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**Amelioration of butylated hydroxytoluene against inorganic mercury induced cytotoxicity and mitochondrial apoptosis in PC12 cells *via* antioxidant effects**

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## **Abstract**

Mercury (Hg) is a toxic metal, well-known for its dangerous health effects on human. Butylated hydroxytoluene (BHT) is a phenolic component generally consumed as a food additive as an antioxidant. However, BHT induced antioxidant properties against heavy metals-influenced toxicity are little studied. We hypothesized that BHT has a regulatory effect on Hg-induced cytotoxicity. The objective of this research was to assess the protecting effects of BHT against inorganic Hg (iHg)-toxicity in PC12 cells, where cells were treated with/without HgCl<sub>2</sub> (Hg<sup>2+</sup>) (5 μM) and BHT (100 μM) for 48 h and analyzed further. Cells treated by Hg caused a significant cell viability reduction, membrane damage, glutathione reduction, DNA fragmentation, ROS generation, with suppressed expressions of akt, mTOR, ERK1, Nrf2 and HO1; and elevated apoptotic expressions of p53, Bax, cytochrome c and active caspase 3. However, BHT and Hg<sup>2+</sup> co-exposure showed prevention against Hg<sup>2+</sup>-toxicity by improving GSH content and inhibiting ROS generation and oxidative stress mediated damages. Additionally, BHT co-treatment inverted the pro-apoptotic proteins by augmenting pro-survival regulatory proteins akt, mTOR, ERK1, Nrf2 and HO1. These findings proved that BHT inhibits Hg<sup>2+</sup>-toxicity, hindering ROS generation and intrinsic apoptosis, *via* enhancing glutathione and antioxidants; and suggested BHT implications as therapeutic.

**Keywords:** Oxidative stress; intrinsic apoptosis; Nrf2; heme oxygenase 1; ERK1; DNA fragmentation

## 1. Introduction

Mercury (Hg) is a universal environmental pollutant which causes a broad spectrum of fatal health effects. Hg is marked as the third in most dangerous and hazardous toxic materials, by the Agency for Toxic Substances and Disease Registry (ATSDR). Severe Hg pollution in the environment (atmosphere, lake water, lake sediments and catchment soils, soil feeding termites) of anthropogenically-disturbed sites (Zhang et al., 2019; Cheng et al., 2020; Diouf et al., 2019) and dreadful contaminations in food sources (fresh and marine fish, rice, clam) (Yoshino et al., 2020; Xu et al., 2020; Sıkdokur et al., 2020) across the world have been reported. Three forms of Hg: elemental, inorganic and organic, can be exposed to human. Hg exposure typically occurs by inhalation or ingestion. Major sources of Hg are industrial emissions, fresh water and ocean fishes, and dental amalgam. For many years, inorganic Hg has been using in various medications, germicidal soaps, teething powders, and skin care products (Guzzi and Porta, 2008). Hg crosses blood brain barrier (BBB) and can cause irreversible effect on the central nervous system. All forms of Hg can cause neurological alteration, cognitive and motor dysfunction, memory loss, behavioral disorders, immune and kidney defects, and neurodevelopmental difficulties (Rahman et al., 2019). Moreover, studies have been shown to induce toxicity by Hg *in vivo* (Goering et al., 2000; Aragão et al., 2018) and *in vitro* (Shenker et al., 2002; Kaur et al., 2006; Teixeira et al., 2018; Hossain et al., 2021a). Hg was affirmed to induce oxidative stress and generate reactive oxygen species (ROS) *in vivo* and *in vitro* (Shenker et al., 2002; Aragão et al., 2018).

Butylated hydroxytoluene (BHT) is a widely recognized antioxidant, which was primarily used as a food additive, and also in other industries as industrial

additive/preservative, personal care product, household product constituents, plastic/rubber components and medical/research uses. It is a hindered phenolic compound, also well-known as 2,6-di-tert-butyl-p-cresol or 2,6-di-tert-butyl-4-methylphenol. It is not found in nature, but produced from p-cresol and isobutylene synthetically (Branen, 1975). It is categorized by the United States Food and Drug Administration (FDA) as “Generally Recognized As Safe” . Depending on the food fat content, a total of 0.02% of BHT is permitted (Babich, 1982). and is one of the most frequently consumed antioxidants in fats-containing-foods (Yehye et al., 2015). By donation of hydrogen to free-radicals, it can prevent lipid oxidation (Babich, 1982).

Several studies have been indicated BHT induced protective effects from metals (ferric ion (Ko and Godin, 1990), cadmium (Peters et al., 1995), chromium (Pourahmad and O'Brien, 2001), copper (Hosseini et al., 2014)) and other toxic compounds (copper oxide (Assadian et al., 2017), mancozeb (Tsang and Trombetta, 2007), melatonin (Gulcin et al., 2003)) induced toxicity *in vivo* and *in vitro*, though details mechanisms were not reported yet. Studies have indicated that the antioxidant property of BHT is the major cause of BHT-protection against reactive oxygen species (ROS) induced toxicity. Very few studies (Peters et al., 1995; Pourahmad and O'Brien, 2001; Hosseini et al., 2014) are present evaluating the protective effects of BHT against heavy metals induced cytotoxicity and the preventive role of BHT in the cells. The exact molecular mechanisms have not been elucidated yet. Moreover, because of the widespread usage of BHT, it is necessary to study about the safety of this antioxidant and critical evaluation of the safe limit of BHT. Most importantly, an effective solution to defend cells from Hg induced severe toxicity is very crucial and urgent. Therefore, the ameliorative effects of BHT against iHg induced toxicity and its molecular mechanisms

have been studied in this research. Here, we hypothesized that BHT has a protective role on iHg-toxicity. The bio-molecular mechanisms involved in these protective effects were also investigated in the light of antioxidant property of BHT. In this research, PC12 cells were used as a model cell line for toxicity investigation (Hossain et al., 2018). To achieve our hypothesis, for the very first time, the protective effects of BHT were evaluated on iHg mediated toxicity in PC12 cells using various toxicity analysis methods.

## **2. Methodology**

### *2.1. Chemicals*

DMEM cell culture media was purchased from Sigma-Aldrich, USA. PC12 cells were bought from American Type Culture Collection (USA and Canada). BHT and HgCl<sub>2</sub> were obtained from MP Biomedicals, LLC (France) and Wako Pure Chemical Corporation (Japan), respectively. Polyclonal antibodies against mammalian target of rapamycin (mTOR) (2972, Cell Signalling Technology), p-mTOR (Cosmo Bio Co.), Heme oxygenase 1 (GTX101147, GeneTex), extracellular signal-regulated kinases (ERK1) (610031, BD Transduction Laboratories), phosphorylated protein kinase B (akt) (9267, Cell Signalling Technology), akt (4691, Cell Signalling Technology), Nuclear factor erythroid 2-related factor 2 (Nrf2) (PM069, Medical and Biological Laboratories Co., Japan), p53 (2524, Cell Signalling Technology), Bax (2772, Cell Signalling Technology), Bcl-xL (612011, BD Transduction Laboratories), caspase 7 (610812, BD Transduction Laboratories), cleaved caspase-3 (NB100-56113SS, BioDynamics Laboratory Inc.), caspase 3 (GTX110543, GeneTEX), LC3 (83506, Cell Signaling Technology),  $\beta$ -actin (GeneTEX), were bought. Cytochrome c release Apoptosis Kit

was bought from Calbiochem®. All chemicals used in this study were of analytical grade.

