

# *Bacopamonnieri* Protects SH-SY5Y Cells Against *tert*-Butyl Hydroperoxide-induced Cell Death via the ERK and PI3K Pathways

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## ABSTRACT

**Objective:** Oxidative stress plays an important role in the pathological processes of various neurodegenerative diseases. *Bacopa monnieri* (BM) has a potent antioxidant property. Therefore, the purpose of this study was to evaluate the neuroprotective potential of BM against SH-SY5Y neuroblastoma cell death induced by the pro-oxidant insult, *tert*-Butyl hydroperoxide (TBHP), and to identify possible mechanisms related to its neuroprotective action.

**Methods:** The neuroprotective effect of BM was evaluated by the degree of protection against TBHP-induced cell death in human SH-SY5Y cells that was measured by calcein-AM assay. ERK1/2 and Akt phosphorylation was evaluated by immunoblotting.

**Results:** We found that BM exhibited protection against TBHP-mediated cytotoxicity. The neuroprotective effect of BM was abolished in the presence of either ERK1/2 or PI3K inhibitors. In addition, western blotting with anti-phospho-ERK1/2 and anti-phospho-Akt antibodies showed that BM increased both ERK1/2 and Akt phosphorylation.

**Conclusion:** These results suggest that BM by activation of ERK/MAPK and PI3K/Akt signaling pathways protects SH-SY5Y cells from TBHP-induced cell death.

**Keywords:** *Bacopa monnieri*, *tert*-Butyl Hydroperoxide, SH-SY5Y cells, ERK1/2, PI3K

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## INTRODUCTION

Oxidative stress has long been implicated both in the physiological process of aging and in a variety of neurodegenerative diseases, including Alzheimer's disease.<sup>1,2</sup> Reactive oxygen species (ROS) can cause apoptotic cell death through DNA damage, oxidation of

proteins, and peroxidation of lipids.<sup>3</sup> Therefore, therapeutic strategies to prevent ROS-induced cell death might be a useful strategy for the treatment of neurodegenerative diseases associated with oxidative stress.

*Bacopa monnieri* (BM), family Scrophulariaceae, is a small perennial, creeping herb with numerous branches, and grows naturally in wet, shallow water, and marshy areas within tropical regions of the world.<sup>4</sup> It has been used in Ayurvedic medicine as a nerve tonic for promoting mental health and improving memory and other related brain functions.<sup>5</sup> Among the specific components

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believed to contribute to the activity of BM are five saponin glycosides that include bacoside A3, bacoside II, bacopasaponin X, bacopasaponin C and bacoside I.<sup>6-8</sup> Mechanistically, BM has been reported to have anti-inflammatory, anti-depressant and antioxidant effects.<sup>9-12</sup> Previous studies have suggested that the antioxidant properties of BM are modulated by metal chelation, free radical scavenging, and lipid peroxidation inhibitory activities as well as through enhancement of antioxidant enzymes.<sup>13,14</sup> It has been shown to provide neuroprotective effect against an oxidative stress inducer, aluminium.<sup>15,16</sup> However, it is not clear whether other cellular mechanisms, such as cell signaling events, may be relevant to BM's protective effects. Therefore, the purpose of this study was to evaluate the neuroprotective potential of BM against SH-SY5Y neuroblastoma cell death induced by the pro-oxidant insult, TBHP, and to identify possible mechanisms related to its neuroprotective action.

## MATERIALS AND METHODS

### BM extract preparation

BM was collected from Petchaburi province, Thailand, and was identified by Associate Professor Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimen (Phrompittayarat 001) was kept at the Pharmaceutical Botany Mahidol Herbarium, Mahidol University, Thailand. The aerial part of the Brahmi plant was cut, dried and then roughly powdered. The dried powder was soaked in water for 24 h. The water was then squeezed out and percolated with 95% ethanol. The plant material was extracted again under the same condition. The extracts yielded from the sequential processing were combined and subsequently dried under reduced pressure. The percent yield obtained was 10% of the starting dried material. The extract contained 6.25% (w/w) of total saponins comprising 0.87% bacoside A3, 1.82% bacoside II, 0.80% bacopasaponin X, 1.73% bacopasaponin C and 1.03% bacoside I. The total saponin content was determined using high pressure liquid chromatography as previously reported.<sup>6,8</sup> The extract was stored at -20°C in an amber bottle until used.

