

Tau protein attenuation ability of *Bacopa monnieri* extract on nerve growth factor-deprived PC12 cells in normal-serum and serum-free medium

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Objective To study the effect of the *Bacopa monnieri* (BM) extract on Tau protein in nerve growth factor (NGF)-deprived PC12 cells.

Methods The cells were cultured in normal-serum medium for one week before transferring to low-serum medium for the next 7 days. Then, the two cultures were raised in normal-serum or serum-free medium and treated for up to 7 days with and without 50 to 300 µg/mL of BM extract for the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay, or for 2 days with and without 50, and 100 µg/mL of BM extract for the immunoblot assay. The average percentage of cellular viability for each treatment was compared daily to that of the corresponding controls. Immunoblot assays were performed by using total Tau, dephosphorylated Tau (Tau-1), and glyceraldehydes dehydrogenase antibodies, which then quantified by Scion Image software. Tau-1 immunoreactivity was normalized by total Tau. All data were analyzed by one-way ANOVA.

Results BM extract (150 and 200 µg/mL) significantly enhanced viability in NGF-deprived PC12 cells raised for four days in normal-serum medium, while BM (50 µg/mL) tended to accentuate viability in NGF-deprived PC12 cells raised for 2-4 days in serum-free RPMI (SF) medium. BM extract (100 µg/mL) could abate the amount of both total Tau and phosphorylated Tau expression in NGF-deprived PC12 cells raised in both media. Additionally, BM extract enhanced Tau-1 immunoreactivity in normal-serum NGF-deprived PC12 cells in a dose dependent manner.

Conclusion This was the first study to report that BM extract could survive and attenuate the amount of both total Tau and phosphorylated Tau in NGF-deprived PC12 cells in normal-serum and serum free medium. **Chiang Mai Medical Journal 2012;51(3):59-69.**

Keywords: *Bacopa monnieri*, Tau protein, phosphorylated Tau protein, NGF-deprived PC12 cells, neurodegenerative disorders

Introduction

Due to greater longevity in the world population, geriatric healthcare has become highly important in present day medicine. Dementia, especially with Alzheimer's disease (AD), is one of the major problems in geriatric medicine. The most common type of dementia is attributed to cerebral atrophy and AD, which are very common and important degenerative diseases of the brain. In Thailand, the caring cost for 1 dementia patient could range from 100-150 \$/month up to 200-800 \$/month [Anonymous]. While diminishing medication costs is sought, appropriate herbal medicine for improving cognitive and memory function might provides reasonably priced alternative treatment for this disease.

Two outstanding characteristics of Alzheimer's disease, i.e., neurofibrillary tangles (NFTs) and neuritic plaques, are the most putative pathologies of the condition. NFTs comprise pathological hyperphosphorylated Tau protein [1]. Tau protein is a microtubule associated protein found in many animals, and is expressed mainly in human neurons [2]. Abnormal phosphorylated Tau causes Tau disengagement from the microtubule and it is likely to misfold. A structure translation leads to hyperphosphorylated Tau becoming more organized and eventually developing into NFTs. Both neuritic plaques and NFTs are found in all associated areas of the cortical cortex, and in comparison, NFTs and quantitative neuronal losses, but not neuritic plaques, correlate best with the severity of dementia [3]. Tau protein levels correlate with memory performance in many studies of both humans and animals. Concentration of total Tau elevates in AD [4], while increasing in both cerebrospinal fluid (CSF) total Tau and CSF phosphorylated Tau, and their levels are found consistently in patients with mild cognitive impairment when compared to healthy cognitive controls [5]. Furthermore, neuronal atrophy also is a prominent macroscopic characteristic of the disease. Correlatively, recent findings reported elevated Tau hyperphosphorylation in apoptotic cells by both DNA damage [6] and removal of trophic factor [7-11]. Tau phosphory-

lation is related to neuronal apoptosis at 156-163 (HT7) and 202-205 (AT8) amino acid residues [12].

Bacopa monnieri or Brahmi (BM) is a versatile traditional Ayurvedic herb that has been used for centuries as an anti-inflammatory, analgesic, antipyretic, antiepileptic, and sedative agent [13-15]. The greatest importance of BM is especially the use of its plant, plant extract and isolated bacoside components that act as an enhancing agent for cognition and memory function. Several findings suggest that the cognition-promoting functions of BM may be attributed partially to the antioxidant effect of the bacosides [16]. By inhibiting acetylcholinesterase (AChE) as an antioxidant, BM could exert a neuroprotective effect that relieves neuronal oxidative stress [17], which might in turn contribute to neuronal apoptosis. As mentioned earlier, it is of interest whether BM can decrease Tau protein expression, which is another prominent pathology of AD. If so, this advantage might alleviate the medication cost of this disease.

