CHAPTER I

INTRODUCTION & LITERATURE REVIEW

บทนำและการทบทวนวรรณกรรม/สารสนเทศ (Information) ที่เกี่ยวข้อง:

โรคสมองเสื่อมคือภาวะที่ประสิทธิภาพการทำงานของสมองถดถอยลงอย่างต่อเนื่อง จากภาวะที่เคยและกำลังเป็นอยู่กลับไปสู่ภาวะเด็กเล็ก 3-4 ปี ในระยะเริ่มต้นและภาวะทารกแรกเกิดในภาวะสุดท้ายของโรค American Academic of Neurology กล่าวว่าคนอเมริกันที่มีอายุ 65 ปี ป่วยเป็นโรคสมองเสื่อมร้อยละ 10 และร้อยละ 50 เมื่ออายุเกิน 65 ปี ความสัมพันธ์ระหว่างโรคสมองเสื่อมกับอายุเริ่มปรากฏตั้งแต่อายุ 60 ปีขึ้นไปโดยความชุกของโรคสมองเสื่อมจะเพิ่มเป็น 2 เท่าทุกอายุ 5 ปี ในอดีตโรคสมองเสื่อมไม่ได้เป็นปัญหาด้านสาธารณสุขของประเทศไทยเพราะคนไทยส่วนมากเสียชีวิตก่อนที่โรคสมองเสื่อมจะแสดงอาการคือ 60 ปีขึ้นไป ปัจจุบันโรคสมองเสื่อมได้กลายเป็นปัญหาสำหรับประเทศแล้วเพราะค่าเฉลี่ยอายุของคนไทยได้ขยับสูงขึ้น คือในปี พ.ศ. 2535 ค่าเฉลี่ยอายุของชายไทยเป็น 66.4 ปี หญิงไทย 71.8 ปี แต่ใน 10 ปีต่อมา คือ พ.ศ. 2545 ค่าเฉลี่ยอายุของชายไทยเป็น 69.9 ปี หญิงไทย 74.9 ปี ซึ่งในอนาคตอายุเฉลี่ยจะเพิ่มขึ้นอย่างต่อเนื่องและโรคสมองเสื่อมจะเป็นปัญหาที่เพิ่มมากขึ้น ทำให้ภาระในการรักษาพยาบาลซึ่งรวมถึงค่ารักษาพยาบาลจะเพิ่มมากขึ้นในอนาคต การพัฒนาสมุนไพรพื้นบ้านที่ช่วยสามารถป้องกันและบรรเทาอาการสมองเสื่อมจึงนับเป็นแนวทางหนึ่งในการลดภาระการรักษาพยาบาลนี้ ดังนั้นคณะผู้วิจัยจึงมีความสนใจในการค้นคว้าและวิจัยเกี่ยวกับสมุนไพรพรมมิซึ่งเป็นพืชพื้นบ้านที่มีรายงานว่ามีสรรพคุณหลายอย่างรวมทั้งการเพิ่มความทรงจำและความสามารถในการเรียนรู้

ภาพที่ 1 แสดงการคาดการณ์ความชุกภาวะสมองเสื่อมในประเทศไทย พ.ศ.2548-2593

CHAPTER II

OBJECTIVES

วัตถุประสงค์ของโครงการวิจัย: งานวิจัยนี้จึงมีจุดประสงค์เพื่อทดสอบฤทธิ์ของสารสกัดพรมมิว่าจะมีผลต่อปริมาณ Tau และ phosphorelated Tau protein หรือไม่ในเซลล์ PC12 ซึ่งใช้เป็นตัวแทนของเซลล์ประสาท และทดสอบผลของสารสกัดพรมมิที่มีต่อระบบการทำลายโปรตีนแบบ autophagy ในเซลล์ PC12

CHAPTER III

MATERIALS AND METHODS

วิธีการดำเนินการวิจัย และการเก็บข้อมูล:

1. การเลี้ยงเซลล์

    - PC12 cells จาก ATCC ในสภาวะปกติเลี้ยงในอาหาร RPMI 1640 medium ที่มี 10% Horse serum, 5% Foetal bovine serum, 50 unit/ mL Penicillin G, และ 50 μg/mL Streptomycin (Normal-serum medium)
    - PC12 cells เลี้ยงในสภาวะปกติเป็นเวลา 7 วัน จากนั้นเหนี่ยวนำให้เกิด appotosis ในอาหาร RPMI 1640 medium ที่มี 3% Horse serum, 2% Foetal bovine serum, 50 unit/ mL Penicillin G, และ 50 μg/mL Streptomycin (Low-serum medium)
    - PC12 cells เลี้ยงในสภาวะปกติเป็นเวลา 7 วัน จากนั้นเลี้ยงในสภาวะเหนี่ยวนำให้เกิด appotosis อีก 7 วัน แล้วจึงเลี้ยงในสภาวะที่ทำให้เกิด appotosis ในอาหาร RPMI 1640 medium ที่มี 50 unit/ mL Penicillin G, และ 50 μg/mL Streptomycin (Non-serum medium)

2. การทดสอบ MTT

    ให้สารสกัดพรมมิที่ความเข้มข้นแตกต่างกัน 6 ความเข้มข้น คือ 50-300 μg/ mL แก่ PC12 cells ที่เลี้ยงใน 3 สภาวะคือ สภาวะปกติ (Normal-serum medium) สภาวะเหนี่ยวนำให้เกิด appotosis (Low-serum medium) และสภาวะที่ทำให้เกิด appotosis (Non-serum medium) เป็นเวลา 1-7 วัน วัดความมีชีวิตรอดของเซลล์ด้วยวิธี MTT ตั้งแต่วันที่ 1-7 ของการให้ยาโดยเปรียบเทียบกับเซลล์ที่เลียงในทั้ง 3 สภาวะที่ไม่ให้ยา

3. การตรวจสอบปริมาณของโปรตีน Tau ด้วยวิธี Immunoblotting

    - เลี้ยง PC12 cells ใน 3 สภาวะคือ สภาวะปกติ (Normal-serum medium) สภาวะเหนี่ยวนำให้เกิด appotosis (Low-serum medium) และสภาวะที่ทำให้เกิด appotosis (Non-serum medium) โดยให้สารสกัดพรมมิที่ความเข้มข้นแตกต่างกัน 2 ความเข้มข้น คือ 50 และ 100 μg/ mL เป็นเวลา 2 วัน

- จากนั้นสกัดโปรตีนจากเซลล์ทั้ง 3 สภาวะ
- นำโปรตีนจากเซลล์ทั้ง 3 สภาวะมาทำ SDS-PAGE แล้ว transfer proteins ไปที่ PVDF membrane
- Probe protein ด้วย anti-Tau antibody (recognizes total tau protein), anti-Tau 1 antibody (regognizes dephosphorylated tau protein), และ anti-GAPDH antibody
- Probe ด้วย secondary antibody ที่ Tag ด้วย HRP enzyme
- ตรวจสอบผลด้วยวิธี Chemiluminescence