### SH-SY5Y neuroblastoma cell culture

The human neuroblastoma, SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12; Invitrogen Life Technologies) supplemented with 15% heat-inactivated fetal bovine serum (Atlanta biological), 1% penicillin-streptomycin solution (Cellgro) and cultured at 37°C in humidified incubator with 5% CO<sub>2</sub>. Cells were plated at a density of 1 × 10<sup>4</sup> cells per well in 96-well plates, and maintained for 24 h before they were differentiated with 10 μM retinoic acid (Sigma Aldrich) for 7 days.

### Treatment protocol

In order to induce oxidative stress, TBHP was freshly prepared prior to each experiment. BM was prepared in DMSO/ethanol. Treatments were conducted such that the final concentration of either DMSO or ethanol was less than 0.5% which did not affect the cells viability (data not shown). In order to monitor the protective effects of BM, some cells were co-treated with TBHP together with BM. To see the possible pathways related to its protection, cells were treated with BM and TBHP in the presence of ERK 1/2 or PI3K inhibitors. The survival of neurons was determined 24 h after treatment by calcein AM assay.

### Cell viability assay

Cell survival was determined by using the calcein AM assay kit (Molecular Probes). Cells were incubated with calcein AM reagents with the concentration at 4 μM for 30 min in CO<sub>2</sub> incubator at 37°C. Live cells were estimated by the assessment of calcein fluorescence (a product of cleavage of the calcein-AM ester by ubiquitous intracellular esterases), measured using a plate reader with the fluorescence at 485 nm excitation, 535 nm emission wavelengths.

### Immunoblotting

Cells were collected and washed with PBS. Cells were lysed with ice-cold lysis buffer containing 50 mM of Tris-HCl pH 7.4, 150 mM of NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Sigma Aldrich).

The lysates were incubated on ice for 30 min. and centrifuged at 12,000 g for 20 min. Supernatants were collected and followed by protein concentration determination using Bradford Assay. An equal amount of total protein (50-125  $\mu\text{g}$ ) were applied to 10% SDS-PAGE, and subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was incubated in blocking buffer (Tris-buffered saline, pH 7.4, 0.2% Tween 20 and 5% skim milk) for 4h at 4°C. This was followed by incubation with primary antibodies (Cell Signaling Tech): anti-phospho-ERK1/2 (1:1,000), anti-phospho-Akt (1:1,000) and anti GAPDH (1:1,000) antibodies overnight at 4°C. The membrane was washed three times for 5 min each using TBST (TBS and 0.05% Tween 20). After that, it was incubated with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:5,000, Thermo Scientific) for 2h at room temperature. Immunoreactivity was detected using enzyme-linked chemiluminescence, and then exposed on Hyperfilm ECL (GE Healthcare). Quantitative assay of antigen expression was based on the density measurements of protein bands, using the Scion Image program.

### Statistical analysis

Results are presented as bar graphs depicting the mean  $\pm$  standard error of the mean (S.E.M.) of at least six independent experiments, using GraphPad Prism software (San Diego, CA). The data were analyzed using a one-way analysis of variance (ANOVA). Individual group differences were assessed using Bonferroni correction for post-hoc analyses. Differences were considered to be significant when p values were less than 0.05.

