A Thesis for the Degree of Master of Philosophy in Pharmacology

Protection Against MPP^+-induced Neurotoxicity in SH-SY5Y Cells by Epiceanothic Acid and Betulinic Acid from Jujube(*Ziziphus jujuba* Mill.) via Enhancing TFEB-dependent Lysosomal Biogenesis

신경독성 MPP^+으로 유도한 SH-SY5Y 세포에서

TFEB 의존성 리소좀 생성 증가에 의한

산조인 유래 유효성분물질의 신경 보호 효과

August, 2020

By

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UNDER THE DIRECTION OF PROF. WOONGCHON MAR

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Abstract

Protection Against MPP^+-induced Neurotoxicity in SH-SY5Y Cells by Epiceanothic Acid and Betulinic Acid from Jujube(*Ziziphus jujuba* Mill.) via Enhancing TFEB-dependent Lysosomal Biogenesis

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The incidence of neurodegenerative diseases has been increased with the continuing growth of the old population. Although the causes of neurodegenerative diseases have not been clearly elucidated, recent research studies have suggested that lysosomal dysfunction and impairment of autophagy-lysosomal pathways (ALP) might play an important role in the neuronal cell death that occurs in Parkinson′s disease.

N-methyl-4-phenylpyridinium iodide (MPP^+) induces several cellular alterations, such as inhibition of mitochondrial complex-Ⅰ, production of reactive oxygen species (ROS), impairment of the lysosomal autophagic degradation, and dopaminergic cell death. Also, this lysosomal damage triggers a selective cellular response which is endo-lysosomal damage response (ELDR).

It was reported that inhibition of master growth regulator mTOR complex 1 (mTORC1) activates transcription factor EB (TFEB) by promoting its nuclear translocation. TFEB, a master regulator of lysosomal biogenesis, links mTORC1 signaling to transcriptional control of lysosome homeostasis.

We investigated the neuroprotective effects of isolates from *Zizyphus jujuba*. Five isolates (Jujuboside A, Jujuboside B, 6″-Feruloylspinosin, Epiceanothic acid, Betulinic acid) showed a neuroprotective effect against MPP^+-induced SH-SY5Y cell death. Especially, Epiceanothic acid and Betulinic acid enhanced expression of TFEB, proteins involved in ALP, clearance of autophagosome and selective macroautophagy, termed lysophagy, and protected the leakage of cathepsin D from lysosomes into cytosol against MPP^+-induced SH-SY5Y cells.

These results demonstrate that Epiceanothic acid and Betulinic acid protect neuronal cells by reducing the MPP^+-induced lysosomal membrane permeabilization (LMP), suggesting that they might be possible candidates for the treatment of neurodegenerative diseases.

Key words : Parkinson′s disease, neuroprotection, lysosome biogenesis, TFEB, lysophagy,

Zizyphus jujuba

Student number : 2018-28060

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Lysosomal Biogenesis

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown origin mainly characterized by the loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) and the presence, in the affected brain regions, of ubiquitinated intraneuronal proteinaceous cytoplasmic inclusions called Lewy bodies (Dauer and Przedborski, 2003). The presence of Lewy bodies in PD suggests that autophagosome (AP) accumulation and aggregated proteins (α-synuclein) may contribute to the pathogenesis of the disease. Increased number of AP has been observed in cultured cells intoxicated with parkinsonian neurotoxins, such as MPP^+, rotenone, and 6-OHDA (Chun et al., 2001), and in postmortem PD brain samples (Yazdani et al., 2006).

The lysosome is an acidic organelle and the major digestive compartment in all eukaryotic cells. Lysosomes contain membrane proteins such as lysosome-associated membrane protein 1 (LAMP1), as well as more than 60 resident soluble hydrolases including cathepsins, which are proteases and function effectively in the acidic environment of the lysosomal lumen (pH 4-5) (Appelqvist et al., 2013). Lysosomes are the key degradative compartments of the cell. Lysosomal cathepsins, which are hydrolase enclosed in the lysosomes, help to maintain the homeostasis of the cell's metabolism by participating in the degradation of autophagic material (Serrano-Puebla et al., 2016).

However, the Destabilization of the lysosomal membranes by the lysosomotropic detergents or reactive oxygen species (ROS) promotes apoptosis (Cathepsin released into the cytosol and initiate the lysosomal pathway of apoptosis) (Repnik et al., 2014).

The autophagy-lysosome pathway (ALP) is the major system for the degradation of cellular constituents in eukaryotic cells. Autophagy refers to the global process by which intracellular components are degraded by lysosomes. The degradation and recycling of macromolecules by the ALP a critical role in the regulation of homeostasis as well as in the normal cellular remodeling associated with development and differentiation (Mizushima et al., 2010).

Endo-lysosomal damage response (ELDR) is critical not only for lysosomal homeostasis but also as a defense against invading pathogens and may prevent the propagation of neurotoxic aggregates (Papadopoulos et al., 2017).

One element of this response is the recruitment of factors that help to alleviate and repair the damage, including the chaperone Hsp70, which stabilizes protective lysosomal proteins (Petersen et al., 2010). The second element is the transcriptional induction of new lysosomal components to compensate for the loss of lysosomal capacity. This branch is triggered by a lysosome-located mammalian target of rapamycin (mTOR), which normally keeps transcription factor EB (TFEB) inactive by phosphorylation (Settembre et al., 2013). The third element is called lysophagy which ensures the efficient clearance of the damaged lysosome by selective macroautophagy (Radulovic et al., 2018)

The mTOR, as part of the mTORC1 complex, is a kinase that localizes to lysosomes. This localization is critical for the ability of mTORC1 to integrate signals from growth factor signaling, cellular stress, and nutrient abundance to control various cellular processes including promoting cell growth, regulating metabolism, and repressing autophagy (Yu et al., 2010).

TFEB was mainly localized to the cytoplasm with focal concentrations associated with lysosomes under basal conditions, and it translocated to the nucleus when lysosome function was inhibited or impairment. 14-3-3 proteins as binding partners of TFEB that prevented its nuclear accumulation under conditions of optimal lysosome function. Furthermore, TFEB was recruited to lysosomes through interaction with mTORC1, and mTORC1-dependent phosphorylation of TFEB was required for its interaction with 14-3-3 to prevent nuclear translocation. Collectively, these findings support a model for lysosome homeostasis in which lysosome status is communicated to TFEB through mTORC1 to prevent nuclear localization of TFEB when lysosome function is optimal. In response to impaired lysosome function, this pathway promotes translocation of TFEB to the nucleus where it increases the expression of genes encoding lysosomal proteins, (LAMP-1) (Roczniak-Ferguson et al., 2012).

Sensing of lysosomal membrane permeabilization exploits the exposure of glycosylated proteins to cytosolic components as a unique feature of ruptured lysosomes. A group of cytosolic galectins has emerged as specific sensors for ELDR caused by distinct factors, such as lysosomotropic agents.

Among these sensors, galectin-1, -3, -8, and -9 bind exposed β-galactosides at the inner lysosomal membrane and elicit downstream events. Especially Galectin-3 (Gal-3) is used as a general and sensitive reporter for endo-lysosomal damage (Hasegawa et al., 2015). Gal-3 recruits the tripartite motif (TRIM) protein TRIM16, a subfamily of the RING-type E3 ubiquitin ligases, which serve as a platform for autophagic initiation factors (ULK1, ATG16L1) that in turn induce phagophore formation. Also extensive ubiquitination of lysosomal proteins (Chauhan et al., 2016).

Lysosomal protein ubiquitination and the TRIM16 - Gal-3 complex act in concert to recruit initiation factors of the autophagic machinery, such as ATG16L1 and ULK1, to trigger local phagophore formation. Besides, ubiquitination, likely K63-linked ubiquitin chains, recruits the autophagic receptor p62, which bridges the ubiquitinated cargo to the LC3 positive phagophore to mediate engulfment by the autophagosomal membrane (Fraiberg et al., 2016).

Among several neurotoxin models of PD, cellular models induced by MPP^+, the active metabolite of MPTP, have been the most widely studied to elucidate the mechanisms of selective dopaminergic neuron death (Dagda et al., 2013).

Increased numbers of autophagosomes and impairment of lysosome membrane stability in these models have been reported by several groups (Miyara et al., 2016). We investigated the effect of five isolates from *Zizyphus jujube* against exposure MPP^+ (250 μM,24h) on autophagy-lysosome processes in SH-SY5Y.

Jujube belonging to the Rhamnaceous family grows mostly in Europe, southern and eastern Asia, and Australia, especially the inland region of northern China. Jujube has a long history of usage as fruit and remedy. The main biologically active components are vitamin C, phenolics, flavonoids, triterpenic acids, and polysaccharides. And biological effects are the anti-cancer, anti-inflammatory, anti-obesity, immunostimulant, antioxidant, hepatoprotective, and gastrointestinal protective activities and inhibition of foam cell formation in macrophages (Gao et al., 2013).

This study may be useful for PD patients and potential drug or food interactions. Also, Our results highlight the integration of the lysosomal biogenesis pathways and have important implications for cell homeostasis and cell survival.

2. Material and Method

2-1. Chemicals and Reagent

MPP^+, CCK-8, Lysosome isolation kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD, USA). Hybond® - polyvinylidene difluoride (PVDF) membranes were purchased from Amersham Pharmacia Biotechnology Inc. (Piscataway, NJ, USA). PRO-PREP protein extraction solution and WEST-Queen ECL solution were purchased from iNtRON Biotech Inc. (Kyunggil, Korea). First antibody (DAPI, Beta-actin, LC3-beta, α-synuclein, mTOR, p-mTOR, ULK-1, p-ULK-1, TFEB, p-TFEB, LAMP-1, Cathepsin D, TRIM16, Gal-3, K-63 ubiquitin, p62), secondary antibody, and FITC-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)

2-2. Cell culture

The human neuroblastoma cell line SH-SY5Y (ATCC NO. CRL-2266) was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO2 atmosphere.

2-3. Measurement of cell viability

SH-SY5Y cells were seeded at a density of 1x105 cells/200μL/well in 96-well plates for 24 h, and the cells were treated with isolated compounds from Z. jujuba and MPP^+ for 24h. Cell viability was determined using a CCK-8 and measured by ELISA (Koo et al., 2011).

2-4. Measurement of protein expression

The cells were collected, washed with PBS, and lysed with a PRO-PREP protein extraction solution at -20°C for 20 min. After centrifugation at 13,000 x g for 30 min, the supernatant was used as the total protein extracts. Western blot analysis was accomplished as the previously described method (Ham et al., 2013).

2-5. Measurement of the fluorescence microscope

The culture dish was coated with 0.2% gelatin at 37°C for 30 min and dried at room temperature (RT) on a clean bench. The SH-SY5Y cells were plated at a density of 5x104 cells/200μL/well in coated dishes and treated with isolated compounds from Z. jujuba and MPP^+ for 24h. After treatment, the cells were washed with 1x PBS/Tween-20 buffer (Ph 7.4) (PBST) once. The cells were fixed with 4% paraformaldehyde for 30 min at RT.

After washing with PBST, the blocking steps were performed with 1% BSA in PBST. Next, the cells were incubated with the primary antibody at 4°C overnight. The next day, the cells were washed with PBST 3 times and incubated with FITC-conjugated secondary antibody for 1h at RT. After 1h, the cells were washed with PBST 3 times, and DAPI staining was performed for 5 min at RT. Lastly, the PBST washing and mounting steps were conducted.

