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Original Article

Artemisia Leaf Extract protects against neuron toxicity by TRPML1 activation and promoting autophagy/mitophagy clearance in both *in vitro* and *in vivo* models of MPP+/MPTP-induced Parkinson's disease

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ABSTRACT

Background: Parkinson's disease (PD) is a neurodegenerative disorder involving the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Cellular clearance mechanisms, including the autophagy-lysosome pathway, are commonly affected in the pathogenesis of PD. The lysosomal Ca^{2+} channel mucolipin TRP channel 1 (TRPML1) is one of the most important proteins involved in the regulation of autophagy. *Artemisia argyi* Lev. et Vant., is a traditional Chinese herb, that has diverse therapeutic properties and is used to treat patients with skin diseases and oral ulcers. However, the neuroprotective effects of *A. argyi* are not explored yet.

Hypothesis: This study aims is to investigate the neuroprotective effects of *A. argyi* in promoting the TRPML1-mediated autophagy/mitophagy-enhancing effect

Methods: In this study, we used 1-methyl-4-phenyl-pyridinium (MPP+)-induced PD model established in an SH-SY5Y human neuroblastoma cell line as well as in a 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP)-induced PD model in C57BL/6 J mice. MTT assay was conducted to measure the cell viability and further MitoSoX and DCFDA assay were used to measure the ROS. Western blot analysis was used to access levels of TRPML1, p-DRP1 (ser616), p-AKT, PI3K, and β-catenin, Additionally, IF and IHC analysis to investigate the expression of TRPML1, LC3B, β-catenin, TH+, α-synuclein. Mitotracker stain was used to check mitophagy levels and a lysosomal intracellular activity kit was used to measure the lysosomal dysfunction. Behavioral studies were conducted by rotarod and grip strength experiments to check motor functions.

Abbreviations: A. Argyi (AA), *Artemisia Argyi* Lev. et Vant.; DCFDA, 2,7-Dichlorofluorescein diacetate; DRP1, dynamin-related protein 1; LC3B, light chain 3B; MPP+, 1-methyl-4-phenyl-pyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine; MTT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PI3K, Phosphoinositide 3-kinases; PD, Parkinson's disease; ROS, Reactive Oxygen Species; SNpc, substantia nigra pars compacta; TH+, Tyrosine Hydroxylase; TRPML1, Transient receptor potential mucolipin 1.

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Results: In our *in vitro* study, *A. argyi* rescued the MPP+-induced loss of cell viability and reduced the accumulation of mitochondrial and total reactive oxygen species (ROS). Subsequently, it increased the expression of TRPML1 protein, thereby inducing autophagy, which facilitated the clearance of toxic accumulation of α-synuclein. Furthermore, *A. argyi* played a neuroprotective role by activating the PI3K/AKT/β-catenin cell survival pathway. MPP+-mediated mitochondrial damage was overcome by upregulation of mitophagy and downregulation of the mitochondrial fission regulator p-DRP1 (ser616) in SH-SY5Y cells. In the *in vivo* study, *A. argyi* ameliorated impaired motor function and rescued TH+ neurons in the SNpc region. Similar to the results of the *in vitro* study, TRPML1, LC3B, and β-catenin expression was enhanced in the SNpc region in the *A. argyi*-treated mice brain.

Conclusion: Thus, our results first demonstrate that *A. argyi* can exert neuroprotective effects by stimulating TRPML1 and rescuing neuronal cells by boosting autophagy/mitophagy and upregulating a survival pathway, suggesting that *A. argyi* can further be exploited to slow the progression of PD.

Introduction

Parkinson's disease (PD) is a progressive, degenerative neurological disease characterized by a large number of motor and non-motor symptoms (Lew 2007) and is the fastest-growing neurodegenerative disorder, which is estimated to increase to 1.24 million by 2030 (Wang et al., 2015). PD involves dysfunction or loss of dopaminergic (DAergic) neurons in the substantia nigra par compacta (SNpc) of the midbrain along with inclusions of Lewy bodies and cytoplasmic aggregations of the protein α-synuclein (Kalia and Lang 2015). The precise mechanisms involved in the degeneration of neurons are still unclear, but an array of modulators are predicted to be involved, including oxidative stress, mitochondrial dysfunction, DNA damage, lipid peroxidation, and neuronal excitotoxicity (Marino et al., 2020). Neuronal tumor cells from the human neuroblastoma SH-SY5Y line treated with neurotoxins like 1-methyl-4-phenyl-pyridinium (MPP+) are presently used to mimic the DAergic neuronal death in PD. In animal models, 1-methyl-4-phenyl-1, 2,3,6-tetrahydro-pyridine (MPTP) is used to simulate the PD condition (Xie et al., 2010; Ito et al., 2017). Current treatments include dopamine replacement therapies and other drugs which cannot reverse the disease progression (Löhle and Reichmann 2010) but its long-term usage can cause severe side effects as well as therapy-related motor complications like dyskinesia (Calabresi et al., 2010). Therefore, the search for safer and more reliable alternative treatments is demanded.

TCM has been used for several years by the Chinese community to treat several diseases, and many drugs evaluated in recent clinical trials have been extracted from plants because of the fewer adverse effects and multi-targeted effects of such drugs (Li et al., 2017; Li and Le 2021). Increasing evidence suggests that TCM can ameliorate neurodegenerative symptoms by upregulating the autophagy pathway (Xia et al., 2020; Wang et al., 2021). Herbs from the *Artemisia* genus have been widely used for their medical properties (Kim et al., 1997; Hussain 2020). *Artemisia argyi* Lev. et Vant. (*A. argyi*) is a perennial herb (Kang et al., 2016) that has been proven to possess anti-inflammatory and anti-oxidant properties (Zeng et al., 2014; Li et al., 2018). Previous literature has shown that the extract from *A. argyi* leaves has therapeutic effects on abdominal pain, dermatitis (Yun et al., 2016) oral ulcer (Yin et al., 2017) obesity and diabetes (Yamamoto et al., 2011) however little to no study has shown the neuroprotective effects of *A. argyi*. Therefore, in the present study, we investigated the neuroprotective effect of *A. argyi* against MPP+/MPTP-induced toxicity in SH-SY5Y cells and B6 mice.

Recent studies have suggested that mucolipin TRP channel 1 (TRPML1) or mucolipin-1, a cation-permeable channel localized on the membrane of lysosomes, plays a major role in regulating autophagy (Scotto Rosato, Montefusco et al. 2019; Tedeschi et al., 2019). It is widely distributed in the brain and other tissues and plays an important role in lysosomal storage, signal transduction, membrane transportation, and acidic homeostasis (Santoni et al., 2020). Previous studies have shown that increased lysosomal Ca^{2+} activity can clear α-synuclein accumulation and protect DAergic neurons in Parkinson's disease (Tedeschi et al., 2019). Moreover, increased TRPML1 expression has been also shown to clear damaged mitochondria and remove excess reactive oxygen species (ROS), which can help reduce neuronal toxicity (Zhang et al., 2016; Zhang et al., 2021). Since this channel is of utmost importance in PD, we hypothesized that it may have been compromised in the disease. There is evidence indicating impaired autophagy (Anglade et al., 1997) and mitophagy (Liu et al., 2019) in vulnerable brain regions in PD patients, which are correlated with α-synuclein accumulation in the same brain samples (Fowler and Moussa 2018; Hou et al., 2020). Thus, defective autophagy can aggravate disease phenotypes, suggesting that autophagy may be an attractive therapeutic target for the prevention and treatment of PD-associated neurodegeneration. In particular, increasing the efficiency of autophagy could ameliorate the toxic effects of aggregates in PD.

On other hand, activation of phosphatidylinositol-3 kinase (PI3K)/Akt (protein kinase-B) has shown neuroprotective effects against oxidative stress and apoptosis in the progression of PD (Jiang and Peng 2021). Furthermore, activated Akt can phosphorylate and stabilize b-catenin which can then translocate to the nucleus and activate several anti-apoptotic genes like Bcl2 thereby promoting cell survival (Fang et al., 2007). Therefore, the current study provides evidence to support the neuroprotective effects of *A. argyi* by up-regulating TRPML1 to modulate autophagy thereby activating the PI3K/ Akt/b-catenin pathway both *in vitro* and *in vivo*.