4. การตรวจสอบปริมาณของโปรตีน Tau ด้วยวิธี Immunoblotting
   - เลี้ยง PC12 cells ใน 3 สภาวะคือ สภาวะปกติ (Normal-serum medium) สภาวะเหนี่ยวนำให้เกิด appotosis (Low-serum medium) และสภาวะที่ทำให้เกิด appotosis (Non-serum medium) โดยให้สารสกัดพรมมิที่ความเข้มข้นแตกต่างกัน 2 ความเข้มข้น คือ 50 และ 100 μg/ mL เป็นเวลา 2 วัน
   - จากนั้นทำการ Fixing, Permeabilizing, Blocking และเติม Primary Antibody (anti-Tau antibody (recognizes total tau protein), anti-Tau 1 antibody (regognizes dephosphorylated tau protein))
   - Probe ด้วย secondary antibody
   - ตรวจสอบผลด้วยวิธี Fluorescence
5. การตรวจสอบ lysosomal enzyme activity
   - เลี้ยง PC12 cells ใน 3 สภาวะคือ สภาวะปกติ (Normal-serum medium) สภาวะเหนี่ยวนำให้เกิด appotosis (Low-serum medium) และสภาวะที่ทำให้เกิด appotosis (Non-serum medium) โดยให้สารสกัดพรมมิที่ความเข้มข้นแตกต่างกัน 2 ความเข้มข้น คือ 50 และ 100 μg/ mL เป็นเวลา 2 วัน
   - จากนั้นสกัดโปรตีนจากเซลล์ทั้ง 3 สภาวะ
   - นำโปรตีนจากเซลล์ทั้ง 3 สภาวะมาตรวจสอบระดับการทำงานของ lysosomal enzyme 3 ชนิด คือ Cathepsin B, D, และ L โดยใช้ Cathepsin assay kit

6. การตรวจสอบปริมาณโปรตีนโดย 2D-gel Electrophoresis
    i. เลี้ยง PC12 cells ใน 3 สภาวะคือ สภาวะปกติ (Normal-serum medium) สภาวะเหนี่ยวนำให้เกิด appotosis (Low-serum medium) และสภาวะที่ทำให้เกิด appotosis (Non-serum medium) โดยให้สารสกัดพรมมิที่ความเข้มข้นแตกต่างกัน 2 ความเข้มข้น คือ 50 และ 100 μg/mL เป็นเวลา 2 วัน
    ii. จากนั้นสกัดโปรตีนจากเซลล์ทั้ง 3 สภาวะ
    iii. นำโปรตีนจากเซลล์ทั้ง 3 สภาวะมาทำ IEF แล้วจึงนำแผ่นเจลนั้นมาทำ SDS-PAGE

CHAPTER IV

RESULTS

MTT cell viability

1. MTT cell viability of NGF-deprived PC12 cells treated by BM extract in normal-serum (NR) RPMI1640 medium

The effect of BM extract on viability of NGF-deprived PC12 cells cultured in normal-serum RPMI 1640 medium was determined by measuring the reducing of MTT to purple crystal formazan by living-cell mitochondrial enzyme. The more purple formazan crystals appear, the more cells survive. Cells were treated with 0 (control), 50, 100, 150, 200, 250, and 300 µg/ mL BM extract for up to 7 days and measured for their viability daily. The experiment was performed in three-independent replications with triplication in an individual experiment. Their viabilities of each treatment in an individual experiment were calculated for their averages, which were further calculated again for mean and standard error (SE) of the same treatment from different independent experiments. The viability percentages of the BM treated cells were compared with the viability percentage of the control from the 1st day and analyzed by one-way ANOVA with α = 0.05.

On the first day, BM extract at all concentrations (0-300 µg/ mL) displayed the cellular viabilities closely to that viability of the cells grown in the absolute normal-serum RPMI1640 medium (the control) (Table 1). On the second day, BM extract at all concentrations (0-300 µg/ mL) could enhance the cellular viability higher than that of the control on the 1st day, especially, at the 50 µg/ mL concentration. Later, BM extract at all concentrations could bring the cellular viabilities closely to that of the control on the 1st day again on the 3rd day. BM extract at 50-200 µg/ mL concentrations could still dose-dependently rise up the cellular higher than that of the control on the 1st day on the 4th day. At this time, BM extract at 150 and 200 µg/ mL concentrations could be contributed to the significant increment (Fig. 1). Since the 5th day, BM at nearly all concentration caused the cellular viability closely to or

higher than that of the control on the 1st day, except for the 300 μg/ mL concentration.

2. MTT cell viability of NGF-deprived PC12 cells treated by BM extract in apoptotic-induced serum-free RPMI1640 medium

NGF-deprived PC12 cells were brought up in the serum-free RPMI 1640 medium for apoptotic induction and cell viability was measured by MTT assay daily. The cells were treated with 0 (control), 50, 100, 150, 200, 250, and 300 μg/ mL BM extract for up to 7 days. The experiment was reproduced independently for six times with triplication in each individual experiment and their cellular viabilities were processed in the same way as those raised up in NR medium.

On the first day, BM extract at 50-200 μg/ mL concentration revealed the cellular viabilities higher than that of the cells brought up in absolute serum-free RPMI1640 medium (the control) (Table 2). Among BM extract at all seven concentration (0-300 μg/ mL), BM extract at 50 μg/ mL concentration could elicit the highest cellular viability on the 2nd and the 3rd days (Fig. 2). BM extract at 150-300 μg/ mL concentration rose up the cell death higher than that of the control cells on these two days. The BM extract at 100 μg/ mL concentration could also enhance the cellular viability slightly higher than the viability of the cells brought up in absolute serum-free RPMI1640 medium on the 2nd day, but lower than that viability of those cells on the 3rd and 4th days (Fig. 2). Since the 5th day, BM extract at all six concentrations (50-300 μg/ mL) have diminished the cellular viabilities lower than that of the control on the first day.

3. MTT cell viability of differentiated SH-SY5Y cells treated by BM extract in camptothecin apoptotic-indeced condition

The effect of BM extract on viability of differentiated SH-SY5Y cells was determined by measuring the reducing of MTT to purple crystal formazan by living-cell mitochondrial enzyme. The more purple formazan crystal, the more cellular survival. Cells were treated with 0.025% DMSO (vehicle), 5 μM camptothecin, and BM extract at the 50 and 100 μg/ mL concentrations for 12 to 72 hours and measured for their viability daily. The experiment was performed in three-independent replications with triplication in an individual experiment. Their viabilities of each treatment in an

individual experiment were calculated for their averages, which were further calculated again for mean and standard error (SE) of the same treatment from different independent experiments. The viability percentages of the BM treated cells were compared with the viability percentage of the absolute DMSO-treated cells at 12 hours and analyzed by one-way ANOVA with α = 0.05.

At 24-48 hours, DMSO attenuated the cellular viabilities of the differentiated SH-SY5Y cells lower than that viability of the cells treated by absolute 0.025% DMSO at 12 hours (the vehicle control) (Table. 3). From 12 to 48 hours, 5 μM camptothecin could slightly decrease the cellular viability lower than that of the vehicle control at 12 hours. BM extract at all concentration (50-250 μg/ mL) tended to have the same pattern of cellular viability as that pattern of the cells treated by absolute 5μM camptothecin, which displayed the cellular viability less than those of the vehicle cells at 12-48 hours (Fig. 3).