2-6. Statistical analysis

All experimental data are expressed as mean value ± standard deviation. Statistical significance between multiple groups was determined by one-way ANOVA (PRISM Graph Pad, San Diego, CA, USA). When ANOVA had a significant difference, post hoc Bonferroni's multiple comparison tests were conducted. Data represent the mean SD of three independent experiments (***$p<0.001$ vs control group, #$p<0.05$, ##$p<0.01$, and ###$p<0.001$ vs MPP^+-induced group)

3. Result

3.1 Lysosomal depletion precedes AP accumulation and cell death in low MPP^+-induced SH-SY5Y cells.

Numerous adverse effects of high-dose MPP^+ on SH-SY5Y cells have been reported, while low dose effects are not as well studied. To establish a mild MPP^+ toxicity cellular model for PD, SH-SY5Y cells were exposed to low-dose MPP^+ (50-250μM) for 24h, and cell viability was evaluated, using the CCK-8 assay. These MPP^+ concentrations decreased cell viability in a concentration-dependent manner.

(Fig. 1) Based on this result, concentrations of 50 and 250μM MPP^+ for 24h, which decreased cell viability to 92.1% ± 4.1% and 73.2% ± 3.7% of the control.

When lysosomal ruptured, Cathepsin D is released into the cytosol from the lysosome (Oberle et al., 2010). So we examined the effects of MPP^+ on lysosomal depletion processes by measuring Lysosome Associated Membrane Protein 1(LAMP1) and Cathepsin D, using Lysosome Isolation Kit and Western blot assay. LAMP1 was used as a lysosomal marker. Cathepsin D was used as a lysosomal hydrolase marker. These MPP^+ concentrations decreased LAMP1 level and increased cytosol Cathepsin D level in a concentration-dependent manner.

(Fig. 2) Based on this result, concentrations of 50 and 250μM MPP^+ for 24h, which decreased LAMP1 to 89.3% ± 2.3% and 12.4% ± 2.2% of the control.

Next examined the effects of MPP^+ on autophagic processes by measuring the expression of the autophagosome marker microtubule-associated protein 1 light chain 3 (LC3)-II , the expression of the autophagic receptor marker p62. Also, The expression of the aggregated protein marker, α-synuclein. LC3 is synthesized as pro-LC3, and its C-terminal is immediately cleaved by cysteine protease autophagy-related 4 to generate the cytosolic form LC3-I. In the autophagic process, LC3-I is conjugated to the membrane lipid phosphatidylethanolamine to become the autophagosome membrane-bound form LC3-II (Kabeya et al., 2000). These MPP^+ concentrations increased p62, α-synuclein, and LC3-II levels in a concentration-dependent manner.

(Fig. 3) Based on this result, concentrations of 50 and 250μM MPP^+ for 24h, which increased LC3-II to 23.1% ± 3.1% and 116.4% ± 3.9% of the control.

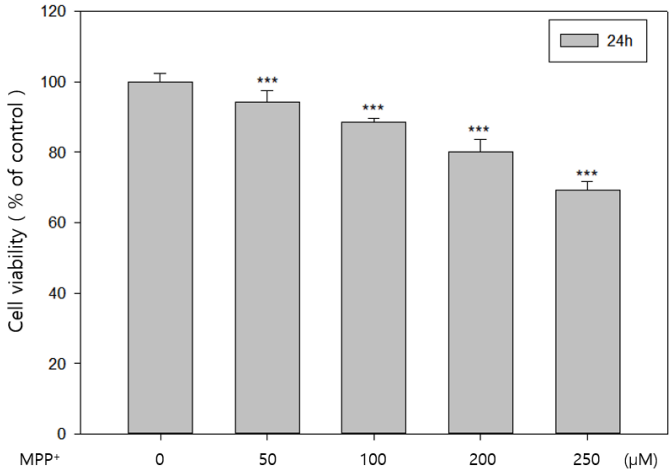


Fig. 1 The effects of low MPP^+ concentrations on the viability of SH-SY5Y cells.

SH-SY5Y cells were cultured in 96-well plate and exposed to low concentrations (50-250μM) of MPP^+ for 24h. cell viability was measured using a CCK-8 assay. Data are expressed as the mean percentage (± SD) of the controls of three independent experiments. ***$p < 0.001$ versus control

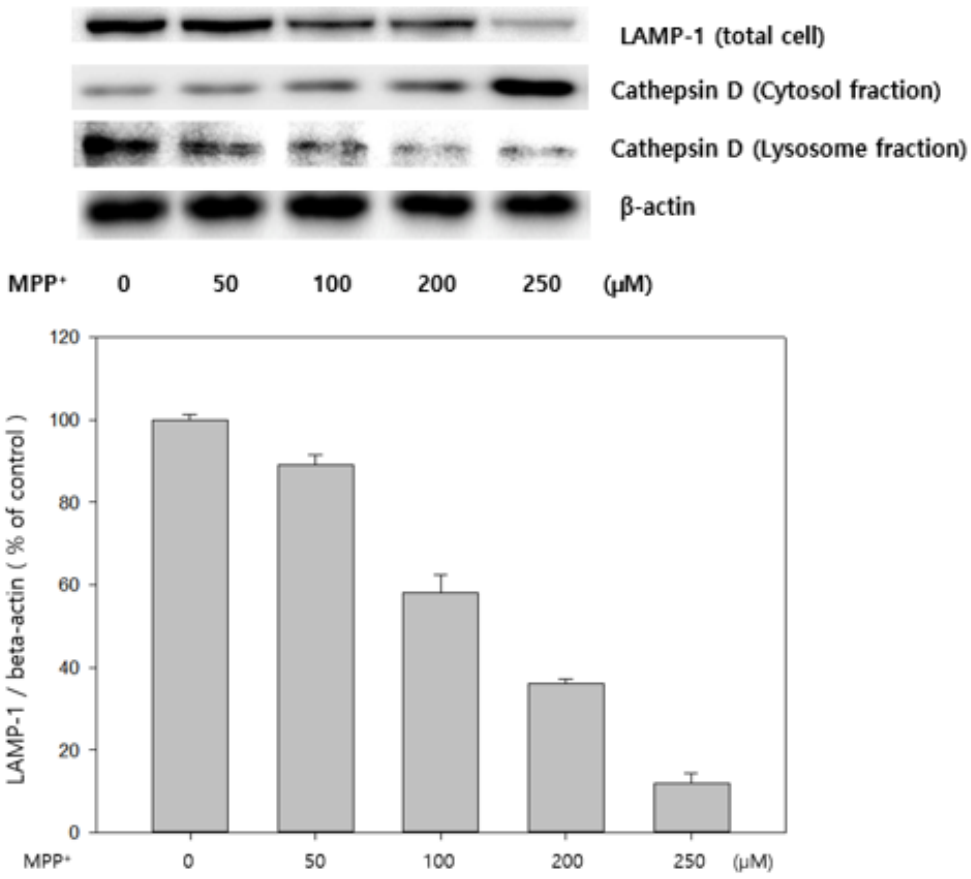


Fig. 2 The effects of low MPP^+ concentrations on the lysosome of SH-SY5Y cells

SH-SY5Y cells were exposed to low concentrations (50-250μM) of MPP^+ for 24h. The level of LAMP1 and Cathepsin D were determined by western blot; β-actin was used as a housekeeping protein. Cathepsin D levels in pellet and supernatant lysosomal fractions from UT and MPP^+-induced cells. Representative data from three independent experiments are shown.

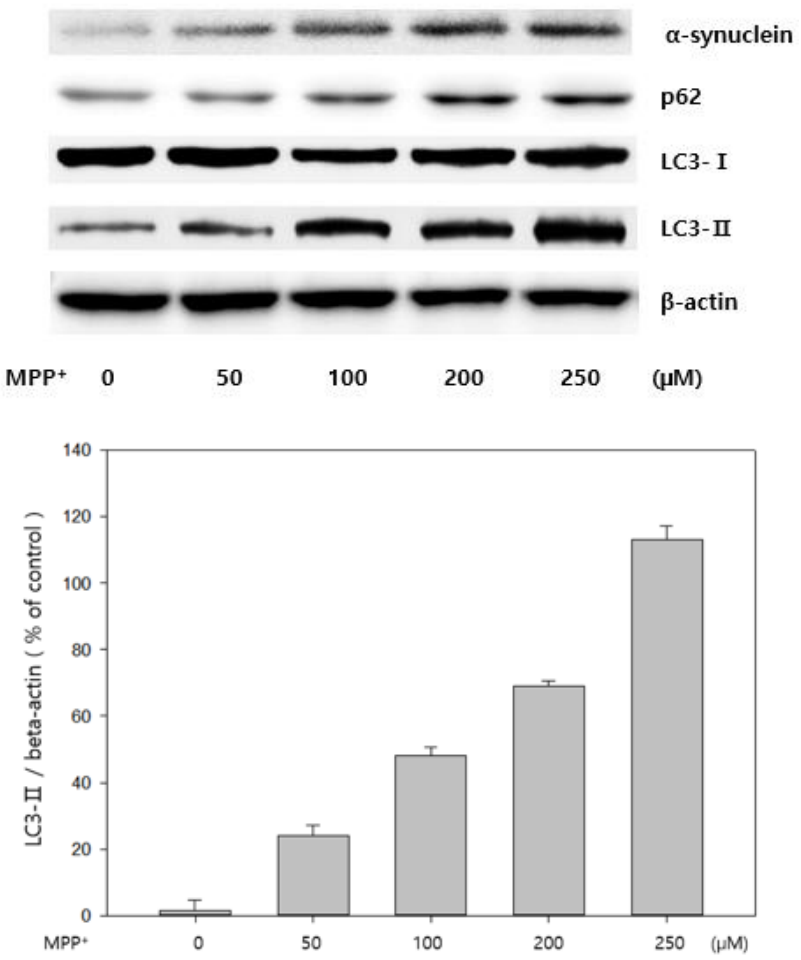


Fig. 3 The effects of low MPP^+ concentrations increased the number of autophagosomes of SH-SY5Y cells.

SH-SY5Y cells were exposed to low concentrations (50-250μM) of MPP^+ for 24h. The level of p62, α-synuclein, LC3-I and LC3-II were determined by western blot; β-actin was used as a housekeeping protein. Representative data from three independent experiments are shown.

3.2 Protective effect of five isolates against MPP^+-induced SH-SY5Y cell death.

Cells were cultured in 96-well plates, and each compounds (Fig. 4) was simultaneously treated with MPP^+ (250μM) for 24h. Cell viability was measured by CCK-8 assay.

(Fig. 5) Based on this result, Each EC_{50} value is (a) Jujuboside-A 0.52μM, (b) Jujuboside-B 0.77μM, (c) 6”-Feruloylspinosin 97.12μM, (d) Epiceanothic acid 0.69μM, and (e) Betulinic acid 40.24μM

Data are expressed as the mean percentage (± SD) of the controls of three independent experiments.