Materials and methods

Cell culture and treatment

The SH-SY5Y neuroblastoma cell line was obtained from the American Type Culture Collection (ATCC). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (Gibco, Gaithersburg, MD, USA). Cells were cultured in an atmosphere of 5% CO_2 at 37 °C, consistent with our previous study (Liu et al., 2020). Briefly, cells were seeded in a 96-well plate (12,000 cells/well) or chamber slides (22,000 cells/well) and incubated overnight to reach the desired confluency. To induce a PD-like phenotype, the cells were treated with 1 mM MPP+ iodide (Sigma Chemical Co., St. Louis, MO, U.S.A.) diluted in dimethyl sulfoxide (DMSO) for 24 h.

Preparation of ethanol crude extract of A. argyi

Artemisia argyi was provided by the Department of Chinese Medicine, Hualien Tzu Chi Hospital, Taiwan. The healthy, mature, and fresh *A. argyi* leaves (Chinese mugwort) were collected locally from Taiwan. The leaves (150 g) were washed, dried under the shade, and then crushed in a grinder with 500 ml of 75% ethanol. The solution was placed in an ultrasonic water bath at 40 kHz, 30 °C for 1 h, and centrifuged at 1300 rpm for 30 min at 4 °C. The centrifugate was filtered using a 0.45 μm pore size filter and concentrated in a hot air oven at 40

°C until the solvent is evaporated (Zainol et al., 2018).

HPLC-MS/MS analysis

HPLC-MS/MS Analysis was performed to identify the main compounds of A. argyi ethanol extract. It was analyzed using AB Sciex Instruments QTRAP 5500 (Agilent Corporation, CA, USA) equipped with Agilent 1260 G1312B Binary Pump, a Phenomenex Kinetex-Phenyl-Hexyl-100A (100 mm x 2.1 mm i.d., 2.6 um; flow rate 0.1 ml/min) column maintained at -20°C. The chromatographic data were interpreted using Analyst 1.5 software (Thermo Fisher Scientific). The analysis showed the presence of 0.53 ng/ml of Swertisin, 0.94 ng/ml Isoliquiritigenin, 0.014 ng/ml 3,4-Dimythoxyflavone, 138.9 ng/ml Eupatilin, 108.6 ng/ml Nerolidol.

3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The cytotoxicity of the compounds administered to the cells was evaluated using the MTT (Sigma-Aldrich, St. Louis, MO, USA) after treatment as described previously (Chang et al., 2021). The cells were plated in a 96-well plate in triplicates and then were treated first with 1 mM MPP+ for 24 hrs followed with *A. argyi* at different concentrations for another 24 hrs. After that, 0.5 mg/ml MTT was added to each well and incubated for 2–3 hrs. Then the blue formazan crystals were diluted in 100 ul of DMSO and the absorbance was checked at 570 nm using a microplate ELISA reader (Bio-Tek Instruments, Winooski, VT, USA). The dosages of 60 μg/ml and 80 μg/ml were fixed for all further experiments as it was found efficient to overcome the toxic effects of MPP+.

Lysosomal intracellular activity assay

The lysosomal activity was measured with a self-quenched substrate, using Bafilomycin 1 × as control, based on the manufacturer's instructions (Lysosomal Intracellular Activity Assay kit, Abcam, Cambridge, United Kingdom; Catalog No. ab234622). Briefly, the SH-SY-5Y cells were incubated with a self-quenched substrate for 1 h at 37 °C. The cells were then washed with the ice-cold assay buffer provided in the kit. The treated cells were then fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 30 mins. The mounting media with 4,6-diamidino-2-phenylindole (DAPI; ab104139) and observed with the Axio Observer A1 digital fluorescence microscope (Olympus, Tokyo, Japan)

Immunofluorescence (IF) staining

SH-SY5Y cells were fixed in paraformaldehyde and permeabilized with 0.1% Triton X-100 and 1% sodium citrate for 2 min on ice. After blocking with 2% bovine serum albumin (BSA), the cells were incubated with the primary antibody (LC3B, #2775; TRPML1, ab272608; α-synuclein [4D6], ab1903; α-synuclein (phospho S129) (EP1536Y), ab51253: 1:200) diluted in 2% BSA at 4 °C overnight. Subsequently, the cells were washed in PBS and incubated with a secondary Alexa Fluor® 594 goat anti-rabbit IgG (*H + l*) 1:500 (A11012, Invitrogen) in 2% BSA at room temperature for 1 h. Finally, the cells were counterstained with a mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; ab104139) and observed with the Axio Observer A1 digital fluorescence microscope (Olympus, Tokyo, Japan)

Immunoblotting assay

Fifty micrograms of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a 0.45-μm polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich). After transfer, the membranes were blocked using 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) for 1 h at room temperature and then incubated with the primary antibodies (1:1000 PI3K [sc-374534], 1:1000 β-catenin [sc-7963], 1:1000 LEF [sc-374412], 1:1000 p-AKT [sc-514032], 1:1000 BCL-2 [610539], 1:1000 DRP1 [sc-271583], 1:1000 p-DRP1 (ser616) [#3455], and 1:5000 GAPDH [sc-32233] diluted in 1% BSA) overnight at 4 °C. The membranes were then incubated with a secondary antibody with appropriate hosts for 1 h at room temperature. The ECL substrate (Millipore, Billerica, MA, USA) was poured onto the membrane, and the membrane was viewed under UVP iBright 1500 (ThermoFisher Scientific). The intensity was measured using ImageJ software (NIH, Bethesda, MD, USA), as previously described (Lin et al., 2019).

MitoSoX assay

Mitochondrial ROS levels were measured using a MitoSoX Red mitochondrial superoxide indicator kit (M36008; Invitrogen, Carlsbad, CA, USA). The cells were fixed and permeabilized according to the protocol described above. The cells were incubated with 4 μM MitoSoX reagent (diluted in warm 1X PBS) for 30 min at 37 °C in the dark. Subsequently, the cells were washed with warm PBS three times, mounted with mounting media containing 4,6-diamidino-2-phenylindole (DAPI; ab104139), and observed with the Axio Observer A1 digital fluorescence microscope (Olympus, Tokyo, Japan). Similarly, MitoSox Red fluorescence intensity was measured using a Synergy HTX Multi-Mode Microplate Reader (Bio-Tek).

MitoTracker assay

The treated cells were incubated with 150 nM MitoTracker™ Red CMXRos (M7512, Invitrogen, Carlsbad, CA, USA) (diluted in serum-free media) for 30 min at 37 °C in the dark. Then, the cells were fixed and permeabilized according to the above protocol and incubated with LC3B (1:200) [#2775, abcam] antibody overnight at 4 °C. The slides were then incubated with the appropriate fluorescent secondary antibody and mounted with a fluoromount aqueous mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; ab104139) and observed with the Axio Observer A1 digital fluorescence microscope (Olympus, Tokyo, Japan)

2,7-Dichlorofluorescein diacetate (DCFDA)/dichlorodihydrofluorescein-diacetate (H2DCFDA) staining

Endogenous ROS levels were measured using the DCFDA/H2DCFDA-Cellular ROS Assay Kit (ab113851, Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. DCFDA (25 μM) was added to the culture plate and incubated for 45 min at 37 °C in the dark. Unstained cells were used as a blank control. Fluorescence intensity was measured using a Synergy HTX Multi-Mode Microplate Reader (BioTek).

Animals

Twelve male C57BL/6 J mice weighing 25–30 gs (10 weeks old) were obtained from BioLASCO Co., Taiwan. They were maintained in filter-top holding cages with free access to food and water in an environmentally controlled room maintained at a temperature of 23 °C, with a humidity of 55%, and a 12-h light-dark cycle (light period, 7 AM to 7 PM). After 1 week of acclimatization to the new environment, the mice were randomly assigned to one of three groups: (i) control ($n = 4$), (ii) MPTP ($n = 4$), and (iii) MPTP + AA ($n = 4$). An acute mouse model of PD was created by injecting four doses of MPTP (25 mg/kg, diluted in DMSO) intraperitoneally at 2-h intervals (Meredith and Rademacher 2011). Animals in the *A. argyi* group received AA at a concentration of 100 mg/kg diluted in 25% ethanol for 2 weeks. Throughout the 3-week treatment regimen, the groups were monitored using behavioral tests.