Immunoblotting

1. **Immunoblotting of NGF-deprived PC12 treated by BM extract in normal-serum (NR) RPMI1640 medium**

To determine the effect of BM extract treatment on total Tau and dephosphorylated Tau-1, NGF-deprived PC12 cells treated with 0 (control), 50, and 100 μg/ mL BM extract for 2 days were immunoblotted with the phophorylation-independent total tau protein (Tau 5), dephosphorylated-dependent Tau (Tau-1), and house-keeping GAPDH protein antibodies in triplicate independent experiments. Tau 5 antibody recognizes all Tau and Tau-1 antibody recognizes Tau only when Ser^{195}, Ser^{198}, Ser^{199}, Ser^{202}, and Thr^{205} (numbering based on longest human brain isoforms) are not phosphorylated, and therefore exhibits decreased immunoreactivity when there is an increase in phosphorylation at Tau-1 epitope (Shelton and Johnson, 2001). Density of each protein band from the triplicate independent experiments was quantified by Scion Image program calculated for an average. The relative amount of total tau protein was normalized to its corresponding average of GAPDH protein. After that, the percentage of total tau protein was quantified by comparing each relative amount of total tau protein with the relative amount of total tau protein of the control. The final data were analyzed by one-way ANOVA with α = 0.05.

Immunoblot showed that BM extract at both 50 and 100 μg/ mL concentrations could reduce the amount of total Tau expression in NGF-deprived PC12 cells, grown up in normal-serum RPMI1640 medium. However, BM extract at 100 μg/ mL concentration could be contributed to the dephosphorylated Tau (at Tau-1 site) increment in these cells (Fig. 4). Because of the grater increment in dephosphorylated Tau (at Tau-1 site) and more decrement in total Tau (Tau 5), the BM extract could dose-dependently accentuate Tau-1 immunoreactivity (Fig. 6). This might suggest that BM extract could attenuate Tau phosphoryltion (at Tau-1 site) in NGF-deprived PC12 cells, grown up in normal-serum RPMI1640 medium.

2. Immunoblotting of serum-free apoptotic-induced NGF-deprived PC12 treated by BM extract

Immunoblot showed that BM extract at both 50 and 100 μg/ mL concentrations could reduce the amount of total Tau expression in NGF-deprived PC12 cells, grown up in serum-free RPMI1640 medium. However, BM extract at 100 μg/ mL concentration could be contributed to the dephosphorylated Tau (at Tau-1 site) increment in these cells (Fig. 5). The BM extract at 100 μg/ mL concentration higher increased both total Tau (Tau 5) and dephosphorylated Tau (at Tau-1 site) higher than BM extract at 50 μg/ mL concentration did. Thus, BM extract at 100 μg/ mL concentration could slightly improve Tau-1 immunoreactivity (Fig. 6). This might also suggest that BM extract could diminish Tau phosphoryltion (at Tau-1 site) in NGF-deprived PC12 cells, apoptotic-induced by serum-free RPMI1640 medium.

3. Immunoblotting of camptothecin apoptotic-induced differentiated SH-SY5Y cells, treated by BM extract

To determine the effect of BM extract treatment on total Tau and dephosphorylated Tau-1, differentiated SH-SY5Y cells grown with only RPMI1640 medium, 0.025% DMSO (vehicle), 5 μM camptothecin, and BM extract at the 50 and 100 μg/ mL concentrations for 12 to 72 hours were immunoblotted with the phophorylation-independent total tau protein (Tau 5), dephosphorylated-dependent Tau (Tau-1), and house-keeping GAPDH protein antibodies in triplicate independent experiments. Tau 5 antibody recognizes all Tau and Tau-1 antibody recognizes Tau only when Ser^{195}, Ser^{198}, Ser^{199}, Ser^{202}, and Thr^{205} (numbering based on longest human brain isoforms) are not phosphorylated, and therefore exhibits decreased

immunoreactivity when there is an increase in phosphorylation at Tau-1 epitope (Shelton and Johnson, 2001). Density of each protein band from the triplicate independent experiments was quantified by Scion Image program calculated for an average. The relative amount of total tau protein was normalized to its corresponding average of GAPDH protein. After that, the percentage of total tau protein was quantified by comparing each relative amount of total tau protein with the relative amount of total tau protein of the control. The final data were analyzed by one-way ANOVA with α = 0.05.

At 12 hours of the experiment, 0.025% DMSO (vehicle) and 5 μM camptothecin could slightly reduce both the amount of total Tau (Tau 5) and the amount of dephosphorylated Tau (at Tau-1 site) expression, compared to the absolute RPMI1649 medium. BM extract at both 50 and 100 μg/ mL concentrations could be able to ebb down the amount of total Tau expression lower than the 0.025% DMSO could. On the other hand, BM extract at both concentration, could rise up the amount of dephosphorylated Tau (at Tau-1 site) expression greater than the 0.025% DMSO could, especially for BM extract at 100 μg/ mL concentration (Fig. 7). Therefore, BM extract could vivid dose-dependently enhance up Tau-1 immunoreactivity in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin for 12 hours (Fig. 8).

At 24 hour of the experiment, 0.025% DMSO (vehicle) and 5 μM camptothecin could enhance up both the amount of total Tau (Tau 5) and the amount of dephosphorylated Tau (at Tau-1 site) expression, compared to the absolute RPMI1649 medium. BM extract at 50 μg/ mL concentration could enhance up only the amount of total Tau expression, compared to the absolute 0.025% DMSO. However, extract at 100 μg/ mL concentration could increase up both the amount of total Tau and dephosphorylated Tau expression, compared to the absolute 0.025% DMSO, with the greater extent in the amount of dephosphorylated Tau expression (Fig. 9). BM extract could impact on reduction of Tau-1 immunoreactivity in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin for 24 hours (Fig. 10).

At 48 hour of the experiment, 0.025% DMSO (vehicle) could slightly reduce both the amount of total Tau (Tau 5) and the amount of dephosphorylated Tau (at

Tau-1 site) expression, compared to the absolute RPMI1649 medium. The absolute 5 μM camptothecin could enhance up the amount of total Tau expression but ebb down the amount of dephosphorylated Tau (at Tau-1 site) expression, compared to the absolute RPMI1649 medium. BM extract at both 50 and 100 μg/ mL concentrations could be able to diminish down the amount of total Tau expression lower than the 0.025% DMSO could (Fig. 11). Thus, BM extract could again dose-dependently accentuate up Tau-1 immunoreactivity in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin for 48 hours (Fig. 12). However, this increment of Tau-1 immunoreactivity at 48 hours of the experiment was at little extent than that increment at 12 hours of the experiment.

Determination of gene expression

Tau cDNA content in the SH-SY5Y cultured cells brought up with the low-serum RPMI 1640 medium only (the control cells), 0.025% DMSO, 5 μM camptothecin, and BM extract at the 50 and 100 μg/ mL concentrations for 12 to 72 hours was evaluated by PCR analysis, using primers specific designed to amplify region covering the Exon 10 of the longest isoform of the tau mRNAs. Two PCR products were predicted, the 254 bp fragment deriving from 4R isofroms (expressing Exon 10) of the tau mRNAs and the other 200 bp fragment deriving from 3R isofroms (excluding Exon 10) of the tau mRNAs. The density of each PCR band product was quantified. The relative density of each PCR band product was determined by normalizing with the density of PCR band product of each corresponding GAPDH cDNA to determine the mRNA expression of the *tau* gene.

