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Geniposide ameliorated fluoxetine-suppressed neurite outgrowth in Neuro2a neuroblastoma cells

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ABSTRACT

Aim: Fluoxetine (FXT), a selective serotonin reuptake inhibitor (SSRI), is one of the most common psychiatric medications clinically prescribed; while over-produced serotonin may suppress neurite development. The role of major iridoids like geniposide (GPS) and genipin (GNP) from *Gardenia jasminoides* Ellis fruit (family *Rubiaceae*) in ameliorating the anti-neurite outgrowth effect of FXT is poorly understood. In this study, the effects of these iridoids on FXT-suppressed neurite outgrowth in Neuro2a neuroblastoma cells were investigated.

Main methods: Neuro2a cells were treated with FXT and GPS. The effect of GPS-FXT co-treatment on neurite outgrowth was observed using inverted phase-contrast microscope imaging system, while neurite outgrowth markers — microtubule-associated protein-2 (MAP2) and growth-associated protein 43 (GAP43) were analyzed using RT-PCR, Western blot and immunofluorescence staining. The transcription factor-cAMP response element binding (CREB), and signaling pathways — mitogen-activated protein kinase (MAPK) and protein kinase B/mammalian target of rapamycin (AKT/mTOR) were also analyzed with the help of Western blot.

Key findings: The results showed that FXT decreased the neurite outgrowth in Neuro2a cells and also down-regulated gene and protein expression of MAP2 and GAP43. It also downregulated the protein expression of phosphorylated-CREB, MAPK, and AKT/mTOR signaling pathways. In contrast, GPS counteracted the effects of FXT. GPS-FXT co-treatment increased the percentage of neurite-bearing cells by 3.6-fold at 200 μM as compared to FXT treatment only.

Significance: This study has provided the possible molecular mechanism showing how FXT exerted its detrimental side-effects on the neurite differentiation, and via the same mechanism how GPS attenuated these side effects.

1. Introduction

Nowadays, the global population of aging has become more serious. Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are gradually receiving the attention of scientists [1]. The findings have attributed to the cause of dysfunction of neurite differentiation and cell death leading to neuropsychiatric disorders and neurodegenerative diseases [2,3]. Neurite outgrowth is the primary marker associated with neurite differentiation, which acts as the crucial role adhering to the development of neuronal function. Patients affiliated with these neurodegenerative diseases usually may lose physical vitality and life quality, which in turn causes the patient more susceptible to depression and would require additional antidepressants in their treatments [4].

The first SSRIs named fluoxetine (FXT) is approved for the treatment of major depressive disorder, which has the potential to increase 5-HT level in the serotonergic synaptic space [5]. FXT was considered to be effective, once-daily medication for the treatment of depression with widespread adoption by physicians, but according to US Food and Drug Administration (FDA), most SSRIs like FXT have many side effects, including insomnia, tremors, nausea, nervousness and sexual problems [6]. Many investigations also revealed the occurrence of tissue toxicity following FXT therapy, such as sexual dysfunction, hepatotoxicity, nephrotoxicity, etc. [7,8]. Not only does FXT have physiological problems, but studies exploring the role of FXT in neuronal functions are also inconclusive and contradictory. Recently, FXT has been found to

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contribute to the neurite outgrowth in PC12 nerve cells [9]. Previously, studies also indicated that FXT has a potential role in therapeutic applications in various neurological disorders [10]. However, chronic exposure to FXT in rats caused long-term changes in gene expression involved in myelination, which then shapes brain connectivity and contributes to symptoms of psychiatric disorders [2]. Also, FXT affects the synaptic transmission and synapse formation between neurons which is due to suppression of Ca^{2+} entry and a corresponding breakdown of the neuronal cytoskeleton and functional communications between the synaptic pairs [11]. Additionally, it was conveyed that 15 ng/mL of FXT down-regulated the growth of NSC34 motor neurons [12]. FXT is also shown to affect the differentiation and decrease the maturation of the midbrain dopaminergic system in mouse [13]. Although FXT is the fifth-ranked prescription used to treat depression as well as neurodegenerative patients, the possible adverse effects of FXT on neurite outgrowth are still not fully understood and remain to be an active subject of research.

Neurite outgrowth markers such as MAP2, GAP43, TUBB3, and MAPT are usually expressed in differentiated neurons [14–16]. In addition, transcription factor CREB and MAPK signaling pathway participate in the various array of cellular programs, including movement, apoptosis, division, and differentiation [17]. AKT/mTOR signaling pathway also contributes to the normal neuronal growth by promoting its differentiation, synaptic formation, and neurite elongation and branching [18]. Hence, these markers, transcription factor and signaling pathways were evaluated to investigate the effect of FXT and GPS on the neurite differentiation.

Complementary alternative medicines (CAMs) can be applied to ameliorate the adverse effects of conventional medications [19]. A particular type of traditional Chinese herbal medicine, *Gardenia jasminoides*, has been shown to enhance the neuroprotective effect in rat's brain-derived neurotrophic factor levels, being suggested as an antidepressant-like medicine [20]. This herbal medicine also has the potential to reduce oxidative stress [21], which is often the leading cause of neurodegenerative diseases and mental illnesses like depression [22]. The essential phytoconstituents found in *G. jasminoides* like geniposide (GPS) and genipin (GNP), can be used as a potential adjuvant to treat depression since they possessed antidepressant-like effect [22,23]. Geniposide can improve the memory of APP/PS1 Alzheimer's disease mouse model [24], it also has neuroprotective effects on PC12 cells [25,26] and SH-SY5Y human neuroblastoma cell line [27]. Genipin was reported to promote prominent neuronal growth in PC12 cells [28].

In this study, our work would show that FXT could inhibit the neurite outgrowth in Neuro2a cells, and GPS ameliorated such adverse effects. In addition, we investigated the molecular mechanism regarding FXT-suppressed neurite differentiation and demonstrated that GPS efficiently counteracted the side effects of FXT through regulation of the target mechanism in Neuro2a cells.

2. Materials and methods

2.1. Materials

Tissue culture media, Fetal Bovine Serum (FBS) and supplements were purchased from gibco. β-Actin was purchased from Thermo Fisher (Waltham, MA, USA). Chemicals GPS, GNP, and FXT were purchased from Sigma Chemical Co. (St Louis, MO, USA). MAP2, GAP43, and LaminB were purchased from Proteintech (Chicago, IL, USA). Anti-CREB, anti-P-CREB, anti-ERK, anti-P-ERK, anti-JNK, anti-P-JNK, anti-p38, anti-P-p38, anti-mTOR, anti-P-mTOR, anti-AKT, and anti-P-AKT were purchased from Cell Signaling Technology. HRP-conjugated secondary antibodies were purchased from BIO-RAD (Madrid, Spain).

2.2. The culture of cells and subculture

Cell culture Neuro2a (ATCC, USA) neuroblastoma cell cryotubes were thawed in a 37 °C water bath and seeded into DMEM medium containing 10% bovine serum, 4.5 g/L glucose, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1% Non-Essential Amino Acid (NEAA) and 1% PS antibiotic solution (100 unit/mL penicillin and 100 g/mL streptomycin) and cultured in a constant temperature (37 °C) incubator containing 5% CO_2. The culture medium was changed every two to three days until the cells grow up to 90% confluence. The cells were then digested with 0.05% Trypsin-EDTA (TE). The shrunken cells that adhered to the inner wall were knocked off gently to facilitate the TE interaction. Culture medium was added to terminate the TE action. The cells were subsequently centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, and the fresh medium was added at a ratio of 1:2, 1:3 or 1:6. The precipitated cells were evenly dispersed and then transferred to 75 cm^2 culture flasks.