## 2.2. Cell culture

DMEM medium supplemented with 10% FBS were used to culture the PC12 cells. The cells were maintained at 37°C for 48 h. The medium was exchanged with serum/serum-free DMEM with/ without different concentrations of HgCl<sub>2</sub> (Hg<sup>2+</sup>) (0-5 µM) and butylated hydroxytoluene (BHT) (0-1,000 µM), separately. The IC<sub>50</sub> value for HgCl<sub>2</sub> was analyzed. Upon analyzing IC<sub>50</sub> value (4.37 µM) of Hg, the final concentration for Hg<sup>2+</sup> was decided as 5 µM for the co-exposure. 100 µM of BHT was selected as the final concentration for the co-treatment, because till 100 µM BHT did not show any toxic effect on the cells. The final combinations were decided as 5 µM of Hg<sup>2+</sup> and 100 µM of BHT for the co-treatment. Therefore, cells were treated with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48h and further tested for various cytotoxicity assessment analysis.

## 2.3. Cell viability analysis

The viability of the cells was dignified by trypan blue exclusion analysis as described by Kihara et al. (2012). Cells were incubated with/without BHT (100 µM) and iHg (5 µM), or co-incubated with BHT (100 µM) and iHg (5 µM) for 48h in the DMEM supplemented with FBS (10%). After the incubation, cells were harvested and washed using 1x PBS buffer. Cells were tainted with trypan blue dye. To measure the stained and non-stained cells, an automated cell counter (Bio-Rad, USA) was employed. These experiments were executed at least ten times to justify reliability.

## 2.4. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase activity analysis kit (Promega, USA) was used to assess the membrane integrity of the cells after 48h of treatment with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M) or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together. After the 48 h of incubation of the cells with the various treatments, the culture medium of the cells was measured for LDH activity, as stated in Kihara et al. (2012). Reproducibility was verified by performing these analyses four times.

### *2.5. Genomic DNA extraction*

Cells were incubated with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M) or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together for 48h, and then were harvested and washed with 1x PBS buffer. The technique explained by Kawakami et al. (2008) was used to extract DNA from the cells with High Pure PCR Template preparation kit (11796828001, Roche Diagnostics, Germany).

### *2.6. Electrophoresis of genomic DNA*

The DNA was extracted as explained by Kawakami et al. (2008) and the intact genomic DNA of the cells was assayed by agarose gel electrophoresis assay. Isolated DNA of the cells treated or co-treated with/without BHT (100  $\mu$ M) and iHg (5  $\mu$ M) were measured using 1.5% agarose gel electrophoresis as defined by Hossain et al. (2018) and Kawakami et al. (2008). The fluorescence intensity of electrophoresed DNA was analyzed using 'Quantity one' software. The density of the intact DNA was later measured using ImageJ software. The reproducibility was ensured by performing this experiment at least four times.

### *2.7. Reactive oxygen species (ROS) measurement*

Dihydroethidium (DHE) is a redox-sensitive fluorescent probe and oxidized by ROS.



DHE was detected to investigate ROS generation using ROS detection cell-based assay kit (Cayman chemical, USA). Cells were treated with/without  $\text{Hg}^{2+}$  (5  $\mu\text{M}$ ), BHT (100  $\mu\text{M}$ ), and combination of BHT and Hg for 48 h, then cells were harvested and incubated with ROS staining buffer. DHE fluorescent was measured according to manufacturer's instructions at 488nm laser. BD FACSVerse™ flow cytometer (BD Biosciences) was used to analyze DHE fluorescence. The experiments were executed for three times to ensure reliability.

#### *2.8. Free sulfhydryl (SH) levels analysis*

Intracellular free SH or glutathione (GSH) levels assay was measured. PC12 cells were exposed or co-exposed with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) for 48 h, and then were harvested and washed with 1x PBS buffer. The protein extraction and free SH level measurement were performed as stated by Rahman et al. (2017). The assay was conducted three times to verify reliability.

#### *2.9. Flow cytometry study*

Flow cytometry was used to assess the cell death process using Annexin V-FITC apoptosis detection kit (BioVision, USA). Cells were incubated or co-incubated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) for 48h, and then flow cytometry was done as according to manufacturer's instruction. The experiments were performed three times for reliability.

#### *2.10. Western blot for protein expression determination*

Cells were treated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ), or treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) together for 48h. After incubation, cells were harvested and washed by 1x PBS. As described in Rahman et al. (2017) protein extraction from the

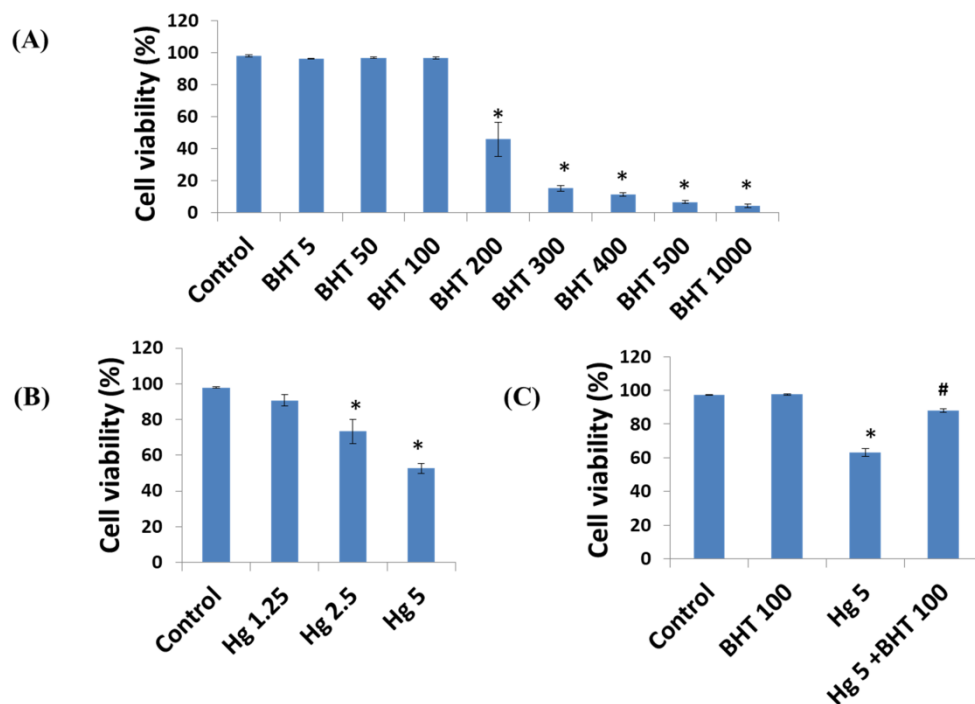
cells and electrophoresis were done. Enhanced chemiluminescence was used to detect the protein bands and; those bands were evaluated by a ChemiDoc XRS (Bio-Rad, USA) and were quantified using ‘Quantity one’ software. The density of the protein band was later analyzed using ImageJ software. The reproducibility was ensured by performing these experiments at least four times.