As shown in fig. 13 A. (upper panel), no any PCR band product was detected from each condition of 12 and 24 hour culturing cells (lane 1 to lane 10). When the cells were cultured for 48 hours (Fig. 13 B., upper panel), the 3R isofroms of tau mRNAs were expressed from in the SH-SY5Y cultured cells brought up with the absolute low-serum RPMI 1640 medium (lane 1) and 0.025% DMSO (vehicle) (lane 2) but the 4R isofroms of tau mRNAs were expressed from in the SH-SY5Y cultured cells brought up BM extract at the 50 concentrations (lane 4). At this time the 4R isoforms were revealed with the highest expression. When the cells were cultured for 72 hours (fig. 13 B., upper panel), all cultured cells expressed 3R isofroms of tau mRNAs (lane 6, 7, 9, and 10) except for the cells brought up with 5μM camptothecin which

displayed no any PCR band product. At this time, high tau mRNA expression was detected from in the SH-SY5Y cultured cells brought up with the absolute low-serum RPMI 1640 medium (lane 6) and 0.025% DMSO (lane 7).

Table 1. MTT viability percentage of NGF-deprived PC12 cells, treated by BM extract for 7 days in normal-serum RPMI1640 (NR) medium, compared to the cells treated with no any BM extract on the 1st day. The more purple formazan crystal, the more MTT viability. Each group n =3-9. All data represented Mean+SEM and were analyzed by statistic one-way ANOVA with $\alpha = 0.05$. $^{a}P < 0.05$

Days	BM extract concentration (µg/ mL)						
	0	50	100	150	200	250	300
1	100.00±17.28	98.93±11.89	91.54±5.68	103.34±12.18	118.87±27.18	96.86±17.20	94.78±22.59
2	123.00±9.76	141.51±12.38	131.76±8.19	126.43±4.36	132.47±16.32	129.20±7.09	101.98±9.03
3	106.01±9.96	110.97±11.08	101.55±2.56	101.83±9.54	113.02±10.14	100.15±6.56	69.36±2.09
4	105.50±10.27	132.75±9.19	156.04±4.48	171.92±9.80[a]	162.83±18.65[a]	150.54±13.25	51.16±11.82
5	66.15±0.29	69.83±5.53	86.36±14.16	73.45±1.95	86.40±3.70	85.96±5.21	36.66±1.92
6	96.30±18.20	114.49±21.22	121.08±20.43	126.52±24.01	149.54±13.88	123.23±1.33	44.88±4.56
7	83.40±10.03	103.03±21.54	105.12±19.90	79.33±19.37	101.49±11.50	85.64±11.07	48.18±8.00

Table 2. MTT viability percentage of NGF-deprived PC12 cells, treated by *Bacopa monnieri* for 7 days in apoptotic-induced serum-free RPMI1640 medium, compared to the cells treated with no any BM extract on the 1st day. The more purple formazan crystal, the more MTT viability. Each group n =3-18. All data represented Mean+SEM and were analyzed by statistic one-way ANOVA with $\alpha = 0.05$.

Days	BM extract concentration (µg/ mL)						
	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	13.39±0.90	14.34±0.64	15.77±0.60

Table 3. The MTT viability percentage of differentiated SH-SY5Y cells, grown up in only low-serum RPMI 1640 medium for 12 to 72 hours, compared to the viability of these cells at 12 hours and the MTT viability percentage of the cells, treated by only 0.025% DMSO (vehicle), 5 μM camptothecin, and BM extract at the 50 to 250 μg/ mL concentrations for 12 to 72 hours, compared to the viability of the vehicle-treated cells at 12 hours (a). Each group n = 3. All data represented by Mean ± SEM and analyzed by statistic one-way ANOVA with $\alpha = 0.05$. [a] $P < 0.05$.

Time	RPMI 1640	0.025% DMSO	5 μM camptothecin	BM extract concentration (μg/ mL) 50	100	150	200	250
12	100.00±5.85	100±7.90	93.55±8.17	92.11±8.42	93.25±8.67	88.06±7.44	87.96±8.18	88.88±5.55
24	74.65±5.95	79.94±5.46	65.14±2.95[a]	62.11±3.90[a]	63.86±2.95[a]	65.13±2.50[a]	64.25±2.00[a]	62.20±2.98[a]
48	86.97±1.49	79.66±2.20	79.39±4.56	76.55±2.81	82.24±6.43	80.64±2.97	79.10±5.71	78.62±5.90

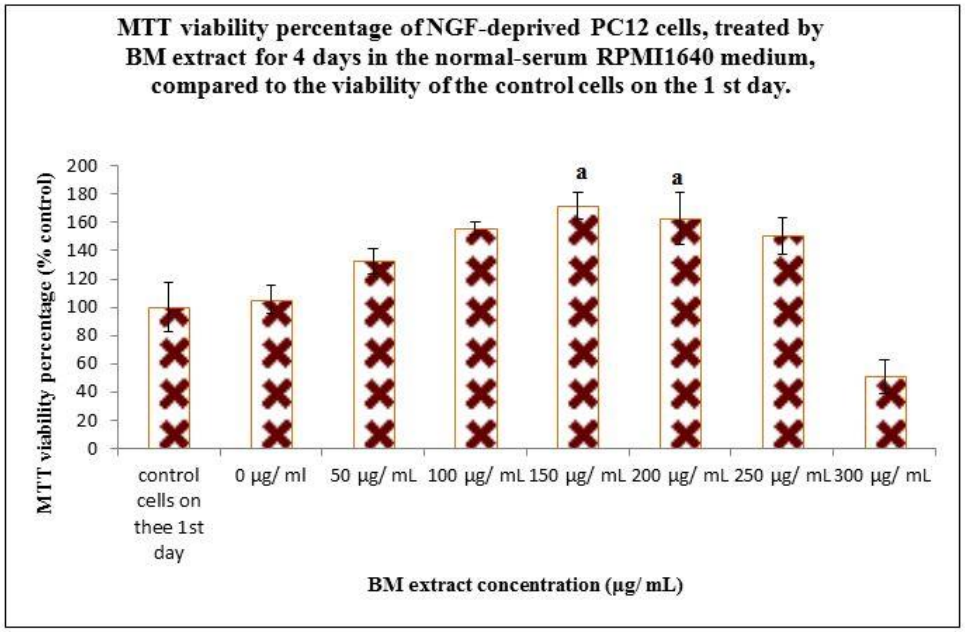

Figure 1. MTT viability percentage of NGF-deprived PC12 cells, treated by BM extract for 4 days in normal-serum RPMI1640 (NR) medium, compared to the cells treated with no any BM extract on the 1st day. Each group n =3-9. Each bar represents Mean+SEM. All data were analyzed by one-way ANOVA with $\alpha = 0.05$. [a] $P < 0.05$

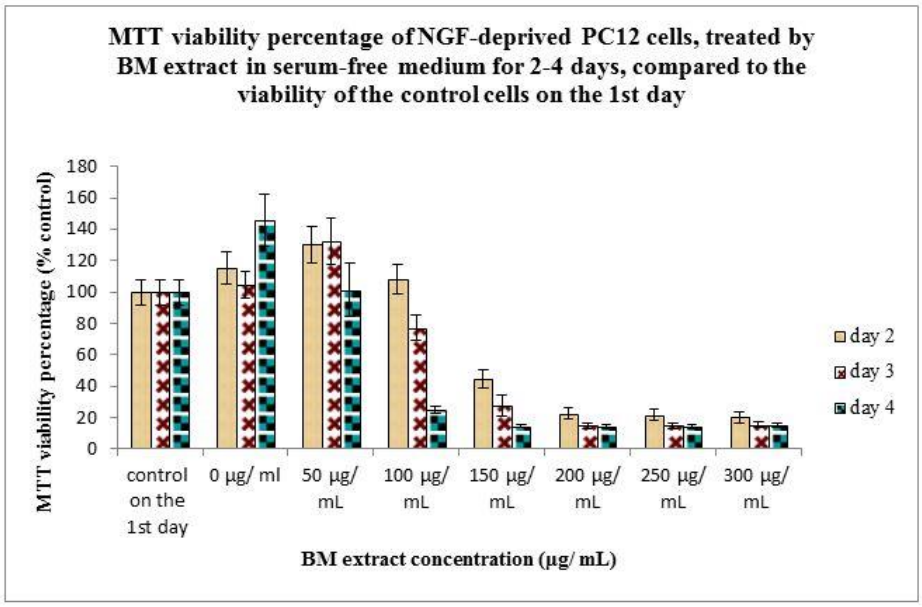

Figure 2. MTT viability percentage of NGF-deprived PC12 cells, treated by *Bacopa monnieri* for 2-3 days in apoptotic-induced serum-free RPMI1640 medium, compared to the cells treated with no any BM extract on the 1st day. Each bar represented Mean+SEM, n =3-18. All data were analyzed by statistic one-way ANOVA with $\alpha = 0.05$.