In this study, we proposed that the property of BM, which improves cognition and memory function, should be beneficial in alleviating the enhancement of phosphorylated Tau protein in NGF-deprived PC12 cells raised in serum-free medium. We also investigated the viability percentage of NGF-deprived PC12 cells raised in serum-free medium and treated with BM extract, and the expression of total tau and dephosphorylated tau proteins in those cells. The results revealed the ability of BM to enhance the viability percentage of NGF-deprived PC12 cells and accentuate Tau-1 immunoreactivity.

Methods

Antibodies

Mouse anti-Tau monoclonal antibody (Tau-5, Millipore, MA, USA) is a phosphorylation-independent type that specifically detects total Tau protein, while mouse anti-Tau-1 monoclonal antibody (Millipore) is dephosphorylation-dependent, and specifically determines the dephosphorylated epitope at amino acids 189-207 [18, 19]. There have been reported that approximately 30 phosphorylated sites are observed in the AD brain [20]. Mouse anti-glyceraldehyde-3-

phosphate dehydrogenase monoclonal antibody (Millipore) is specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); a ubiquitous glycolytic enzyme present at reasonably high levels in almost all tissue. With the same method, goat anti-IgG (H&L) peroxidase conjugated affinity purified antibody (Millipore, MA, USA) was used for secondary reagent.

Cell culture preparation and treatment

PC12 cells (ATCC, VA, USA) were maintained for 7 days in a poly-D-lysine (Sigma Aldrich, MO, USA)-coated flask in normal-serum RPMI1640 media (Gibco, CA, USA), containing 10% heat-inactivated horse serum (Gibco), 5% heat-inactivated fetal bovine serum (Gibco), 2.05 mM of L-glutamine (Gibco), and 1x penicillin/streptomycin (21). Then, they were cultured for another 7 days in RPMI1640 media containing 3% heat inactivated horse serum, 2% heat inactivated fetal bovine serum, 2.05 mM of L-glutamine, and 1x penicillin/streptomycin [21]. The cells were then re-flasked in fresh poly-D-lysine-coated flasks and maintained for 2-3 days in apoptotic serum-free RPMI 1640 media containing 1x Insulin-transferrin-sodium selenite (Sigma Aldrich), 0.2% induced bovine albumin fraction V (Calbiochem, Darmstadt, Germany), 2.05 mM of L-glutamine, and 1x penicillin/streptomycin to allow for the regrowth of neuronal processes [10]. The serum-free media were then refreshed by new serum-free media, with and without alcoholic BM extract ranging from 50-300 µg/mL [17]. The other group of cells was maintained in normal-serum medium throughout the experiment. Each group of cells would be processed in the same way in further experiments.

Preparation of BM extract

Brahmi or BM was collected from Phetburi province, Thailand and identified by Assoc. Prof. Dr. Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The specimen (Phrompittayarat 001) was kept at the PBM Herbarium, Mahidol University, Thailand. BM was cut into small pieces and dried in a hot air oven at 50 °C for 12 hours. The dried plant material was powdered coarsely and soaked in water. After 24 hours, the water was squeezed mechanically out of the plant material, which was then percolated for 8 hours with circulating 95% ethanol. The residue was extracted twice more using the same procedure. The combined extract, containing 5% (w/w) of total saponins, and a mixture of bacoside A₃, bacoside II, bacopasaponin X, bacopasaponin C and bacopaside I, was filtrated and dried under reduced pressure [22, 23]. The total saponin content was determined by using HPLC, as previously reported [24]. The extract was stored at -20 °C in a dark bottle until use. Brahmi extract was provided generously by Assoc. Prof. Dr. Kornkanok Ingkaninan, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Science, Naresuan University.

To prepare stock BM solution, 50 mg of ethanolic BM extract paste were dissolved firstly in a 2 mL 1:1 ratio mix-

ture of sterilize DMSO (Amresco, OH, USA): filtered absolute 95% ethanol; aliquoted and stored in a dark tube at -20 °C. This stock BM solution was diluted further with sterile D-PBSA to obtain proper concentrations of fresh working solution, immediately before the experiment. The working solution contained both DMSO and ethanol of less than 0.03% for exclusion from the cytotoxicity of these substances [9].