Table 1
Primer design.

Gene	Primer	Size (bp)
MAP2	Forward 5′-ACCACACCIGCAGTGGAGAA-3′ Reverse 5′-AATCTGGACCTGGTTCCTGC-3′	215
MAPT	Forward 5′-CceTGGAGGAGGGAATAAGAAG-3′ Reverse 5′-AGGTGCCGTGGAGATGTGT-3′	135
GAP43	Forward 5′-GGAGAAGAAGGGTGAAGGGG-3′ Reverse 5′-GGACGGGGAGTTATCAGTGG-3′	100
TUBB3	Forward 5′-ACGCATCTCGGAGCAGTT-3′ Reverse 5′-CGGACACCAGGTCATTCA-3′	125
β-Actin	Forward 5′-TACAGCTTCACCACCACAGC-3′ Reverse 5′-AAGGAAGGCTGGAAAAGAGC-3′	206

2.3. MTT test for cell viability

Neuro2a neuroblastoma cells were cultured in 6-well plate at an initial density of 2×10^5 cells per well containing complete growth medium (10% FBS) and incubated for 24 h. To induce cell differentiation and study the effect of different compounds, the complete medium was carefully replaced with differentiation medium (2% FBS medium) with FXT or co-treated FXT/GPS and FXT/GNP for 24 h. Later, the used medium was removed and washed twice with PBS solution. Added 1 mL of 0.5% MTT solution and incubated for 30 min to 3 h. After that, the MTT solution was removed, and 0.3–0.5 mL of DMSO was added and left to stand in the dark for 30 min. DMSO solution was sucked out, and the absorbance was measured at 570 nm with the ELISA Reader (Thermo Fisher Scientific Inc., Waltham, USA). Calculated the cell viability as follows: Viability (%) = [Abs 570 nm (sample) / Abs 570 nm (control)] × 100%.

2.4. Neurite outgrowth identification

The same Olympus CKX41 inverted phase-contrast microscope imaging system with microscopic magnification 200× was used, and three fields of view were randomly selected. The number of cells with neurites and the total number of cells was calculated using the Image J cell counter (National Institutes of Health, USA, Kurt De Vos). Neuron bearing cells (NBC) were defined as cells with neuron protuberances longer than twice the length of the cell body. The number of cells with neurites were counted and divided by total cells. The formula was as follows: NBC (%) = [number of neurite-bearing cells / total number of cells in the field] × 100%.

2.5. Gene expression in Neuro2a cells

2.5.1. Extraction of RNA

The TRIzol kit was used to extract the RNA from the cells. After cell dosing reaction time terminated, the cells were washed twice with a sterile PBS solution, and 1 mL/well Tri-Reagent (Sigma, Poole,

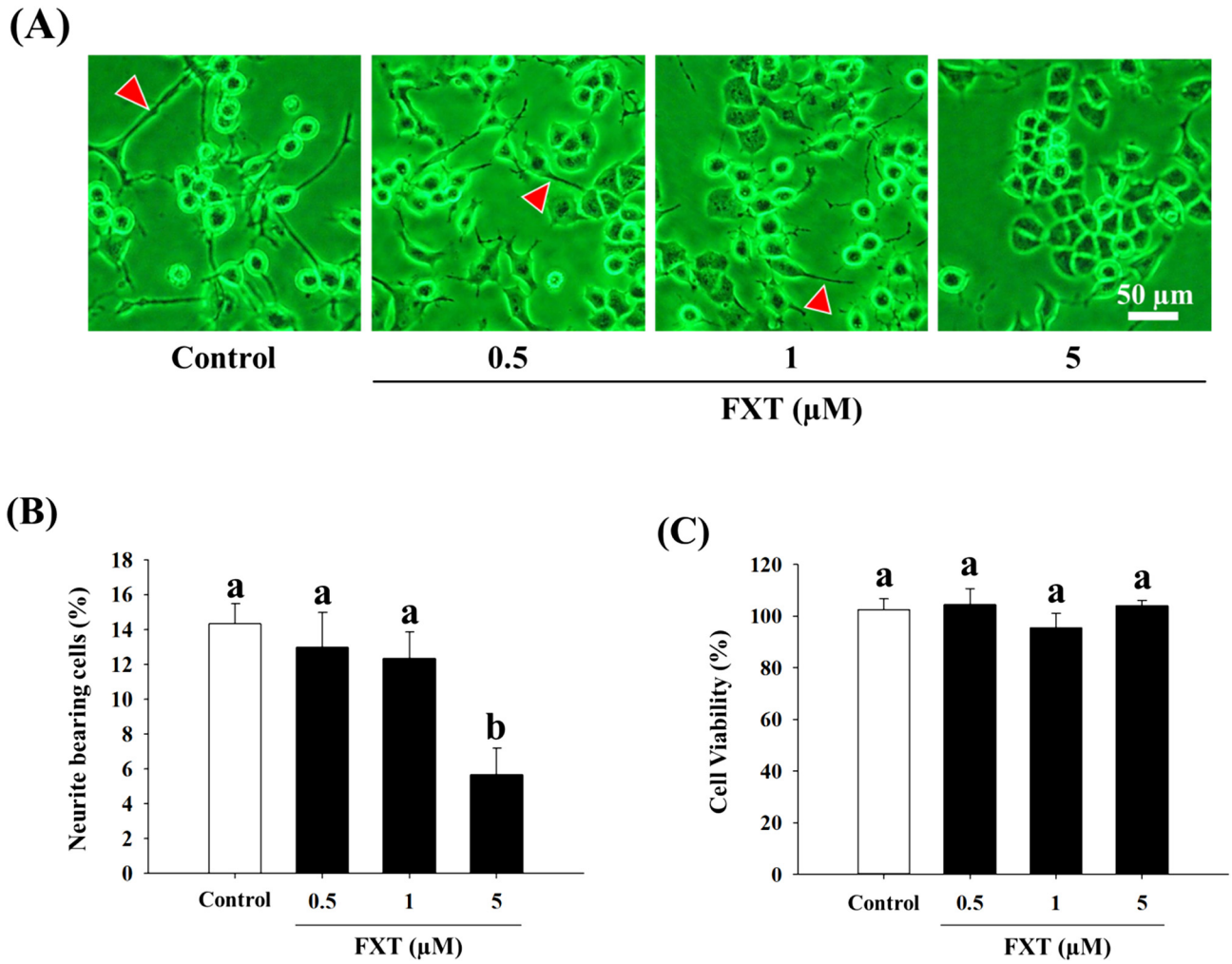

Fig. 1. Effect of FXT on the neurite outgrowth and cell viability in Neuro2a cells. (A) Neurite outgrowth observed under a phase-contrast light microscope (Olympus CKX41) at a magnification of 200×. Scale bar: 50 μm. (B) Percentage of neurite-bearing cells (%) is the number of neurite-bearing cells divided by the total number of cells in a field and then multiplied by 100%. (C) Effect of FXT on the cell viability of Neuro2a cells. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with FXT (0.5, 1, 5 μM) and PBS as vehicle control for 24 h. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$).