Rotarod test

The rotarod test was performed on the mice by using the Biosep Rota rod Model LE8205. The mice were pre-trained for 1 week before the experiment. On the third week after MPTP treatment, the mice were tested on the rotarod. The latency to fall was recorded (Deacon 2013).

Grip strength

The grip strength of mice was measured using a grip strength meter (Ugo Basile, Cat. No. 47200; Gemonio, Italy) to measure the grip strength (peak force and time resistance) of the forelimbs. The mouse was held by the tail and lowered to just above the grid. Once the mouse grabbed the metal grid, it was then gently pulled back by its tail, and the maximal value of grip strength was recorded. Each mouse was tested six times and the values averaged were used for data analysis. (Zheng et al., 2021)

Immunohistochemistry (IHC)

The sectioned samples were fixed with 4% PFA, permeabilized, and blocked with 0.3% Triton X-100 and 2% BSA in PBS for 1 h. The cells were then incubated with the primary antibody (Tyrosine Hydroxylase (TH), ab112; β-Catenin (E-5), sc-7963; LC3B, #2775; TRPML1, ab272608) 1:200 diluted in 2% BSA overnight at 4 °C. Thereafter, the slides were incubated with the secondary antibody Alexa Fluor® 488 goat anti-rabbit IgG ($H + l$) 1:500 (A11008, Invitrogen) diluted in 2% BSA and 1 × PBS for 1 h at room temperature. Finally, the cells were counterstained with a mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; ab104139) and observed with the Axio Observer A1 digital fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using GraphPad Prism v5.0.3.477 (GraphPad Software Inc., San Diego, CA). T-test followed by a non-parametric Mann–Whitney U test was used for comparisons between groups. All data are presented as mean ± standard deviation (S.D.). p values less than 0.05 were considered statistically significant, with * representing $p< 0.05$, ** indicating $p< 0.01$, and *** indicating $p< 0.001$.

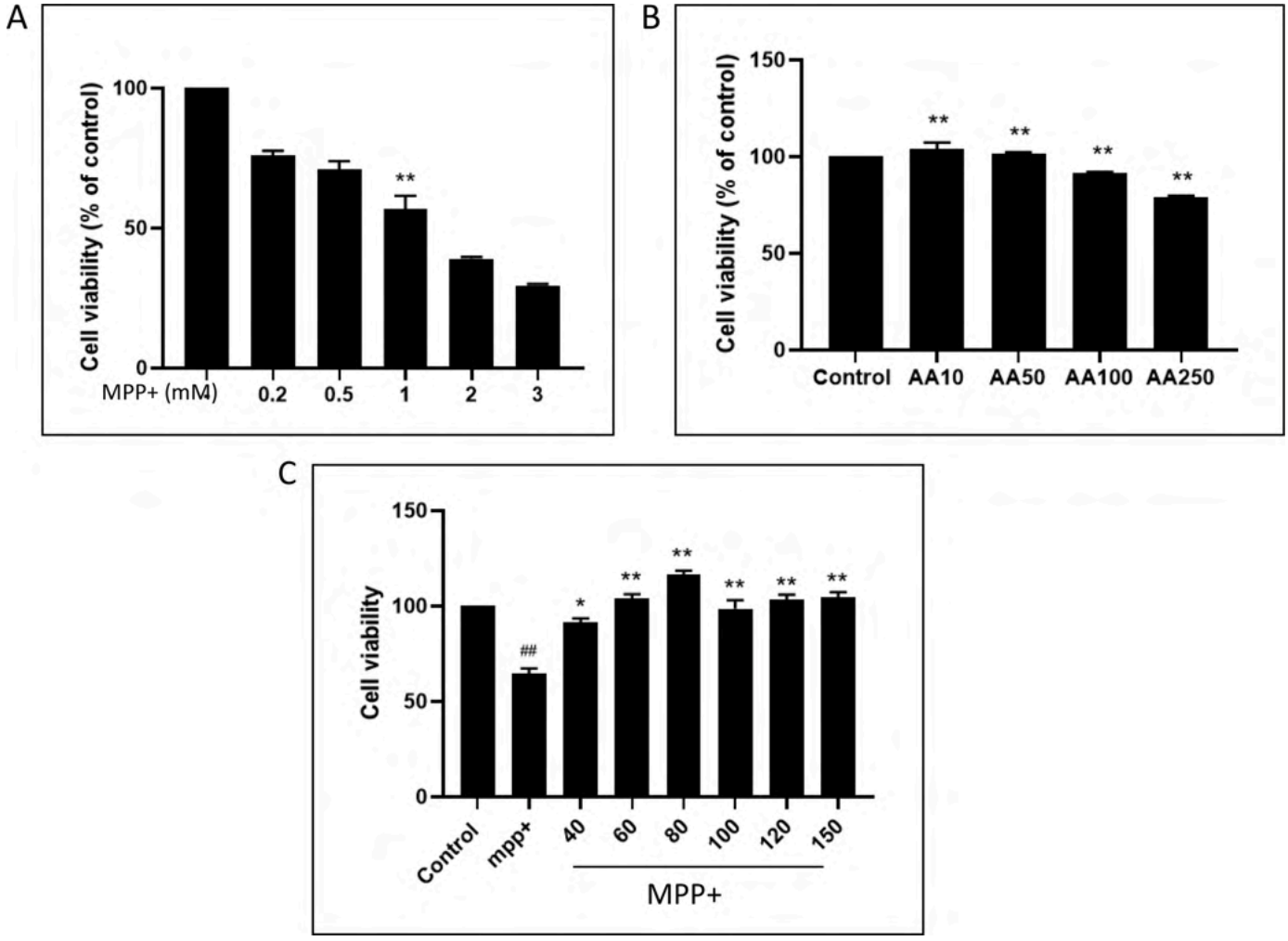

Fig. 1. Effects of 1-methyl-4-phenylpyridinium (MPP+) and *A. argyi* (AA) on SH-SY5Y cell viability. (A) Cells were incubated with different concentrations of MMP+ ranging from 0.2 mM to 3 mM for 24 h for the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. (B) Cells were treated with 10 to 250 μg/ml of *A. argyi* for 24 h to check cell toxicity. (C) Cells were pretreated with 1 mM of MPP+ for 24 h followed by incubation with different concentrations of AA for another 24 h. Cell viability was assessed and expressed as the percentage of the viability of the control group. Data are expressed as the mean ± standard deviation. *, #$p< 0.05$, ***$p< 0.001$; *indicates the p-value for the comparison between MPP+ and AA groups, # indicates the p-value for comparison between the MPP+ group and control.

Results

A. argyi ameliorated MPP+-mediated cytotoxicity in sh-sy5y cells

The viability of SH-SY5Y cells was monitored using the MTT assay. As shown in (Fig. 1A), MPP+ treatment at concentrations of 0.2–3 mM significantly reduced the viability of SH-SY5Y cells in a dose-dependent manner in comparison with untreated cells. Based on the MTT data, we chose an MPP+ concentration of 1 mM to generate the PD model. As shown in Fig. 1B, the treatment of *A. argyi* had no significant cytotoxic effect on cell viability upto 80 μg/ml. Further to finalize the effective doses of *A. argyi*, cells were incubated with MPP+ (1 mM) for 24 hrs, followed by post-treatment with different concentrations of *A. argyi* for another 24 h. We found that *A. argyi* treatment at 60 and 80 μg/ml significantly attenuated MPP+-induced loss of cell viability (Fig. 1C).

A. argyi attenuated MPP+-induced oxidative stress in SH-SY5Y cells

Numerous studies have suggested that ROS represent a common denominator associated with neurodegenerative diseases; therefore, we analyzed mitochondrial superoxide levels using MitoSoX and intracellular ROS levels by using the DCFDA assay. *A. argyi* treatment resulted in a significant reduction in MitoSoX red fluorescence emission, indicating reduced levels of superoxide in the mitochondria in AA-treated groups in comparison with those in the MPP+-treated groups (Fig. 2A). Similarly, the DCFDA assay showed a significant reduction of 31.12% (60 μg/ml) and 36.57% (80 μg/ml) in the total ROS levels in the *A. argyi*-treated groups in comparison with those in the MPP+-treated group (Fig. 2B). These results suggest that *A. argyi* diminished MPP+-induced ROS generation.