Cell viability test

Neuronal survival was measured by the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (BioBasic, Canada) or (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; a yellow water-soluble tetrazolium dye that is reduced by a mitochondria dehydrogenase enzyme from live cells to a purple water-insoluble formazan product. Firstly, 0.5-10x10³ cells were plated into 96 round-bottomed well plates in an appropriate medium and incubated in a humidified atmosphere of 37 °C for 1-3 days, in such a way that the cells were in exponential growth phase when the BM extract was added. The cells were then exposed to an appropriate medium with and without BM for 1-7 days. Then, 100 µL of MTT (1 mg/mL in appropriate media) was added daily to each well and incubated further in the dark for 2 hours. At the end of the MTT treatment, the medium was removed and 100 µL of DMSO (Amresco) were added to each well in order to dissolve the purple formazan crystal. The color was quantified using a microplate reader (LabSystem iEMS reader, Helsinki, Finland) at 540 nm. The viability percentage was analyzed by one-way ANOVA with $\alpha = 0.05$.

Immunoblotting

Cells were harvested by scraping from the flasks lysed with ice-cold RIPA buffer, 25 mM of Tris-HCl pH 7.6, 150 mM of NaCl (Merck, Darmstadt, Germany), 1% NP-40 (USB, OH, USA), 1% sodium deoxycholate (BioBasic), 0.1% SDS (USB), and a protease inhibitor cocktail (Sigma Aldrich) with at least 5x10⁶ cells/mL of RIPA buffer, and incubated on ice for 30 min. After centrifugation for 15 min at 14,000 xg, total proteins were determined using quick start™ Bradford reagent (Biorad, CA, USA). An equal amount of total protein (50-125 µg) was resolved on 10-12% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). This membrane was incubated overnight in a blocking buffer containing 10 mM of Tris (USB Corporation, OH, USA) pH 7.5, 150 mM of NaCl, and 0.05% (v/v) Tween 20 (BioBasic) plus 5% dry milk (wt/v) at 4 °C. This was followed by incubation with primary antibodies: anti-Tau (clone Tau-5) (1:500), anti-Tau-1 (1:500), or anti-GAPDH (1:200) in 1% skimmed milk TBST for 3 hours at room temperature. Each membrane was then washed 3 times in TBST (5 min/each), before being incubated in goat anti-mouse IgG (H&L) peroxidase conjugated secondary antibody (1:5000) in 1% skimmed milk TBST for 1 hour

at room temperature. The immunoblots were analyzed using the ECL plus Western blotting detection system (GE Healthcare) following the manufacturer's instructions, 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. Tau 1 immunoreactivity was derived from normalizing the relative amount of Tau-1 expression by the relative total amount of Tau (Tau 5) expression present in each sample. All of the data were analyzed by statistical one-way ANOVA with $\alpha = 0.05$.

Results

1. BM at 150 and 200 $\mu\text{g/mL}$ concentration significantly enhanced MTT viability of NGF-deprived PC12 cells raised for four days in normal-serum RPMI1640 medium.

On the first day, BM extract in all concentrations (50-300 $\mu\text{g/mL}$) displayed cellular viabilities, which were close to that of the cells grown in absolute normal-serum RPMI1640 medium (the control) (Table 1). On the second day, BM extract in all concentrations (50-300 $\mu\text{g/mL}$) enhanced higher cellular viability than that of the control on the 1st day, especially, at the 50 $\mu\text{g/mL}$ concentration. Later, BM extract in all concentrations caused cellular viabilities close to that of the control on the 1st day, and again on the 3rd day. BM extract at 50-200 $\mu\text{g/mL}$ concentrations raised cellular viabilities higher than that of the control on the 1st and 4th day. At this time, BM extract at 150 and 200 $\mu\text{g/mL}$ concentrations contributed to significant increment. After the 5th

day, BM in nearly all concentration caused the cellular viability to be close or higher than that of the control on the 1st day, except for the 300 $\mu\text{g/mL}$ concentration.

2. BM at 50 $\mu\text{g/mL}$ concentration tended to accentuate MTT viability in NGF-deprived PC12 cells raised in serum-free RPMI1640 (SF) medium for 2-4 days.

On the first day, BM extract at 50-200 $\mu\text{g/mL}$ concentration revealed higher cellular viabilities than that of the cells raised in absolute serum-free RPMI1640 medium (the control) (Table 2). Among BM extract in all six concentrations (50-300 $\mu\text{g/mL}$), 50 $\mu\text{g/mL}$ elicited the highest cellular viability on the 2nd and 3rd days. BM extract at 150-300 $\mu\text{g/mL}$ concentrations increased cell death to a point higher than that in control cells on these two days. The BM extract at 100 $\mu\text{g/mL}$ concentration also enhanced cellular viability slightly more than that of the cells raised in absolute serum-free RPMI1640 medium on the 2nd day, but less on the 3rd and 4th days. After the 5th day, the BM extract in all six concentrations (50-300 $\mu\text{g/mL}$) diminished less cellular viability than that of the control on the first day.

3. BM extract reduced the amount of total Tau expression and attenuated Tau phosphorylation (at Tau-1 site) in NGF-deprived PC12 cells grown in normal-serum RPMI1640 medium.

