and inducing oxidative stress. Rotenone's toxicity has been demonstrated in various invitro [Gao et al., 2002, Freestoneet al., 2009, Qualls et al., 2014] and in-vivo [Caboni et al., 20114, Radad et al., 2006, Sanders and Greenamyre, 2013] studies. While the reasons for the selective loss of the nigral dopaminergic neurons still remain to be clarified, oxidative stress and neuroinflammation are believed to be major contributors. Increased reactive oxygen species (ROS) production within the microglia by enzymes such as NADPH oxidase, as well as ROS produced by nearby cells, are known to elicit activation of the neuroinflammation [Gloire et al., 2006]. This in turn leads to increased production of proinflammatory enzymes such as inducible NOS (iNOS), the cytokines TNF-α and IL-1β and NO, all of which can contribute to neurodegeneration [Zhou et al., 2014, Stuckenholz et al., 2013]. Inflammatory mediators such as TNF-α, IL-1β, COX-2 and iNOS were over expressed in the Nrf2 knockout mice and these mice were hypersensitive to neuroinflammation induced by 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) [Rojo et al., 2010].

The currently available treatment for PD only relieves the symptoms without delaying the degeneration itself. Drugs that can modify the disease progression are being actively sought. *Bacopa monnieri* (BM) is an important plant in Ayurveda commonly known as brahmi. Due to the therapeutic importance of BM in indigenous systems of medicine, systematic chemical examinations of the plant have been carried out by several groups of researchers. Ayurvedic medicine classifies BM as belonging to a group of plant medicines known as medhya rasayana- that improve mental health, intellect and memory (medhya) and promotes longevity and rejuvenation (rasayana) [Singh and Singh, 1980]. BM has been used for centuries as a brain tonic, memory enhancer, revitaliser of sensory organs, anti-anxiety, cardio-tonic, diuretic, anti-depressant and anticonvulsant agent. It has a very important role in Ayurvedic therapies for the treatment of cognitive disorders of aging [Uabundit et al., 2010] and also possesses anti-inflammatory, analgesic, antipyretic, epilepsy, anticancer and antioxidant activities [Russo and Borrelli, 2005]. Neuro-inflammatory effects of BM with rotenone toxicity in neuroblastoma is unknown. Therefore, the aim of this study was to investigate the inhibitory effect of BM on rotenone-induced pathogenesis of PD in neuroblastoma SH-SY5Y cells by restoration of the neuroinflammation marker and decreasing reactive oxygen species (ROS) production.

MATERIALS AND METHODS

Material

Rotenone, 2, 7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). DMEM/F12 cell culture medium, trypsin, EDTA, fetal bovine serum (FBS) and 100X antibiotic and antimycotic solution were purchased from Invitrogen Corporation (Van Allen Way, Carlsbad, California, USA).

Nitric oxide colorimetric kit, TNF- alpha and others ELISA kit were purchased from Abcam (Cambridge, MA, USA).

Cell Culture and treatment

SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). SH-SY5Y cells were cultivated in a humidified incubator with 5% CO2 at 37°C in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and HAM's F12 (Invitrogen Corporation, Van Allen Way, Carlsbad, California, USA) with penicillin/streptomycin (100 IU/ml), gentamicin (50 µg/ml), and 10% heat inactivated fetal bovine serum (FBS). Cells were grown in cell culture flasks were > 80% confluent. Media was changed twice weekly and the cultures were split at a ratio of 1:5 once a week.

Drug treatment

Once cells were confluent they were harvested and plated into 96 well plates at ~ 12,000 cells per well. The cells were allowed 24 hours to settle and adhere to the bottom before drug treatment. Then fresh media containing various concentrations of drugs (rotenone and *Bacopa monnieri* (BM), from Sigma-Aldrich and Natural remedies) were added to aspirated wells.

Rotenone exposure and BM treatment

In order to determine the concentration-responses, rotenone (5, 25, 50, 100, and 200 µM) were added to the cell media for 24 h and 48 h. Cell viability was then assessed using MTT (3,[4,5-dimethyliazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. BM (5, 10, 25, 50, 100 and 200µg/ml), were added to the cells at the same time for 3 hours pre and 3 hours post treatment for determining cell viability.