### 2.11. Statistics

Data were presented as the mean  $\pm$  standard error of mean (SEM) of independent experiments (at least three). Analysis was performed using one-way ANOVA, followed by two-way ANOVA and unpaired Student’s *t*-test for statistical analysis. Either  $p < 0.05$  or  $p < 0.01$  difference was reflected as significant statistically.

## 3. Results

### 3.1. Assessment of cell viability



**Fig. 1** (A) Cell viability of PC12 cells cultured in medium with 0-1,000  $\mu$ M of BHT, (B)

Cell viability of PC12 cells cultured in medium with 0-5  $\mu\text{M}$  of iHg, (C) Cell viability of PC12 cells co-exposed with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) for 48 h incubation. Error bars indicate mean  $\pm$  SEM (n= at least 6). \* denotes  $p < 0.05$ , significantly difference from control group and # denotes  $p < 0.05$ , significantly difference from only iHg-treated group.

To examine the BHT-effect on cells, cells were incubated with various concentrations (0-1,000  $\mu\text{M}$ ). Fig. 1A shows the cell viability when incubated with 0-1,000  $\mu\text{M}$  of BHT. Up to 100  $\mu\text{M}$  of BHT concentrations the viability did not show any significant variance from the control cells (treated with no treatment). However, in over 100  $\mu\text{M}$  of BHT concentrations, the viability of the cells were drastically ( $p < 0.01$ ) reduced in opposition to the control group. This result indicated that upto 100  $\mu\text{M}$  concentration of BHT is not toxic or harmful to PC12 cells. For this reason 100  $\mu\text{M}$  of BHT was chosen for the combined treatment with iHg.

For selecting the iHg concentration for the combined treatment, PC12 cells were incubated with various concentrations of iHg. Fig. 1B displays the viability of cells incubated with 0, 1.25, 2.5 and 5  $\mu\text{M}$  of iHg concentrations. Cell groups treated with 1.25  $\mu\text{M}$  of iHg did not show any significant ( $p < 0.01$ ) difference in comparison with the control group, whereas cell groups treated with 2.5 and 5  $\mu\text{M}$  of iHg, both displayed significant ( $p < 0.01$ ) cell viability loss in opposition to the control group, with IC<sub>50</sub> value of 4.37 (approximately 5). For this reason, 5  $\mu\text{M}$  of iHg was chosen for the combined treatment with BHT.

To evaluate the ameliorative effect of BHT on iHg induced toxicity in cells, cells were incubated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ), or co-treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) for 48h; and cell viability assay was performed. Fig. 1C displayed the viability of cells treated or co-treated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ).

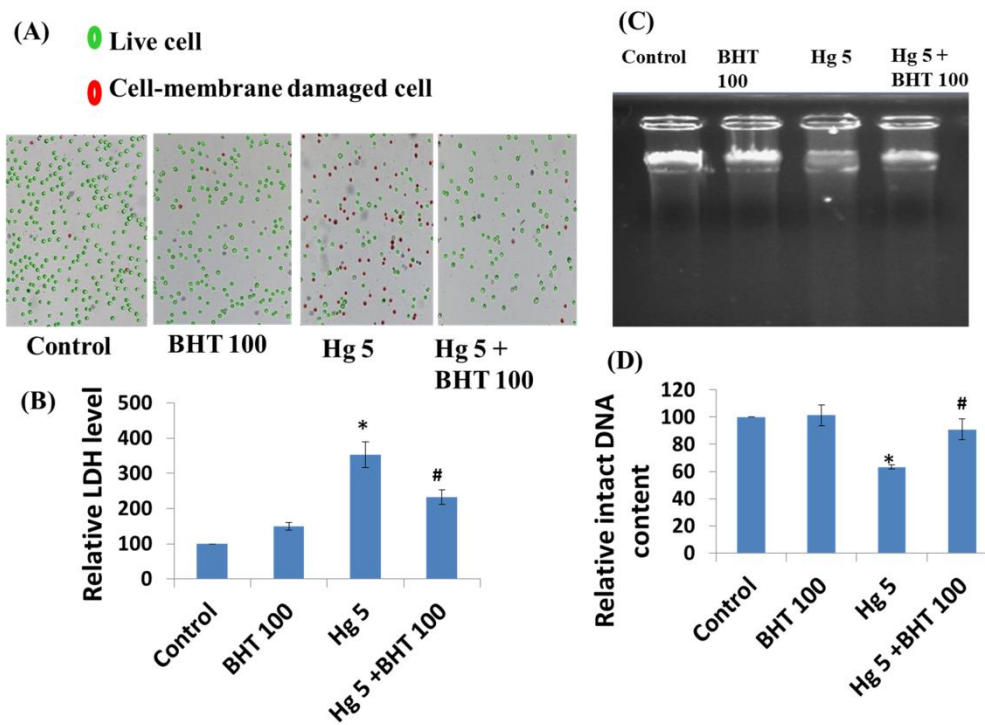
The viability of the cells incubated with BHT (100  $\mu\text{M}$ ) alone did not expressed any significant difference from the control group, but the cells incubated with only iHg (5  $\mu\text{M}$ ) showed a significant ( $p<0.01$ ) cell viability drop in opposition to the control. Interestingly, the cells treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) together displayed a significant ( $p<0.01$ ) rise in cell viability when compare with the cells treated with only iHg (5  $\mu\text{M}$ ). These findings suggested that BHT has a protective regulatory effect on iHg-toxicity in PC12 cells.

### 3.2. Cell-membrane integrity

To assess the cytotoxicity of iHg, cell-membrane damage was detected by trypan blue assay. Fig. 2A showed the pictures of cells treated or co-treated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) for 48h and then stained with trypan blue. Here, red colored cells are indicating the cell membrane damaged cells and green colored cells are indicating cell membrane intact cells. The cells incubated with only iHg, presented a significant increase of red colored (cell- membrane damaged or dead) cells, in opposition to control cells. However, the cells co-incubated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ), showed a significant decrease of red colored cells, when compared with the cells treated with only iHg (5  $\mu\text{M}$ ).