A. argyi can enhance TRPML1 levels and promoted lysosomal activity

TRPML1 deficits have been shown to increase neuronal toxicity and have been implicated in PD. Immunostaining and western blotting showed decreased expression of TRPML1 in MPP+-induced cells, while treatment with *A. argyi* significantly enhanced the expression of TRPML1 in a dose-dependent manner (Fig. 3A, 9A). Similarly, lysosomal activity was reduced in the MPP+-treated group, while *A. argyi* treatment resulted in increased lysosomal activity in comparison with the activity levels in both MPP+- and BA1-treated groups (Fig. 3B). These data suggest that *A. argyi* can upregulate TRPML1 activity and therefore repair lysosomal dysfunction.

A. argyi promoted autophagy-dependent clearance of α-synuclein

Immunostaining showed increased α-synuclein accumulation and reduced LC3B expression in MPP+-treated cells in comparison with untreated cells. Treatment with *A. argyi* decreased α-synuclein accumulation, and increased LC3 expression showed a puncta-like appearance in a dose-dependent manner in comparison with the cells treated with MPP+ alone (Fig. 4A). These data suggest that *A. argyi* can upregulate autophagy and remove α-synuclein aggregates. To confirm this finding, active form of α-synuclein protein was cultured with cells, and the results showed that *A. argyi* can also reduce the levels of active α-synuclein aggregates in a dose-dependent manner, in comparison with the findings obtained after culturing with α-synuclein alone (Fig. 4B). Similarly, LC3B expression in the SNpc region of brain sections was significantly enhanced in the AA-treated group, in comparison with the expression in the MPTP group (Fig. 9B).

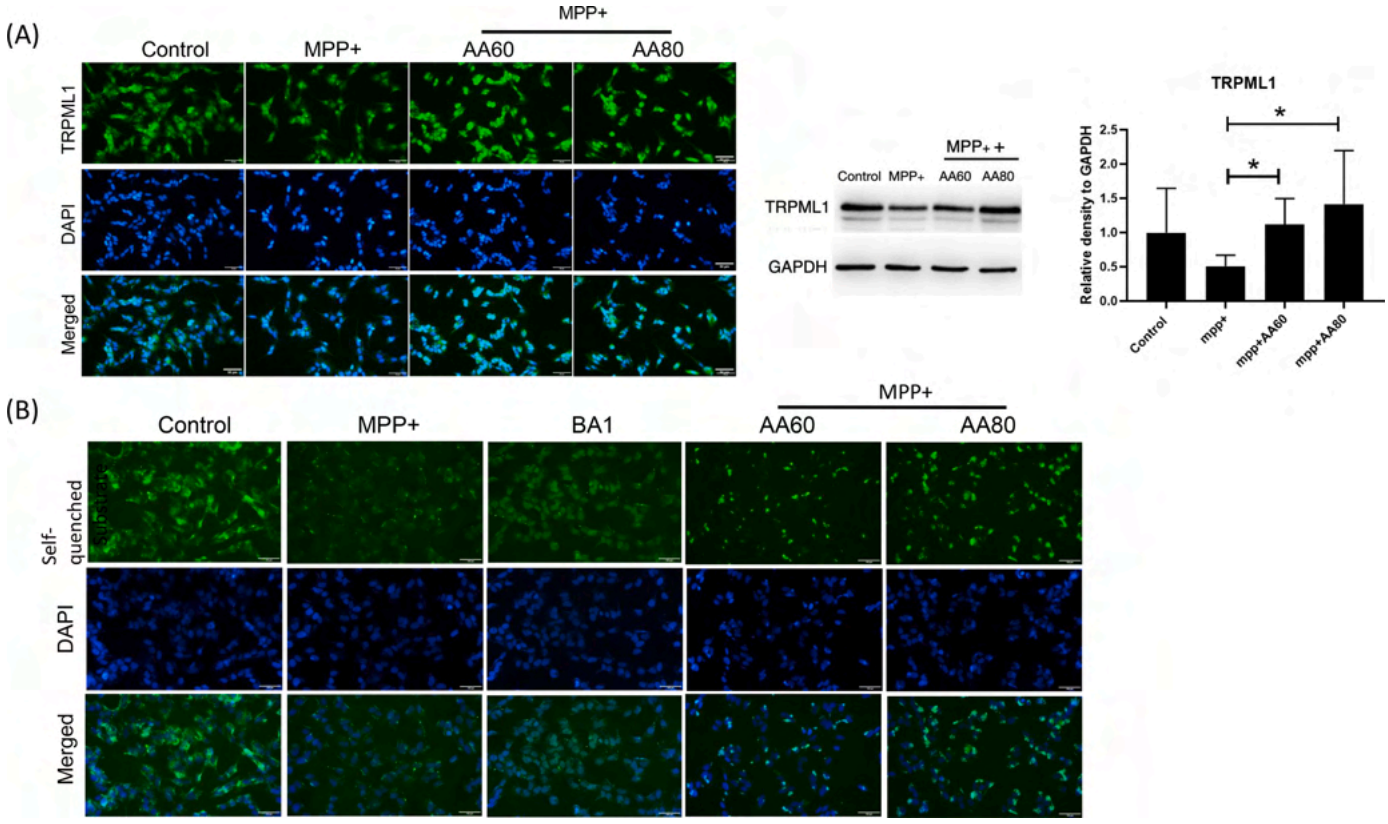

Fig. 2. *A. argyi* (AA) can upregulate mucolipin TRP channel 1 (TRPML1) and thereby promote lysosomal activity. (A) Immunohistochemical staining was performed to detect TRPML1(green) and DAPI (blue) to detect nucleus. Scale bar-100 μm. Western blot analyses showed increased TRPML1 expression in the AA-treated group. (B) Immunohistochemical analyses showed increased lysosomal intracellular activity(green) and nuclear (blue). BA1 was used as a negative control. Scale bar-100 μm.