Based on these results, nontoxic concentrations of rotenone and effective dose of BM were combined for the combination studies. Control wells were treated with pure media in five independent replicates of the experiment. 24 h later cell viability was determined by MTT assay.

MTT Assay

Cell viability was determined by MTT assay [Ansari et al., 2016]. This assay is based on the enzymatic reduction phenomenon of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye. The assay provides a direct relationship between the viable cells and color formation (absorbance). In brief, the cells ($5x10^3$) were seeded in 100 µL complete medium in each well of 96-well culture plate for 24 hrs at 37 ^{0}C and 5% CO_2. Stocks of compounds were prepared in Dimethyl sulfoxide (DMSO) and diluted to the desired concentrations (25 to 500µg/ml) in medium and added to the wells in triplicate as per experimental design. After 24h of treatment, 10 µL of MTT (5 mg/mL) solution was added in each well and the plates were further incubated for 2h at 37 ^{0}C until formazan blue crystals developed.

Then the supernatant was discarded from each well and 100µL of DMSO was added to solubilize formazan crystals for 10 min at 37^{0}C. The absorbance was recorded at 540 nm by a microplate reader (BIORAD-680). The percentage viability was calculated by using the formula % Cell viability = [OD of treated) / (OD of control)] X 100.

Measurement of ROS generation

Cells were seeded in 96-well tissue culture plates (10,000 cells/well) in complete DMEM F-12 media. Intracellular ROS generation was estimated by the method of Wan et al. [2016] using 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye by measuring the conversion of non-fluorescent DCFH-DA to fluorescent dichloro fluorescein (DCF) within the cell using SYNERGY-HT multiwell plate reader (Bio-Tek, Winooski, USA). Briefly, cells seeded in black 96-well plate at a density of 10,000 cells/well were incubated with 10 µM DCFH-DA for 30 min at 37 ^{0}C followed by incubation with desired treatments of rotenone and BM for 24 h. The fluorescent intensity of 2'7'-dichlorofluorescein (DCF) was measured using a plate reader at 485 nm excitation and 538 nm emission.

Nitric Oxide Assay

Nitric oxide (NO) was assessed by measuring the levels of oxidized forms (nitrites and nitrates) in samples using the Nitric Oxide Colorimetric Assay Kit (Abcam) as per manufacturer protocol. Briefly, 2×10^6 cells were plated in 25 cm^2 flasks. Control and treated cells were washed with cold PBS, resuspended in ice cold assay buffer (provided with kit), cells were homogenized by pipetting up and down for few times, centrifuged the samples for 5 min at 4 ^{0}C at top speed, collected supernatant and transferred to a clean tube to finally performed deprotenization. Obtained protein samples were exposed to nitrate reductase and cofactor after which plate was incubated at room temperature for 1 h to transform nitrate to nitrite. Griess reagents were applied to convert nitrite to a deep purple azo compound and the color was developed for 10 min at room temperature. Absorbance was recorded at 540 nm using a multimode-detection microplate reader synergy HT (Biotek).

ELISA assay for TNF-α, IL-1β, IL-6

Supernatants of cell cultures with treatment were collected and stored at -80^{0}C for subsequent quantification of cytokine content TNF-α, IL-1β, IL-6 using conventional double antibody sandwich ELISA with microplate reader, ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.

Statistical analysis

Statistical differences between groups were determined by one-way ANOVA with Statistical Package, Graph Pad Prism Version 6.01. Data were expressed as mean ± standard error (SEM) for and differences were considered statistically significant, when p values were less than 0.05 ($p< 0.05$).

RESULTS

Rotenone only toxicity

Fig. 1 depicts the effects of various concentrations of rotenone (5, 25, 50, 100, and 200 µM) treatment for 24 h and 48 h, on SH-SY5Y cell viability. There was a clear dose-response effect, with higher concentrations of rotenone resulting in greater cell toxicity. Maximum toxicity (about 50%) was achieved in 24 with the 100 µM rotenone ($p<0.001$).

Thus, this concentration of rotenone was chosen to test the potential protective effects of BM (Fig. 1). Rotenone at low concentration (5 or 25 μM) did not result in any toxicity and shows more toxicity in 48h, so 100 uM concentrations were selected for the combination studies.