To double check the modulation of cell-membrane damage, another cytotoxicity assessment method LDH activity assay was conducted. Fig. 2B showed the LDH activity in the culture media incubated or co-incubated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) for 48 h. The activity of LDH in the cells incubated with only iHg (5  $\mu\text{M}$ ), resulted in a significant rise ( $p<0.01$ ) in opposition to that of the control group, which indicating a severe cell membrane damage. But the activity of LDH when co-treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ), showed a significant ( $p<0.05$ ) drop in opposition

to the cells incubated with iHg (5  $\mu$ M) alone; which indicating improvement in the cell-membrane. These findings together with cell viability assay evidenced that BHT protects cells from iHg induced cytotoxicity and membrane damage.



**Fig. 2** (A) Detection of cell wall damage using trypan blue staining when cells were treated with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M), or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together for 48 h. (B) LDH levels in the cultured medium for cells treated with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M), or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together for 48 h. (C) Agarose gel electrophoresis of DNA isolated from PC12 cells treated with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M), or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together for 48 h and (D) Intensity of electrophoresed band obtained from DNA of the cells. Error bars indicate mean  $\pm$  SEM (for A and B, n= 5; whereas for C and D, n=4). \* denotes p < 0.05, significantly difference from control group and # denotes p < 0.05, significantly difference from only iHg-treated group.

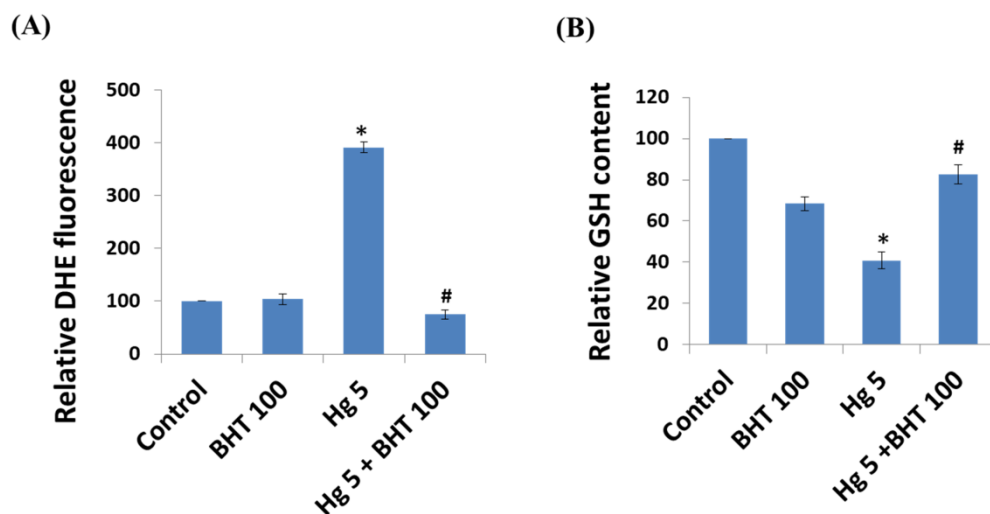
### 3.3. DNA fragmentation

Genomic DNA extraction and agarose gel electrophoresis experiment was implemented

to evaluate the protective effect of BHT on iHg influenced cytotoxicity. Fig. 2C showed the image of electrophoresed extracted DNA of the cells incubated or co- incubated with/without BHT (100  $\mu$ M) and iHg (5  $\mu$ M); and Fig. 2D showed the quantification of the intact DNA intensity. The amount of relative intact DNA was significantly ( $p<0.05$ ) reduced in the cells incubated with only iHg (5  $\mu$ M) comparing to those control cells. On the other hand, the cells co-treated with BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together, revealed a significant ( $p<0.05$ ) increase in the relative intact DNA level in opposition to the cells incubated with only iHg. These findings suggested that BHT co-treatment with iHg, improves the amount of intact DNA and inhibited iHg encouraged DNA-fragmentation in cells.

#### *3.4. ROS generation*

Dihydroethidium (hydroethidine or DHE) was investigated to measure ROS generation Fig. 3A showed that relative DHE fluorescence or ROS amount in the cells treated with iHg (5  $\mu$ M) was significantly ( $p<0.05$ ) increased compared to the control group. On the other hand, the relative DHE fluorescent (ROS amount) was significantly ( $p<0.05$ ) decreased in cells co-treated with BHT (100  $\mu$ M) and iHg (5  $\mu$ M), when compared with the group treated with only iHg (5  $\mu$ M). These findings indicated that iHg induced cytotoxicity in PC12 cells *via* ROS generation and BHT co-treatment reduced iHg-induced ROS formation.



**Fig. 3** (A) Relative geometric mean of DHE fluorescence and (B) GSH content of the cells treated with/without Se (5  $\mu$ M), iHg (5  $\mu$ M) or Se and iHg (5  $\mu$ M+5  $\mu$ M) together. Error bars are mean  $\pm$  SEM (n=3). \* denotes  $p < 0.05$ , significantly difference from control group and # denotes  $p < 0.05$ , significantly difference from only Hg-treated group