England) was added. The cells were repeatedly mixed with pipette until the solution was no longer sticky. The solution was placed in a sterile Eppendorf and allowed to stand at room temperature for 3 min, then added 0.2 mL chloroform and shaken vigorously for 15 s, then left at room temperature for 5 min. It was centrifuged at 12,000 rpm for 15 min at 4 °C. The RNA was carefully pipetted into a microcentrifuge tube and added 0.5 mL of isopropanol and mixed gently 10 to 15 times. After standing for 5 min, it was centrifuged at 12,000 rpm for 15 min at 4 °C. Carefully poured off the supernatant to obtain a white precipitate, the RNA extract. Rinsed the RNA extract with 1 mL of 95% alcohol per 1 mL of Tri Reagent, centrifuged at 12,000 rpm for 15 min at 4 °C and removed the supernatant, and repeated the alcohol rinse twice to remove the alcohol. The residual alcohol was completely evaporated (about 5 to 10 min) by suction drying, and the dry RNA extract was redissolved by adding to 30–50 μL of DEPC (diethylpyrocarbonate) treated water. Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) was used to measure the extraction performance (OD ratio of A260/A280 = 1.7–1.9). The isolated RNA was stored at −80 °C.

2.5.2. Reverse transcription polymerase chain reaction (RT-PCR)

The same concentration of RNA obtained from Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) was used for reverse transcription reaction to prepare cDNA. The reagents (HiScript I TM First Strand cDNA Synthesis Kit, Bionovas, USA) were as follows: 7 μL RNA (1–10 μg), 10 μL 2× Fast premix, 2 μL Primer mix, and total volume would be 19 μL. After being placed in a dry bath at 65 °C for 5 min, immediately placed on ice for 1 min, and then added 1 μL of reverse transcriptase and start the PCR machine. Set the temperature at 42 °C for 30 min, then 85 °C for 5 min and stored at −20 °C for further use.

2.5.3. Polymerase chain reaction (PCR)

In this experiment, the neurite outgrowth marker genes *MAP2*, *MAPT*, *GPA43*, and *TUBB3* of Neuro2a neuroblastoma cells were analyzed by PCR. The reagents were configured as follows: 2 or 3 μL cDNA, 2 μL Forward Primer (10 μM), 2 μL Reverse Primer (10 μM), 12.5 μL 2× PCR Master Mix, 5.5 or 4.5 μL ddH_2O, 1 μL of 25% DMSO and the total volume would be 25 μL. The PCR conditions setting were as follows: 1 cycle of pre-denaturation at 95 °C for 15 min. Then, the next 30 cycles were performed with denaturation at 95 °C for 15 s, annealing for 15 s (47 °C for *β-actin*, 62 °C for *MAP2* and *MAPT*, 57 °C for *GAP43* and 55 °C for *TUBB3*), and extension at 72 °C for 10 s. Next one cycle was the final extension at 72 °C for 10 min. The last cycle was cooling down at 4 °C for 10 min. The sequences of primer genes were as follows (Table 1):

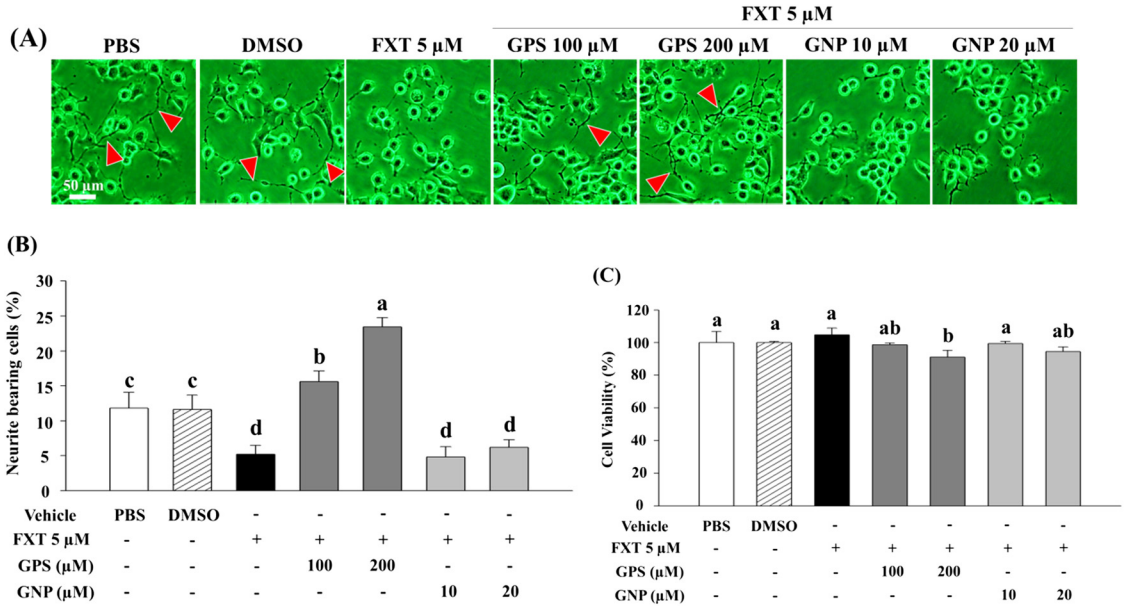

Fig. 2. Effect of GPS- or GNP-FXT co-treatment on neurite outgrowth and cell viability in Neuro2a cell. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with PBS/DMSO (vehicle control), FXT (0.5, 1, 5 μM), GPS (100 and 200 μM),) and GNP (10 and 20 μM) for 24 h. (A) Neurite outgrowth was observed at a magnification of 200×. Scale bar: 50 μm. (B) The percentage of neurite-bearing cells (%) is the number of neurite-bearing cells divided by the total number of cells in a field and then multiplied by 100%. (C) Effect of GPS- or GNP-FXT co-treatments on the viability of Neuro2a cells. The cells viability of Neuro2a was analyzed by using MTT assay. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$).

2.5.4. DNA separation using electrophoresis

In our study, 1.7% of the agar concentration was used, and an appropriate amount of agar was added to the 0.5× TAE buffer, and heated in a microwave until the solution clarified. 10 μL/100 ml of safe view DNA stain was added and let it cool down at room temperature for about 10 min and poured into the plastic cast tray. The agar was placed in the electrophoresis tank with sufficient amount of 0.5× TAE buffer, then injected the product of the polymerase chain reaction into the well and applied 100 V for 30–40 min. The image was photographed by a Luminescence/UV Image System, and the results were compared with the DNA ladder. The position of the bright band was the same as the expected size, and the photograph was archived and quantitatively analyzed. The density of genes of cells was quantified using the Image J software (National Institutes of Health, USA, Kurt De Vos).

2.6. The Western blot of neurite outgrowth signal protein and transcription factor protein analysis in Neuro2a cells

2.6.1. Protein extraction

After the time for cell differentiation and treatment with different drugs had finished, the culture solution was removed. After washing the cells twice with PBS, added 300 μL RIPA buffer (Genestar, USA). After left the mixture for 5 min on ice, carefully scraped and collected the cells and vortexed for 15 s and placed on ice for 10 min. Centrifuged it thrice at 13,000 rpm for 5 min at 4 °C. The supernatant was placed in a new Eppendorf tube and was kept at −80 °C until use.