Immunoblot showed that BM extract at both 50 and 100 $\mu\text{g/mL}$ concentrations reduced the

Table 1. MTT viability percentage of NGF-deprived PC12 cells, treated by BM extract for 7 days in normal-serum RPMI1640 (NR) medium. The viability percentages were compared to that of the cells treated without BM extract on the 1st day. Each group, $n = 3-9$. All data represented mean $\pm$ SEM and were analyzed by one-way ANOVA.

Time (Day)	BM extract concentration ($\mu\text{g/mL}$)						
	0	50	100	150	200	250	300
1	100.00 $\pm$ 17.28	98.93 $\pm$ 11.89	91.54 $\pm$ 5.68	103.34 $\pm$ 12.18	118.87 $\pm$ 27.18	96.86 $\pm$ 17.20	94.78 $\pm$ 22.59
2	123.00 $\pm$ 9.76	141.51 $\pm$ 12.38	131.76 $\pm$ 8.19	126.43 $\pm$ 4.36	132.47 $\pm$ 16.32	129.20 $\pm$ 7.09	101.98 $\pm$ 9.03
3	106.01 $\pm$ 9.96	110.97 $\pm$ 11.08	101.55 $\pm$ 2.56	101.83 $\pm$ 9.54	113.02 $\pm$ 10.14	100.15 $\pm$ 6.56	69.36 $\pm$ 2.09
4	105.50 $\pm$ 10.27	132.75 $\pm$ 9.19	156.04 $\pm$ 4.48	171.92 $\pm$ 9.80 ^a	162.83 $\pm$ 18.65 ^a	150.54 $\pm$ 13.25	51.16 $\pm$ 11.82
5	66.15 $\pm$ 0.29	69.83 $\pm$ 5.53	86.36 $\pm$ 14.16	73.45 $\pm$ 1.95	86.40 $\pm$ 3.70	85.96 $\pm$ 5.21	36.66 $\pm$ 1.92
6	96.30 $\pm$ 18.20	114.49 $\pm$ 21.22	121.08 $\pm$ 20.43	126.52 $\pm$ 24.01	149.54 $\pm$ 13.88	123.23 $\pm$ 1.33	44.88 $\pm$ 4.56
7	83.50 $\pm$ 10.03	103.03 $\pm$ 21.54	105.12 $\pm$ 19.90	79.33 $\pm$ 19.37	101.49 $\pm$ 11.50	85.64 $\pm$ 11.07	48.18 $\pm$ 8.00

a: $p = 0.007$

Table 2. MTT viability percentage of NGF-deprived PC12 cells, treated by *Bacopa monnieri* for 7 days in serum-free RPMI1640 medium. The viability percentages were compared to that of the cells treated with no any BM extract on the first day. Each group, n =3-18. All data represented mean $\pm$ SEM and were analyzed by one-way ANOVA.

Time	BM extract concentration (μg/mL)						
(Day)	0	50	100	150	200	250	300
1	100.00 ± 8.00	126.03 ± 7.58	124.55 ± 6.49	116.20 ± 14.59	122.10 ± 15.45	99.44 ± 9.54	40.03 ± 9.37
2	115.29 ± 9.91	130.50 ± 11.63	108.16 ± 9.66	44.76 ± 6.50	22.61 ± 3.79	21.66 ± 3.56	20.27 ± 3.39
3	104.87 ± 8.42	132.60 ± 14.73	77.19 ± 7.94	27.52 ± 6.99	14.71 ± 1.85	14.69 ± 1.58	15.45 ± 1.51
4	145.49 ± 16.62	101.20 ± 16.88	24.99 ± 2.50	14.39 ± 1.00	13.83 ± 1.39	13.80 ± 1.37	15.44 ± 1.29
5	76.74 ± 9.25	40.45 ± 8.55	16.74 ± 2.06	18.31 ± 4.35	14.53 ± 0.70	15.09 ± 1.07	16.67 ± 0.79
6	76.98 ± 6.89	56.04 ± 18.22	18.86 ± 1.77	16.73 ± 1.48	17.73 ± 1.48	18.19 ± 1.53	18.39 ± 1.54
7	63.34 ± 7.98	26.48 ± 5.90	15.50 ± 1.85	14.29 ± 0.76	13.369 ± 0.90	14.34 ± 0.64	15.77 ± 0.60

amount of total Tau expression in NGF-deprived PC12 cells grown in normal-serum RPMI1640 medium. However, BM extract at 100 μ g/mL concentration contributed to the dephosphorylated Tau (at Tau-1 site) increment in these cells (Fig. 1). Due to the greater increment in dephosphorylated Tau (at Tau-1 site), and more decrement in total Tau (Tau 5), the BM extract accentuated Tau-1 immunoreactivity in a dose dependent manner (Fig. 3). This might suggest that BM extract attenuated Tau phosphorylation (at the Tau-1 site) in NGF-deprived PC12 cells raised in normal-serum RPMI1640 medium.