## RESULTS

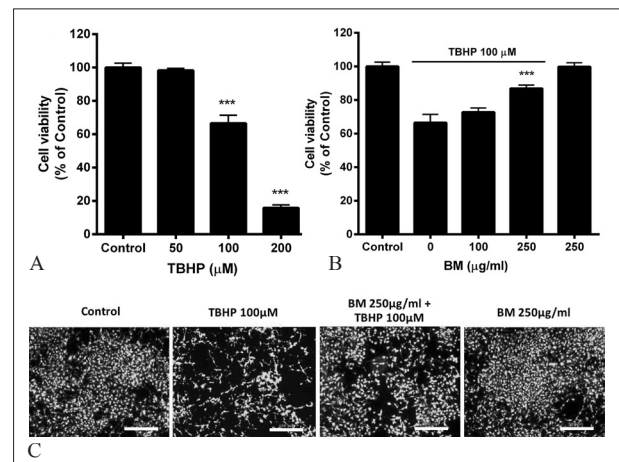
### TBHP-induced cytotoxicity in SH-SY5Y cells

In order to establish an effective concentration of TBHP to subsequently evaluate the protective effects of BM, we first conducted a concentration-response analysis for the effects of TBHP on the viability of neuroblastoma SH-SY5Y cells. Cells were exposed with TBHP (50-200  $\mu\text{M}$ ) for 24 h and cell viability was assessed

using the calcein AM assay. TBHP decreased cell viability in a concentration-dependent manner (Fig 1A). Exposure of 100 and 200  $\mu\text{M}$  of TBHP significantly reduced the survival to  $66.61 \pm 4.82\%$  and  $15.86 \pm 1.76\%$  of control, respectively ( $p$  less than 0.001). Based on these results, we used the 100  $\mu\text{M}$  concentration as a mild-to-moderate insult (approximately 30-40% of dead cells) to assess the protective effects of BM in subsequent studies.

### BM rescued SH-SY5Y cells from TBHP-induced cell death

In order to assess the protective effects of BM, we co-applied increasing concentrations of BM of 100 and 250  $\mu\text{g}/\text{ml}$  along with TBHP. As illustrated in Fig 1B, TBHP (100  $\mu\text{M}$  for 24 h) treatment decreased cell survival to  $66.61 \pm 4.82\%$ . Co-application with BM (250  $\mu\text{g}/\text{ml}$ ) significantly prevented cell death, restoring cell survival to  $86.96 \pm 1.93\%$  ( $p$  less than 0.001). BM by itself, at the highest concentration tested (250  $\mu\text{g}/\text{ml}$ ) did



**Fig 1.** Neuroprotective effects of BM against TBHP toxicity in SH-SY5Y cells. **(A)** Concentration-dependent effect of TBHP on cell survival in SH-SY5Y cells. Cells were exposed to different concentrations of TBHP for 24 h. Cell viability was assessed using the calcein AM assay. Vehicle treated cells served as the control. \*\*\*  $p < 0.001$  as compared to Control;  $n=7$ . **(B)** Neuroprotective effects of BM on TBHP-induced cytotoxicity in SH-SY5Y cells. Cells were treated with 100  $\mu\text{M}$  TBHP for 24 h. Some cells were treated with 100 and 250  $\mu\text{g}/\text{ml}$  BM together with 100  $\mu\text{M}$  TBHP. \*\*\*  $p < 0.001$  as compared to TBHP alone;  $n=7$ . **(C)** Microscope-assisted visualization of SH-SY5Y cell viability. Bright fluorescent signal indicated cell survival. Scale bar = 500  $\mu\text{m}$

not cause any apparent neurotoxicity. Microscopy-assisted evaluation of cell viability confirmed that BM (250 µg/ml) was protective against TBHP (Fig 1C). Based on these data, we selected the 250 µg/ml concentrations of BM to test in subsequent experiments.