2.6.2. Quantification of protein

Protein quantification was performed by the Bradford protein-binding assay, which was based on the properties of Coomassie Brilliant Blue G-250. The albumin with a known concentration of 24 mg/mL was serially diluted to 12.0, 6.0, 3.0, 1.5, 0.75 and 0.0 mg/mL. A wavelength of 570 nm was used to read the optical density. Protein concentration was calculated from the standard curve.

2.6.3. SDS-PAGE electrophoresis for protein separation

The SDS-PAGE protocol was carried out by following the manufacturer's instruction (ThermoFisher Scientific). The protein sample was mixed with 5× protein loading dye and heated at 100 °C for 10 min, and cooled on ice for later use. Each sample was sequentially injected into the pores of the colloid, and the voltage used was 70 V. After the sample was passed through the stacking gel, the voltage was adjusted to 100 V. Adjust the electrophoresis time as needed.

2.6.4. Western blotting

The electrophoresis film was rinsed with transfer buffer, and PVDF membrane (Millipore, Bedford, MA, USA) was cut to meet the size of electrophoresis film. The membrane was soaked with methanol for 5–10 min. Full-wet transfer equipment was used for protein transfer at 500 mA for 2 h. Removed the membrane and immersed it in a blocking buffer of 5% skim milk powder and incubated for 1 h at room temperature. Then rinsed with 1× TBST buffer for 5 min. After repeating 3 times, the desired primary antibody (primary antibody; 1:1000) was added for 12 to 16 h at 4 °C. On the next day, the cells were washed with 1× TBST buffer for 5 min (3 times), and the secondary antibody (1:40,000) was added. The reaction was shaken at room temperature for 2 h and then washed 3 times with 1× TBST buffer for 5 min. Then immersed the PVDF membrane in chemiluminescence HRP substrate (Millipore, Bedford, MA, USA) was applied for approximately 15 s for visualization and analysis. The density of proteins of cells was quantified using the Image J software (National Institutes of Health, USA, Kurt De Vos).

2.6.5. Immunofluorescence staining

Neuro2a neuroblastoma cells were cultured in 6 well plates (2×10^5 cells/mL) pre-placed with coverslips, and after the cell dosing reaction time was terminated, the old cell culture medium was removed, and the Neuro2a cells were washed twice with PBS solution. Neuro2a cells were fixed by adding 0.5 mL of 4% formaldehyde for 10 min and washed 2–3 times with PBS. The cells were permeabilized with 0.2 mL 0.1% Triton X-100 for 10 min. The cells were washed twice with PBS, and non-specific binding was blocked with 1% BSA for 30 min and washed twice with PBS. Primary antibody (1:100–1:400) was prepared with 1% BSA. The antibodies used were as follows: β-

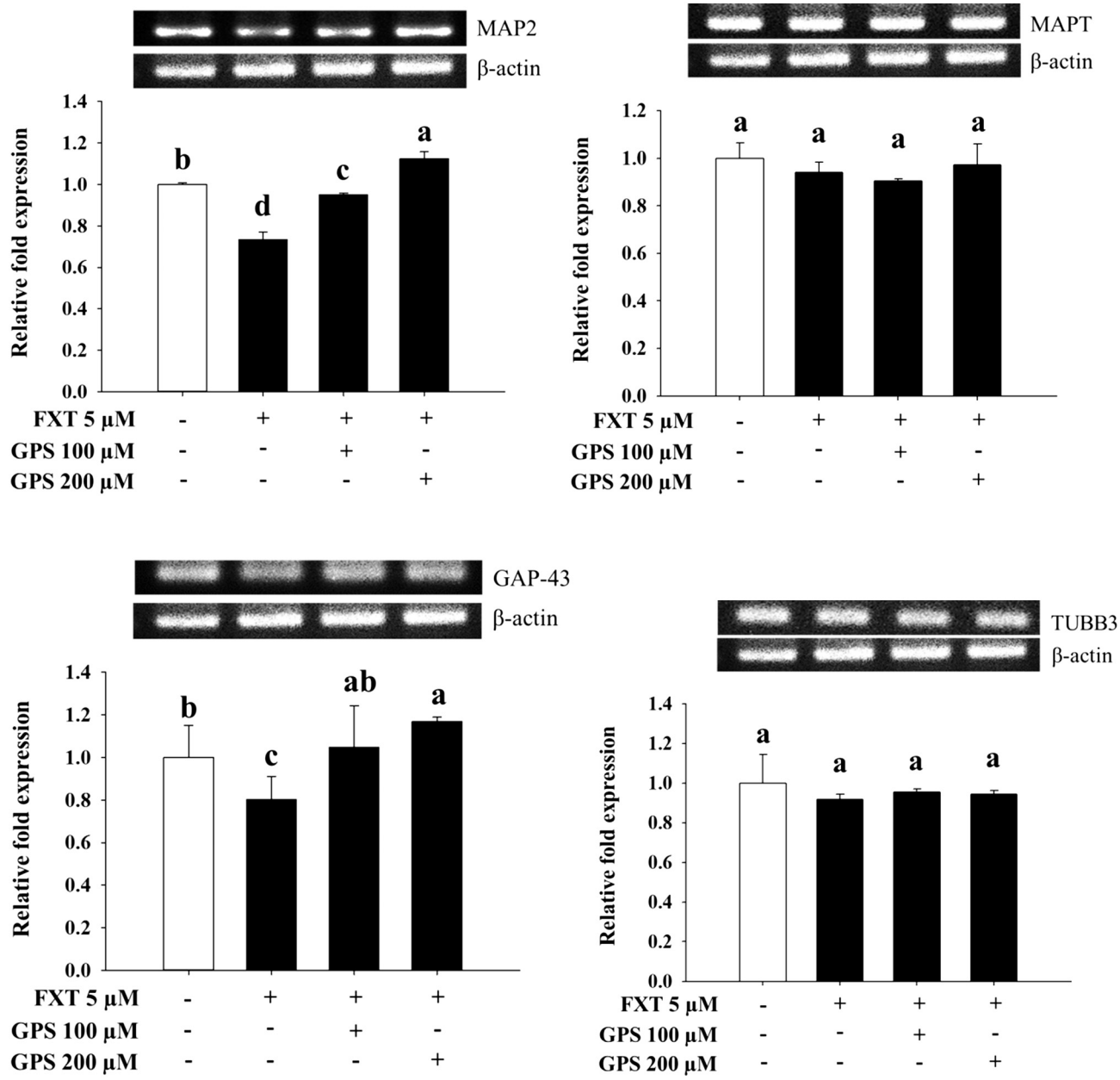

Fig. 3. Effect of GPS-FXT co-treatment on mRNA level of neurite outgrowth markers in Neuro2a cells. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with FXT. GPS (100 and 200 μM) and PBS as vehicle control for 24 h. The RT-PCR analysis was performed to neurite markers gene expression of neuro2a cells. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$).

actin (Fisher, Waltham, MA, USA); MAP2 and GAP43 (Proteintech Group, Inc. Chicago, IL, USA). About 0.2 mL of primary antibody was added and reacted at 4 °C for 16 h, then washed twice with PBS. The following steps should be protected from light. A secondary antibody containing FITC (Jackson ImmunoResearch, USA) was added for 1–2 h and washed twice. The cells were stained with 0.1–1 μg/mL DAPI (Biolegend, USA) for 1–5 min and washed twice. Photographs were taken with a fluorescent microscope, and FITC was excited with blue fluorescence. DAPI staining was excited by UV. The number of neurite branch cells were examined using the Neurite_Analyzer plug-in attached to Image J cell counter (National Institutes of Health, USA, Kurt De Vos). The results of immunofluorescence staining and DAPI staining were merged, and the file format was saved as Tif format.