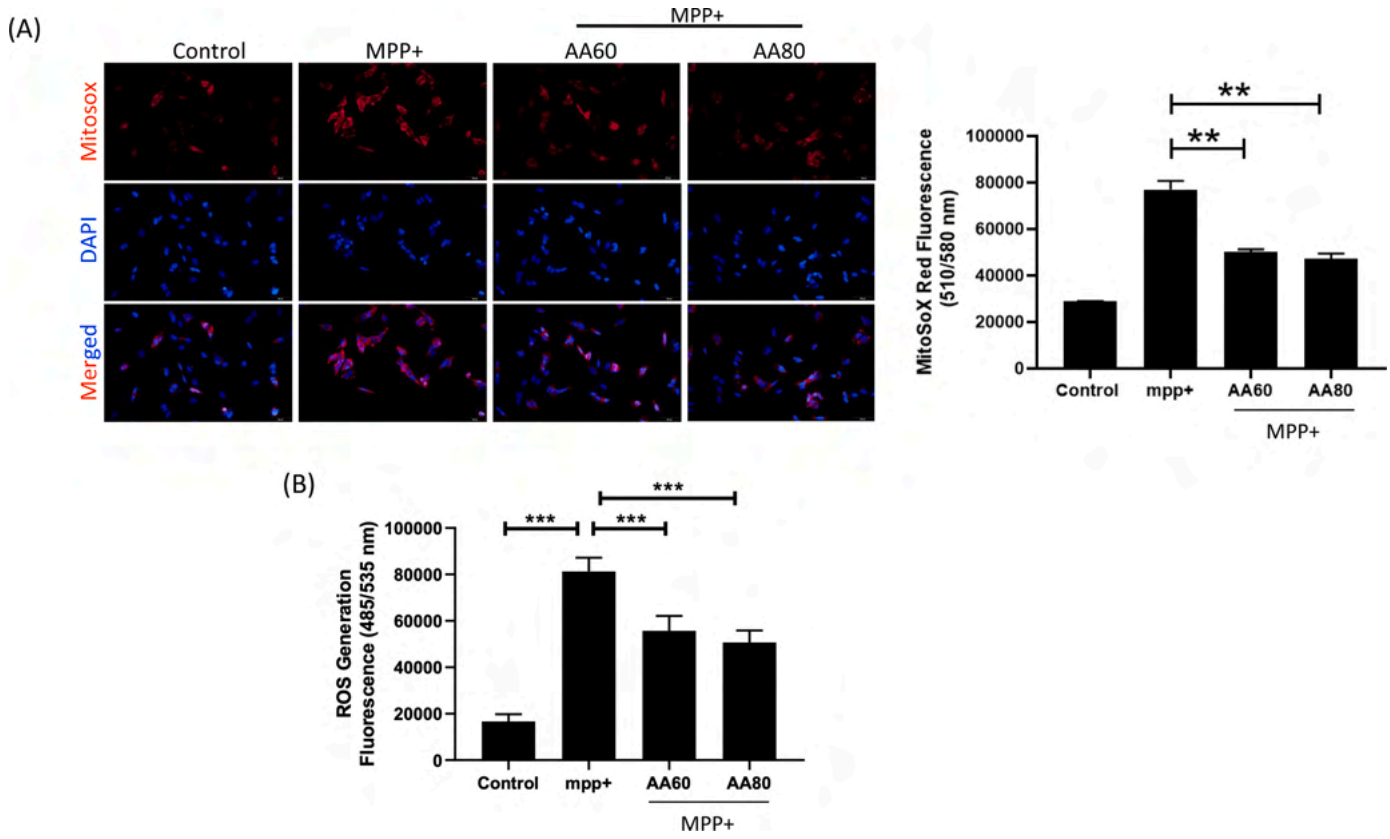

Fig. 3. Attenuation of 1-methyl-4-phenylpyridinium (MPP+)-induced mitochondrial oxidative stress in SH-SY5Y cells. (A) MitoSoX assay was used to stain ROS (red) and DAPI was used to stain nucleas (blue) Scale bar- 100 μm. Bar chart showing the fluorescence intensity (B) 2,7-Dichlorofluorescein diacetate (DCFDA) assay showed reduced levels of reactive oxygen species (ROS). Data are expressed as the mean ± standard deviation; ***$p<$ 0.001.

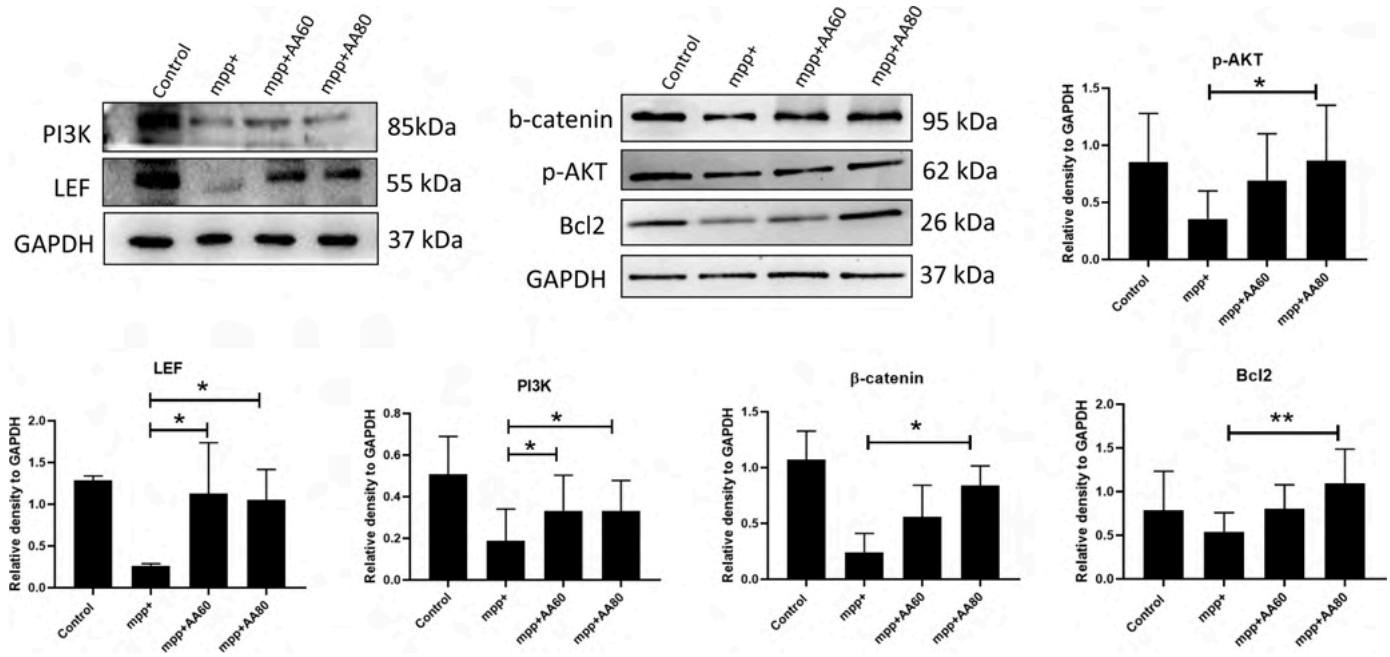

Fig. 4. Upregulated autophagy can inhibit pathogenic α-synuclein in 1-methyl-4-phenylpyridinium (MPP+)-induced SH-SY5Y cells. SH-SY5Y cells were treated with 1 mM MPP+ for 24 hrs followed by incubation with different concentrations of *A. argyi* (AA) for another 24 hrs (A) Immunofluorescence (IF) analyses show the expression of LC3B (green) and total α-synuclein (red). Arrowheads showing the expression of LC3B. Scale bar- 50 μm (B) IF analyses showing active α-synuclein protein (green) expression in SH-SY5Y cells. Scale bar- 100 μm. DAPI (blue) staining was used to determine the position of the nuclei. Data are expressed as the mean ± standard deviation; *, #$p<$ 0.05, ***$p<$ 0.001; *indicates the p-value for the comparison between α-synuclein and other groups, # indicates the p-value for comparison between the MPP+ group and other groups.

A. argyi increased the levels of cell survival markers and upregulated the β-catenin signaling pathway in SH-SY5Y cells

To determine the mechanism underlying *A. argyi*-mediated neuroprotection, immunoblotting was performed. As shown in Fig. 5A, B, in comparison with untreated cells, MPP+-treated cells showed markedly reduced expression of PI3K, p-AKT, β-catenin, LEF, and BCL-2. However, the protein expression of all markers was significantly upregulated in the AA-treated group. Similarly, the expression of β-catenin in the SNpc region of brain sections was significantly enhanced in the AA-treated group in comparison with that in the MPTP group (Fig. 9C). These results suggest that *A. argyi* induces the activation of the PI3K/p-AKT/β-catenin/bcl-2 signaling pathway in MPP+-induced cell death.

A. argyi can also protect SH-SY5Y cells through upregulation of mitophagy

Mitochondrial damage and dysfunction are known to be key contributing factors in PD. We analyzed the co-localization of Mito-Tracker Red with LC3B as an index of mitophagy and observed no co-localization per cell after treatment with MPP+. However, the AA-treated group clearly showed co-localization of LC3B with mitochondria, indicating autophagic mitochondrial sequestration (Fig. 6A). Immunoblot analyses also showed that both Drp1 and p-Drp1(ser616) were significantly downregulated in the AA-treated group in comparison with the MPP+-treated group (Fig. 6B). These data indicate that *A. argyi* treatment restored MPP+-mediated impaired mitophagy to remove damaged mitochondria and also protected the mitochondria by down-regulating mitochondrial fission protein.

Behavioral analysis in the MPTP-induced PD mice model

We quantified the grip strength and rotarod latency starting one day after the last drug treatment. As shown in Fig. 6A, forelimbs were significantly impaired in MPTP-treated mice (Fig. 7A). Similarly, rotarod analyses indicated defects in motor function, with the MPTP-treated mice showing a significantly lower latency to fall in comparison with their control littermates (Fig. 7B). However, mice treated with *A. argyi* showed improved strength in the forelimbs and a longer latency to fall compared to only MPTP-treated mice. Thus, these results suggest that *A. argyi* can improve motor functions in an MPTP-induced PD mouse model.

A. argyi prevented the loss of DAergic neurons in the SNpc in MPTP-injected mice

We analyzed the neuroprotective effects of *A. argyi* on DAergic neurons in MPTP-induced PD mice. The number of TH+ neurons in the SNpc region was assessed by IHC, and the results suggested that the MPTP-induced group had significantly fewer DAergic neurons than the control group. In contrast, the number of TH+ neurons were significantly greater in the AA-treated group in comparison with the MPTP-only group (Fig. 8). These results indicate that *A. argyi* reduces MPTP-induced loss of SNpc DAergic neurons.