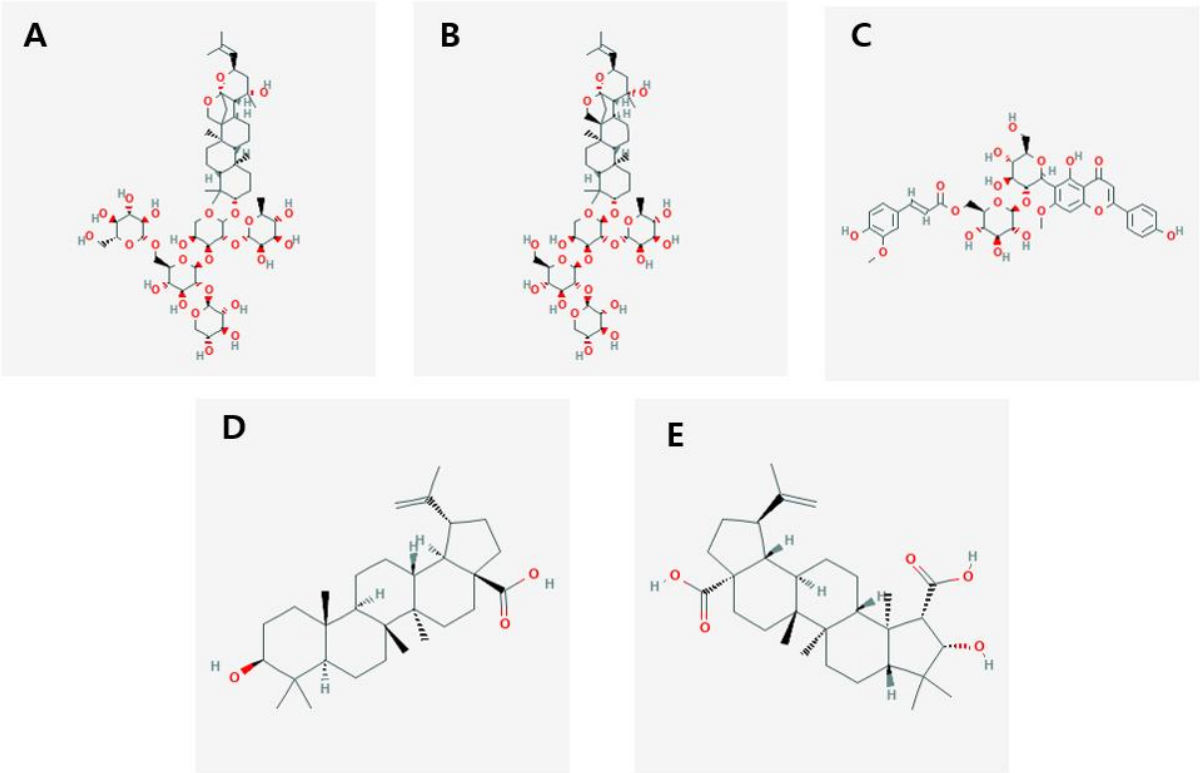


Fig. 4 Chemical structure of five isolates from Jujube (*Ziziphus jujube* Mill.)

(a) Jujuboside-A, (b) Jujuboside-B, (c) 6”-Feruloylspinosin, (d) Epiceanothic acid, (e) Betulinic acid

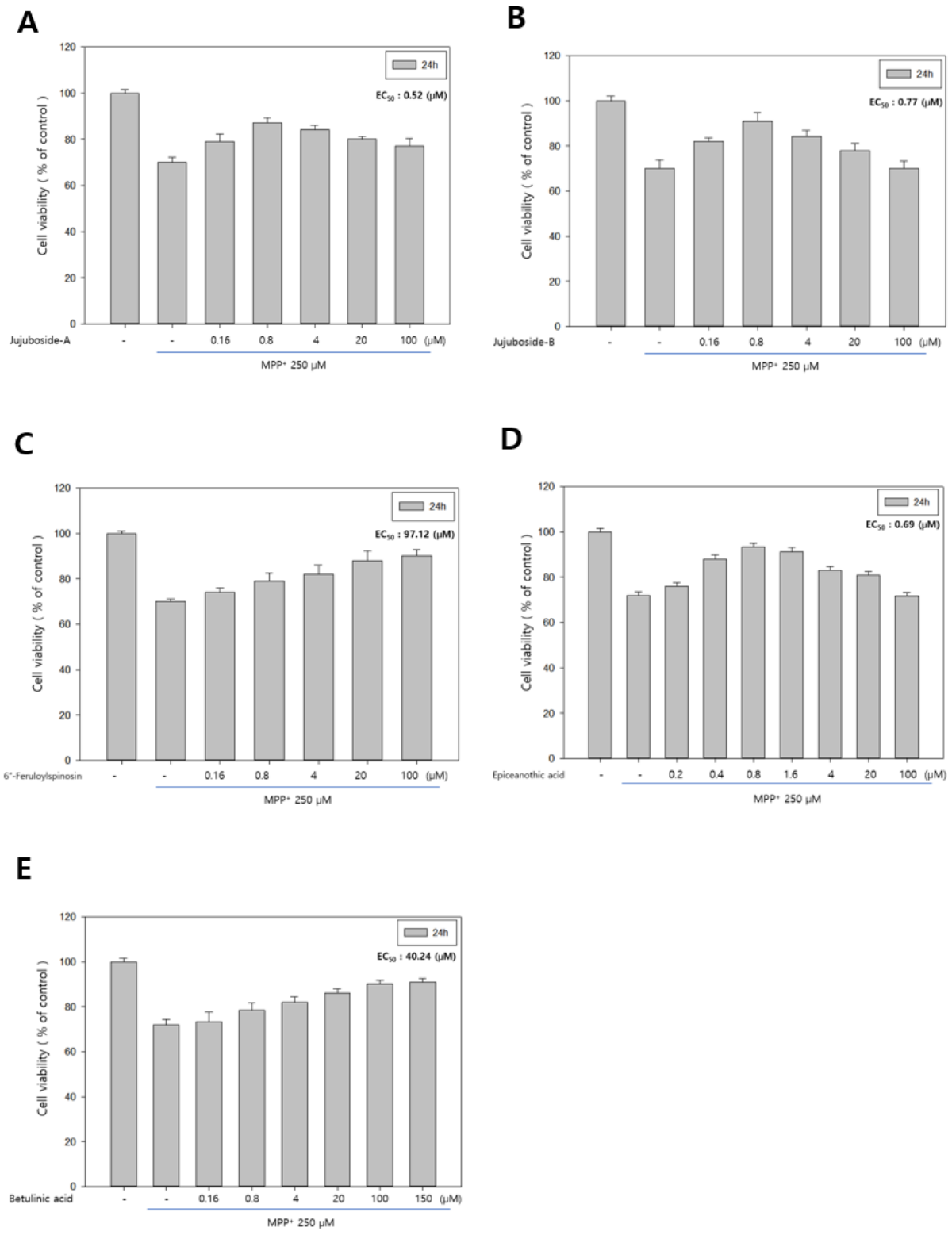


Fig. 5 Protective effect of five isolates against MPP^+-induced SH-SY5Y cell death.

(a) Jujuboside-A, (b) Jujuboside-B, (c) 6”-Feruloylspinosin, (d) Epiceanothic acid, (e) Betulinic acid

3.3 Inhibitory effect of Epiceanothic acid and Betulinic acid on mTOR activity in MPP^+-induced SH-SY5Y cells.

Based on studied, Mammalian targets of rapamycin (mTOR) play a crucial role in the regulation of autophagy flux, including the formation of phagophore and autophagosome, the degradation of autolysosomes, and the reformation of autophagic lysosomes. Also, Unc51-like kinase 1 (ULK1) is a critical initiator of autophagy, and its activity is mainly regulated by being phosphorylated by mTORC1. Upon cellular stress, ULK1 is released from mTORC1, which has been inhibited, and is activated through being phosphorylated. So we tested effect of Epiceanothic acid and Betulinic acid on mTOR activity in MPP^+-induced SH-SY5Y cells.

Treatment of SH-SY5Y cells with MPP^+ 250μM alone didn't effect on mTOR, p-mTOR, ULK1, p-ULK1 expression. However, treatment with MPP^+ + Epiceanothic acid (0.2, 0.4, 0.8μM) (Fig. 6-a), and MPP^+ + Betulinic acid (4, 20, 100μM) (Fig. 6-b) decreased the mTOR activity and increased ULK1 activity in a concentration-dependent manner.

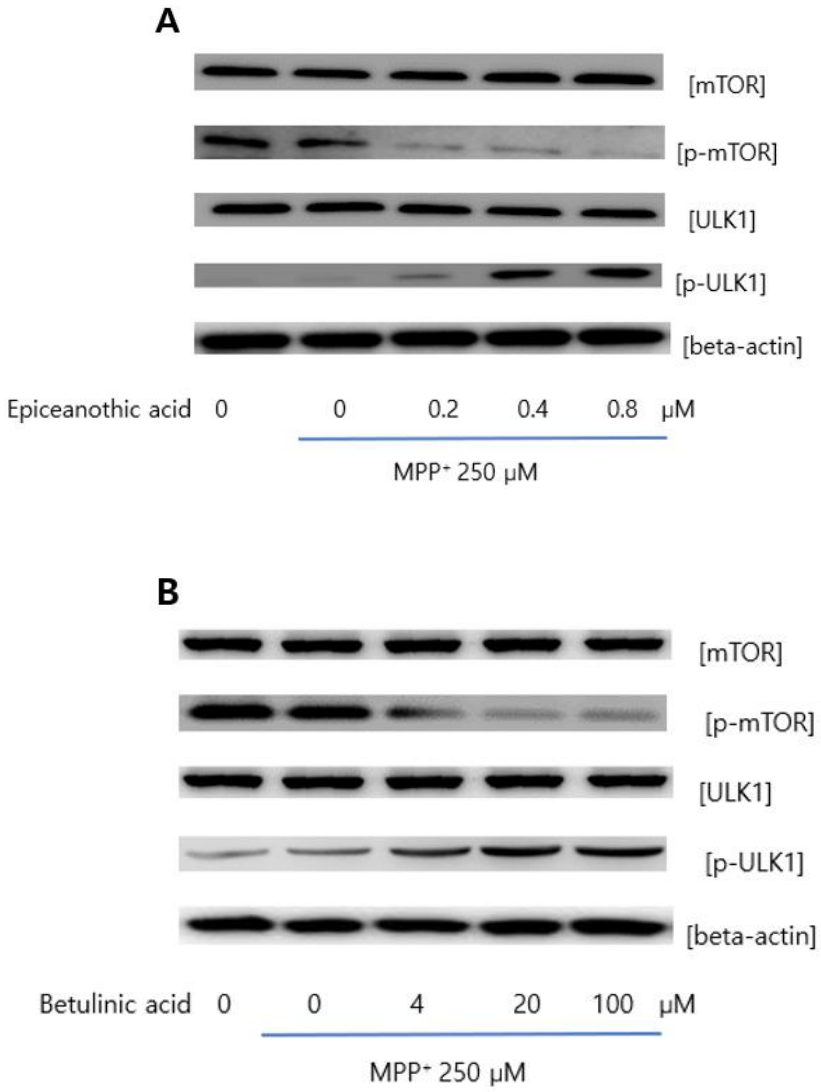


Fig. 6 Epiceanothic acid and Betulinic acid inhibited mTOR activity and enhanced ULK1 activity in MPP^+-induced SH-SY5Y cells

SH-SY5Y cells were simultaneously treated with different concentrations of compounds with MPP^+ (250μM) for 24h. The level of mTOR, p-mTOR, ULK1, and p-ULK1were determined by western blot; β-actin was used as a housekeeping protein. Representative data from three independent experiments are shown.

3.4 Protective effect of Epiceanothic acid and Betulinic acid on lysosome condition in MPP^+-induced SH-SY5Y cells.

We found that Epiceanothic acid and Betulinic acid inhibited mTOR activity in MPP^+-induced SH-SY5Y cells. Also, Before we found that Lysosomal depletion occurred in MPP^+-induced SH-SY5Y cells. Based on studied, TFEB, a master regulator of lysosomal biogenesis, regulated by mTORC1. When inhibition of mTORC1, activates TFEB by promoting its nuclear translocation. The lysosome maintains cellular homeostasis and mediates a variety of physiological processes, including cellular clearance, lipid homeostasis, energy metabolism, plasma membrane repair, and pathogen defense. All these processes are essential to cells. So we tested the effect of Epiceanothic acid and Betulinic acid on lysosome condition in MPP^+-induced SH-SY5Y cells.