2.7. Statistical analysis

Experimental data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) version 15.0. The experimental values were expressed as a mean ± standard error (mean ± SD) and analyzed by one-way analysis of variance which was one-way ANOVA. Difference analysis was performed in each test group, and the difference between the mean values of each test group was analyzed using Duncan's multiple range test. A p value of < 0.05 ($p < 0.05$) was considered statistically significant. Different letters in the results represent that there were a considerable difference and vice versa.

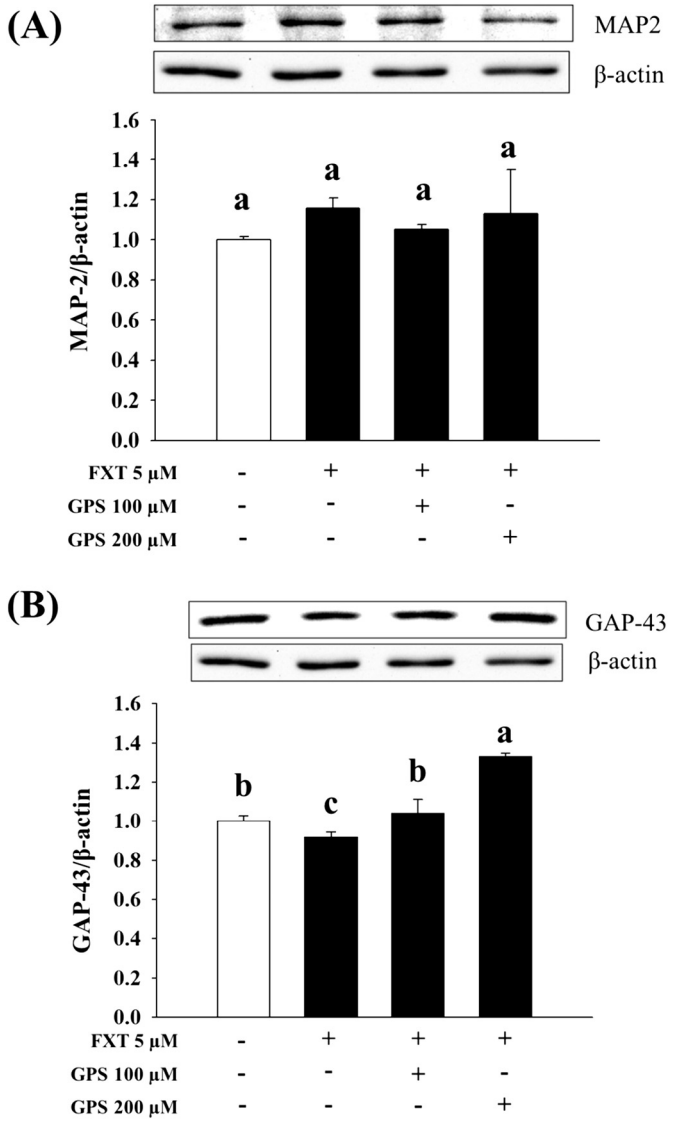

Fig. 4. Effect of GPS-FXT co-treatment on the protein expression of neurite outgrowth markers in Neuro2a cells. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with FXT (5 μM), GPS (100 and 200 μM) and PBS as vehicle control for 24 h. Western blot analysis was performed using specific antibodies against (A) MAP2 and (B) GAP43. Actin was used as a control for equal loading. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$).

3. Results

3.1. Effect of FXT on the neurite outgrowth and cell viability in Neuro2a cells

The effect of different concentrations of FXT on neurite outgrowth and viability of Neuro2a cells was evaluated. Fig. 1 shows the changes in Neuro2a cells that were treated with FXT at 0.5, 1 and 5 μM, respectively. It can be observed that FXT reduced the neurite-bearing cells significantly by 61% at 5 μM (Fig. 1B), compared with the control group, while there were no significant changes at 0.5 and 1 μM. There were also no significant changes in the cell viability (Fig. 1C, indicated by the lowercase a, $p > 0.05$). Based on the result, 5 μM of FXT was used in this study as it affected the neurite outgrowth but did not have toxic effects on the cell viability (no significant changes on a number of cells).

3.2. Effect of GPS- or GNP-FXT co-treatment on neurite outgrowth and cell viability in Neuro2a cell

In the preliminary study, we firstly screened the use of bioactive compound dosage affecting cell viability, followed by using the non-cytotoxic dosages in investigating the protective effects of Geniposide against FXT-induced neuronal dysfunction. Preliminary data indicated that GNP exhibited significant cytotoxic effects on neurite branched cells as the GNP dosage used was higher than 20 μM, as well as the GPS dosage used was higher than 200 μM (data not shown). Thus, we conducted the GNP dosages < 20 μM and GPS dosages < 200 μM for the following investigation. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with PBS/DMSO (vehicle control), FXT (5 μM), GPS (100 and 200 μM) and GNP (10 and 20 μM) for 24 h. The co-treatment of GPS or GNP with FXT was shown in Fig. 2, where FXT decreased the number of neurite-bearing cells by 58% as compared to the controls. As compared to the FXT treatment, GPS-FXT co-treatment significantly increased the percentage of neurite-bearing cells ($p < 0.05$), indicating that GPS could diminish FXT-induced adverse effect on neurite-bearing cells. On the other side, GNP-FXT did not significantly increase the neurite branched cell growth after FXT treatment ($p < 0.05$) and did not significantly decrease the cell viability as compared to the controls. Fig. 2C shows there were no significant differences ($p > 0.05$) in cell viability (%) for all treatments and it showed that compounds used at these concentrations did not have a toxic effect on cell viability.

3.3. Effect of GPS-FXT co-treatment on mRNA level of neurite outgrowth markers in Neuro2a cells

As there was no rescue effect of GNP on FXT-suppressed Neuro2a cells, the analysis of neurite outgrowth markers proceeded only with GPS. The effect of GPS-FXT on the neuronal cell axon marker gene MAPT, dendritic marker gene MAP2, the neuronal growth marker gene GAP43, and mature neuronal marker protein TUBB3 was studied. Based on the results, it showed that only MAP2 and GAP43 were affected significantly with the application of GPS-FXT co-treatment (Fig. 3). The level of MAP2 and GAP43 was down-regulated by approximately 26% and 20% ($p < 0.05$), respectively when FXT was applied. However, with the addition of 100 and 200 μM GPS, the MAP2 level was improved and increased significantly by 27% and 51% ($p < 0.05$) respectively compared with FXT treatment alone. The same pattern was also observed in GAP43, where the addition of 100 and 200 μM GPS increased it by 30% and 56% respectively, compared with FXT treatment alone. The FXT-GPS co-treatments did not change the level of MAPT and TUBB3 significantly ($p > 0.05$).