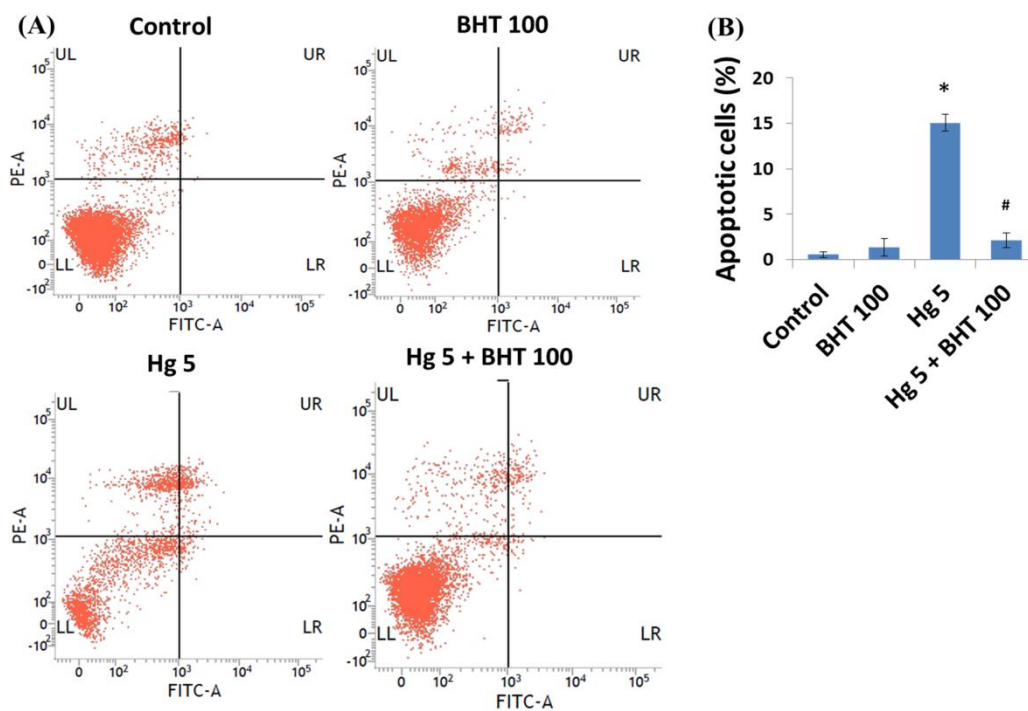
### 3.5. GSH levels

Free SH group or glutathione level in the cells is a vital element for antioxidant defense mechanisms. Reduced GSH is altered to its oxidized form (GSSG) for defending the cells from ROS influenced oxidative stress. To assess the antioxidant defense state of the cells and the contribution of ROS mediated stress in iHg-treated cells, intracellular GSH level was evaluated. Fig. 3B showed the GSH level in cells incubated or co-incubated with/without BHT (100  $\mu$ M) and iHg (5  $\mu$ M). The GSH level in the cells incubated with only iHg (5  $\mu$ M) was significantly ( $p < 0.01$ ) dropped in opposition to the control cells, indicating significant upsurge in ROS amount in the cells. Again, while compared with the cells treated with only iHg (5  $\mu$ M), the GSH level of the cells co-treated with BHT (100  $\mu$ M) and iHg (5  $\mu$ M) revealed a significant ( $p < 0.01$ ) uplift, indicating significant decrease in ROS amount in the cells. These findings suggested

that iHg persuades ROS arbitrated oxidative stress and BHT protects the cells from ROS mediated oxidative stress and cell death by improving the GSH content of the cells.

### 3.6. Apoptosis analysis

To know about the cell death mechanisms flow cytometry analysis was employed. The cells exposed to only iHg (5  $\mu$ M), presented a significant growth ( $p < 0.05$ ) in apoptotic cells in opposition to the cells treated with no treatment (Fig. 4). Again, the cells co-treated with BHT (100  $\mu$ M) and iHg (5  $\mu$ M), disclosed a significant decrease in apoptotic cells in opposition to the cells treated with only iHg (5  $\mu$ M). These findings suggested that BHT provides protection from iHg mediated toxicity and inhibited apoptotic cell death.



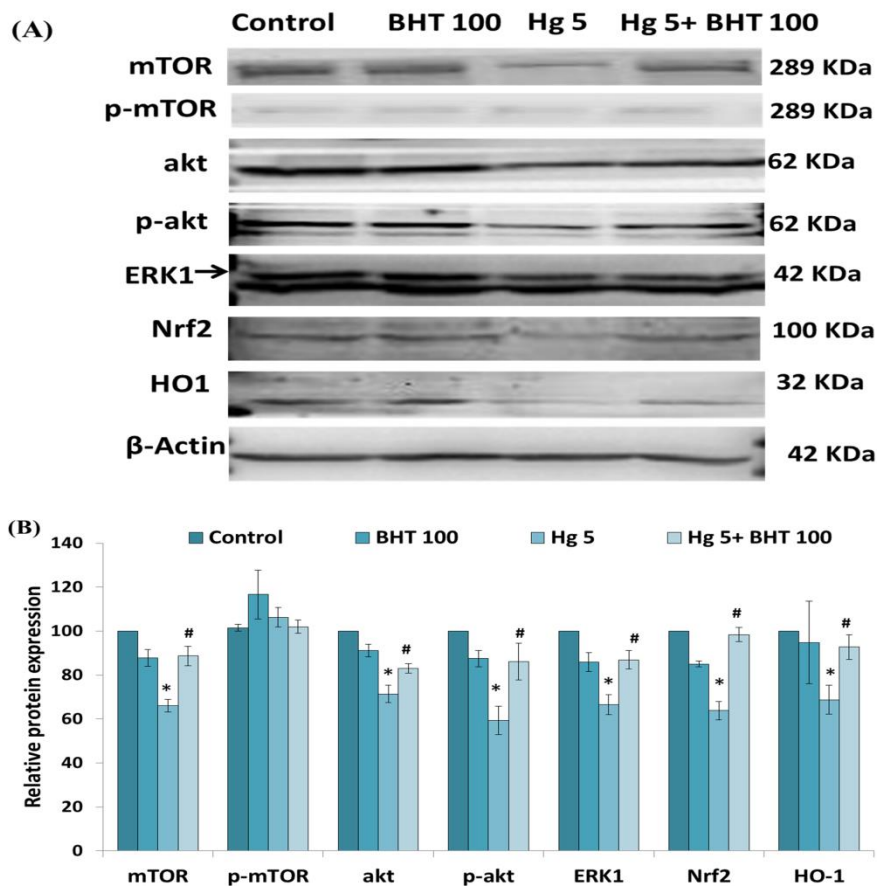
**Fig. 4** (A) Flow cytometry analysis of cultured PC12 cells, stained with Annexin V-FITC/PI after treatment with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M), or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together for 48 h; and apoptotic cells (B). Error bars are mean  $\pm$  SEM



(n=3). \* denotes  $p < 0.05$ , significantly difference from control group and # denotes  $p < 0.05$ , significantly difference from only iHg-treated group.

### 3.7. Expression of proteins

The molecular mechanisms involved in BHT mediated protection and iHg induced toxicity in cells were investigated using western blotting analysis. The protein samples were extracted from the cells treated or co-treated with/without BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) and western blotting was performed. The protein expressions of p-akt, akt, p-mTOR, mTOR, ERK1, Nrf2, HO1, p53, Bax, Bcl-xL, caspase 7, cleaved caspase-3, cytochrome c, caspase 3, LC3 and  $\beta$ -Actin were shown in Figs. 5 and 6.



**Fig. 5** (A) Protein expressions and (B) quantification of mTOR, p-mTOR, akt, p-akt, ERK1, Nrf2, HO1 and  $\beta$ -actin in the cultured cells treated with/without BHT (100  $\mu\text{M}$ ), iHg (5  $\mu\text{M}$ ), or BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) together for 48 h. Error bars are mean  $\pm$

SEM (n=3). \* denotes  $p < 0.05$ , significantly difference from control group and # denotes  $p < 0.05$ , significantly difference from only iHg-treated group.