Treatment of SH-SY5Y cells with MPP^+ + Epiceanothic acid (0.2, 0.4, 0.8, 1.6μM), and MPP^+ + Betulinic acid (4, 20, 100, 150μM) enhanced lysosome biogenesis detected by immunoblotting (Fig. 7-a,b) and immunofluorescence (Fig. 7-c,d) of the TFEB protein.

Treatment of SH-SY5Y cells with MPP^+ + Epiceanothic acid (0.2, 0.4, 0.8, 1.6μM), and MPP^+ + Betulinic acid (4, 20, 100, 150μM) increased the number of lysosomes and detected by immunoblotting (Fig. 8-a,b) and immunofluorescence (Fig. 8-c,d) of the LAMP1 protein.

Treatment of SH-SY5Y cells with MPP^+ + Epiceanothic acid (0.2, 0.4, 0.8μM), and MPP^+ + Betulinic acid (4, 20, 100μM) alleviated LMP detected by immunoblotting of the Cathepsin D protein. (Fig. 8-a,b)

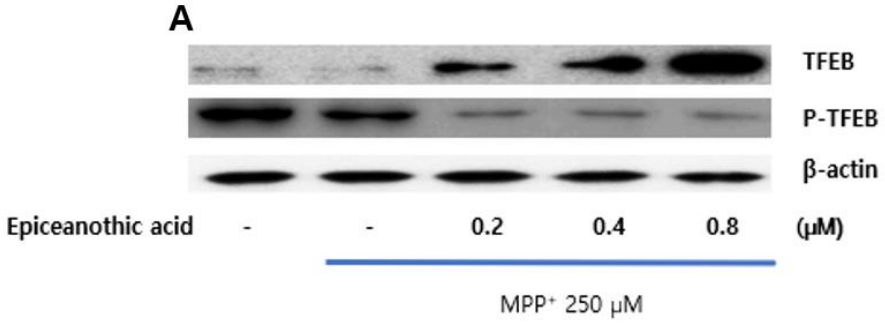
(Fig. 7-e) Based on this result, Each TFEB EC_{50} value using immunoblotting is MPP^+ + Epiceanothic acid 0.39μM, and MPP^+ + Betulinic acid 84.82μM.

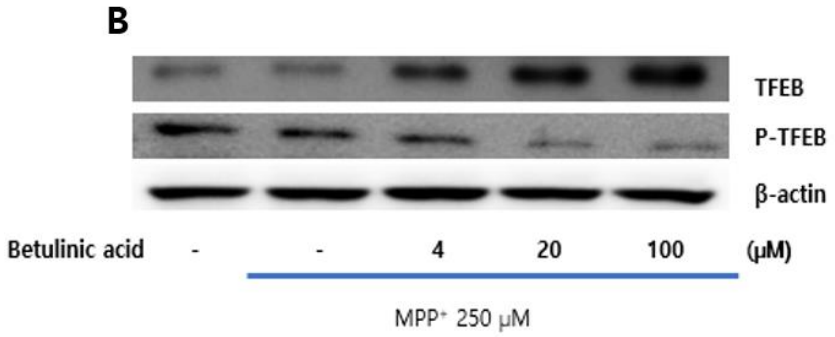
(Fig. 7-f) Each TFEB EC_{50} value using immunofluorescence is MPP^+ + Epiceanothic acid 0.03μM, and MPP^+ + Betulinic acid 2.34μM.

(Fig. 8-e) Each LAMP1 EC_{50} value using immunoblotting is MPP^+ + Epiceanothic acid 0.49μM, and MPP^+ + Betulinic acid 98.21μM.

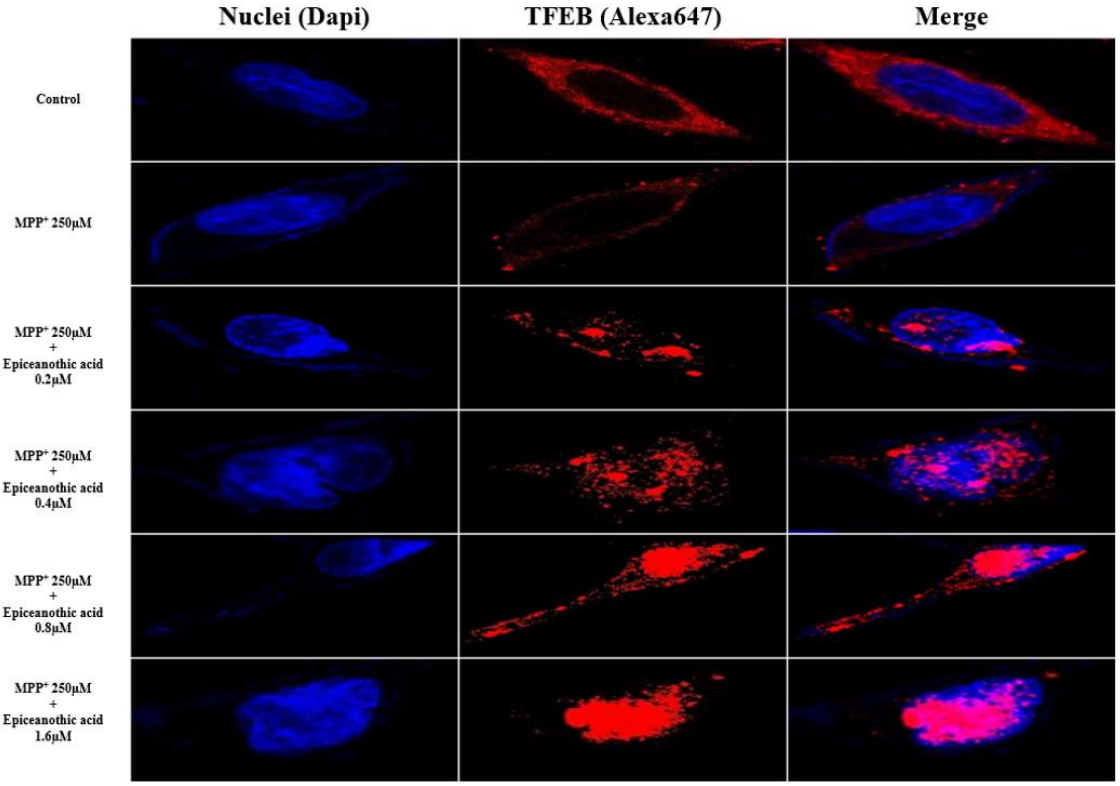
(Fig. 8-f) Each LAMP1 EC_{50} value using immunofluorescence is MPP^+ + Epiceanothic acid 0.93μM, and MPP^+ + Betulinic acid 108.1μM.

(Fig. 8-g) Each lysotracker EC_{50} value is MPP^+ + Epiceanothic acid 0.42μM, and MPP^+ + Betulinic acid 92.4μM.

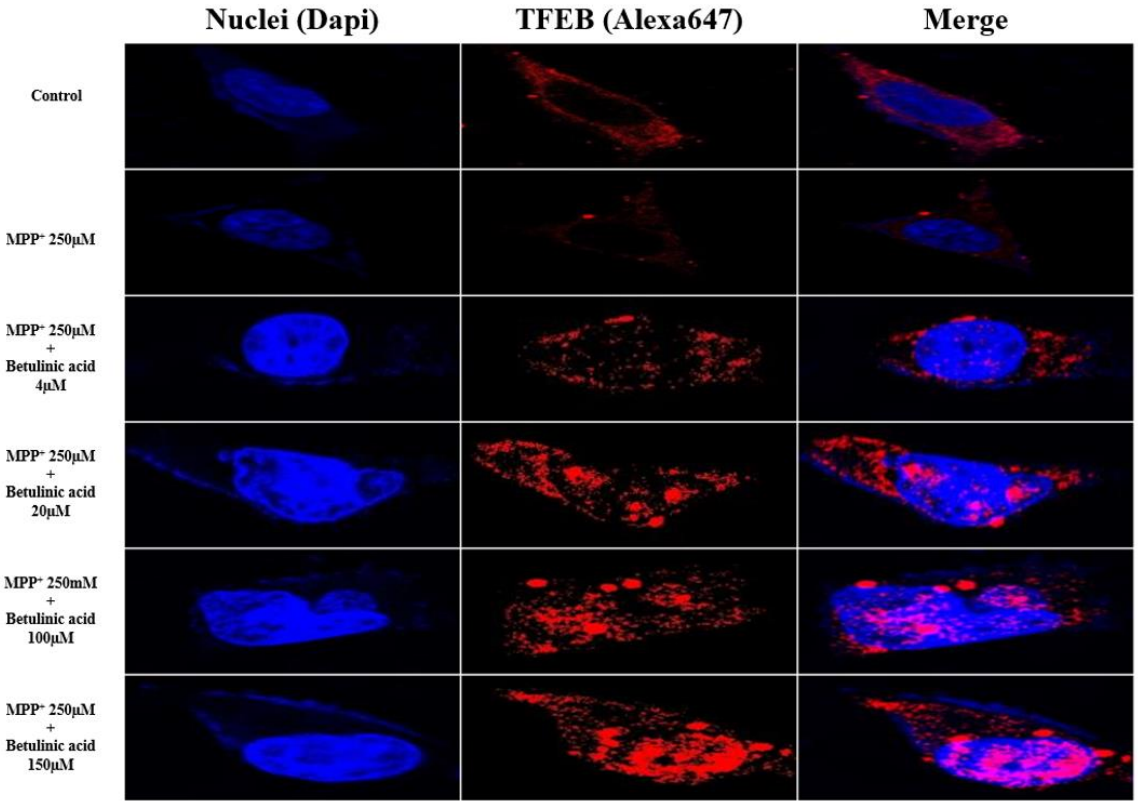

A
TFEB
P-TFEB
β-actin
Epiceanothic acid
-
-
0.2
0.4
0.8
(μM)
MPP+ 250 μM


B
TFEB
P-TFEB
β-actin
Betulinic acid
-
-
4
20
100
(μM)
MPP+ 250 μM

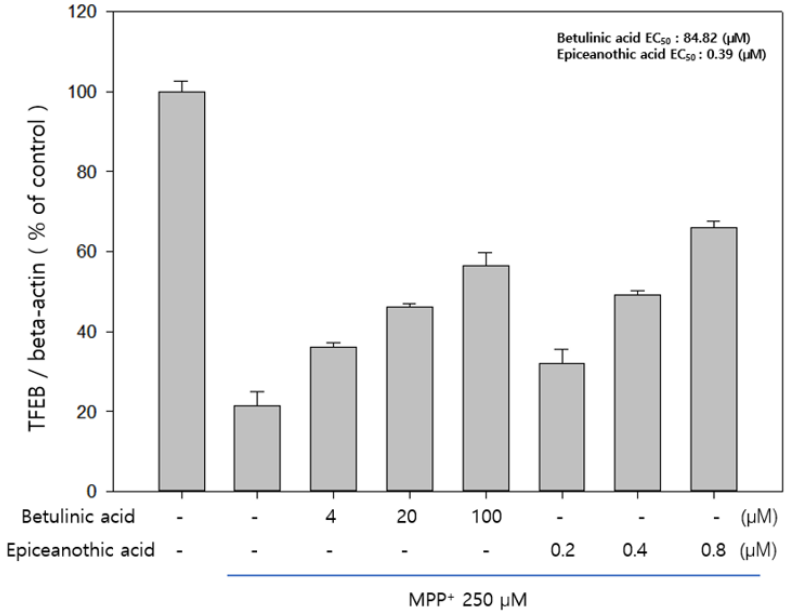
C


Nuclei (Dapi)
TFEB (Alexa647)
Merge
Control
MPP+ 250μM
MPP+ 250μM + Epiceanothic acid 0.2μM
MPP+ 250μM + Epiceanothic acid 0.4μM
MPP+ 250μM + Epiceanothic acid 0.8μM
MPP+ 250μM + Epiceanothic acid 1.6μM