4. BM extract reduced the amount of total Tau expression and diminish Tau phosphorylation (at Tau-1 site) in NGF-deprived PC12 cells raised in serum-free RPMI1640 medium.

Immunoblot showed that BM extract at both 50 and 100 μ g/mL concentrations reduced the amount of total Tau expression in NGF-deprived PC12 cells grown in serum-free RPMI1640 medium. However, BM extract at 100 μ g/mL concentration contributed to the dephosphorylated Tau (at Tau-1 site) increment in these cells (Fig. 2). The BM extract at 100 μ g/mL concentration increased both total Tau (Tau 5) and dephosphorylated Tau (at the Tau-1 site) more than 50 μ g/mL concentration. Thus, BM extract at 100 μ g/mL concentration could improve Tau-1 immunoreactivity slightly (Fig. 3). This also might suggest that BM extract could diminish Tau phosphorylation (at the Tau-1 site) in NGF-deprived PC12

cells raised in serum-free RPMI1640 medium.

Discussion

BM extract could enhance the MTT viability of NGF-deprived PC12 cells raised in normal-serum RPMI1640 medium. This result was congruent with a recent study by Limpeanchob N, et al in that the primary cortical cultural neurons, induced by A β 25-35, underwent more cell death when treated with high dose BM extract. At the dose of 100 μ g/mL, BM extract did not alter its cellular viability when induced by A β ₂₅₋₃₅ for 24 hr, but when the treatment was prolonged, this concentration could enhance significant viability by 37% and 20% at 48 hr and 72 hr, respectively [17]. In that study, the 50% inhibitory concentration (IC50) was 242.8 $\pm$ 1.5 μ g/mL, and the concentration of BM extract at 100 μ g/mL did not affect cell viability. Some differences from the study by Limpeanchob N, et al might be attributed to the utilization of a different cell culture system. The PC12 or pheochromocytoma cell line is derived from rat adrenal medullar, which originates from the same neural crest as other peripheral neurons. PC12 is an NGF-dependent cell that can be induced to proliferate and differentiate into sympathetic neurons when expose to NGF [25]. Many studies have reported that NGF-dependent neurons may impair the basal forebrain selectively during aging or AD [26-30]. This might contribute to insignificant enhance-

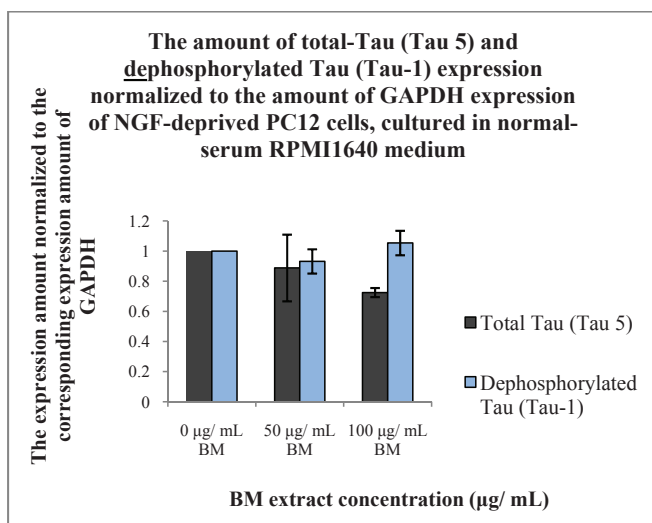
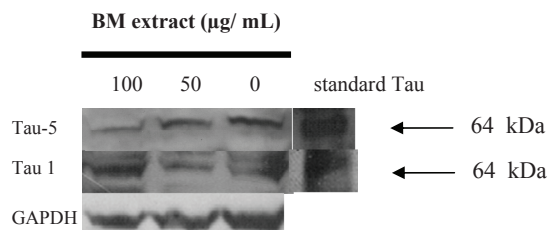


Figure 1. The relative amount of total tau (Tau 5) and dephosphorylated Tau (Tau-1) expression of NGF-deprived PC12 cells in normal-serum RPMI1640, treated with BM extract for two days. Each group, $n=3$. All data represented by $\text{mean} \pm \text{SEM}$ and were analyzed by statistic one-way ANOVA.