3.4. Effect of GPS-FXT co-treatment on protein expression of neurite outgrowth markers in Neuro2a cells

As the mRNA level of MAPT and TUBB3 was unaffected by GPS-FXT, only MAP2 and GAP43 protein expression analysis proceeded. Fig. 4 shows 5 μM of FXT was used for FXT test alone, and 100 and 200 μM of GPS were used as co-treatment with FXT. Fig. 4A shows that with the addition of 5 μM FXT alone, and with the addition of 100 and 200 μM GPS-5 μM FXT co-treatment, the MAP2 level did not change significantly ($p > 0.05$). Fig. 4B shows that the application of FXT (5 μM) decreased the level of GAP-43 protein by 8% (compared with control) but the application of 100 and 200 μM GPS-5 μM FXT co-treatment, increased the GAP-43 level about 11% and 43% respectively compared to the FXT treatment alone. This phenomenon showed, with the addition of GPS, the adverse effects of FXT can be improved.

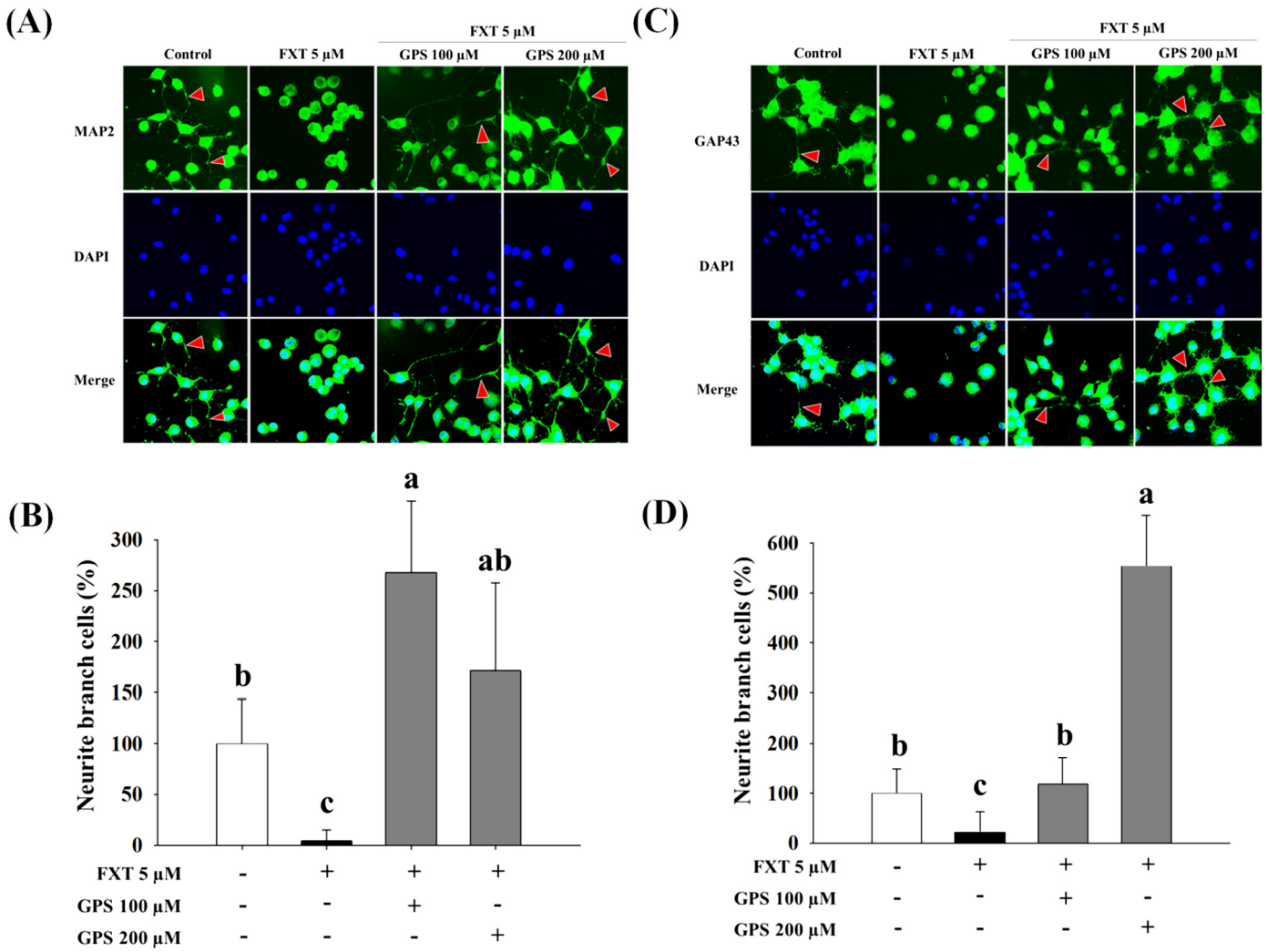

Fig. 5. Immunofluorescence staining on neurite outgrowth markers MAP2 and GAP-43 in Neuro2a cells. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with FXT (5 μM), GPS (100 and 200 μM) and PBS as vehicle control for 24 h. Immunostaining analysis was performed using specific antibodies against MAP2. (A & C) Neurite outgrowth was observed at a magnification of 40×. Blue: DAPI; green: MAP2 (5A) and GAP43 (C). (B & D) The percentage of neurite branch cells (%) is the number of neurite branch number divided by the total number of cells in a field and then multiplied by 100%. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Immunofluorescence staining on neurite outgrowth markers MAP2 and GAP-43 in Neuro2a cells

Fluorescence staining further explored the expression of neuronal growth marker proteins and to prove that neurite outgrowth markers MAP2 and GAP-43 in Neuro2a cells were affected by the FXT and can be improved by the addition of GPS. The expression of neurite outgrowth markers, MAP2 and GAP43, was quantitatively represented as the number of neurite branching cells. Fig. 5A and C show the immunofluorescence staining done on MAP2 and GAP43 respectively. After FXT (5 μM) treatment, the marker expression presented as neurite branching cells was significantly inhibited, regardless of MAP2 or GAP43 by 95% and 80% respectively (compared with control). Fig. 5B shows that 100 and 200 μM of GPS increased the number of MAP2 marker significantly by around 53.0 and 33.0-fold respectively, compared with FXT treatment alone ($p < 0.05$). From Fig. 5B, GPS-FXT co-treatment did not possess dose-dependent effect on MAP2 ($p > 0.05$), but still counteracted the effect of FXT. Fig. 5C and D show that GPS increased GAP43 marker significantly with an increase of GPS concentration (100 and 200 μM) by 4.5 and 26.5-fold respectively compared with FXT treatment alone ($p < 0.05$).