D


Nuclei (Dapi)
TFEB (Alexa647)
Merge
Control
MPP+ 250μM
MPP+ 250μM + Betulinic acid 4μM
MPP+ 250μM + Betulinic acid 20μM
MPP+ 250mM + Betulinic acid 100μM
MPP+ 250μM + Betulinic acid 150μM

E


Betulinic acid EC50 : 84.82 (μM)
Epiceanothic acid EC50 : 0.39 (μM)
TFEB / beta-actin (% of control)
120
100
80
60
40
20
0
Betulinic acid - - 4 20 100 - - - (μM)
Epiceanothic acid - - - - - 0.2 0.4 0.8 (μM)
MPP+ 250 μM

F

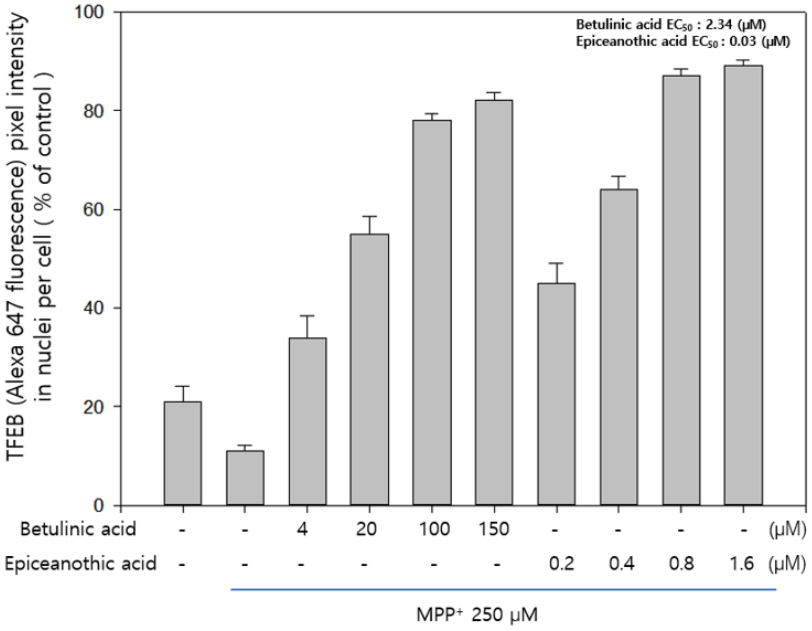
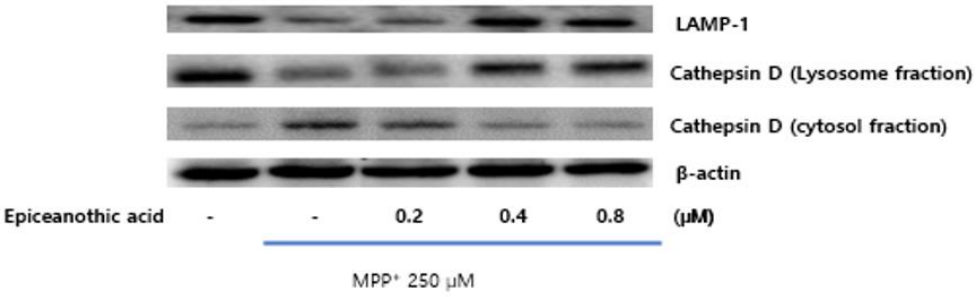

Betulinic acid EC50 : 2.34 (μM)
Epiceanothic acid EC50 : 0.03 (μM)
TFEB (Alexa 647 fluorescence) pixel intensity in nuclei per cell (% of control)
100
80
60
40
20
0
Betulinic acid - - 4 20 100 150 - - - - (μM)
Epiceanothic acid - - - - - - 0.2 0.4 0.8 1.6 (μM)
MPP+ 250 μM

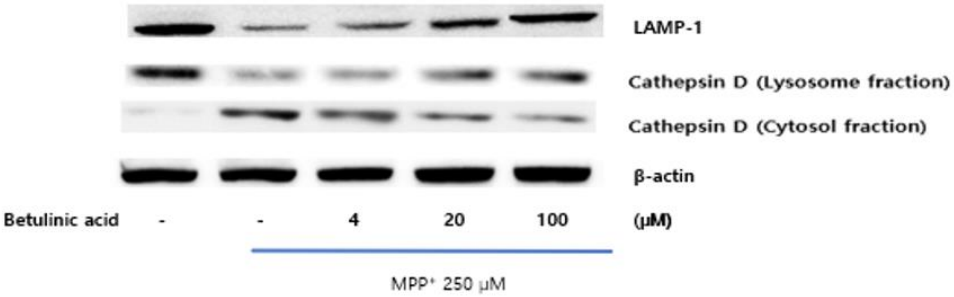
Fig. 7 Epiceanothic acid and Betulinic acid enhanced TFEB nuclear location in MPP+-induced SH-SY5Y cells.

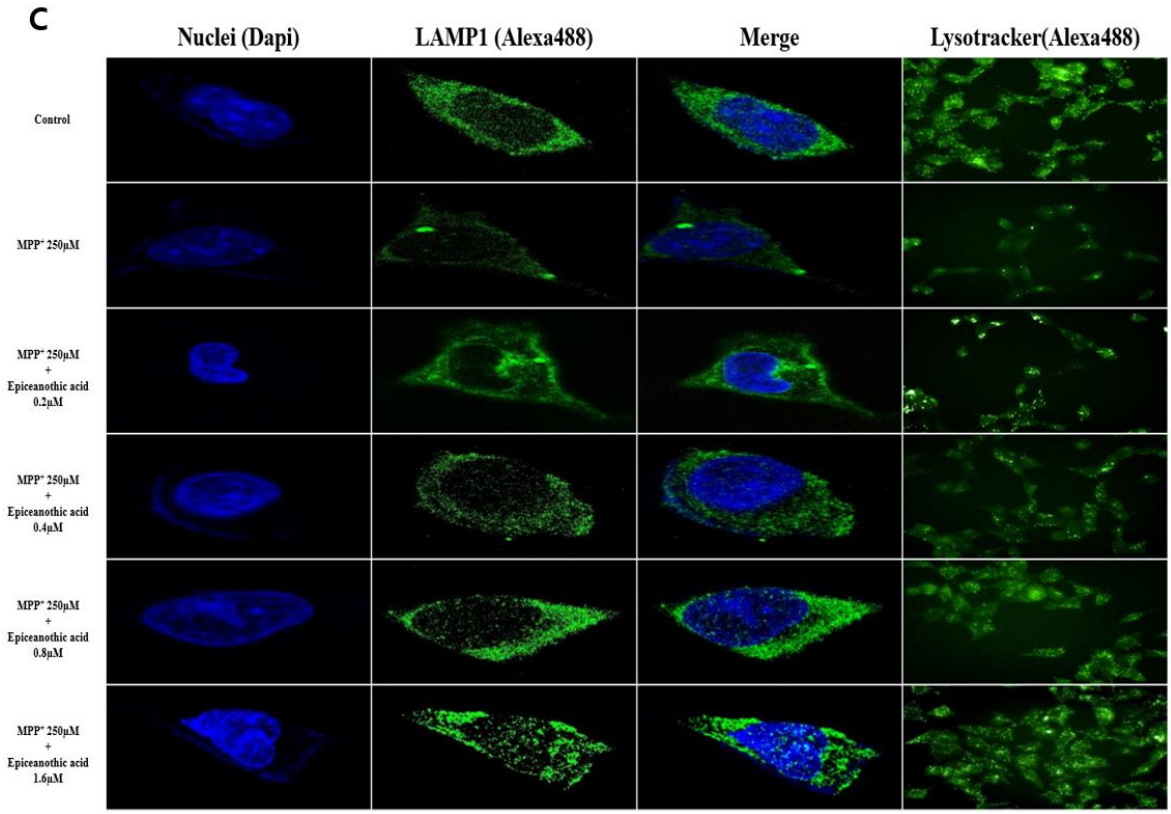
SH-SY5Y cells were simultaneously treated with different concentrations of compounds with MPP^+ (250μM) for 24h. The immunoblotting level of TFEB and p-TFEB were determined by western blot; β-actin was used as a housekeeping protein. The immunofluorescence level of TFEB were determined by CONFOCAL fluorescence microscope (600x). The nuclei were visualized DAPI (blue) staining. TFEB were detected with Alexa 647 (red)-conjugated antibody. Quantification of the number of cells in the four condition. Representative data from three independent experiments are shown. Data are expressed as the mean percentage (± SD) of the controls of three independent experiments.

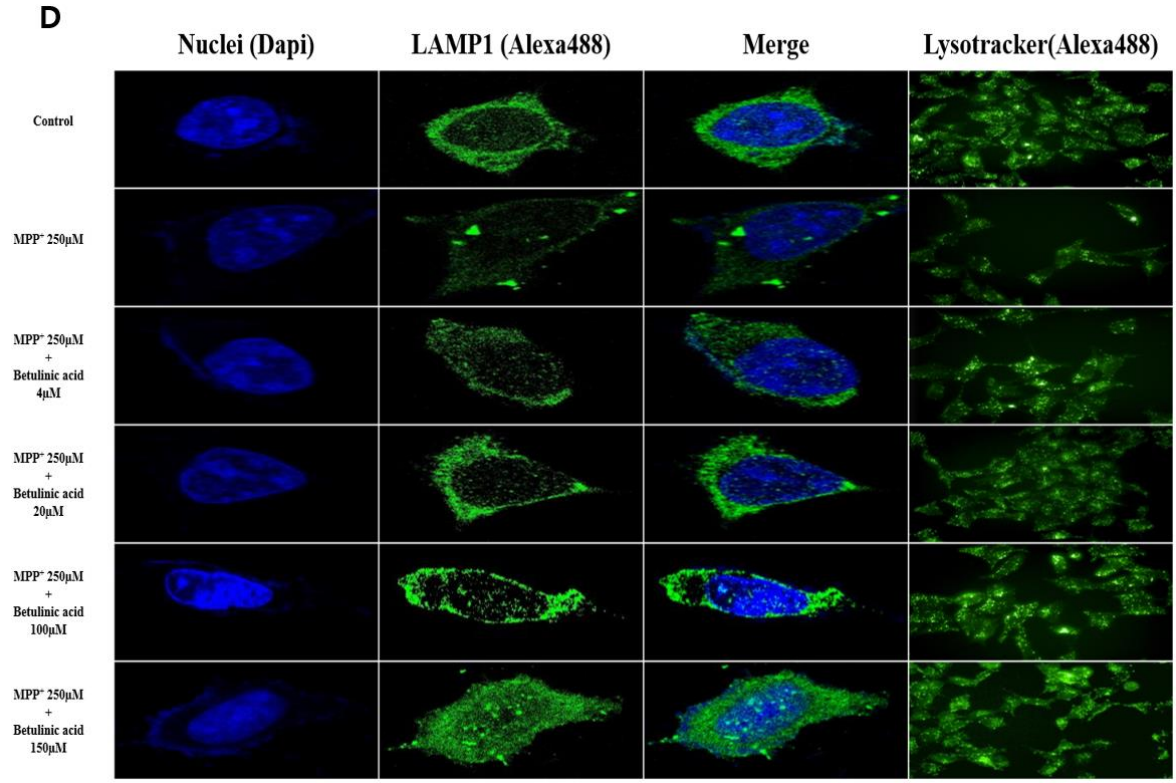
A


LAMP-1
Cathepsin D (Lysosome fraction)
Cathepsin D (cytosol fraction)
β-actin
Epiceanothic acid - - 0.2 0.4 0.8 (μM)
MPP⁺ 250 μM