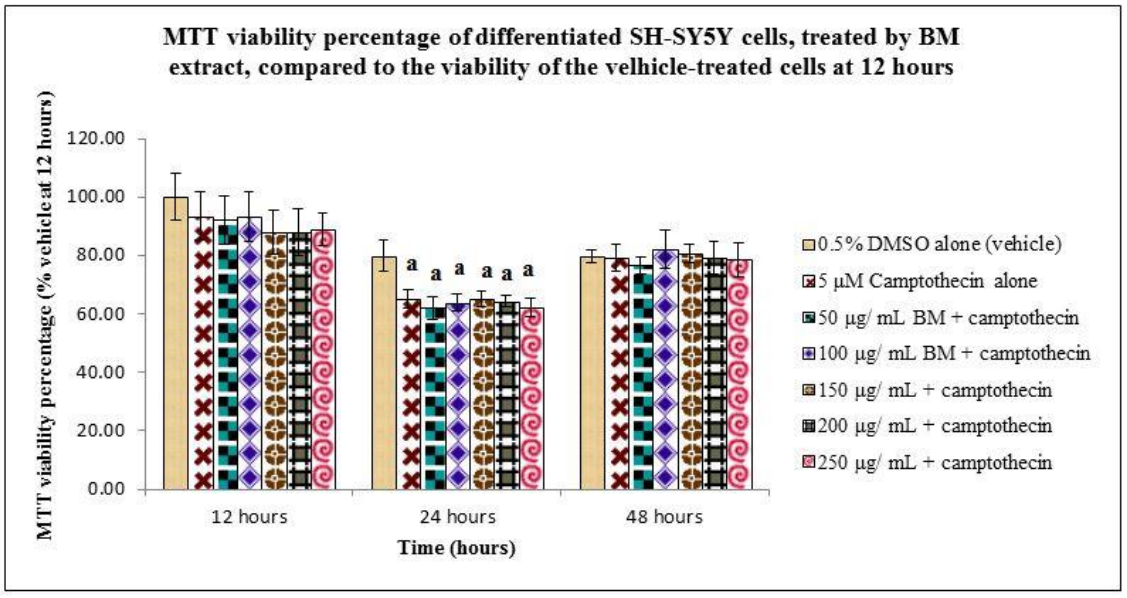

Figure 3. The MTT viability percentage of the cells, treated by only 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 to 250 µg/ mL concentrations for 12 to 48 hours, compared to the viability of the vehicle-treated cells at 12 hours (a). Each group n = 3. All data represented by Mean ± SEM and analyzed by statistic one-way ANOVA with $\alpha = 0.05$. $^{a}P < 0.05$.

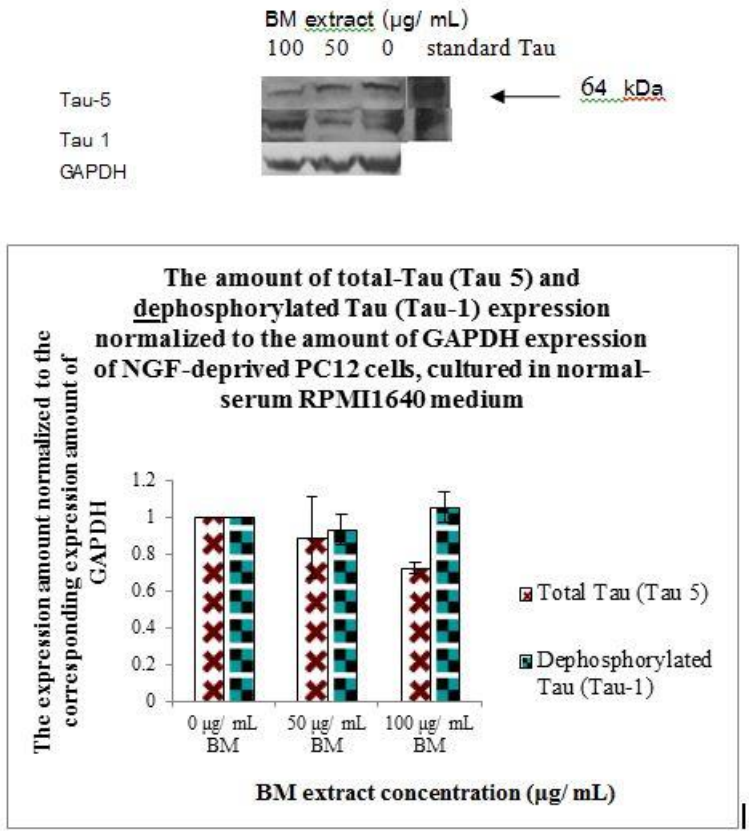

Figure 4. The relative amount of total tau (Tau 5) and dephosphorylated Tau (Tau-1) expression, normalized to the amount of GAPDH expression of NGF-deprived PC12 cells, cultured in normal-serum RPMI1640, treated with BM extract for 2 days. Each group n =3. All data represented by Mean ± SEM

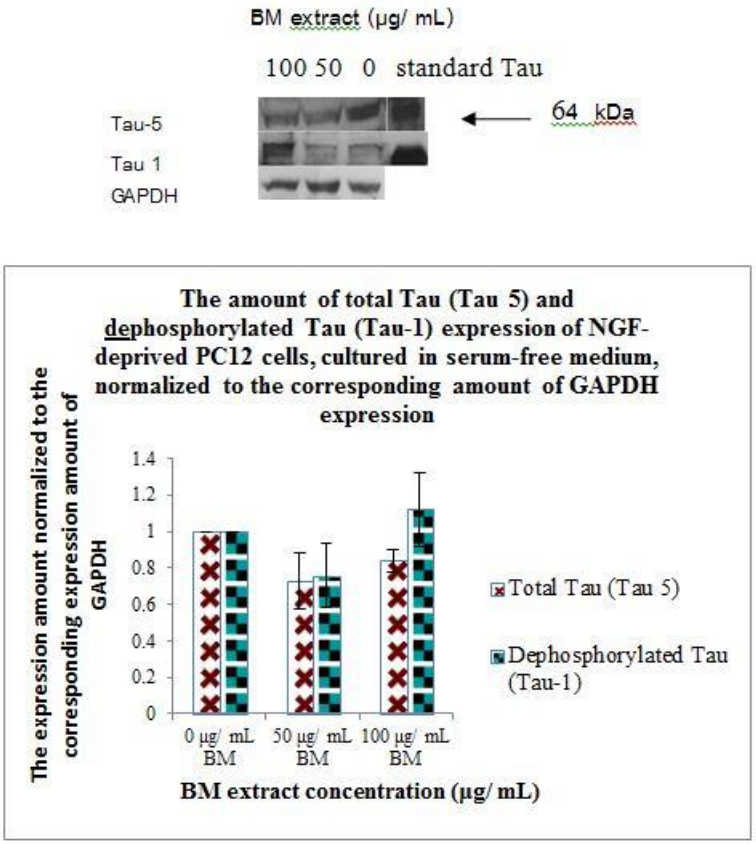

Figure 5. The relative amount of total tau (Tau 5) and dephosphorylated Tau (Tau-1) expression, normalized to the amount of GAPDH expression of NGF-deprived PC12 cells, cultured in serum-free RPMI1640, treated with BM extract for 2 days. Each group n =3. All data represented by Mean ± SEM