3.6. Effect of GPS-FXT co-treatment on CREB and MAPK expression of neurite differentiation in Neuro2a cells

It is known that the transcription factor of neurite outgrowth of Neuro2a cells is CREB. Fig. 6A shows that the application of FXT did significantly down-regulated the expression of p-CREB by 54% compared with the control group ($p < 0.05$). However, the 100 and 200 μM GPS-5 μM FXT co-treatment did increase the expression of the p-CREB by 35% and 96% ($p < 0.05$) respectively, compared with the FXT group alone. Then, the effects of GPS and FXT on the protein expression of cell signaling pathway were also studied. The MAPK pathway of Neuro2a cells regulating neurite outgrowth is one of the pathways of CREB phosphorylation. This study used Western blotting method. The results showed that FXT could inhibit the phosphorylation of ERK, reducing it by about 34% ($p < 0.05$). With the application of GPS, it was found that the expression of ERK increased significantly after 100 and 200 μM GPS treatment by 85% and 55% respectively ($p < 0.05$), while on p38 and p-JNK, there were no significant changes ($p > 0.05$) (Fig. 6B).

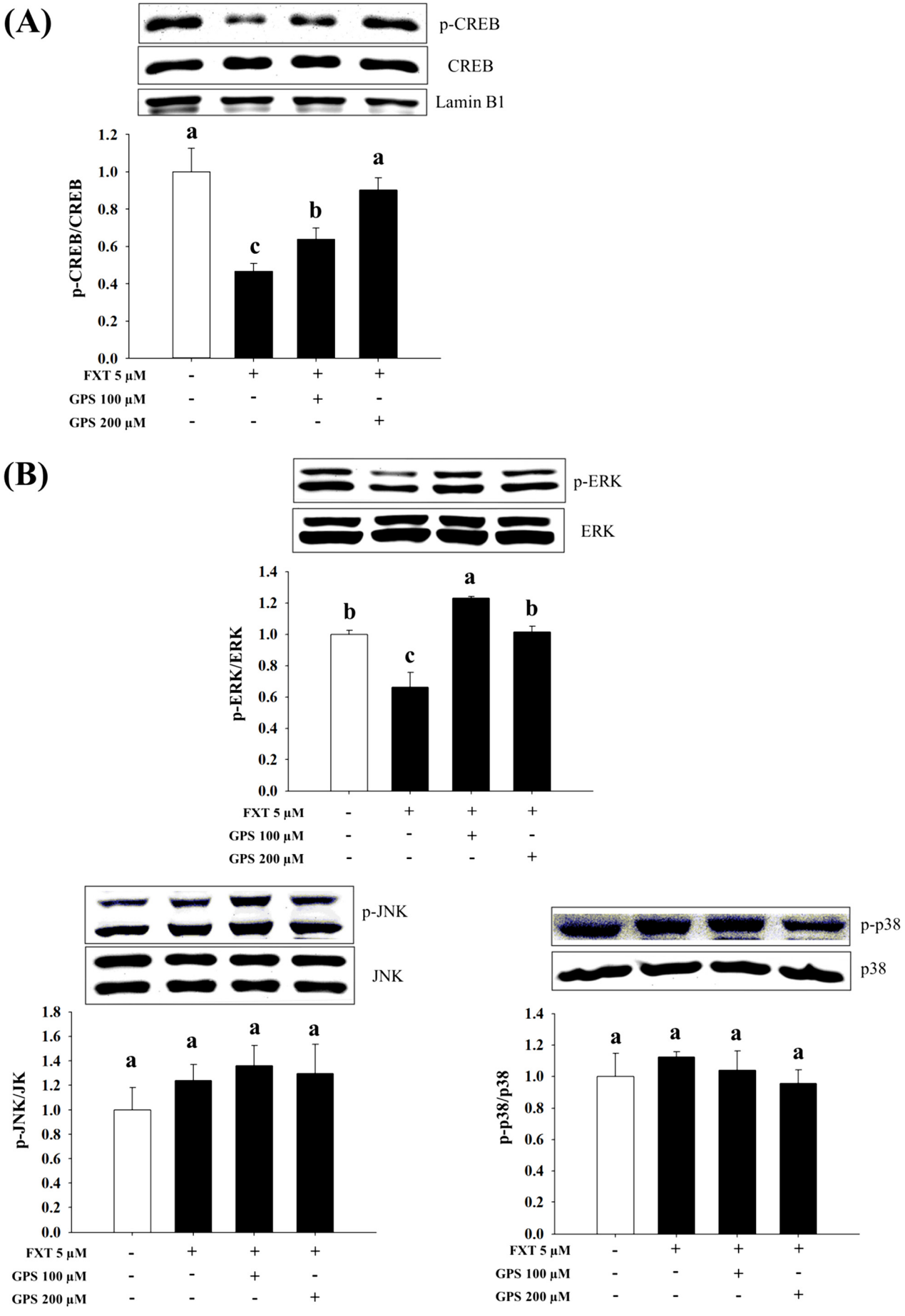

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Fig. 6. Effects of GPS-FXT co-treatment on the expression of neurite differentiation transcription factor CREB (A) and MAPK pathway protein (B) in Neuro2a cells. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with FXT (5 μM), GPS (100 and 200 μM) and PBS as vehicle control for 24 h. Western blot analysis was performed using (A) specific antibodies against CREB and p-CREB, (B) specific antibodies against phospho- and total-antibodies ERK, JNK, and p38. Actin was used as a control for equal loading. Lamin B was used as a control for equal loading. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$).

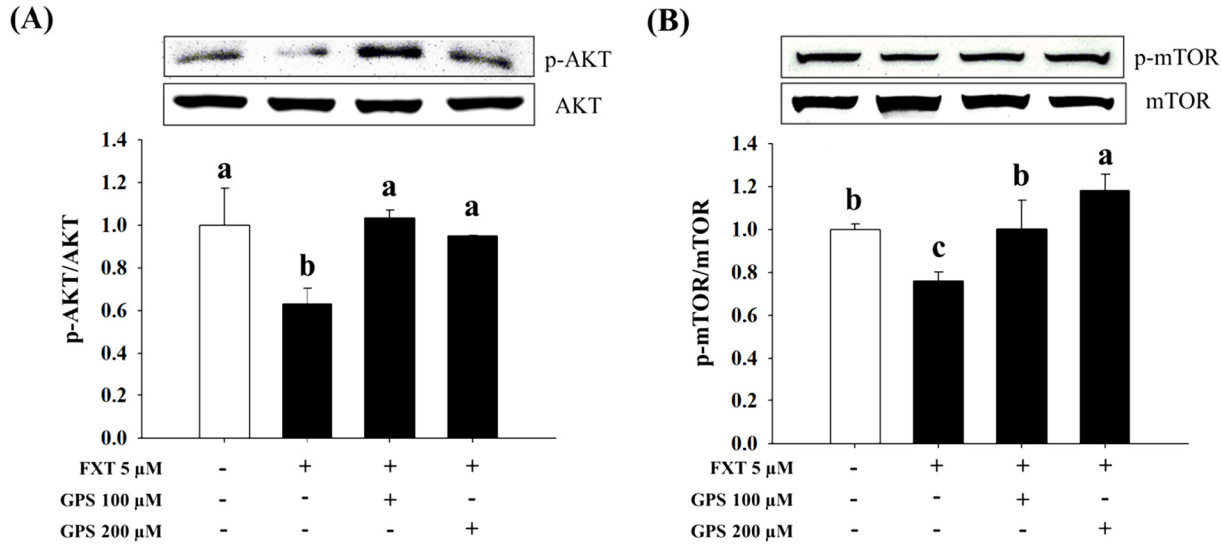

Fig. 7. Effects of GPS-FXT co-treatment on the expression of AKT/mTOR proteins in Neuro2a cells. Neuro2a cells were seeded in 6-well plate for 24 h followed by treating with FXT (5 μM), GPS (100 and 200 μM) and PBS as vehicle control for 24 h. Western blot analysis was performed using specific antibodies against phospho- and total-antibodies AKT and mTOR. Actin was used as a control for equal loading. Each value represents the mean ± SD (n = 3). Different letters indicate statistical significance ($p < 0.05$).