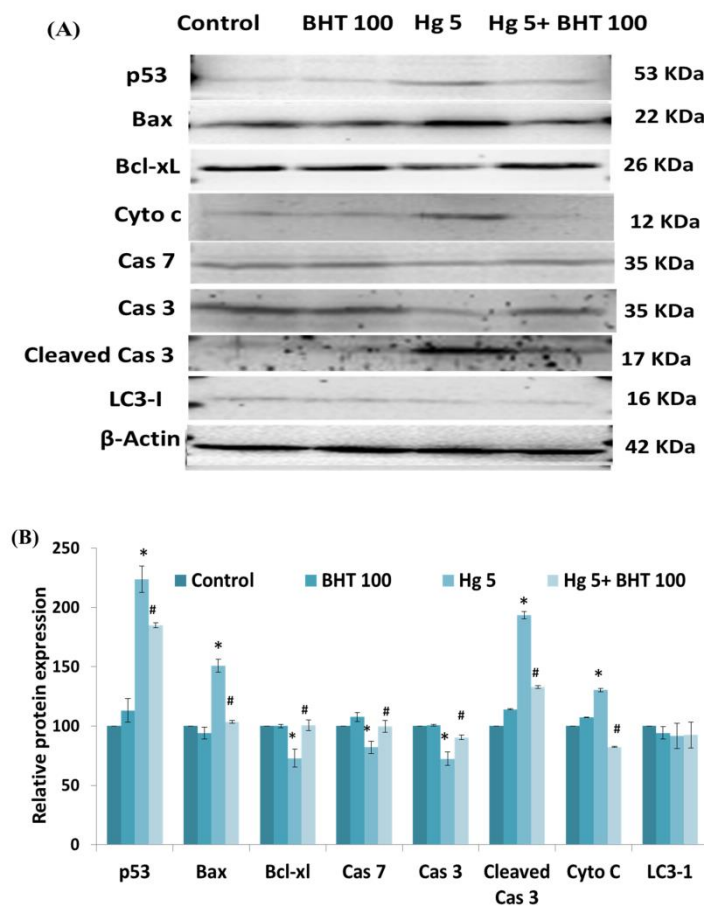
The mTOR plays a principal regulatory role in cellular metabolism, growth, proliferation, differentiation and survival. mTOR and phosphorylated mTOR, both were investigated in this study (Fig. 5). The level of mTOR was significantly ( $p < 0.01$ ) downregulated in cells treated with only iHg ( $5 \mu\text{M}$ ) in opposition to that of the control group. Again, mTOR level was upregulated significantly ( $p < 0.05$ ) when co-incubated with BHT ( $100 \mu\text{M}$ ) and iHg ( $5 \mu\text{M}$ ) in opposition to the cells incubated with only iHg ( $5 \mu\text{M}$ ). Though, the p-mTOR level did not display any significant difference between the groups.

Akt regulates cellular metabolism, survival, cell cycle, and apoptosis by regulating many downstream proteins, for example, NFkB, Bcl-2 family proteins. To investigate the involvement of akt in this study, akt and phosphorylated akt were assessed (Fig. 5). Both p-akt and akt protein levels in the cells incubated with only iHg ( $5 \mu\text{M}$ ), were significantly ( $p < 0.01$ ) reduced in opposition to the control cells proteins. Inversely, both proteins in the cells co-treated with BHT ( $100 \mu\text{M}$ ) and iHg ( $5 \mu\text{M}$ ) were significantly increased compared to the only-iHg ( $5 \mu\text{M}$ ) treated cells.

ERK1/2 plays a crucial role in regulating cell proliferation, differentiation, cycle progression, metabolism, transcription and survival (Roskoski, 2012). The ERK1 level in the cells incubated with only iHg ( $5 \mu\text{M}$ ), were significantly ( $p < 0.01$ ) reduced in opposition to the control cells proteins (Fig. 5). On the contrary, ERK1 in the cells co-exposed to BHT ( $100 \mu\text{M}$ ) and iHg ( $5 \mu\text{M}$ ) were significantly increased compared to the only-iHg ( $5 \mu\text{M}$ ) treated cells.

Nrf2 modulates the expression of antioxidant proteins e.g., glutathione reductase (GR)

and heme oxygenase-1 (HO1) which defend against oxidative damage induced by inflammation and injury. To inquiry about the contribution of Nrf2-HO1 pathway, Nrf2 and HO1 protein levels were checked (Fig. 5). The level of Nrf2 was significantly ( $p<0.01$ ) downregulated in cells exposed to only iHg (5  $\mu\text{M}$ ) significantly ( $p<0.01$ ) reduced in opposition to the control cells; while the Nrf2 level was upregulated significantly ( $p<0.01$ ) in cells co-exposed to BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) in opposition to the cells treated with only iHg (5  $\mu\text{M}$ ). Again, while compared with the control group the HO1 level was significantly ( $p<0.01$ ) reduced in the cells exposed to only iHg (5  $\mu\text{M}$ ). And comparing with the only-iHg (5  $\mu\text{M}$ ) treated group, HO1 level was significantly ( $p<0.05$ ) increased in the group co-treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ).



**Fig. 6** (A) Protein expressions and (B) quantification of p53, Bax, Bcl-xL, cytochrome c, caspase 7, caspase 3, cleaved caspase 3, LC3-I and  $\beta$ -actin in the cultured cells treated with/without BHT (100  $\mu$ M), iHg (5  $\mu$ M), or BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together for 48 h. Error bars are mean  $\pm$  SEM (n=3). \* denotes  $p < 0.05$ , significantly difference from control group and # denotes  $p < 0.05$ , significantly difference from only iHg-treated group.

The p53 protein acts a fundamental part in responding to genomic abnormalities and DNA degradation; thus could cause seizure of cell cycle and apoptosis. The p53 protein level was abruptly ( $p < 0.01$ ) increased in cells incubated with only-iHg (5  $\mu$ M) in opposition to the control cells, whereas p53 level was significantly ( $p < 0.05$ ) decreased in cells co-incubated with BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together as compared with cells incubated with only-iHg (5  $\mu$ M) (Fig. 6).

The Bcl-2 family is well known regulatory proteins of programmed cell death apoptosis, which consists of pro-apoptotic or anti-apoptotic members that either promote or inhibit apoptosis and control apoptosis. These proteins regulate the permeability of mitochondrial outer layer and cytochrome c discharge into cytosol from mitochondria, which is a basic part of intrinsic apoptosis of the cells. In this research, with the cytochrome c level in the cytosol, pro-apoptotic Bcl-2 family protein Bax and anti-apoptotic Bcl-2 family protein Bcl-xL have been studied (Fig. 6). The anti-apoptotic Bcl-xL level was significantly ( $p < 0.05$ ) downregulated in only-iHg (5  $\mu$ M)-exposed cells in opposition to the control cells. Contrarily, Bcl-xL level was significantly ( $p < 0.05$ ) upregulated when cell treated with BHT (100  $\mu$ M) and iHg (5  $\mu$ M) together as compared with the only-iHg (5  $\mu$ M)-treated cells.