Discussion

Recent research has targeted the identification of potent neuroprotective candidate agents for disease-modifying therapy in several neurodegenerative diseases. In this regard, the present study is the first to report the neuroprotective potential of *A. argyi* ethanol extract against MPP+-mediated PD in SH-SY5Y cells and an MPTP-mediated PD mouse model. Our findings explored the neuroprotective effects of *A. argyi*, which ameliorated MPP+-mediated SH-SY5Y cell damage by decreasing MPP+-mediated ROS generation, upregulating TRPML-1 mediated autophagy, attenuating the accumulation of α-synuclein, upregulating the cell survival pathway, and preventing mitochondrial damage. Furthermore, in a mouse model, *A. argyi* also restored motor deficits, recovered DAergic neurons, and upregulated autophagy. However, analyses of the effectiveness of *A. argyi* in differentiated SH-SY5Y cells will help to prove its efficacy in neuron-like cells, which is a limitation of the current study.

Mutations in TRPML1 can affect lysosomal storage, which is known to be responsible for autophagy dysfunction (Kendall and Holian 2021). A recent study showed that the enhanced functioning of TRPML1 can remove excess ROS and attenuate photoreceptor apoptosis by upregulating autophagy (Yan et al., 2021). We found that TRPML1 was under-expressed in PD-modeled neurons, and the forced expression of TRPML1 by *A. argyi* could repair lysosome dysfunction and reduce mitochondrial damage and ROS production in the cell model. Some studies indicate that TRPML1 can reduce the over-acidification of lysosomes by dissipating the excess *H*+ ions to maintain the lysosomal pH and thereby maintain its activity (Santoni et al., 2020). Blocking autophagy flux with MPP+ as well as BA1 which inhibits endocytosis and served as a control significantly reduced the lysosomal proteolysis which

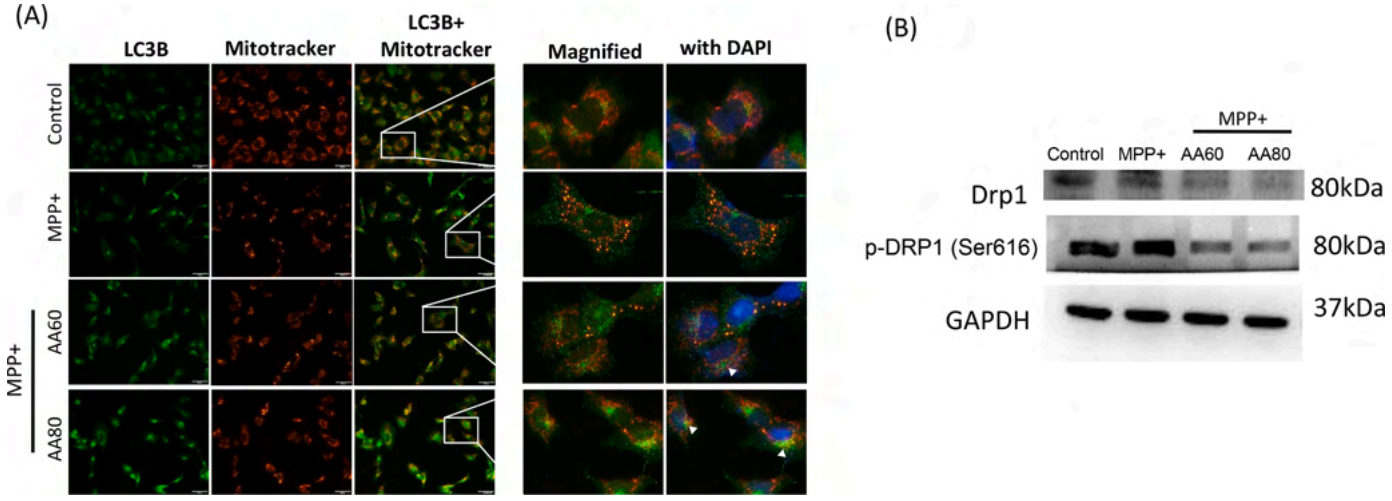

Fig. 5. Effect of *A. argyi* (AA) on the expression of cell survival regulators. SH-SY5Y cells were treated with 1 mM MPP+ for 24 hrs followed by incubation with different concentrations of AA for another 24 hrs. (A) Protein expression levels of anti-PI3K, p-AKT, β-catenin, LEF and BCL-2 was measured using western blot. GAPDH was used as internal control. (B) Statistical analysis relative to GAPDH. Data are expressed as the mean ± standard deviation; $^{*}p < 0.05$, $^{**}p < 0.01$. The band intensities were normalized against internal control using ImageJ software.

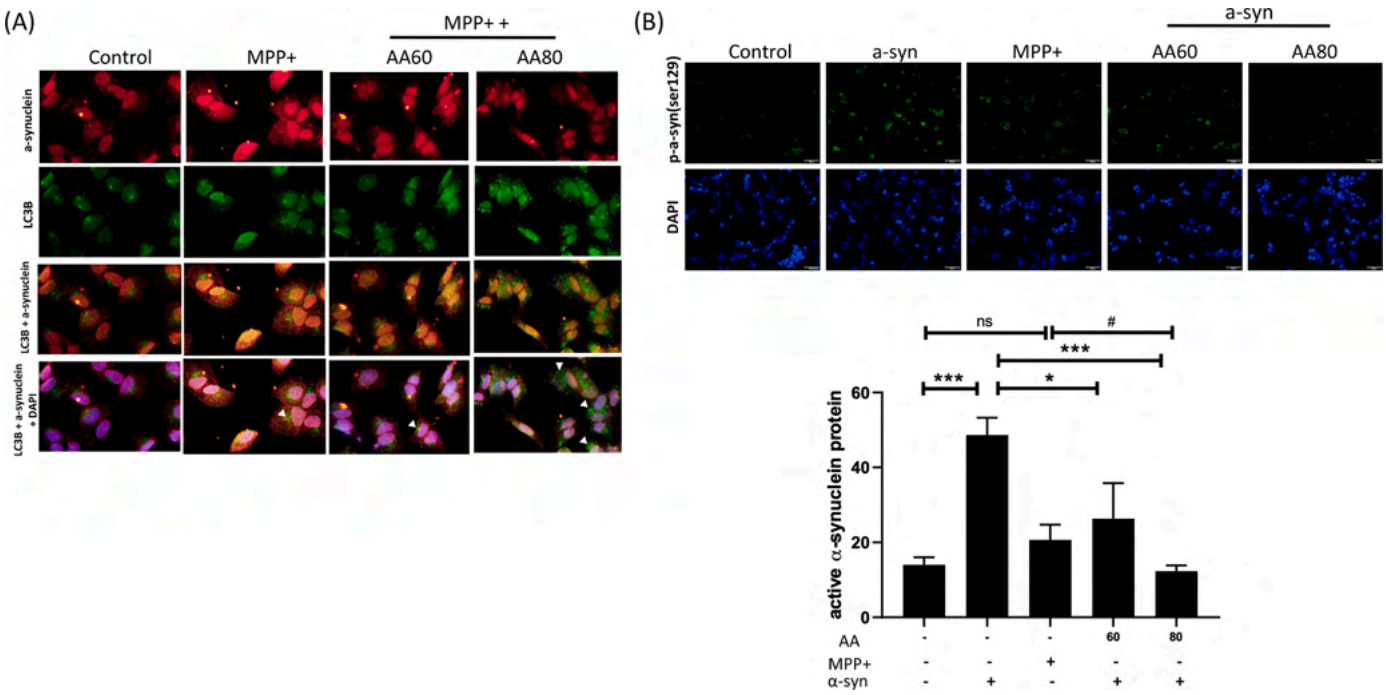

Fig. 6. Protective effect of *A. argyi* (AA) by induction of mitophagy in SH-SY5Y cells. (A) IHC showing localization of the mitotracker (red) with LC3B (an autophagy marker, green) in the AA-treated group. DAPI (blue) staining was used to determine the position of the nuclei. Arrowheads showing the localization of mitochondria. Scale bar- 200 μm (B) Protein expression levels of Drp1, p-DRP1(ser 616) p-DRP1(ser 637). GAPDH was used as internal control.