Effective dose of BM

The effective dose of BM was determined after 24 h and 48 h of incubation (Fig. 2). Treatments with the BM extract for 3 h pre and post, the addition of 100 uM rotenone, protected SH-SY5Y cells against rotenone induced toxicity. This protection was assessed 24 h after the addition of rotenone through the MTT test, and results was significant from 50 μg/ml dose of BM. More significant protection was observed from 50 μg/ml of BM and reached a maximum at 100 μg/ml in pretreatment. BM alone did not cause any cytotoxicity till the highest tested concentration (200 μg/ml).

Rotenone induces oxidative stress and disturbs the antioxidant defenses within Neurobloastoma cells

ROS generation was assessed at 24 h after treatment. To investigate the involvement of the antioxidant effect of the BM extract in the neuroprotective effect against rotenone, we then measured the intracellular levels of ROS using a cell permeable fluorescent probe DCF-DA. As expected, SH-SY5Y cells treated with a high concentrations of rotenone (100 uM) displayed intense fluorescence after staining with DCF dye, and this fluorescence was significantly decreased when cells were pretreated with BM from 100 μg/ml. Although cells showed neuroprotection in post treatment with BM but more significant results were found in pretreated neuro-blastoma cells (Fig. 3).

Effect of BM extract pre and post treatment on nitric oxide (NO)

To assess whether BM extract prevents loss of dopaminergic neurons, we measured the level of NO, a potent mediator of inflammatory response which revealed significant ($p<0.001$) enhancement in NO levels after rotenone exposure. 3 h Pretreatment of BM extract (100 μg/ml) showed more significant neuroprotection as compared to post treatment with BM extract against rotenone (100 uM) induced toxicity (Fig. 4).

Effect of BM extract pre and post treatment on levels of TNF-α, IL-1β, IL-6

Neuroinflammation is an important event in neurodegeneration process and rotenone potentially induces inflammatory proteins. In this study the status of specific cytokines production was assessed i.e. Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6) andTumor necrosis factor- alpha (TNF alpha). We found that higher concentration of all the three cytokines in rotenone induced toxicity. Pretreatment of BM extract (100 μg/ml) had more significant ($p<0.001$) effect as neuroprotectant by decreasing the levels of cytokines in 3 h prior treatment of BM (Fig. 5, Fig. 6 a,b).

DISCUSSION

The results of the present study confirms that rotenone induces toxicity in SH-SY5Y cells model of dopaminergic neurons [Song et al., 2012]. Rotenone's effect has been attributed to inhibition of mitochondrial complex I [Alam and Schmidt, 2002]. Rotenone might also cause inflammation, which appears to prominent contributed to the causation of PD syndrome [Gao et al., 2003, Chinta et al., 2013]. BM, an ayurvedic medicinal plant, has attracted considerable interest owing to its diverse neuropharmacological properties [Jadiya et al., 2011, Hosamani et al., 2016].

Rotenone-induced ROS production through, NF-κB activation [Gao et al., 2013]. Furthermore, ROS was reported to be a second messenger that activates diverse redox-sensitive signaling transduction cascades, including p38 and its associated up-stream transcription of many proinflammatory genes [Droge et al., 2002, Jia et al., 2007]. Rotenone, as a mitochondrial complex I inhibitor, is reported to decrease the mitochondrial membrane potential, leading to ROS production [Li et al., 2003]. Our study provides strong evidence that BM extract restores the rotenone-induced ROS production. This study is for the first time reported from our laboratory in rotenone induced cytotoxicity. Similar study has been reported in MPP+ neurotoxicity [Singh et al., 2012].