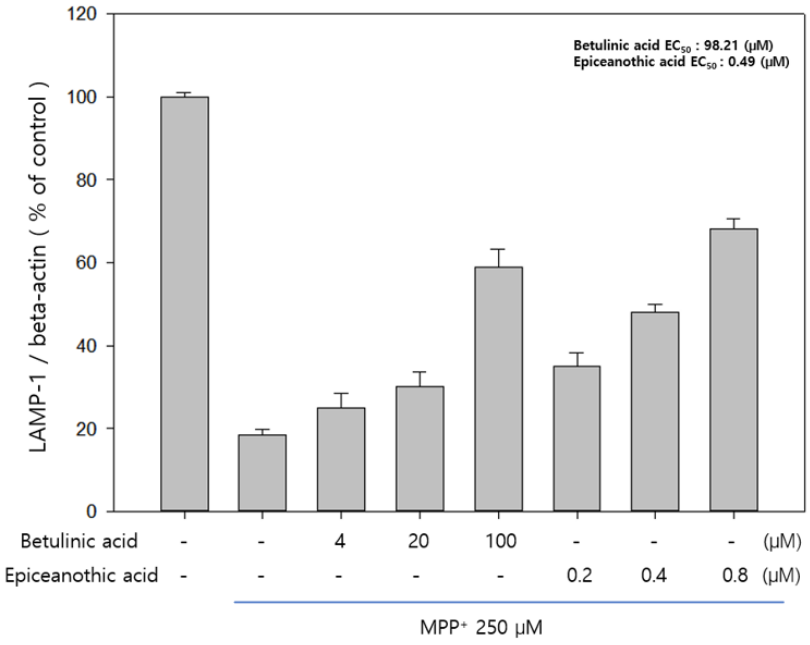
B


LAMP-1
Cathepsin D (Lysosome fraction)
Cathepsin D (Cytosol fraction)
β-actin
Betulinic acid - - 4 20 100 (μM)
MPP⁺ 250 μM

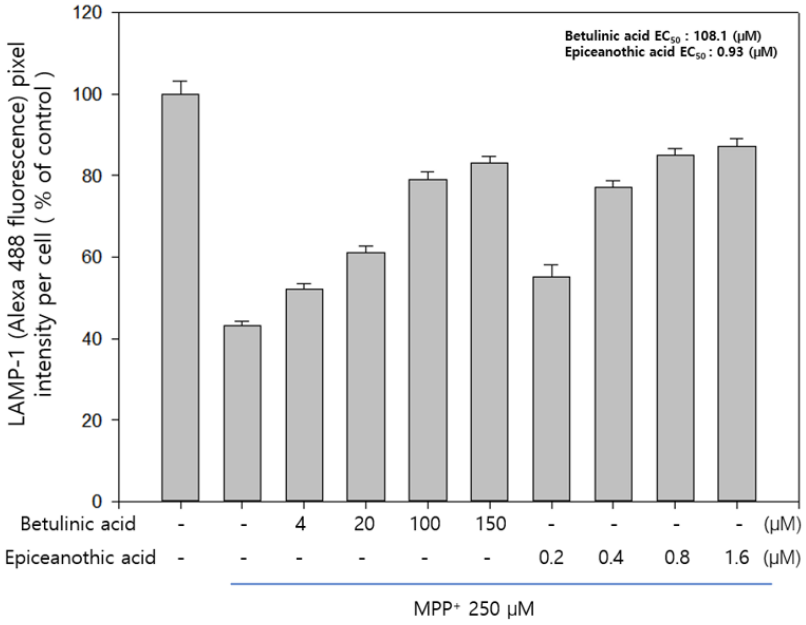

C
Nuclei (Dapi)
LAMP1 (Alexa488)
Merge
Lysotracker(Alexa488)
Control
MPP+ 250μM
MPP+ 250μM + Epiceanothic acid 0.2μM
MPP+ 250μM + Epiceanothic acid 0.4μM
MPP+ 250μM + Epiceanothic acid 0.8μM
MPP+ 250μM + Epiceanothic acid 1.6μM


D
Nuclei (Dapi)
LAMP1 (Alexa488)
Merge
Lysotracker(Alexa488)
Control
MPP+ 250μM
MPP+ 250μM + Betulinic acid 4μM
MPP+ 250μM + Betulinic acid 20μM
MPP+ 250μM + Betulinic acid 100μM
MPP+ 250μM + Betulinic acid 150μM

E


Betulinic acid EC50 : 98.21 (μM)
Epiceanothic acid EC50 : 0.49 (μM)
LAMP-1 / beta-actin (% of control)
120
100
80
60
40
20
0
Betulinic acid - - 4 20 100 - - - (μM)
Epiceanothic acid - - - - - 0.2 0.4 0.8 (μM)
MPP+ 250 μM

F


Betulinic acid EC50 : 108.1 (μM)
Epiceanothic acid EC50 : 0.93 (μM)
LAMP-1 (Alexa 488 fluorescence) pixel intensity per cell (% of control)
120
100
80
60
40
20
0
Betulinic acid - - 4 20 100 150 - - - - (μM)
Epiceanothic acid - - - - - - 0.2 0.4 0.8 1.6 (μM)
MPP+ 250 μM

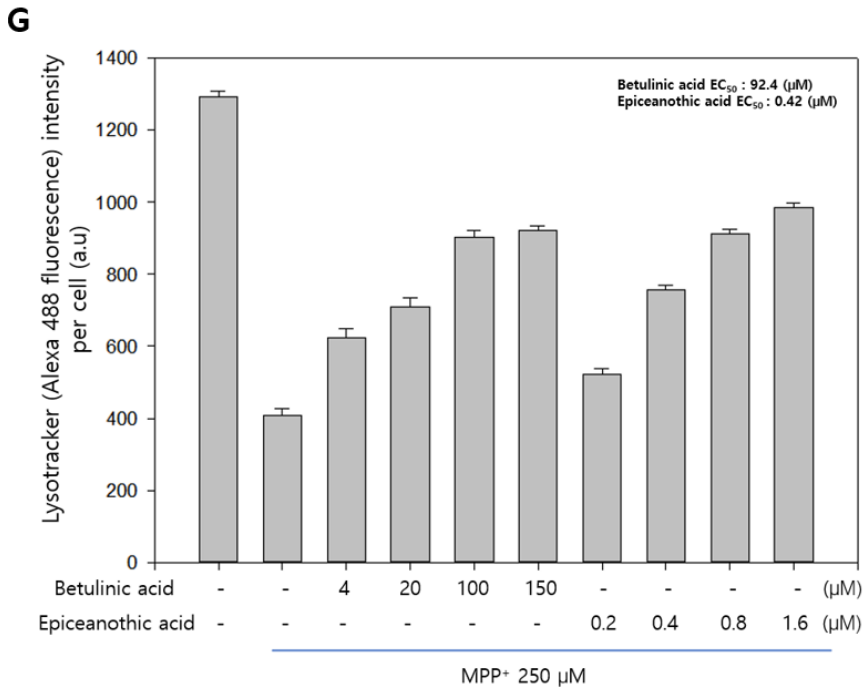


Fig. 8 Epiceanothic acid and Betulinic acid enhanced activation of lysosome biogenesis and alleviating of LMP in MPP+-induced SH-SY5Y cells.

SH-SY5Y cells were simultaneously treated with different concentrations of compounds with MPP^+ (250μM) for 24h. The immunoblotting level of LAMP1 and Cathepsin D were determined by western blot; β-actin was used as a housekeeping protein. Cathepsin D levels in pellet and supernatant lysosomal fractions from UT and MPP^+-induced cells. Lysotracker were determined by OPERETTA fluorescence microscope (400x). The immunofluorescence level of LAMP1 were determined by CONFOCAL fluorescence microscope (600x). The nuclei were visualized DAPI (blue) staining. LAMP1 were detected with Alexa 488 (green)-conjugated antibody.

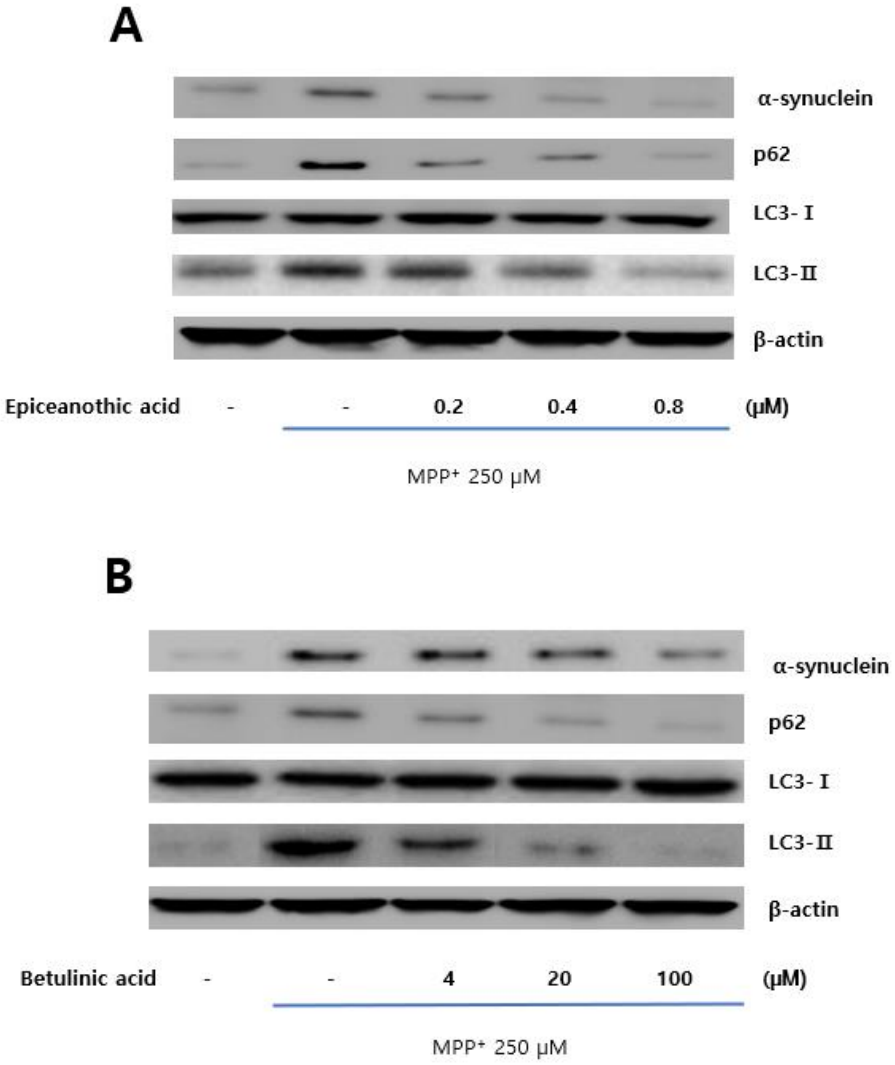
Quantification of the number of cells in the four condition. Representative data from three independent experiments are shown. Data are expressed as the mean percentage (± SD) of the controls of three independent experiments.