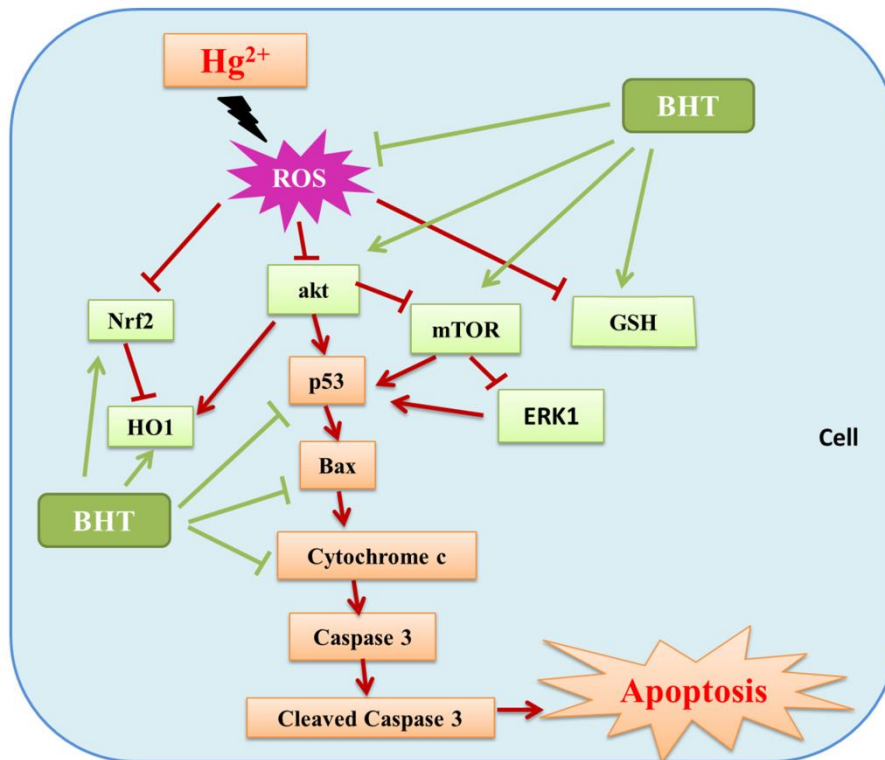
Moreover, the pro-apoptotic Bax level was significantly ( $p < 0.01$ ) upregulated in only-iHg (5  $\mu$ M)-treated cells while compared with that of the control cells. Inversely, Bax

level was significantly ( $p < 0.01$ ) down regulated when cell treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) together as compared with the only-iHg (5  $\mu\text{M}$ )-treated cells (Fig. 6). Similarly, cytochrome c level in the cytosol was significantly ( $p < 0.01$ ) upregulated in cells treated with only-iHg (5  $\mu\text{M}$ ) while compared with that of the control cells; and indicating intrinsic apoptosis occurred in only-iHg (5  $\mu\text{M}$ ) treated cells. Then again, cytochrome c level was significantly ( $p < 0.01$ ) downregulated when cell treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) together as compared with the only-iHg (5  $\mu\text{M}$ )-treated cells; indicating inhibition of intrinsic apoptosis by BHT co-treatment.

Caspase 7 is an effector caspase family protein that executes apoptosis. Activation of caspase 7 is a sequential step of apoptosis induction. Caspase 7 protein level was significantly ( $p < 0.01$ ) downregulated in cells exposed to only iHg (5  $\mu\text{M}$ ) in opposition to that of the control group; indicating caspase 7 activation in only-iHg (5  $\mu\text{M}$ )-treated cells. Again, caspase 7 level was upregulated significantly ( $p < 0.05$ ) when co-exposed to BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) in opposition to the cells treated with only iHg (5  $\mu\text{M}$ ); indicating inhibition of caspase 7 activation in co-treated group cells. Similarly, another vital caspase family protein caspase 3 and its activated form were also investigated in this study (Fig. 6). Caspase 3 protein level was significantly ( $p < 0.01$ ) downregulated in only-iHg (5  $\mu\text{M}$ )-exposed cells in opposition to the control cells; indicating caspase 3 activation in only-iHg (5  $\mu\text{M}$ )-treated cells. Again, caspase 3 level was significantly ( $p < 0.05$ ) upregulated when cell treated with BHT (100  $\mu\text{M}$ ) and iHg (5  $\mu\text{M}$ ) together as compared with the only-iHg (5  $\mu\text{M}$ )-treated cells. The cleaved or activated caspase 3 level was significantly ( $p < 0.01$ ) increased in cell group treated with iHg (5  $\mu\text{M}$ )-only in opposition to the control group and confirmed apoptosis. Inversely, activated caspase 3 level was significantly ( $p < 0.05$ ) decreased in cell group co-exposed with BHT (100  $\mu\text{M}$ )

and iHg (5  $\mu\text{M}$ ) compared to the group treated with only- iHg (5  $\mu\text{M}$ ) and confirmed blockage of apoptosis.

LC3 is a protein related to another programmed cell death autophagy, in which LC3-I is converted to LC3-II to conjugate autophagosomes. To investigate autophagy LC3 protein was assessed (Fig. 6), though only LC3-I was observed while LC3-II was absent indicating no involvement of autophagy. Fig. 7 depicts a graphic diagram of BHT-induced protection on iHg mediated toxicity in PC12 cells.



**Fig. 7** Schematic diagram of BHT-protection on iHg-induced cytotoxicity in PC12 cells.

#### 4. Discussion

There are approximately six thousand scientific articles discussing exclusively on BHT and its derivatives-induced antioxidant activities. BHA is a mixture of 15% 2-tert-butyl-

4-hydroxyanisole (II) and 85% 3-tertbutyl-4-hydroxyanisole (III) (Francois et al., 2012). Recently, BHT is comprehensively applied in the food industry, especially in low-fat foods, packaging materials and fish products (Yehye et al., 2015). After accumulation from food, BHT is primarily metabolized in liver and a small amount metabolized in lungs; and finally excreted via urine (Babich, 1982). The antioxidant property of BHT has been studied by various researchers (Lambert et al., 1996; Dawidowicz and Olszowy, 2011) indicating BHT as an inhibitor of free radical-chain reactions (Yehye et al., 2015). Numerous studies have been published on other antioxidants, where BHT has been used as an antioxidant standard (Dawidowicz and Olszowy, 2010; Sánchez-Vioque et al., 2012). In this study the antioxidant properties of BHT have been studied against a heavy metal induced toxicity (e.g., inorganic Hg mediated cytotoxicity) in PC12 cells using several cytotoxicity assessment assays.

Hg is an extremely toxic substance to human health, which has been evidenced to be hazardous even at a low concentration. In our research, relatively higher concentration was used to induce toxicity in PC12 cells. Cells exposed to iHg concentration (5  $\mu$ M) showed a significant cell viability reduction (Fig. 1), compromised cell membrane integrity (Fig. 2), genomic DNA fragmentation (Fig. 2), reduced glutathione amount decrease (Fig. 3) and ROS generation (Fig. 3). Similar results were also found by previous researches. Kim et al. (2003) reported similar iHg induced cell viability loss, cell membrane damage (LDH release) and blocked DNA proliferation in murine T and B lymphoma cells. Lee et al. (2001) described iHg influenced cell viability loss, ROS generation and GSH content drop in glioma cells. In our previous research (Hossain et al., 2020; 2021a; 2021b), iHg has been proved to be apoptogenic to PC12 cells. Similarly, our present research has appeared to encourage apoptosis in PC12 cells via

mitochondrial pathway (Figs. 3 and 5). iHg caused downregulating p-akt, akt, mTOR, ERK1, Nrf2, HO1, Bcl-xL, inactivate caspase 7 and inactivated caspase 3 expressions; whereas resulted in upregulating protein expressions of p53, Bax, cleaved caspase 3 and cytochrome c (Figs. 4 and 5) synergistically, demonstrating oxidative stress and ROS generation. Kim et al. (2003) reported similar iHg induced apoptosis in lymphoma cells. Teixeira et al. (2018) showed iHg mediated oxidative stress and apoptosis in motor cortex. We demonstrated here that iHg (5  $\mu$ M) induces cytotoxicity and cell death in PC12 cells via compromising cellular antioxidant defense associated with oxidative stress.