Peroxynitrite, is one of the most destructive molecules that forms when NO reacts with superoxide free radical and mediates cell death [Ischiropoulos and Al Mehdi, 1995]. iNOS expression is induced by excessive secretion of inflammatory molecules during infection and cellular injury which is the main contributor in neurodegeneration [Dawson and Dawson, 1998]. The results found in this study are in accordance with the previous investigations showing the role of nitric oxide in neurodegeneration and iNOS inhibitors in neuroprotection [Liberatore et al., 1999]. NO finally converts into peroxy nitrite and causes neuronal cell death by increasing the oxidative stress [Noack et al., 1999]. Rotenone also activates microglia, the resident immune cells in the brain, to release pro-inflammatory cytokines thereby enhancing ROS and lipid oxidation, protein and DNA damage. Alternatively Rotenone also activates NADPH oxidase leading to further increase in ROS and oxidative stress [Tada-Oikawa et al., 2003]. In our study we found that the activity of i-NOS and ROS were restored against rotenone induced cytotoxicity, with treatment of BM extract. Although the pretreatment showed highly significant result in restoring the cell against neurotoxicant as compared to post treatment.

Cytokines play an important role in maintaining the function of neurons, while on the other hand, any disturbance in the homeostasis of cytokines is implicated in many neurological disorders including AD and PD. Studies reveal that TNF-alpha and Interlukin-1 receptors are present on the neurons, and it has also been reported that TNF-alpha and Interlukin-1 m-RNA accumulate in neurons. TNF also play a role in maintaining physiological levels of neurotransmitter release through regulation of adrenergic auto receptor activity [Ignatowski et al., 1997]. Additionally, changes in TNF level correlates with the neuronal survival or cell death [Downen et al., 1999]. IL-6 is produced locally in the brain and reports indicate that increased IL-6 is associated with severe cognitive impairments that may contribute to neuronal loss or injury [Heyser et al., 1997]. On the basis of above reports and our findings, we can say that rotenone is responsible for the altered levels of cytokines and it mediates neuroinflammation and cell death. We observed, an enhanced level of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in neuroblastoma cell line (SH-SH5Y), which was significantly attenuated following BM pre and post treatment. Therefore, the present findings further suggest that BM extract has its anti-inflammatory effects through inhibiting the increased level of ROS and iNOS, as well as proinflammatory cytokines. The BM mediated suppression of inflammation, as observed in the present study, could be facilitated by the strong anti-inflammatory activity of BM that has been reported in other studies [Singh et al., 2012].

Oxidative stress and neuroinflammation play major roles in different neurodegenerative diseases such as stroke, Alzheimer's disease, and PD. Drugs with anti-oxidative and anti-inflammatory characteristics are considered effective therapeutic agents for these neurodegenerative diseases [Qualls et al., 2014, Sanders and Greenamyre, 2013].

Study showed that BM extract has strong antioxidant property which is very useful to develop a potential drug against neurodegenerative diseases [Singh et al., 2012]. It may be suggested that, BM might be maintaining mitochondrial membrane potential to suppress ROS production, an upstream cellular response to the rotenone toxicity. The suppression of rotenone-induced microglial activation by BM extract treatment decreases the production of inflammatory factors, which attenuates inflammatory factor against rotenone induced cytotoxicity in SH-SY5Y cell line. Therefore, BM with its effective antioxidant and anti-inflammatory properties, may be responsible for the neuroprotection in the rotenone induced neurodegeneration.