### BM-mediated protective action involves extracellular signal regulated kinase 1 and 2 (ERK 1/2) activation

In the nervous system, the ERK/MAPK signaling pathway is critical for neuronal differentiation, plasticity and survival.<sup>17-21</sup> U0126, an inhibitor of the ERK1/2 signaling pathway, was used to determine whether the protective effect of BM was mediated by this signaling pathway. Treatment with U0126 (10 µM) abolished BM-mediated protection against TBHP-induced cell death (Fig 2). Cell viability was significantly decreased from 76.51±2.25% to 58.79±2.66% with 10 µM of U0126 (*p* less than 0.001). In contrast, U0124 (10 µM), the inactive analog of U0126, had no effect on the BM-induced protection against TBHP-induced neurotoxicity. Moreover, U0126 by itself did not cause any apparent neurotoxicity.

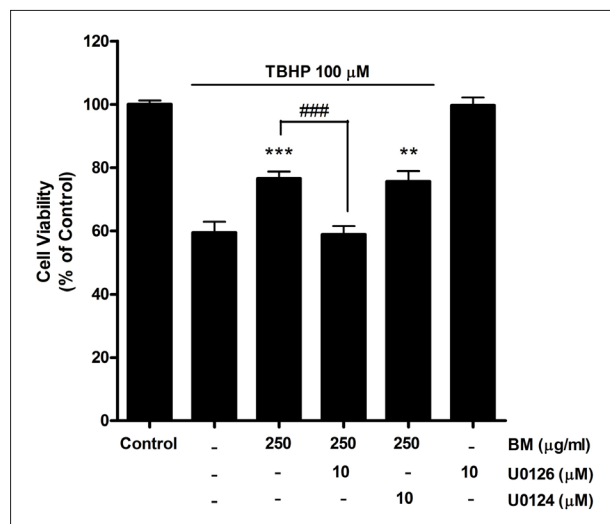
### The protective effects of BM are dependent on the Phosphatidylinositol-3 kinase (PI3K) pathway

The PI3K/Akt signaling pathway has also been reported to play an important role in the promotion of cell survival and the suppression apoptosis.<sup>17,22-24</sup> We used the PI3K inhibitor, LY294002, to determine whether the PI3K pathway was involved in the effect of BM. Treatment with LY294002 abolished BM-mediated protection against TBHP-induced cell death (Fig 3). Cell viability was significantly decreased from 78.27±1.60% to 64.74±2.95% with 15 µM of LY294002 (*p* less than 0.001). In addition, LY294002 by itself did not cause any apparent neurotoxicity.

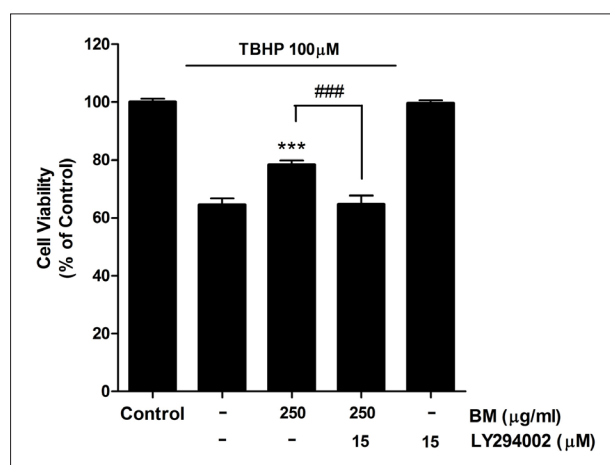
### BM increased the amount of both ERK1/2 and Akt phosphorylation

From our results, we found that protective effects of BM might be dependent on mechanisms of ERK1/2 and PI3K activation. In order to confirm that mechanisms are related to its protective