3.5 Clearance effect of Epiceanothic acid and Betulinic acid on AP accumulation in MPP^+-induced SH-SY5Y cells.

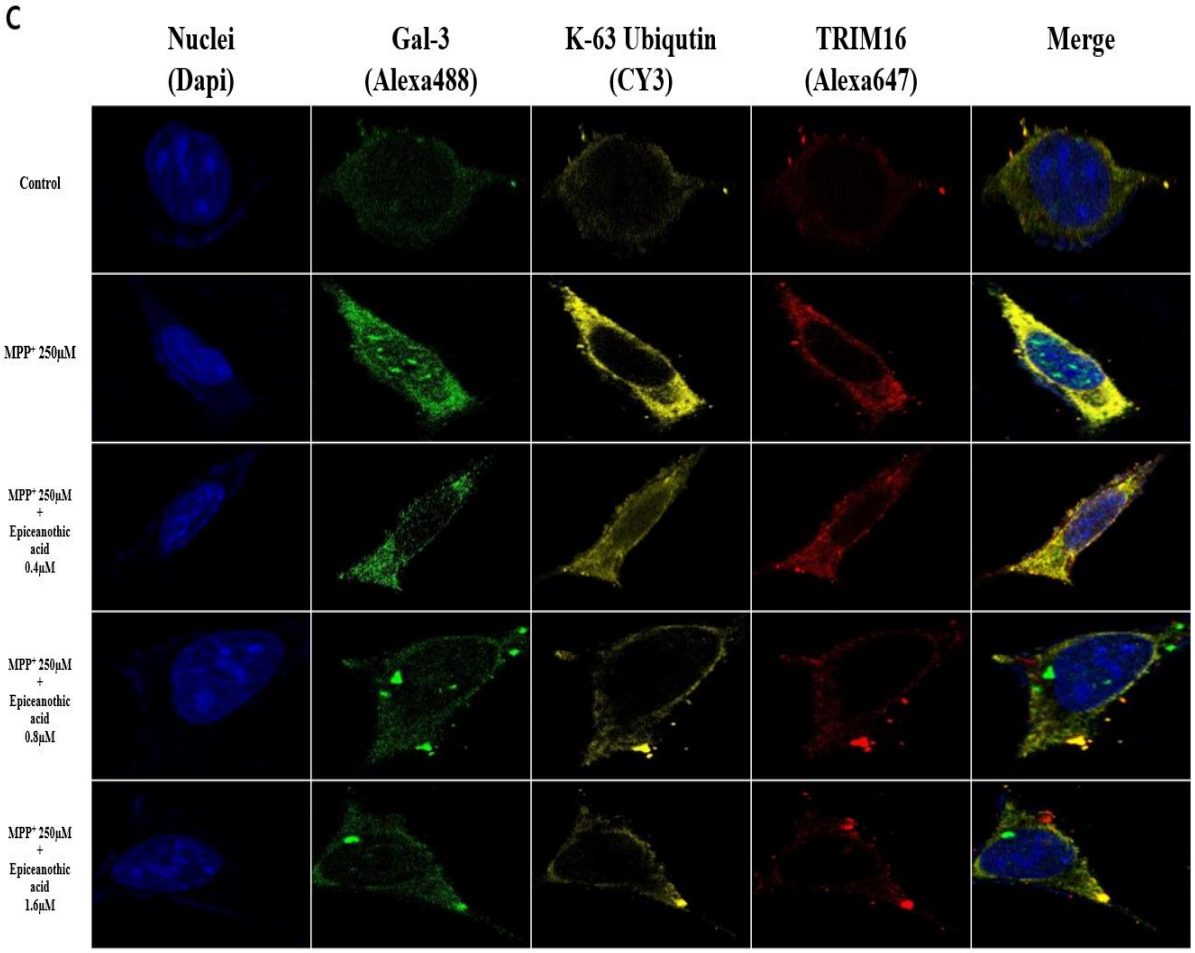
We found that Epiceanothic acid and Betulinic acid increased TFEB nuclear translocation and enhanced lysosome biogenesis in MPP^+-induced SH-SY5Y cells. Also, Before we found that AP accumulation and increased α-synuclein proteins occurred in MPP^+-induced SH-SY5Y cells. Based on studied, AP accumulation by autophagy dysregulation is increasingly recognized as a potential pathogenic factor in neurodegeneration. LMP leads to exposure of intraluminal glycans as a damage signal that is sensed by cytosolic galectins and ubiquitination machinery. Subsequent ubiquitination of the lysosomal membrane recruits autophagic machinery, autophagy receptors and additional regulators that lead to engulfment of the damaged organelle by the phagophore, followed by fusion of the resulting autophagosome with intact lysosomes for degradation. So we tested the effect of Epiceanothic acid and Betulinic acid on AP accumulation in MPP^+-induced SH-SY5Y cells.

Treatment of SH-SY5Y cells with MPP^+ + Epiceanothic acid (0.2, 0.4, 0.8μM), and MPP^+ + Betulinic acid (4, 20, 100μM) decreased AP accumulation detected by immunoblotting of the p62, α-synuclein, and LC3-II proteins. (Fig. 9-a,b)

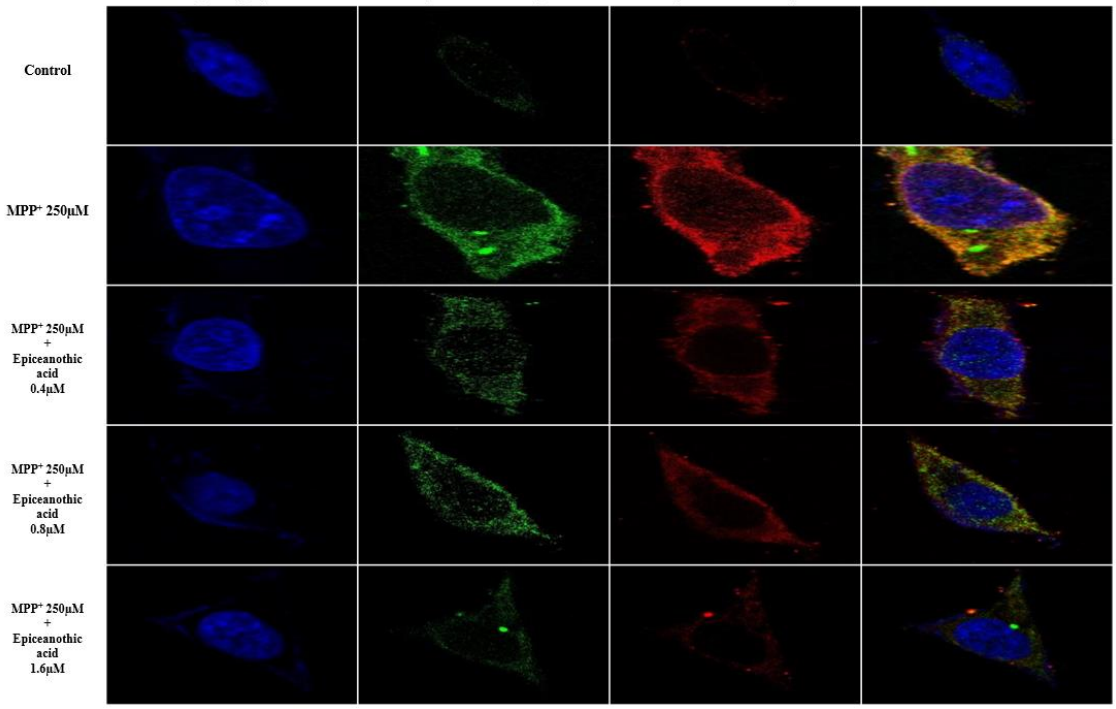
Treatment of SH-SY5Y cells with MPP^+ + Epiceanothic acid (0.4, 0.8, 1.6μM), and MPP^+ + Betulinic acid (20, 100, 150μM) decreased AP accumulation by lysophagy detected by immunofluorescence (Fig. 9-c,d,e,f) of the Gal-3, TRIM16, K-63 ubiquitin, p62, and LC3 proteins.

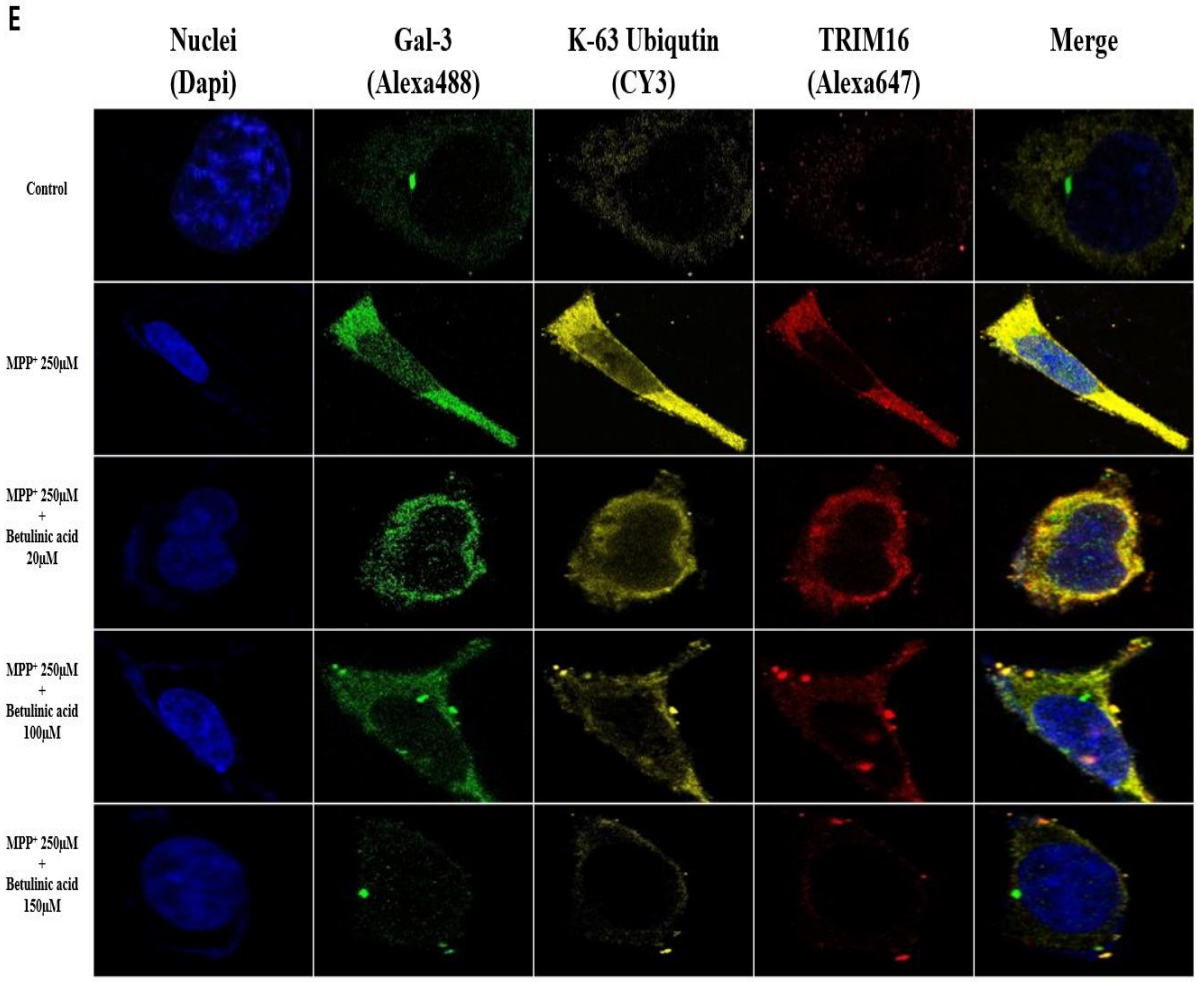


C


Nuclei (Dapi)
Gal-3 (Alexa488)
K-63 Ubiqutin (CY3)
TRIM16 (Alexa647)
Merge
Control
MPP+ 250μM
MPP+ 250μM + Epiceanothic acid 0.4μM
MPP+ 250μM + Epiceanothic acid 0.8μM
MPP+ 250μM + Epiceanothic acid 1.6μM