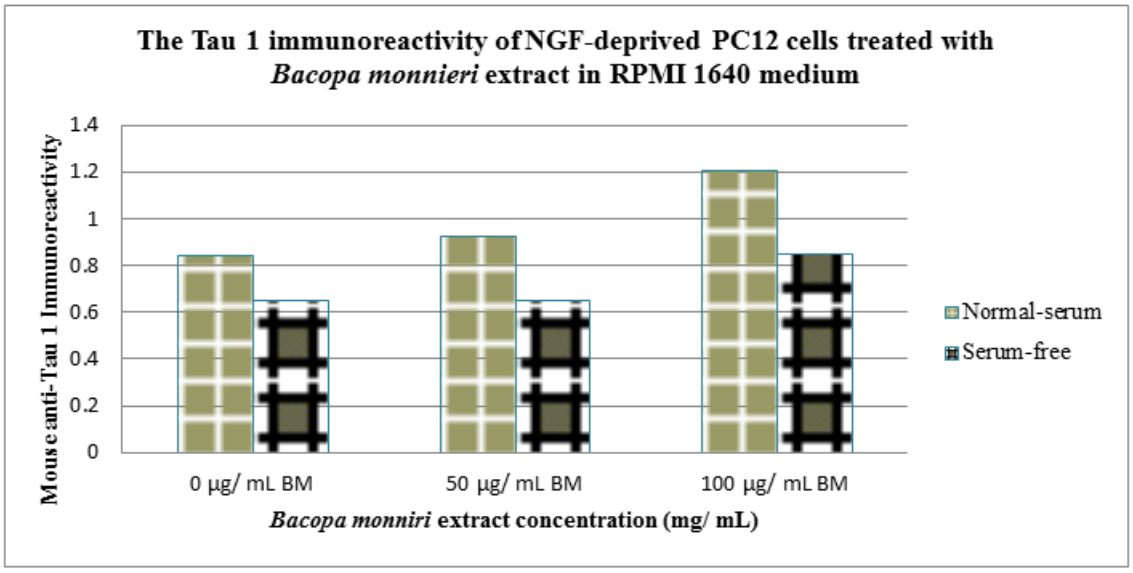

Figure 6. Tau-1 immunoreactivity normalized to the relative amount of total Tau expression of NGF-deprived PC12 cells treated with BM extract in RPMI1640 medium for 48 hours. Each group n =3. All data were represented Mean±SD and were analyzed by statistic one-way ANOVA with $\alpha = 0.05$ and *P*-value = 0.269.

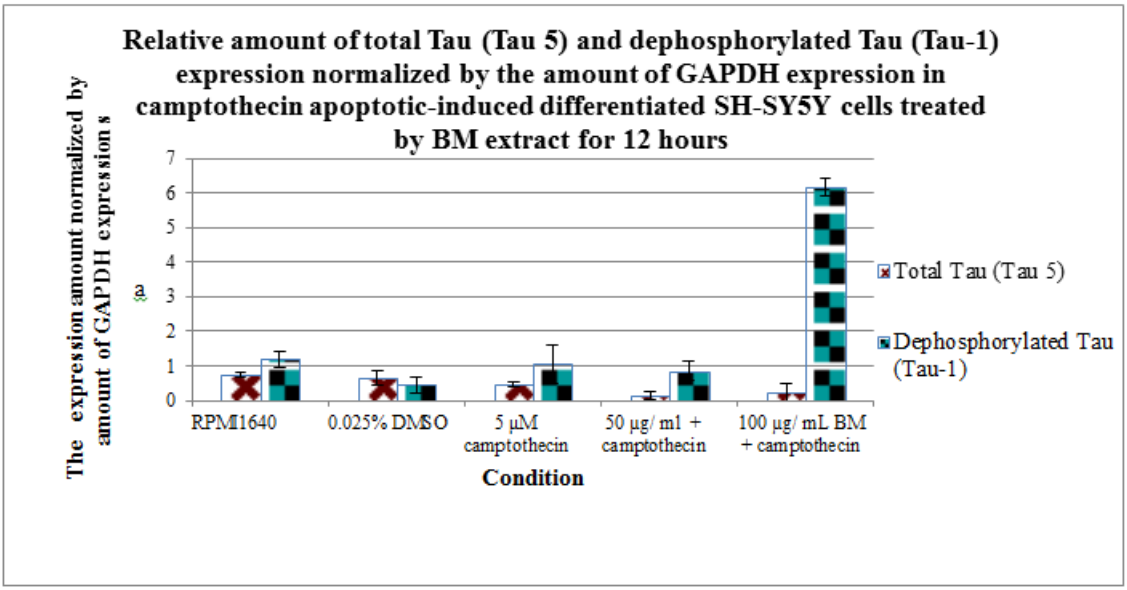

Figure 7. The relative amount of total Tau (Tau 5) and dephosphorylated Tau (Tau-1) expression normalized to the amount of GAPDH expression of differentiated SH-SY5Y cells, grown up in only low-serum RPMI 1640 medium, 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 and 100 µg/ mL concentrations for 12 hours. Each group n =3. All data represented Mean±SD and were analyzed by statistic one-way ANOVA at $\alpha = 0.05$.

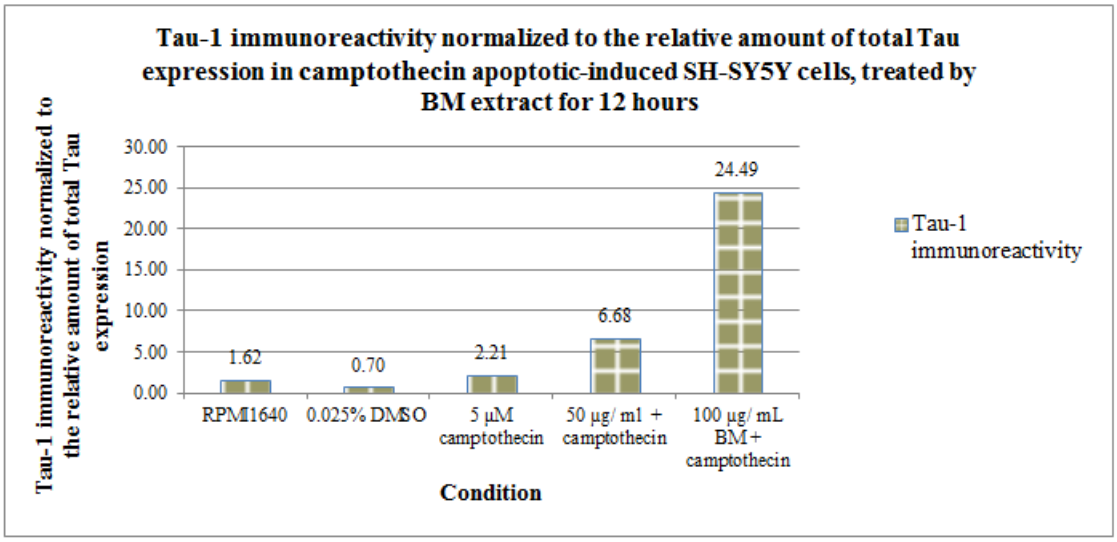

Figure 8. Tau-1 immunoreactivity normalized to the relative amount of total Tau expression of differentiated SH-SY5Y cells grown up in only low-serum RPMI 1640 medium, 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 and 100 µg/ mL concentrations for 12 hours. Each group n =3. All data represented Mean±SD and were analyzed by statistic one-way ANOVA at $\alpha = 0.05$.