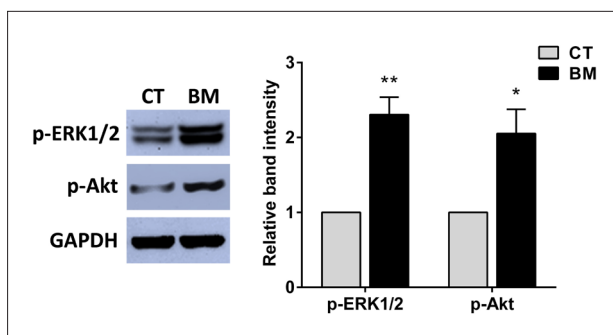
action, we evaluated ERK1/2 and PI3K activities during BM treatment by western blotting. We used a phospho-ERK1/2 (Thr202/Tyr204) antibody to monitor ERK1/2 activity. In addition, we used a phospho-Akt (Ser473) antibody to monitor PI3K activity because phosphorylation of Akt at Ser473 is required for its full activation. Fig 4 shows



**Fig 2.** Effect of the ERK 1/2 inhibitor, U0126, on the neuroprotective effect of BM in SH-SY5Y cells. Application of U0126 attenuated the neuroprotective effect of BM. U0124, the inactive analog of U0126 and serving as a negative control, had no effect on the neuroprotection. Cell viability was assessed using the calcein AM assay. \*\* *p* < 0.01, \*\*\* *p* < 0.001 as compared to TBHP alone. ### *p* < 0.001 as compared to TBHP + BM; *n*=8.



**Fig 3.** Effect of the PI3K inhibitor, LY294002, on the neuroprotective effect of BM in SH-SY5Y cells. Application of LY294002 attenuated the neuroprotective effect of BM. Cell viability was assessed using the calcein AM assay. \*\*\* *p* < 0.001 as compared to TBHP alone. ### *p* < 0.001 as compared to TBHP + BM; *n*=7.



**Fig 4.** Effects of BM on the activation of ERK1/2 and Akt phosphorylation in SH-SY5Y cells. Cells were treated with 250  $\mu\text{g/ml}$  of BM for 30 min. Protein expressions were analyzed by immunoblotting with antibodies specific to phospho-ERK1/2 (p-ERK1/2), phospho-Akt (p-Akt) and GAPDH. The relative band intensities for p-ERK1/2 and p-Akt were determined by normalizing against GAPDH. Results were presented as mean $\pm$ SEM of three determinations in a bar chart. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared to control. CT=control group, BM=BM treated group.

that application of BM (250  $\mu\text{g/ml}$ ) significantly increased phosphorylation of both ERK1/2 and Akt after treatment for 30 min. These results are consistent with the notion that BM-mediated protective action involves ERK1/2 and PI3K/Akt activation.

## DISCUSSION

Since the brain is very metabolically active, its high level of oxygen consumption and unique composition of membranes, which contain a large amount of oxidant-sensitive polyunsaturated fatty acids, make it particularly susceptible to free-radical damage.<sup>25</sup> Oxidative stress is a major cause of cellular injuries in a variety of neurodegenerative disorders.<sup>1,2</sup> Therefore, several studies have been conducted in search for natural products with antioxidant and thus, neuroprotective potential. Because BM has received much attention based on its reported anti-oxidant properties in the brain,<sup>7,15,16</sup> the present study was designed to investigate the potential for the BM extract as a neuroprotectant. Specifically, the studies conducted addressed whether an extract from BM plant can protect SH-SY5Y neuroblastoma cells from TBHP neurotoxicity and further, to determine the potential

mechanism underlying its effects. The human neuroblastoma SH-SY5Y cell line is widely used as model cell system for studying neuronal cell death induced by oxidative stress.<sup>26-28</sup> In this study, we found that treatment of SH-SY5Y cells with BM at the concentration of 250  $\mu\text{g/ml}$  protected these cells against TBHP-induced cytotoxicity. At this concentration, some evidences had supported that the extract concentrations higher than 150  $\mu\text{g/ml}$  almost completely prohibited the generation of lipid peroxide products in primary cortical cells.<sup>7</sup> Several findings suggest that the neuroprotective activities of BM may be attributed partially to the antioxidant effect of the bacoside A, an active ingredient.<sup>29</sup> BM could exert a neuroprotective effect that relieves neuronal oxidative stress, which might in turn contribute to neuronal apoptosis.