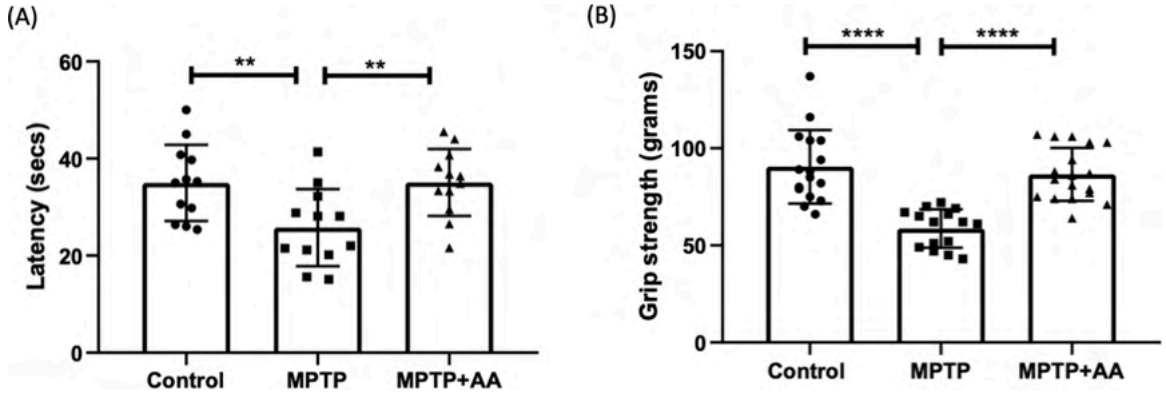

Fig. 7. *A. argyi* (AA) improved motor function in a 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP)-induced Parkinson disease (PD) mouse model. (A) Latency to fall off on the rotarod apparatus. The MPTP- treated mice significantly decreased the latency to fall (**$p < 0.01$) and the treated with AA significantly increased the latency to fall (**$p < 0.01$). (B) In grip strength test, MPTP injection significantly reduced the absolute grip strength (****p <0.0001) which was ameliorated by AA treatment (****p <0.0001). Data are expressed as the mean ± standard deviation. ($n = 6$).

was observed by decreased fluorescence. However, treatment with *A. argyi* recovered the lysosomal activity. This indicate that upregulated TRPML1 is integral to formation of autophagolysosome and maintain the lysosomal function (Kendall and Holian 2021). To further validate the data, we also checked the expression of TRPML1 in the SNpc region which showed increased expression compared to MPTP induced mice.

Impairment of the autophagy pathway has been known to play a central role in neurodegenerative diseases (Ravanan et al., 2017). Previous studies have shown that increased TRPML1 activity can promote autophagy (Tedeschi et al., 2019) and thereby counteract α-synuclein-mediated neuron toxicity (Xilouri et al., 2016). Similarly in our study, TRPML1 also activates autophagy and promotes the degradation of accumulated proteins induced by MPP+ in SH-SY5Y cells. We further found that cells overexpressing α-synuclein active protein also showed reduced α-synuclein toxicity after treatment with *A. argyi*. Similarly, *A. argyi* treatment upregulated LC3B expression in brain tissue sections. In contrast, we did not detect α-synuclein expression in brain sections. This finding was somewhat surprising since previous reports have shown that MPTP administration for over seven days induced α-synuclein aggregation in the substantia nigra of baboons (Kowall et al., 2000). The time required for the development of inclusion bodies or aggregation may have precluded their formation in this study (Maries et al., 2003); therefore, more chronic administration of MPTP could result in the formation of α-synuclein aggregates.

Several studies have indicated that the PI3K/Akt signaling pathway has a remarkable influence on protection against various apoptotic stimuli. Akt is known to be involved in a large number of brain diseases such as PD, cancer, ischemia, and autism. Previous studies have shown that activation of the PI3K/AKT pathway can facilitate the survival of DAergic neurons by inhibiting apoptosis (Wang et al., 2017). Similarly, previous studies also showed that up-regulation of β-catenin can prevent the cells from going into apoptosis, whereas downregulation of β-catenin

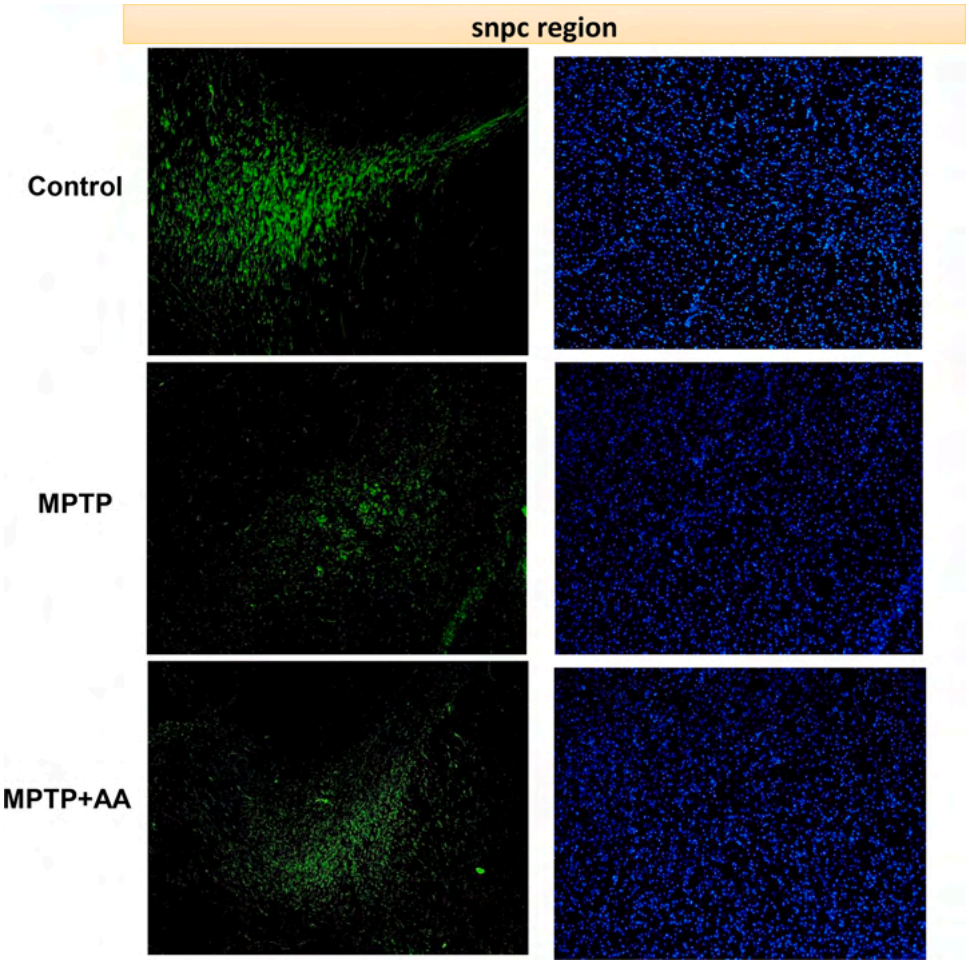

Fig. 8. *A. argyi* can protect dopaminergic neurons and reduce apoptosis in the substantia nigra par compacta (SNpc) of the mice model. Representative data obtained by immunostaining with tyrosine hydroxylase (TH) stain showing TH+ cell bodies in the entire SNpc (left side) (green); DAPI (blue) staining was used to determine the position of the nuclei (right side). Scale bar- 200 μm, ($n = 6$).

can induce MPTP/MPP-induced DAergic cell death (L'Episcopo, Tirolo et al. 2011). Our results are consistent with the previous findings indicating that the MPP+ intervention dramatically downregulated the protein levels of PI3K, p-AKT, and β-catenin in SH-SY5Y cells, and *A. argyi* upregulated the levels of these survival markers to inhibit apoptosis.