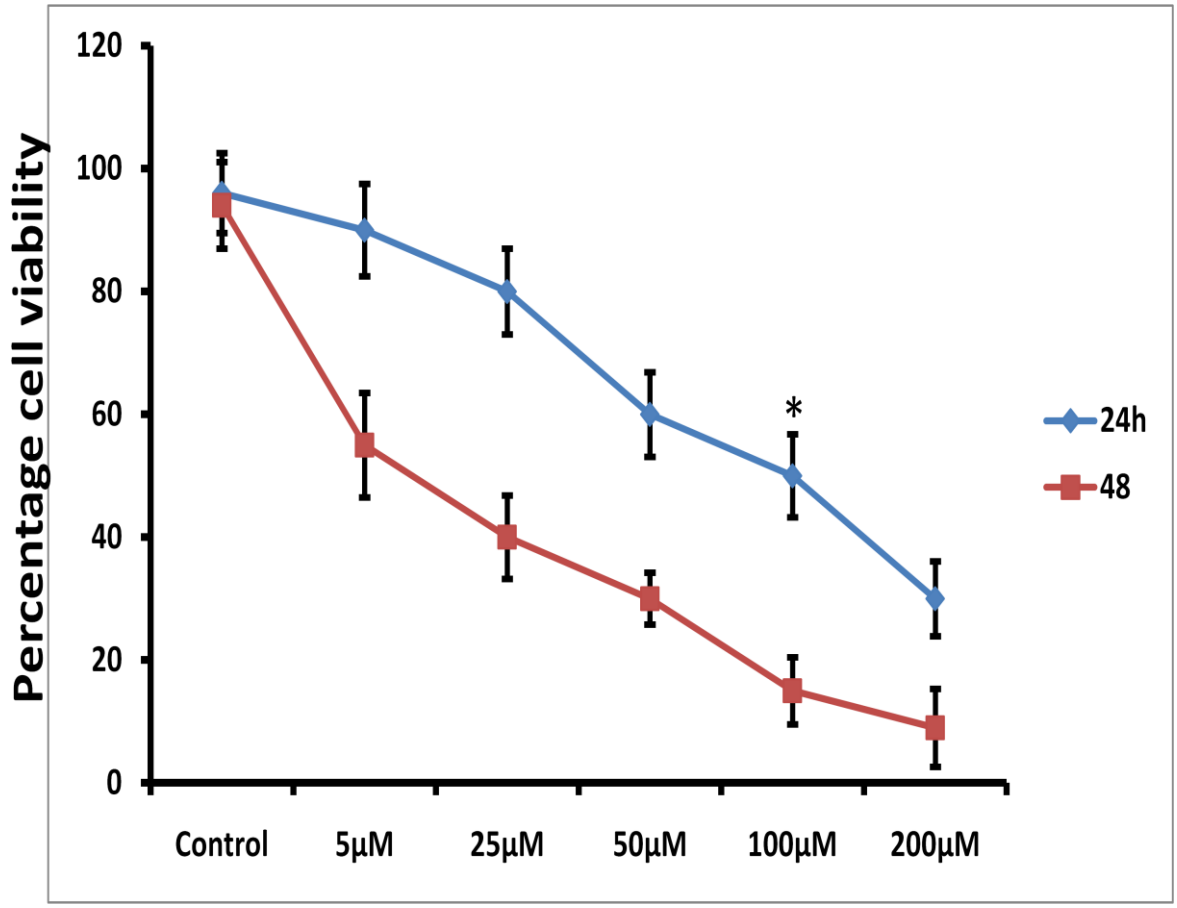

Fig.1. Effect of various concentrations of rotenone on SH-SY5Y cell viability. Cells were exposed for 24 h & 48 h and cell viability was determined by MTT assay. Values represent mean ± SEM of five independent experiments, *p <0.05 considered statistically significant compared to control.