3.7. Effect of GPS-FXT co-treatment on the expression of AKT/mTOR proteins in Neuro2a cells

The effects of FXT and GPS on the AKT/mTOR pathway were also studied. Fig. 7 shows that the application of FXT on the Neuro2a cells could inhibit the phosphorylation of AKT and mTOR signaling protein about 38% and 24% respectively. Then, with the addition of 100 and 200 μM GPS, the level of AKT was increased significantly by 65% and 55% respectively, compared to FXT treatment alone. The mTOR protein also increased significantly with the increase of GPS concentration (100 and 200 μM) by 33% and 55% respectively ($p < 0.05$).

4. Discussion

In the past, primary cells and PC12 cells were mostly used in the study of growth and differentiation of the neuronal cells by psychotropic drugs [29,30], while the same studies concerning neuroblastoma cells are limited. The only work dealing with this subject involves overexpression of 5HT-1A receptor, in which serotonin can promote the survival rate and neuronal growth of Neuro2a cells through the 5HT-1A receptor pathway [31]. Therefore, we used Neuro2a neuroblastoma cells as an experimental model to study the effects of FXT on the neurite outgrowth of neuroblastoma cells. In this study, Neuro2a cells were treated with FXT at the concentration of 0, 0.5, 1 and 5 μM for 24 h, and it was observed that 5 μM of FXT significantly exhibited morphological changes and inhibited the neurite outgrowth, but no significant effects were found on the cell viability (Fig. 1). In another study promotion of neurite outgrowth has been observed in PC12 cells when applied 10 μM of FXT [9]. The reason behind the opposite effect by FXT could be due to the difference in the receptor involved in the outgrowth process. The source of a cell line may affect serotonin receptor subtype, where PC12 cells express the 5-HT3 receptor [32], while Neuro2a cells slightly express 5-HT-1A receptor [24], where both of the serotonin receptors are targets of SSRI drugs [33,34].

We also compared the two gardenia fruit compounds-GPS and GNP for the difference in neurite outgrowth of Neuro2a cells. The results showed that GPS (100–200 μM) could significantly promote the neurite outgrowth compared with the control group. Our results were consistent with other studies, where the length of the neurite outgrowth in PC12 cells increased with the application of GPS (5–100 μM) [35]. The concentration of GPS used in this experiment was higher than GNP as its absorption rate in animal tissue is lower compared to GNP [36], and the cytotoxicity increases with the concentration of GNP > 20 μM. Also, we studied the co-treatment of FXT with GPS and GNP in Neuro2a and observed whether the compounds could ameliorate the suppression effects on neurite outgrowth caused by FXT (5 μM). It appears that GPS restored the neurite outgrowth of Neuro2a significantly and therefore could contribute as adjuvant therapy for prescriptions (Fig. 2). The results could be possibly explained by a study which indicates that PC12 and SH-Y-5Y cells uptake GPS via GLP-1R receptor to achieve the ability to repair neurons [37].

We proceeded on studying how GPS targeted towards the neurite outgrowth markers (Figs. 3–5): MAPT, MAP2, GAP43, and TUBB3. The addition of FXT did not change the level of the MAPT and TUBB3 genes and their proteins significantly ($p > 0.05$), but FXT significantly down-regulated the MAP2 and GAP43 genes and their corresponding protein while GPS counteracted the effect of FXT treatment ($p < 0.05$). Our findings were consistent with the literature [38] describing the close relationship between GAP-43 mRNA/protein and neurite outgrowth. The results in this study also showed a similar phenomenon with previous findings that uridine upregulated the levels of cellular GAP-43 expression in Neuro2a cells, leading to neurite outgrowth [39].

Although FXT could reduce the production of Aβ in transgenic APP/PS1 mice, which might possess the potential to treat Alzheimer's disease [40], our study herein proposed a different viewpoint concerning the CREB expression in FXT-induced neuronal cells. Our study found out that FXT unfortunately inhibited the phosphorylation of CREB (Fig. 6a), a key transcription factor which regulates the growth of nerves, which

is considered to be the therapeutic target of the neurodegenerative disease for Alzheimer's disease [41] and a dominant regulatory role in the nervous system by promoting neuronal survival, precursor proliferation, neurite outgrowth, and neurite differentiation in specific neuronal populations [35]. Differentiation of cells was unfavorable under the treatment of 5 μM FXT due to the phosphorylation of CREB being suppressed. However, the performance of p-CREB by the co-treatment of GPS (200 μM) can be restored to the same level of the control group, leading to the phosphorylation of CREB which can selectively activate numerous downstream genes such as the differentiation-associated protein 43 (GAP-43), thus demonstrating that GPS has a good neuroprotective effect (Fig. 6a).

Considering the importance of MAPK signaling pathway in neurite differentiation, this study analyzed three MAPKs in mammals, which were ERK, JNK, and p38. As a result, phosphorylation of the ERK1/2 protein was significantly inhibited by FXT which is a new finding in neuroblastoma cells, whereas the change in expressions of JNK and p38 were not significant (Fig. 6b). Also, another critical pathway in neuronal cell differentiation, which is the AKT/mTOR signaling pathway [42], was also studied. Our results showed that FXT inhibited phosphorylation of AKT/mTOR, while co-treating with GPS protects these pathways from the inhibition (Fig. 7). The result was consistent with [37] which proved that GPS might protect PC12 and SH-Y-5Y through AKT/mTOR pathway. Read et al. [43] also demonstrated the close relationship between the AKT/mTOR pathway and neurite branching. However, the positive effects of 200 μM GPS on the regulation of neurite outgrowth could be commonly observed higher than positive control, which might be due to the high dose of bioactive compound behavior beneficial to cellular function in this study.

The findings in this study demonstrated that FXT decreased neurite outgrowth in Neuro2a cells and also downregulated gene and protein expression of MAP2 and GAP43. It also downregulated the protein expression of phosphorylated-CREB, MAPK, and AKT/mTOR signaling pathways. GPS treatments ameliorated the suppression effects caused by FXT in Neuro2a cells. In conclusion, our research shows how FXT exerts its detrimental side effects and how GPS counters it back in Neuro2a cells. Hence, using GPS as a complementary alternative medicine should be considered whenever prescribing FXT for clinical use.

Author contributions

M.-K.C. (Min-Kai Chen): Performed the experiment and collected the data. C.-C.P. (Chiung-Chi Peng): Performed the experiment and collected the data. R.S.M (Rida S. Maner): Wrote part of the paper. N.D.Z. (Nor Diana Zulkefli): Wrote part of the paper. S.M.H. (Shang-Ming Huang): Edited the paper. C.L.H. (Chiu-Lan Hsieh): Designed the experiment and proofread the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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