Mitochondrial dysfunction is a critical mechanism associated with the pathogenesis of PD. Mitophagy plays an important role in maintaining mitochondrial homeostasis and preventing cell death (Fivenson et al., 2017). Here, we demonstrated that treatment with *A. argyi* reduced MPP+-mediated mitochondrial damage by upregulating mitophagy. Although research on the connection of TRPML1 with mitochondria is limited, one previous study showed that activated MCOLN1/TRPML1 can facilitate the removal of damaged mitochondria (Zhang et al., 2016). So, we hypothesize that the upregulated mitophagy could be due to increased expression of TRPML1, however further studies need to be performed for direct evidence. Interestingly, we also observed that MPP+ induced mitochondrial fragmentation in the cells (fig 6A, mitotracker alone), while administration of *A. argyi* abolished the disruption of mitochondrial integrity. A recent study revealed that Drp1-mediated fission is required for mitophagy, which exerts a protective effect on neurons. However, abnormal mitochondrial fission or dysregulation in mitochondrial dynamics can cause neuronal cell death, suggesting that Drp1 may play a pivotal role in the pathogenesis of PD (Feng et al., 2020). We found that *A. argyi* inhibited Drp1 phosphorylation at serine 616, reducing excessive mitochondrial fission and preserving mitochondrial morphology when compared with MPP+. In addition, we also observed the increase of Drp1 (ser 616) in the control group in western blot but a normal mitochondria morphology in our staining data (fig 6A). This could be because Drp1 is widely distributed in the central nervous system and is highly expressed in the brain (Luo et al., 2020). It is the main regulator of mitochondrial fission but is alone not responsible for the pathological processes (Hu et al., 2017). Furthermore, some studies show endogenous Drp1 is required to mediate mitochondrial autophagy in the heart and their downregulation can upregulate mitochondrial dysfunction (Shirakabe et al., 2016). Therefore, the balance between mitochondrial fission and fusion is required to maintain the structural integrity and overall health of the cells. So, we hypothesize that though our control group has a higher expression of Drp1, it does not necessarily indicate that it is involved in the implication of disease. However, additional mitochondrial fission and fusion markers still need to be checked to confirm this hypothesis. Taken together, our findings suggest that *A. argyi* can exert neuroprotective effects by mitophagy-mediated elimination of impaired mitochondria generated during excessive mitochondrial fission.

To further confirm the degree of neuroprotection achieved by *A. argyi*, we investigated its effects in an *in vivo* model of MPTP-induced PD. We performed the behavioral assessment of mice, and the group treated with *A. argyi* showed improved mobility among parkinsonian mice. Tyrosine hydroxylase (TH) is a rate-limiting enzyme required for dopamine synthesis, and its loss is considered to contribute to DAergic deficiency and induce the onset of PD (Shams et al., 2012). The present

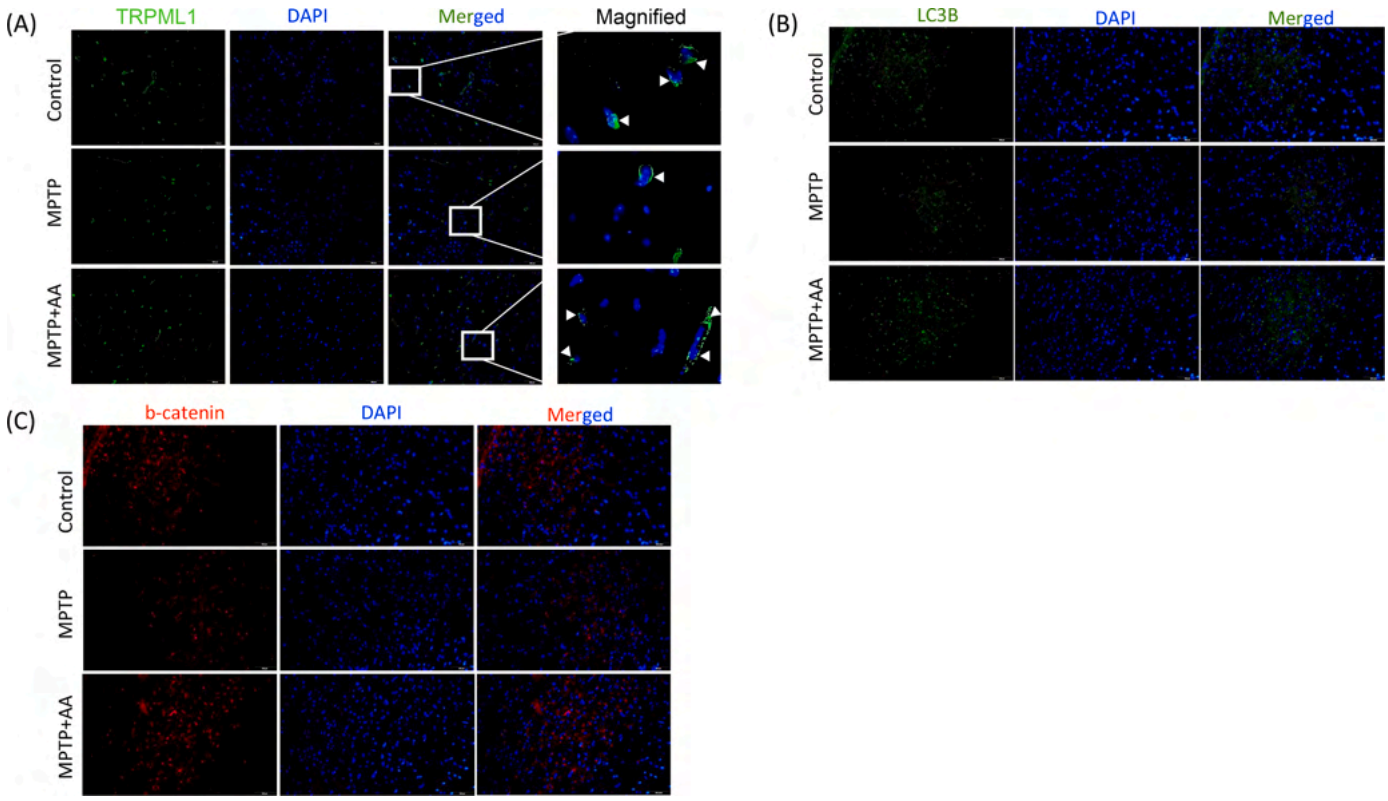

Fig. 9. Expression profile of protein markers in the SNpc in Mouse brain. Representative data obtained by immunostaining with (A) TRPML1(green), arrowheads showing the presence of TRPML1 around the nucleus, (B) LC3B (green), and (C) β-catenin (red). DAPI (blue) staining was used to determine the position of the nuclei. Scale bar- 100 μm, ($n = 6$).

study revealed that *A. argyi* significantly prevented the depletion of TH-positive cells in MPTP-treated mice. These results are similar to those of previous studies, demonstrating that diminished TH expression correlates with behavioral deficits in a toxin-induced animal model of PD (More and Choi 2017). This data suggests that TRPML1 stimulation may protect motor neurons from neurotoxicity by enhancing autophagy. In summary, this is the first study to demonstrate the neuroprotective role of *A. argyi* by upregulating the survival pathway and inducing autophagy in SH-SY5Y cells and mice models.

Conclusion

Our study provided evidence that *A. argyi* efficiently inhibited MPP+-induced cell death in SH-SY5Y cells and exhibited a prominent neuroprotective effect against MPTP-induced brain lesions. However, the active compounds present in the *A. argyi* ethanol extract still need to be explored in further studies to check its efficacy for neuroprotection. Furthermore, exploiting TRPML1 channel can provide mechanistic insight into the molecular targets and how *A. argyi* can modulate the mitochondrial dynamics. Thus, the therapeutic potential of *A. argyi* explored in this study implies that ethanol extract of *A. argyi* leaves is a promising source for the development of new drugs against PD and other neurodegenerative disorders.

Ethics approval and consent to participate

All animal use protocol was approved and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC), Taiwan. All experiments were maintained and approved by Animal center of Tzu Chi University Institutional Animal Care and Use Committees (No. 108-40) (04.2020-03.2021).

CRediT authorship contribution statement

Li-Kung Wu: Conceptualization, Data curation, Investigation, Methodology. **Surbhi Agarwal:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Chia-Hua Kuo:** Methodology. **Yen-Lun Kung:** Formal analysis, Writing – original draft. **Cecilia Hsuan Day:** Data curation. **Pi-Yu Lin:** Writing – original draft. **Shinn-Zong Lin:** Project administration. **Dennis Jine-Yuan Hsieh:** Formal analysis. **Chih-Yang Huang:** Funding acquisition, Project administration, Resources. **Chien-Yi Chiang:** Conceptualization, Funding acquisition, Resources, Writing – review & editing.

Declarations of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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