We explored whether alternative mechanisms may also be recruited in the protective effect of BM against TBHP-induced cell death. In this study, we found that treatment with U0126, an inhibitor of the ERK1/2, abolished the protective effect of BM indicating that the ERK1/2 pathway is involved in BM-mediated protection of SH-SY5Y cells against TBHP-induced neurotoxicity. The hypothesis was confirmed by the observation that ERK1/2 phosphorylation was increased after application of BM. ERK1/2 has been known to be involved in cell survival.<sup>17,19-21</sup> The survival activities mediated by ERK1/2 include the capacity to induce the activation of transcription factors that, in turn, stimulate the expression of various anti-apoptotic proteins. Furthermore, ERK1/2 can also directly affect several cell death/survival regulators.<sup>20</sup> For instance, the ERK signaling pathway can inhibit the pro-apoptotic protein BAD in addition to inducing the expression of pro-survival genes.<sup>30</sup> While our data certainly supports the involvement of the ERK1/2 pathway, we must exercise some caution since U0126 has been reported to inhibit not only the ERK1/2, but also the ERK5 signaling pathway as well.<sup>31,32</sup> Both ERK1/2 and ERK5 belong to the family of mitogen-activated protein kinases (MAPKs). Indeed, ERK5 signaling has been associated with the promotion of cell survival.<sup>33-35</sup> However, there are important differences in the potential role of these two signaling pathways, as it relates to

regulating cellular mechanisms associated with cell viability. For example, ERK1/2 and ERK5 signaling pathways have been reported to regulate the transcription of brain-derived neurotrophic factor (BDNF) differentially. ERK1/2 signaling can induce BDNF expression, whereas the ERK5 pathway appears to be an inhibitory regulator of BDNF gene expression.<sup>32</sup> Since BDNF, like the other neurotrophins, plays an important role in the development, differentiation, and survival of neuronal and non-neuronal cells,<sup>36-39</sup> it is possible that BM exerts its protective effects via the regulation of BDNF and its associated signaling pathways as well.

Another important signaling pathway linked to the promotion of cell viability is the PI3K/Akt signaling pathway. PI3K is a serine/threonine protein kinase that plays critical roles in neuronal growth, differentiation and survival.<sup>24</sup> In general, activation of the PI3K/Akt signaling pathway suppresses apoptosis and promotes cell survival in cultured neurons from the peripheral<sup>23</sup> and central nervous systems.<sup>22</sup> The activation of PI3K leads to phosphorylation and activation of Akt which promotes cell survival by enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic proteins. Phosphorylated Akt directly inhibits the apoptotic machinery at sites both upstream, Bcl-2 family member BAD<sup>40</sup> and downstream, caspase-9<sup>41</sup> of mitochondrial cytochrome c release. Moreover, an activated Akt also phosphorylates and inactivates FKHRL1, a member of the family of Forkhead transcriptional regulators, which when inactivated, is unable to induce the expression of death genes.<sup>42</sup> Our study found that treatment of SH-SY5Y cells with LY294002, the inhibitor of PI3K, attenuated the protective effects of BM against TBHP neurotoxicity and Akt phosphorylation was increased after BM application implicating the PI3K/Akt pathway in the protective effects of BM.

Collectively, our data support the neuroprotective effects of BM against TBHP-induced cell death in differentiated SH-SY5Y cells. We determined that mechanisms, such as the recruitment of the ERK/MAPK and the PI3K signaling pathways, may be involved in the neuroprotective effect of BM. Importantly, BM by itself was

not cytotoxic at any of the concentrations tested, suggesting that consumption of BM may be safe. These studies support the potential of BM as a therapeutic to prevent neuronal dysfunction and/or death associated with age or age-associated diseases such as Alzheimer's disease wherein oxidative stress plays an important role.

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