D


Nuclei (Dapi)
p62 (Alexa488)
LC3 (Alexa647)
Merge
Control
MPP+ 250μM
MPP+ 250μM + Epiceanothic acid 0.4μM
MPP+ 250μM + Epiceanothic acid 0.8μM
MPP+ 250μM + Epiceanothic acid 1.6μM


E
Nuclei (Dapi)
Gal-3 (Alexa488)
K-63 Ubiqutin (CY3)
TRIM16 (Alexa647)
Merge
Control
MPP+ 250μM
MPP+ 250μM + Betulinic acid 20μM
MPP+ 250μM + Betulinic acid 100μM
MPP+ 250μM + Betulinic acid 150μM

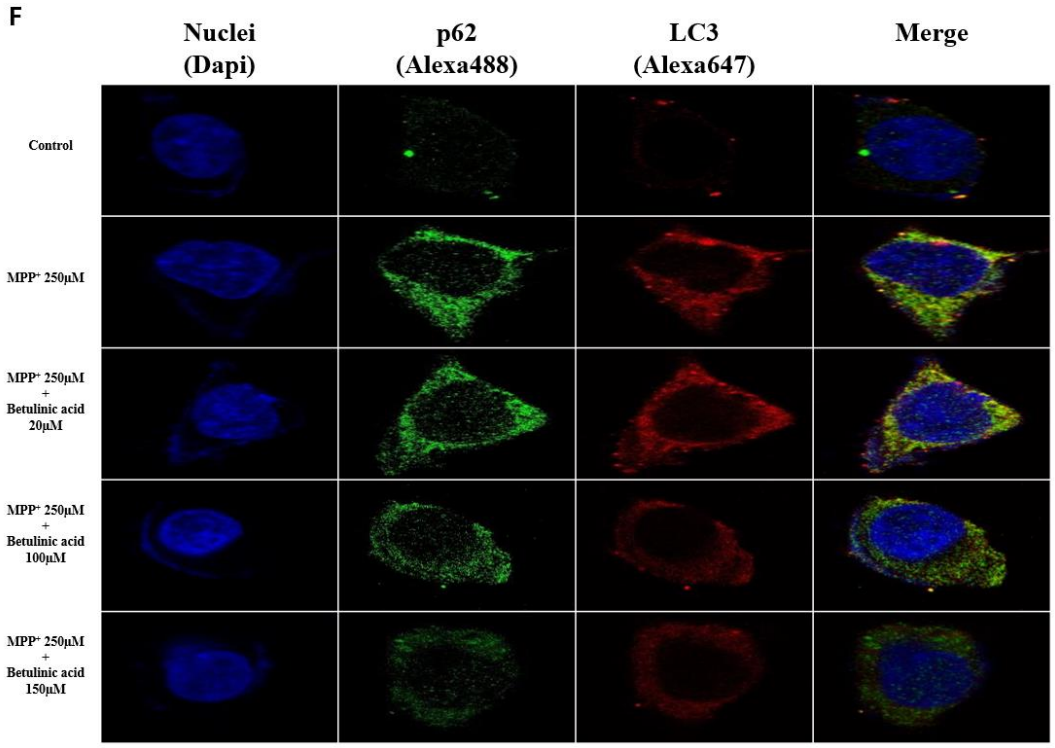

F
Nuclei (Dapi)
p62 (Alexa488)
LC3 (Alexa647)
Merge
Control
MPP+ 250μM
MPP+ 250μM + Betulinic acid 20μM
MPP+ 250μM + Betulinic acid 100μM
MPP+ 250μM + Betulinic acid 150μM

Fig. 9 Epiceanothic acid and Betulinic acid enhanced AP clearance in MPP+-induced SH-SY5Y cells.

SH-SY5Y cells were simultaneously treated with different concentrations of compounds with MPP^+ (250μM) for 24h. The immunoblotting level of p62, α-synuclein, and LC3-II were determined by western blot; β-actin was used as a housekeeping protein. The immunofluorescence level of Gal-3, TRIM16, K-63 ubiquitin, p62, and LC3 were determined by CONFOCAL fluorescence microscope (600x). The nuclei were visualized DAPI (blue) staining. Gal-3 were detected with Alexa 488 (green)-conjugated antibody, TRIM16 were detected with Alexa 647 (red)-conjugated antibody, K-63 ubiquitin were detected with cy3 (yellow)-conjugated antibody, p62 were detected with Alexa 488 (green)-conjugated antibody, and LC3 were detected with Alexa 647 (red)-conjugated antibody. Quantification of the number of cells in the four condition. Representative immunoblotting data from three independent experiments are shown. Data are expressed as the mean percentage (± SD) of the controls of three independent experiments.

4. Discussion

Based on our results, we propose here novel candidates for the therapy contributing to PD-related dopaminergic neurodegeneration. In our study, we demonstrate that **(i)** MPP^+ blocks autophagic flux via the inhibition of autophagosome-lysosome system and the disruption of lysosomal function; **(ii)** Epiceanothic acid and Betulinic acid enhance lysosome condition and autophagic flux in MPP^+-induced SH-SY5Y cells via TFEB-dependent lysosome biogenesis; **(iii)** Epiceanothic acid and Betulinic acid clearance AP accumulation in MPP^+-induced SH-SY5Y cells detected by LC3 protein.

In our study, MPP^+ induced lysosomal dysfunction, permeabilization of lysosomal membranes and disruption of lysosomal structural integrity. It makes undegraded AP, damaged organelles, such as lysosomal deficiency can contribute to neuron cell death by the release of lysosomal hydrolase into the cytosol, some of which, such as Cathepsin D, can remain active at neutral pH and cause the digestion of vital proteins, the activation of additional hydrolases, including caspases. In addition, accumulation of undegraded AP may eventually contribute to cell dysfunction/death by occupying a large portion of the neuron cell body and interfering with cellular functions.

TFEB plays a critical role in enhancing autophagic flux. And this protective effect is independent, at least in part, of its role as a nuclear transcription factor. The current study provides new evidence that the stimulation of TFEB-dependent autophagy-lysosome machinery protects against MPP^+-induced neurotoxicity *in vitro* and *in vivo.*

Autophagy is a highly conserved cellular degradation process through which cells remove damaged organelles and toxic macromolecules. Although under certain circumstances pathologically increased autophagy has been implicated in cell death, under most circumstances autophagy is considered a cytoprotective mechanism. Basal levels of autophagy are important for maintaining cellular homeostasis and appear to be essential for normal cellular function and the survival of terminally differentiated cells, such as neurons. Recently, various studies have demonstrated that dysfunction of autophagy is implicated in neuronal cell loss in neurodegenerative diseases. For example, defects in autophagic flux lead to aggregate-prone proteins such as huntingtin and a-synuclein, which are accumulated in Huntington's and Parkinson's diseases. The role of autophagy in MPP^+ toxicity is still a controversial topic.

Since the discovery of lysophagy as a mechanism to dispose damaged lysosomes, major progress has been made with regard to its regulation. Clearly, we now need to identify the full ubiquitination machinery which likely involves diverse ubiquitin ligases, and put together a full catalog of the proteins ubiquitinated during the endo-lysosomal damage response. So, It will be important to clarify whether lysophagy is also a means to adjust the amount of intact lysosomes and adapt it to cellular needs, as has been shown for other organelles.

From a therapeutic perspective, we believe that Epiceanothic acid and Betulinic acid are a promising target autophagy -lysosome mechanism. In many cases, it enhances the removal of the primary toxic entity causing disease. So, This would have major benefits from a drug toxicity perspective

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국문초록

신경독성 MPP+ 으로 유도한 SH-SY5Y 세포에서

TFEB 의존성 리소좀 생성 증가에 의한

산조인 유래 유효성분물질의 신경 보호 효과

조 성 균

약학과 천연물과학 전공

서울대학교 약학대학 대학원

고령화 인구의 지속적인 성장으로 인해 퇴행성 신경질환의 발생률이 증가하고 있다. 그럼에도 해결방안이 명확하게 밝혀지고 있지 않다. 최근 연구에 의하면 리소좀의 기능장애와 자가포식-리소좀 기전의 손상이 신경세포의 사멸을 일으켜 퇴행성 신경질환의 일환인 파킨슨병을 일으킨다는 보고가 있다.

N-methyl-4-phenylpyridinium iodide (MPP^+)는 미토콘드리아 호흡 복합체-Ⅰ을 저해하고, 활성산소를 생산하고, 리소좀-자가포식 반응을 손상시켜 도파민 세포의 사멸을 유도한다고 알려져 있다. 특히 리소좀의 막에 손상을 일으켜 리소좀 손상 반응을 유도하는 기폭제의 역할을 한다고 한다.

리소좀이 손상을 받으면 안에 있는 Cathepsin D와 같은 분해효소들이 세포질로 나와 다른 세포내소기관에 손상을 준다. 이때 손상 받은 세포내소기관들은 자가 포식이라는 정교한 분해과정을 통해 자가포식소체로 이동하여 리소좀으로 전달 되어 분해 시켜야 한다. 하지만 MPP^+는 분해과정을 담당하는 리소좀을 손상시켜 자가포식소체의 축적을 일으켜 세포 사멸을 촉진시킨다.

TFEB는 리소좀 재생성을 조절하는 인자로 mTOR에 의해 조절되어 LAMP1을 생성한다. Galectin-3는 손상 받은 리소좀을 찾고, 유비퀴틴화 시키는 TRIM16을 모집하여 복합체를 이뤄 자가포식체를 형성하는 단백질과 lysophagy 반응을 유발한다.

본 연구에서는 MPP^+로 유도한 SH-SY5Y 세포에서의 신경보호효과를 가진 산조인 화합물 5가지 (Jujuboside A, Jujuboside B, 6″-Feruloylspinosin, Epiceanothic acid, Betulinic acid)를 이용하여, 리소좀-자가포식과 관련된 기전에 대하여 조사하였다.

그중 Epiceanothic acid와 Betulinic acid는 mTOR의 억제에 의한 TFEB의 발현을 증가 시켜 리소좀을 재생성 하였고, 리소좀-자가포식 반응을 촉진시키는 단백질들에 효과가 있었고, 리소좀에서 방출된 세포질에서의 Cathepsin D의 발현이 줄어드는 효과를 보였다.

결과를 토대로 Epiceanothic acid 와 Betulinic acid는 MPP^+로 유도한 신경세포에서 리소좀 재생성 및 자가포식소체 제거에 의한 신경보호효과가 있고, 파킨슨병의 치료에 대한 유망한 후보 후보물질이 될 수 있음을 시사한다.

주요 단어 : 파킨슨병, 신경 보호, 리소좀 재생성, TFEB, Lysophagy, *Zizyphus jujuba*

학번 : 2018-28060