To protect cells from heavy metal induced toxicity, various antioxidants (selenium, dihydrolipoic acid, zinc) have been used for limiting ROS generation and oxidative stress (Hossain et al., 2018; 2020; 2021a; 2021b; Rahman et al., 2017). Several line of researches have been shown the possibility of BHT-protection upon heavy metals and other toxicants-induced toxicity (Hosseini et al., 2014; Al-Malki, 2010; Assadian et al., 2017; Tsang and Trombetta, 2007; Gulcin et al., 2003). In our research, BHT (100  $\mu$ M) co-treatment with iHg (5  $\mu$ M) showed significant improvement in cell viability, genomic DNA and cell membrane integrity (LDH level decrease) Similarly, Tsang and Trombetta (2007) reported BHT-improved cell viability from mancozeb-induced cytotoxicity in rat hippocampal astrocytes. Lison et al. (1995) demonstrated in a macrophage culture model, BHT decreased the LDH release caused by the cytotoxicity of hard metal particles (cobalt metal and carbide particles). Again, Pourahmad and O'Brien (2001) showed that BHT prevented dichromate induced lipid peroxidation in isolated rat hepatocytes. Assadian et al. (2017) showed BHT mediated protection from lipid peroxidation caused by copper oxide nanoparticles influenced toxicity on human



blood lymphocytes. Ko and Godin (1990) demonstrated that BHT inhibited the ferric ion-influenced peroxidation of erythrocyte membrane lipids. In our study, reduced glutathione content was improved by BHT (100  $\mu$ M) co-treatment with iHg (5  $\mu$ M) indicating ROS formation. Similar to our results, Assadian et al. (2017) showed BHT induced glutathione content increase in human blood lymphocytes after copper oxide nanoparticles-treatment. Pourahmad and O'Brien (2001) showed that BHT prevented dichromate induced reactive oxygen species (ROS) formation in isolated rat hepatocytes. It was assumed that the mechanism of BHT associated antioxidant properties consist of the quenching of ROS and lipid soluble radicals (Yehye et al. 2015).

Antioxidant activity is extensively utilized as factor to illustrate substances with the property of neutralizing free radicals, by which the substance is able to protect a biological system from harmful oxidation. Scavenging property of BHT has been scrutinized critically (Yehye et al. 2015; Gulcin et al., 2003) and considered the major cause of antioxidant activity. Upon BHT co-treatment GSH upregulation and reduction in ROS amount in our results indicated that, BHT amelioration in presence of iHg-toxicity is mediated through ROS scavenging/neutralization.

In our study, the proteins responsible for cellular metabolism, cell survival and cell cycle, akt, p-akt, mTOR, ERK1 were rejuvenated by BHT exposure against iHg induced toxicity. Here, BHT was able to upregulated the anti-apoptotic and pro-survival proteins mTOR, akt and ERK1. These upstream regulator proteins contributed as the initiator proteins of survival in PC12 cells and protected from iHg-induced cell death. One of the major cell survival pathways, Nrf2-HO1 is also potential to contribute in BHT induced protection, as p-akt, Nrf2 and HO1 were upregulated upon BHT co-exposure with iHg. Nrf2 as a transcription factor sensing oxidative stress, regulated the expression

of antioxidant HO1 protein that protect against oxidative damages triggered by injury and inflammation in the cell. Similar Nrf2 and HO1 protein upregulation was also found in another antioxidant dihydrolipoic acid protection against iHg-induced toxicity (Hossain et al., 2020). In this study, as upstream regulator proteins (akt, p-akt, mTOR, ERK1, Nrf2, HO1) were recovered by BHT exposure, downstream effector pro-apoptotic proteins (p53, Bax, caspase 7, cytochrome c and caspase 3) were inhibited, preserving mitochondrial membrane potentiality and modulated apoptosis caused by iHg in PC12 cells. Hosseini et al. (2014) reported BHT mediated protection from lipid peroxidation caused by copper toxicity on isolated liver mitochondria, describing mitochondrial membrane potential loss. Again, Assadian et al. (2017) showed BHT improved mitochondrial membrane potential in human blood lymphocytes after copper oxide nanoparticles-treatment.

In summary, consistent with previous reports, our study elucidated that iHg induced cytotoxicity in PC12 cells *via* antioxidant defense disruption and oxidative damages, which resulted cell death in intrinsic/mitochondrial apoptosis pathway. Inversely, BHT co-treatment with iHg protected the cells from iHg-cytotoxicity by intensifying antioxidant defense molecules and preserving from oxidative stress. BHT augmented glutathione content to modify oxidative stress induced by iHg, indicating preservation of oxidative homeostasis. BHT induced homeostasis led restoration of upstream survival regulating proteins (mTOR, akt, ERK1, Nrf2 and HO1) and limited pro-apoptotic proteins (p53, Bax, cytochrome c and caspase 3). The apoptosis inhibition by BHT upon iHg exposure suggested that, BHT may have ameliorative properties against heavy metal induced cytotoxicity. This is the first study directly enlightening the ameliorative effect of BHT against iHg-toxicity. BHT appeared to be a good antioxidant

against Hg induced toxicity and cellular damages, which suggested a possible therapeutic agent against not only Hg, but also other heavy metal-induced toxicity facilitated by oxidative stress.

## **5. Conclusions**

Our results demonstrated that iHg is highly toxic to PC12 cells, whereas BHT can protect PC12 cells from iHg-induced toxicity and intrinsic apoptosis. The protection provided by BHT in iHg-mediated toxicity is briefly *via* its antioxidant properties. It was suggested that BHT amelioration in presence of iHg is mediated *via* inhibition of ROS generation, GSH stimulation and oxidative stress reduction. Simultaneously, akt-mTOR upregulation contributed as initial protein response in BHT-protection against iHg-toxicity. Our research provided strong evidence of the involvement and contribution of Nrf2-HO1 pathway in BHT mediated amelioration. Further analysis is needed to investigate the chelation capability of BHT. The results obtained from our research can be beneficial for pharmaceuticals and pharmacological uses of BHT as a therapeutic agent against heavy metals and environmental toxic substances. This is the first study directly enlightening the ameliorative effect of BHT against iHg-toxicity on cells.

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