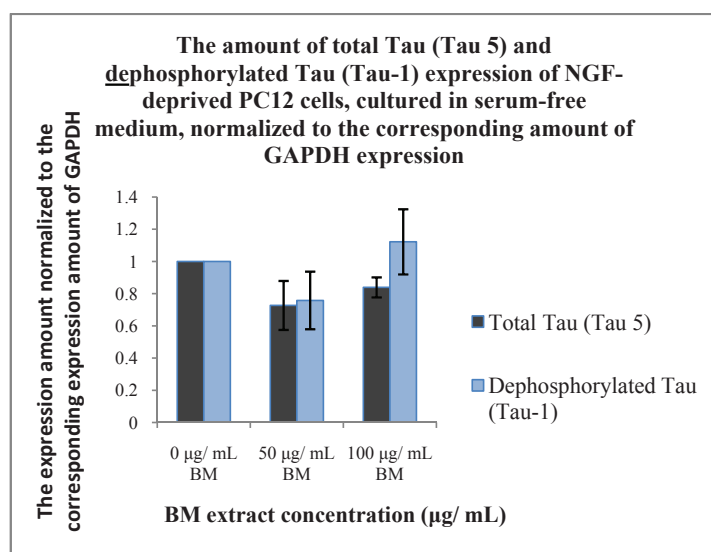
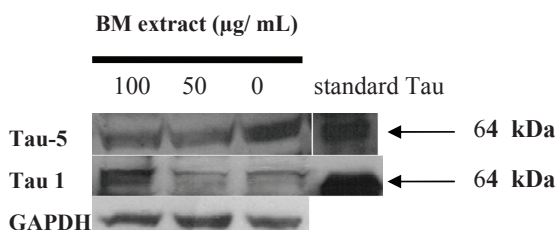


Figure 2. The relative amount of total tau (Tau 5) and dephosphorylated Tau (Tau-1) expression of NGF-deprived PC12 cells in serum-free RPMI1640, treated with BM extract for two days. Each group, $n=3$. All data represented by $\text{mean} \pm \text{SEM}$ and were analyzed by one-way ANOVA.

ment of cellular viability in cells treated with 100 µg/mL concentration of BM extract for 48 hours. However, BM extract at 150 and 200 µg/mL concentrations could accentuate cellular viability significantly in the middle phase (4th day) of the experiment.

BM extract might accentuate the viability of serum-free PC12 cells. This study on the viability measurement of NGF-deprived PC12 cells grown in serum-free medium (Table 2) was congruent with the study by Shelton SB, and Johnson GVW, which showed that the removal of NGF resulted in a progressive loss of cellular viability of PC12 cells maintained in serum-free medium [10]. A small discrepancy could be attributed to personal error or a different measuring technique. In this study, the MTT assay was run on 96-well microplates, which could carry only a minute volume of solution. If only a small mistake is made when pipetting, great fluctuations are prone to occur as a result. Additionally, in their study, they measured cell death from the percentage of LDH (lactate dehydrogenase) released from the dead cells. On the other hand, cellular viability in this study was measured from the reduction of MTT by reductase enzymes in the viable cells.

The capability of BM extract, especially at a low concentration, improved the cellular viability from the model of serum-free PC12 cells in this study, and was again in accordance with the study of Limpeanchop N, et al, [17], with some exceptions. The primary cortical neuronal culture was induced with Aβ peptide and a neurotrophically deprived neuronal PC12 cell line that were both contributors to cellular apoptosis [31-36]. Since NGF can rescue PC12 cells grown in neurotrophic-deprived medium from apoptosis [37, 38], PC12 cells raised in NGF-deprived serum-free medium may undergo greater apoptosis than those in normal-serum medium. Therefore, BM extract might not be able to enhance cellular viability significantly in these apoptotic neuronal cells.

BM extract could attenuate Tau phosphorylation (at the Tau-1 site) in NGF-deprived PC12 cells grown in normal-serum RPMI1640 medium. This was attributed to 100 µg/mL of BM extract

concentration being able to reduce the amount of total Tau (Tau 5) expression, but increase that of dephosphorylated Tau (at Tau 1 site) expression to a greater extent than 50 µg/mL in NGF-deprived PC12 cells grown for 48 hours in normal-serum RPMI1640 medium. Nevertheless, 100 µg/mL of BM extract concentration increase a cellular viability less than 50 µg/mL.

BM extract at 100 µg/mL concentration could diminish Tau phosphorylation (at the Tau-1 site) in NGF-deprived PC12 cells grown in serum-free RPMI1640 medium. As BM extract at 100 µg/mL concentration displayed more cell death than that at 50 µg/mL in NGF-deprived PC12 cells grown for 48 hours in serum-free RPMI1640 medium, the former exhibited more total Tau than the latter did. However, this Tau was high in dephosphorylated form (at the Tau-1 site).