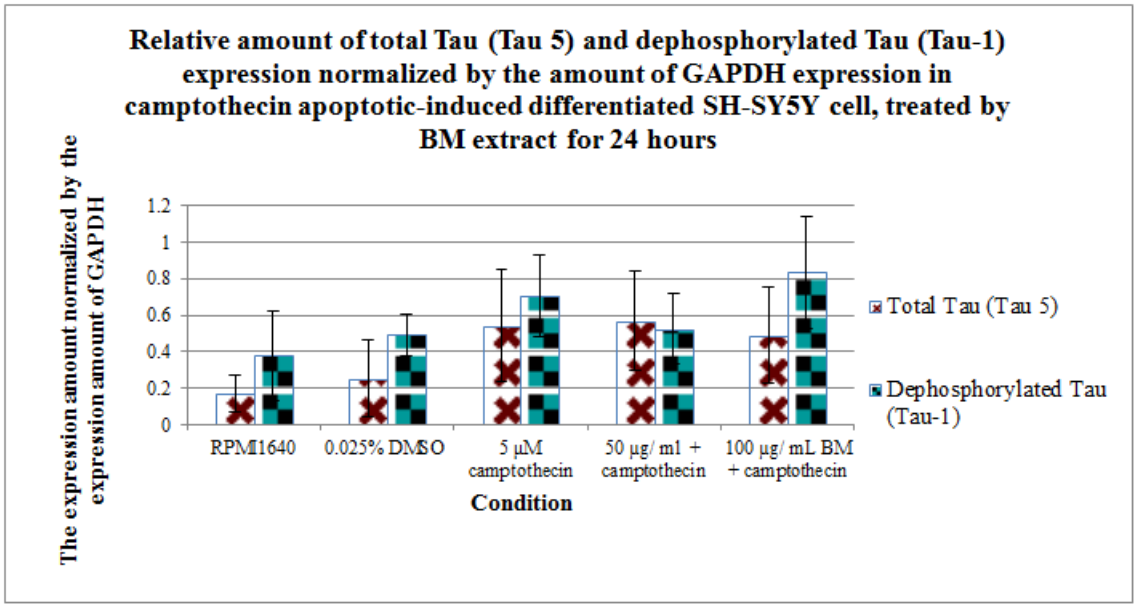

Figure 9. The relative amount of total Tau (Tau 5) and dephosphorylated Tau (Tau-1) expression normalized to the amount of GAPDH expression of differentiated SH-SY5Y cells, grown up in only low-serum RPMI 1640 medium, 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 and 100 µg/ mL concentrations for 24 hours. Each group n =3. All data represented Mean±SD and were analyzed by statistic one-way ANOVA at $\alpha = 0.05$.

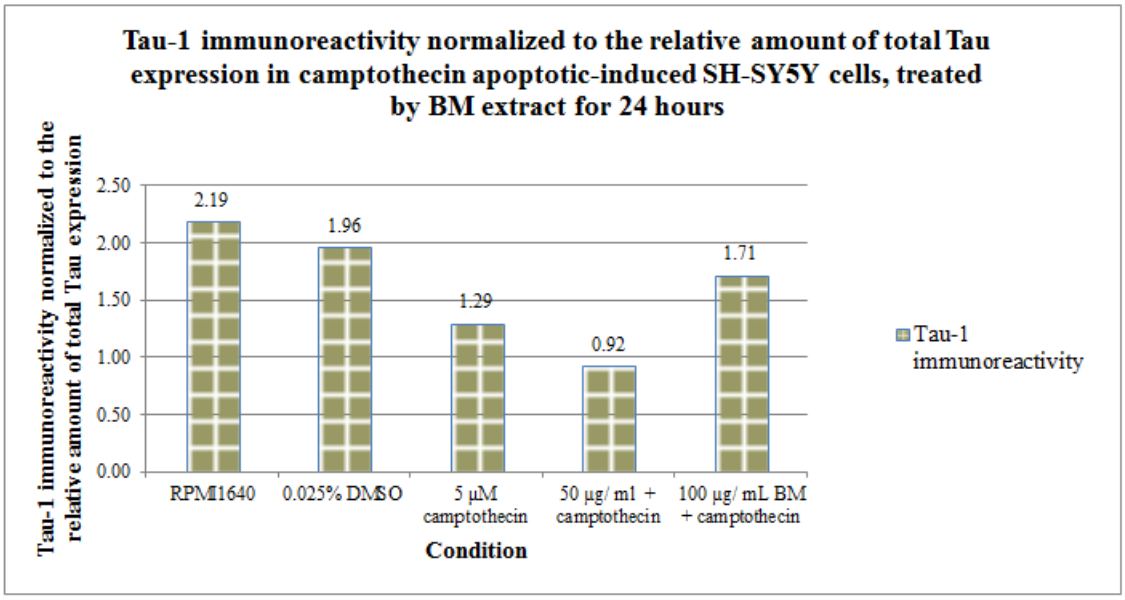

Figure 10. Tau-1 immunoreactivity normalized to the relative amount of total Tau expression of differentiated SH-SY5Y cells grown up in only low-serum RPMI 1640 medium, 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 and 100 µg/ mL concentrations for 24 hours. Each group n =3. All data represented Mean±SD and were analyzed by statistic one-way ANOVA at $\alpha = 0.05$