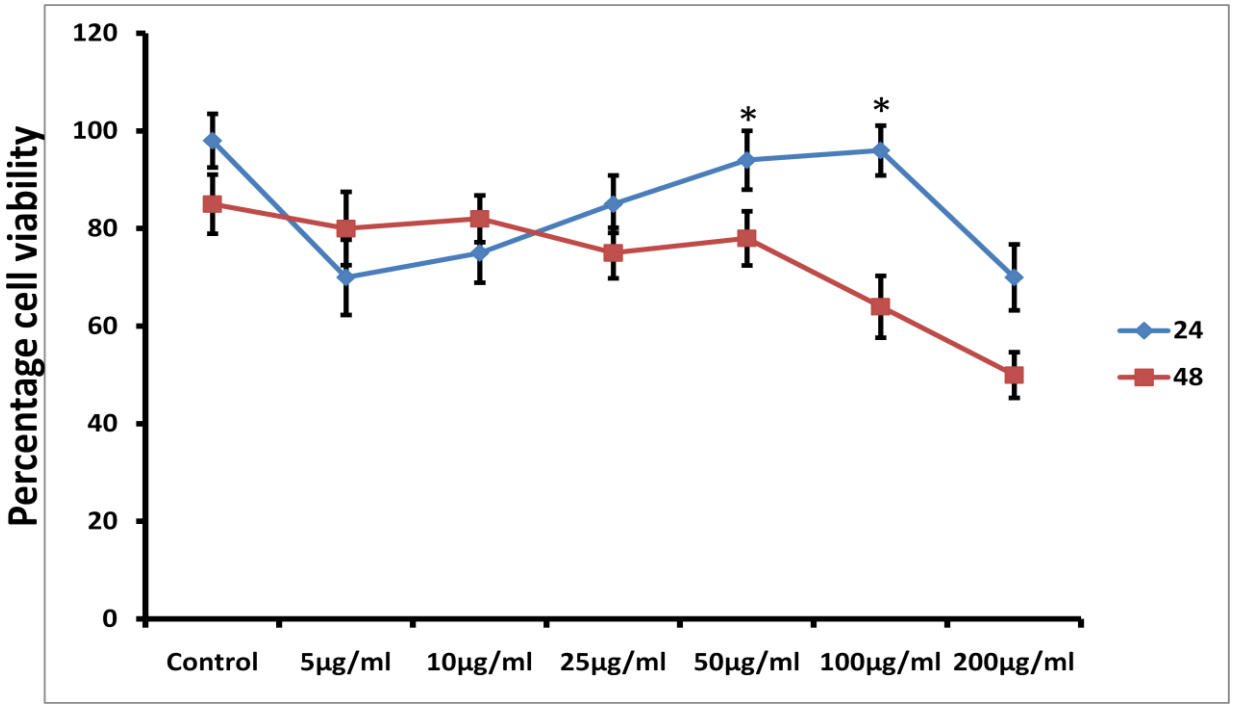

Fig. 2. Effect of various concentrations of BM extract on SH-SY5Y cell viability. Cells were exposed for 24 h & 48 h and cell viability was determined by MTT assay. Values represent mean ± SEM of five independent experiments, *p <0.05 considered statistically significant compared to control.

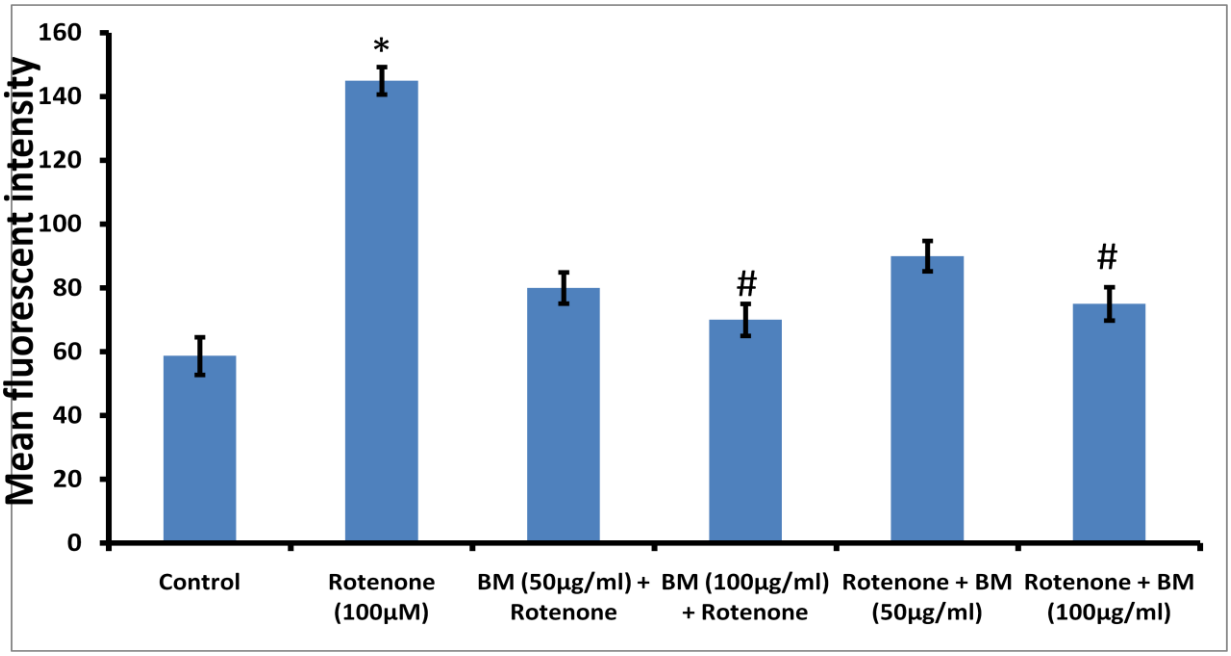

Fig. 3. Showing the Effective of BM extract and rotenone on SH-SY5Y. ROS generation was assessed in terms of relative fluorescence units using 10 µM DCFH-DA in neuroblastoma cells. Values represent mean ± SEM of five independent experiments, *p <0.05 considered statistically significant compared to control, #p <0.05 compared to rotenone.