In this study, BM extract could weaken phosphorylated Tau (at the Tau-1 site) more distinctively in cells raised in normal-serum medium than those grown in serum-free medium (Fig. 3). This was concordant with the study of Shelton SB and Johnson GVW in that a decrement in Tau-1 immunoreactivity was shown, and thus an increment occurred in the phosphorylation of high molecular weight (HMW) Tau and Tau in PC12 cells deprived of serum and NGF [10].

As indicated earlier, BM could not only attenuate total Tau proteins, but also abate Tau protein phosphorylation at the Tau-1 site in NGF-deprived PC12 cells raised in both media. The ability of BM to reduce both total Tau proteins and phosphorylated Tau protein may be attributed to the same antioxidant capability similar to the antioxidant property of curcumin, which can decrease both total Tau (Tau 5) and phosphorylated Tau (at the Tau-1 site or Ser₂₀₂ AT₈ residues) in cytotoxic Aβ-induced PC12 cells [39]. Several studies reported consecutively on the antioxidant property of BM not only in the in vivo system [15, 40] but also the in vitro system [17, 41]. The capability of BM to attenuate tau phosphorylation (Tau-1) may be due to other properties of BM similar to Icariin, a flavanoid compound from the herb, *Epimedium brevicornum Maxim*,

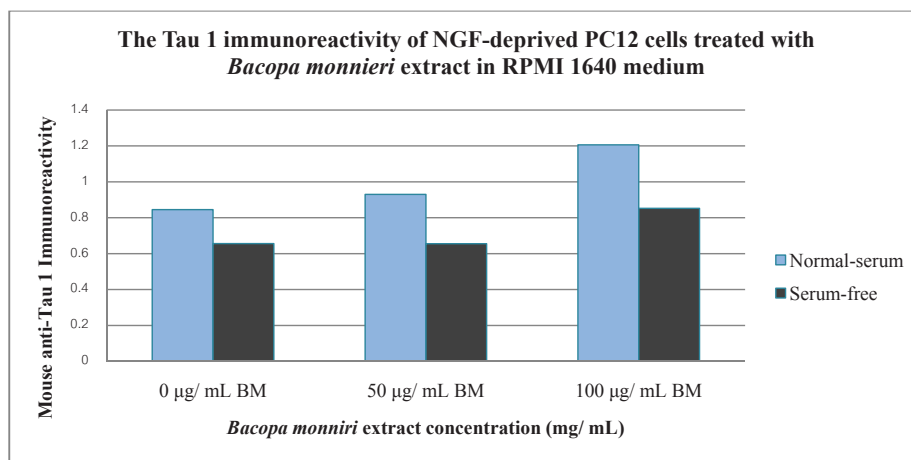


Figure 3. Tau-1 immunoreactivity of total Tau expression of NGF-deprived PC12 cells treated with BM extract in RPMI1640 medium for two days. Each group, n =3. All data were represented mean±SD and were analyzed by one-way ANOVA.

which can reduce $A\beta_{25-35}$ -induced cytotoxicity and apoptosis rate significantly by inhibiting Tau protein phosphorylation at Ser₃₉₆, Ser₄₀₄, and Thr₂₀₅ sites. This is achieved through activating a PI3K/Akt signaling pathway that can have a further inhibiting effect on glycogen synthase kinase (GSK-3 β); a major protein kinase response for tau protein hyperphosphorylation. Recent findings demonstrated that BM extract can diminish $A\beta_{1-40}$ and $1-42$ levels in the cortex by as much as 60%, and reverse Y-maze performance and open field hyperlocomotion behavioral change present in PSAPP mice [42], as well as preclude neurons from $A\beta$ -induced cell death [17]. Accordingly, some studies showed that $A\beta$ peptide can activate GSK3 β [43]. Since BM extract comprises several compound ingredients that contain the putative saponin, Bacoside A, as an active ingredient [44], further research is left to investigate whether attenuation of total Tau protein and phosphorylated Tau protein affects BM extract by Bacoside A participation, and what the precise mechanism is in the PC12 system or other neuronal culture systems.

These data could support the capability of BM extract in protecting neuronal cells from the cell death process, which is attributed to the neurode-

generative effect of Tau protein. BM extract could be beneficial for not only cell survivability, especially at a low dose, but also as a Tauopathy protectant by reducing the Tau level in the early phase. Additionally, this information may be supportive in advocating the benevolent property of BM extract as an alternative therapy for neurodegenerative diseases, including Alzheimer's disease.

Acknowledgments

This work was subsidized by the National Council of Research Thailand fund, and the authors' study was supported by the Commission for Higher Education of Thailand (CHE) for the 2006 shortage program and 2007 exchange program, and the Graduate School, Naresuan University. We also thank Assoc. Prof. Kornkanok Ingkaninan from the Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Science, Naresuan University, Phitsanulok, Thailand for her generosity in providing the crude extract of *Bacopa monnieri*.