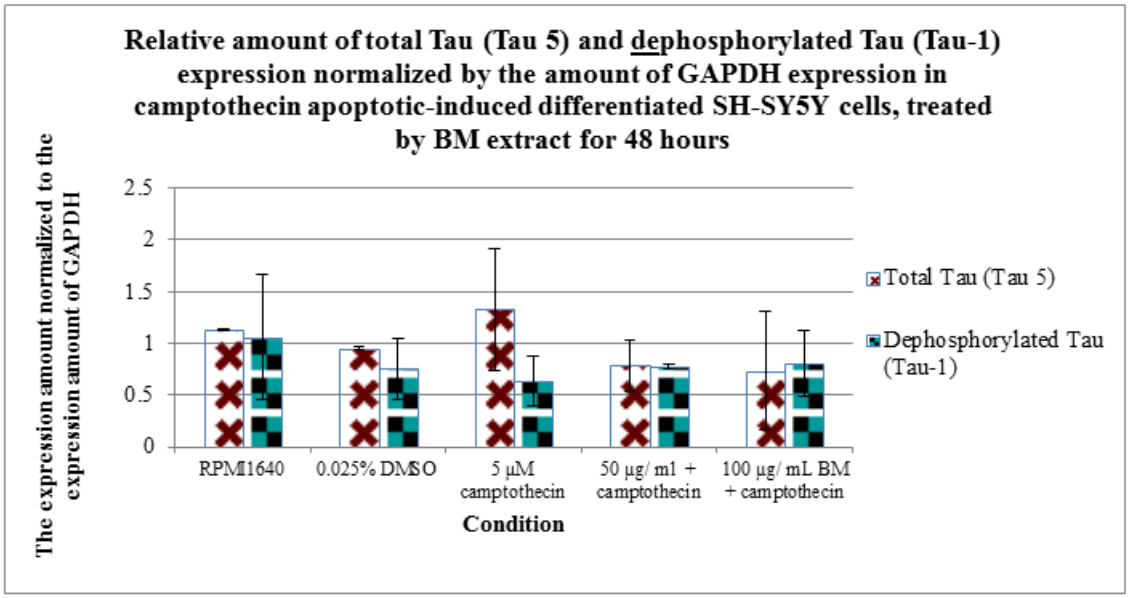

Figure 11. The relative amount of total Tau (Tau 5) and dephosphorylated Tau (Tau-1) expression normalized to the amount of GAPDH expression of differentiated SH-SY5Y cells, grown up in only low-serum RPMI 1640 medium, 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 and 100 µg/ mL concentrations for 48 hours. Each group n =3. All data represented Mean±SD and were analyzed by statistic one-way ANOVA at $\alpha = 0.05$.

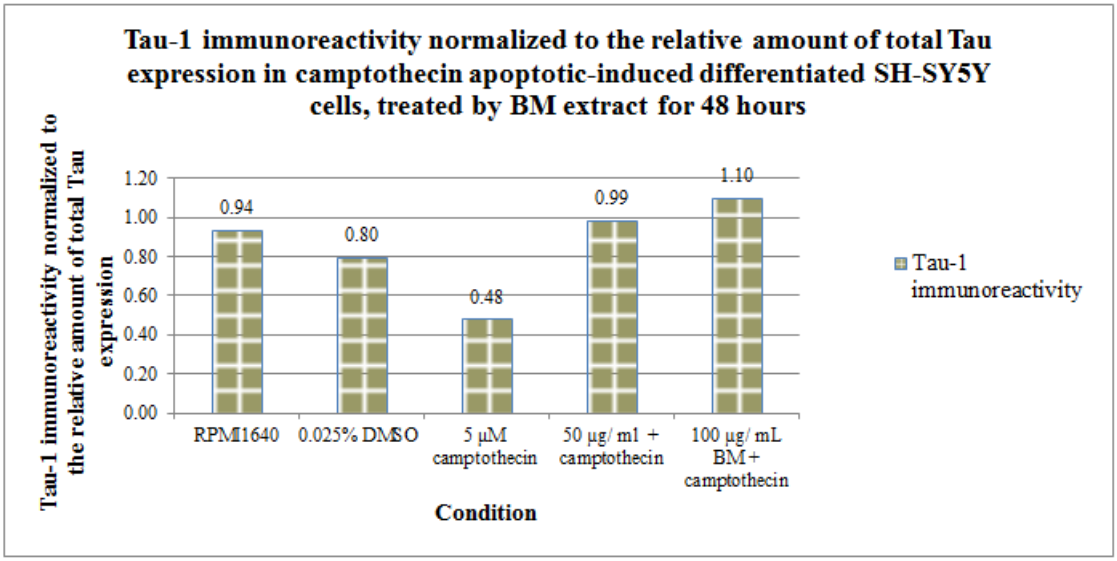

Figure 12. Tau-1 immunoreactivity normalized to the relative amount of total Tau expression of differentiated SH-SY5Y cells grown up in only low-serum RPMI 1640 medium, 0.025% DMSO (vehicle), 5 µM camptothecin, and BM extract at the 50 and 100 µg/ mL concentrations for 48 hours. Each group n =3. All data represented Mean±SD and were analyzed by statistic one-way ANOVA at $\alpha = 0.05$

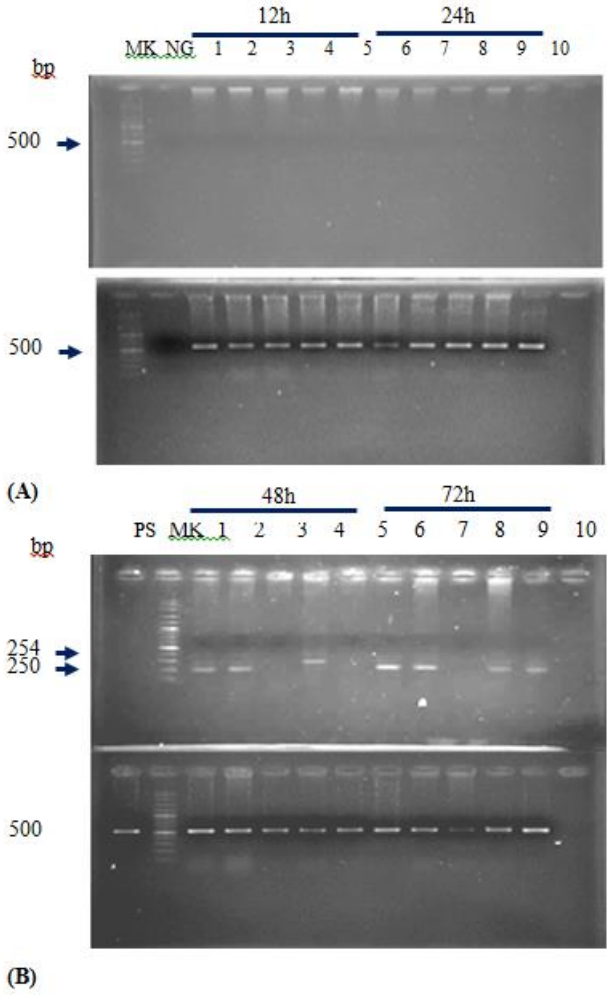

Figure 13. Tau PCR band products (upper panels of A and B) and GAPDH PCR band products (lower panels of A and B) of differentiated SH-SY5Y cells grown up in (left to right) absolute low-serum RPMI 1640 medium (lane 1, and 6), 0.025% DMSO (lane 2 and 7), 5 µM camptothecin (lane 3 and 8), and BM extract at the 50 (lane 4 and 9) and 100 (lane 5 and 10) µg/ mL concentrations for 12 and 24 hours (A.) and 48 and 72 hours (B.). MK = Molecular weight marker, NG = Negative control, PS = Positive control

CHAPTER V

CONCLUSION

The findings in this present study could be concluded that:

- BM extract could improve the cellular viability of the normal neuronal cells and apoptotic-induced neuronal cells.
- BM extract could be able to abate down both the amount of total Tau (Tau 5) and phosphorylated Tau (at Tau-1 site) expression in apoptotic-induced neuronal cells.
- BM extract at both 50 and 100 μg/ mL concentration could be able to reduce the 3R tau mRNA to express in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin.
- These may partially support the capability of BM extract to protect neuronal cells form cell death process, attributed to the neurodegerative effect of Tau protein. BM extract could be beneficial for not only cell survivability, especially at low dose, but also Tauopathy protectant by reducing Tau level in early and deleterious Tau isoform in late phase. Additionally, this information may again be a supportive document advocating to the benevolent property of BM extract to be an alternative therapy for neurodegenerative diseases including Alzheimer's disease.

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