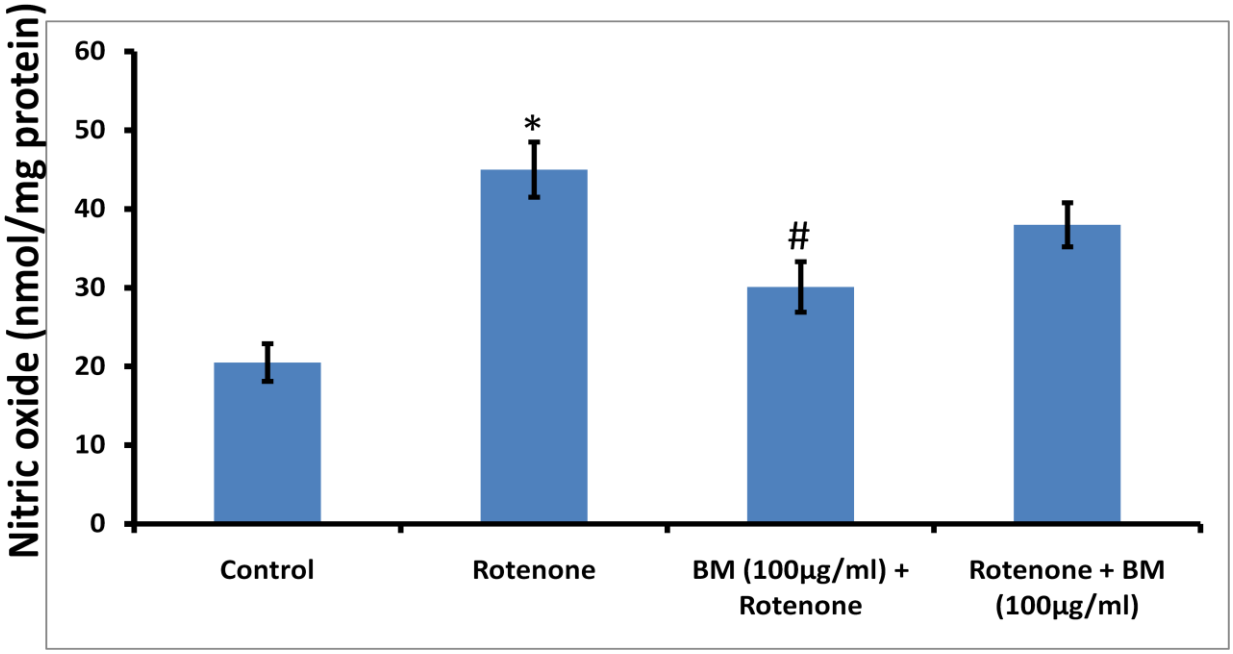

Fig. 4. Effect of BM extract on Nitric oxide concentration against rotenone induced cyto-toxicity. Values represent mean ± SEM. *p <0.05 considered statistically significant compared to control, #p <0.05 compared to rotenone.