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ความสามารถของสารสกัดสมุนไพรในการลดโปรตีน Tau ในเซลล์ PC12 ที่ไม่ได้รับ nerve growth factor ที่เลี้ยงในอาหารที่มีซีรัมปกติและในอาหารที่ปราศจากซีรัม

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วัตถุประสงค์ เพื่อศึกษาผลของสารสกัดสมุนไพรที่มีต่อโปรตีน Tau ในเซลล์ PC12 ที่ไม่ได้รับ nerve growth factor (NGF)

วิธีการ นำเซลล์ PC12 ที่ไม่ได้รับ NGF มาเลี้ยงในอาหารที่มีซีรัมปกติ 1 สัปดาห์ก่อนเปลี่ยนมาเลี้ยงในอาหารที่มีซีรัมต่ำ 1 สัปดาห์ จากนั้นแบ่งเซลล์เป็น 2 กลุ่ม กลุ่มแรกเลี้ยง 7 วันเพื่อใช้สำหรับการวิเคราะห์ $^{3-(4,5\text{-Dimethylthiazol-2-yl})-2,5\text{-diphenyltetrazolium bromide}}$ (MTT) กลุ่มที่สองเลี้ยงในอาหารที่มีซีรัมปกติหรือที่ปราศจากซีรัมที่ไม่มีสารสกัดสมุนไพรหรือมีสารสกัดสมุนไพรความเข้มข้น 50 ถึง 300 $\mu\text{g/mL}$ นาน 2 วันเพื่อใช้สำหรับการวิเคราะห์ immunoblot สำหรับการวิเคราะห์ MTT ในแต่ละวันค่าเฉลี่ยของความรอดชีวิตของเซลล์ที่เลี้ยงในอาหารที่มีสารสกัดสมุนไพรแต่ละความเข้มข้นถูกนำมาเทียบกับความรอดชีวิตของเซลล์ที่เลี้ยงในอาหารที่ไม่มีสารสกัดสมุนไพร การทำ immunoblot โดยใช้แอนติบอดีต่อโปรตีน Tau โปรตีน dephosphorylated Tau และ glyceraldehyde dehydrogenase และวิเคราะห์ปริมาณโปรตีนด้วยโปรแกรม Scion Image แล้วนำมาคำนวณค่า Tau-1 immunoreactivity ผลการทดลองทั้งหมดวิเคราะห์โดยใช้ one-way ANOVA

ผลการทดลอง สารสกัดสมุนไพรความเข้มข้น 150 และ 200 $\mu\text{g/mL}$ เพิ่มความรอดชีวิตของเซลล์ PC12 ที่ไม่ได้รับ NGF ที่เลี้ยงในอาหารที่มีซีรัมปกติ 4 วันอย่างมีนัยสำคัญขณะที่สารสกัดสมุนไพรความเข้มข้น 50 $\mu\text{g/mL}$ มีแนวโน้มที่สามารถลดความรอดชีวิตของเซลล์ PC12 ที่ไม่ได้รับ NGF ที่เลี้ยงในอาหารที่ไม่มีซีรัม นาน 2-4 วัน สารสกัดสมุนไพรความเข้มข้น 100 $\mu\text{g/mL}$ สามารถลดทั้งปริมาณรวมของโปรตีน Tau และปริมาณ phosphorylated Tau ในเซลล์ PC12 ที่ไม่ได้รับ NGF ทั้งที่เลี้ยงในอาหารที่มีซีรัมและในอาหารที่ปราศจากซีรัม นอกจากนี้สารสกัดสมุนไพรสามารถเพิ่ม Tau-1 immunoreactivity ในเซลล์ PC12 ที่ไม่ได้รับ NGF ทั้งที่เลี้ยงในอาหารที่มีซีรัมในลักษณะ dose-dependence

สรุปผลการทดลอง สารสกัดสมุนไพรสามารถเพิ่มความรอดชีวิตของเซลล์ PC12 ที่ไม่ได้รับ NGF และสามารถลดทั้งปริมาณรวมของโปรตีน Tau (Tau 5) และปริมาณ phosphorylated Tau (ที่ตำแหน่ง Tau-1) ในเซลล์ PC12 ที่ไม่ได้รับ NGF ที่เลี้ยงในอาหารที่มีซีรัมและในอาหารที่ปราศจากซีรัม **เชิงให้มวขสาร 2555;51(3):59-69.**

คำสำคัญ: *Bacopa monnieri*, Tau protein, phosphorylated Tau protein, NGF-deprived PC12 cells, neurodegenerative disorders