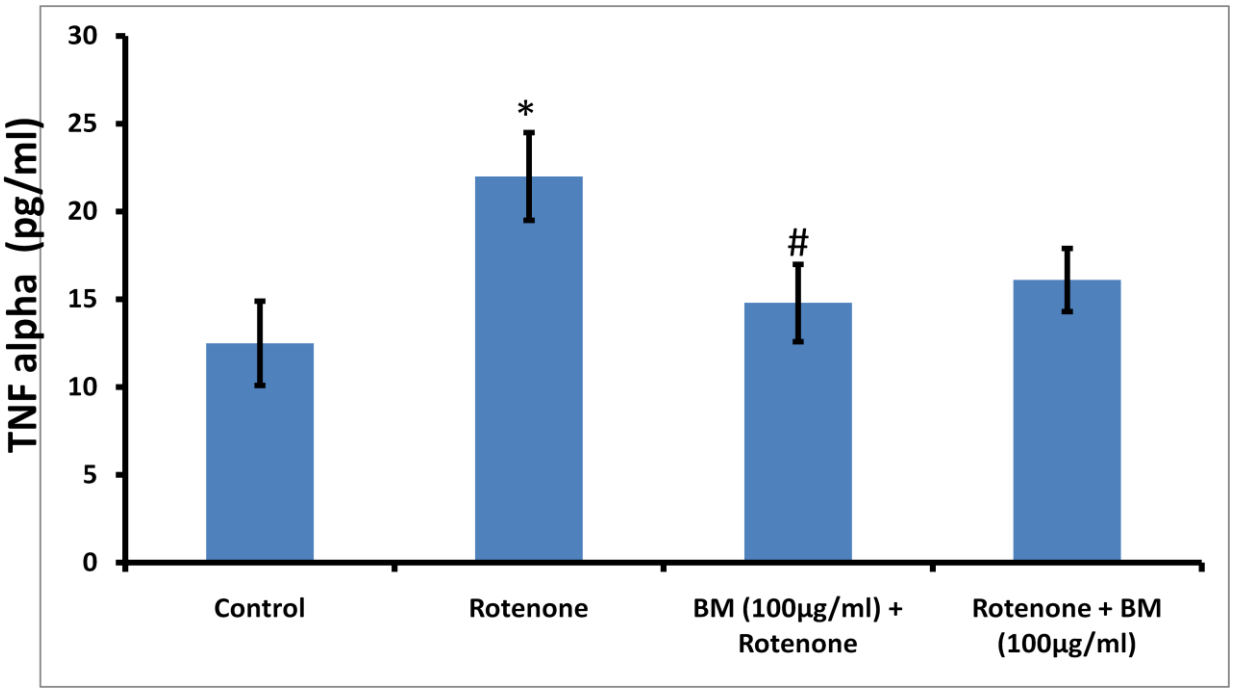

Fig. 5. Effect of BM extract on TNF alpha concentration against rotenone induced cyto-toxicity. Values represent mean ± SEM. *p <0.05 considered statistically significant compared to control, #p <0.05 compared to rotenone.

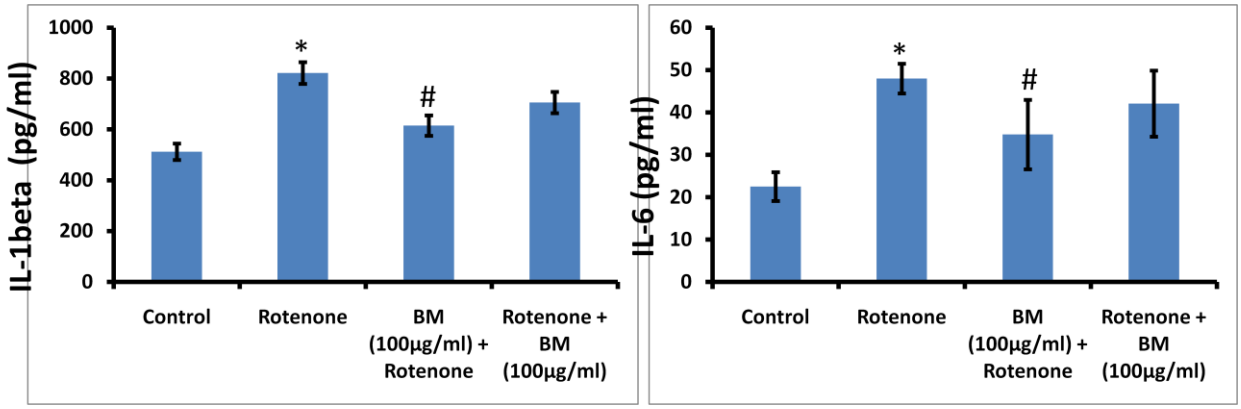

Fig. 6. Effect of BM extract on a) IL-1 beta and b) IL-6 concentration against rotenone induced cyto-toxicity. Values represent mean ± SEM. *p <0.05 considered statistically significant compared to control, #p <0.05 compared to rotenone.

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