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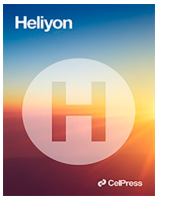

Research article

Phikud navakot extract acts as an ER stress inhibitor to ameliorate ER stress and neuroinflammation

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ABSTRACT

The prevalence of neurological disorders (NDs) such as Alzheimer's disease (AD) is increasing globally, and the lack of effective pharmacological interventions presents a significant health risk. Multiple mechanisms including the activation of oxidative stress, amyloid pathway, ER stress, and neuroinflammation have been implicated in AD; therefore, multi-targeted agents against these mechanisms may be preferable to single-target agents. Phikud Navakot (PN), a Thai traditional medicine combining nine herbs, has been shown to reduce oxidative stress and neuroinflammation of neuronal and microglia cells and the coculture between them, indicating the promising role of PN extract as anti-AD. This study evaluated the neuroprotective effects of PN extract against oxidative stress, amyloid pathway, endoplasmic reticulum stress (ER stress), and neuroinflammation using neuronal and microglia cells, as well as in a *Drosophila* model of AD. Results showed that PN extract reduced oxidative stress, lipid peroxidation, pro-inflammatory cytokines, amyloid pathway, and ER stress induced by aluminum chloride ($AlCl_3$, AD-induced agent) or thapsigargin (TG, an ER stress activator) in both neurons and microglia cells. PN extract also reduced oxidative stress, ER-stress-related genes, and neurotoxic peptides (amyloid beta) in a *Drosophila* model of AD. Data indicated that PN extract may function as an anti-AD agent by targeting multiple mechanisms as described. This research also revealed for the first time that PN extract acted as an ER stress inhibitor.

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List of abbreviations:

Aβ	Amyloid beta peptides
AD	Alzheimer's disease
$AlCl_3$	Aluminum chloride
APP	Amyloid precursor proteins
ATF-6	Activating transcription factor-6
BACE-1	β-secretase 1
Bip	Immunoglobulin heavy chain binding protein
CAT	Catalase
ELISA	Enzyme-linked immunosorbent assay
ERAD	Endoplasmic reticulum-associated degradation
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
ER stress	Endoplasmic reticulum stress
GPx	Glutathione peroxidase
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IRE-1	Inositol-requiring protein-1
JNK	Stress-activated protein kinases/Jun amino-terminal kinases
MAPK	Mitogen-activated protein kinases
NDs	Neurological disorders
p38	p38 mitogen-activated protein kinases
PERK or PEK	Protein kinase RNA-like ER kinase
PN extract	Phikud Navakot extract
RT–qPCR	Reverse transcription-quantitative polymerase chain reaction
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TG	Thapsigargin
TNF-α	Tumor necrosis factor-alpha

1. Introduction

Neurological disorders (NDs) are defects in the central and peripheral nervous systems (CNS and PNS) that cause a variety of complications for individuals, families, and the economy as a whole. The CNS and PNS are responsible for the majority of bodily and cognitive functions, and the progression of NDs cannot be halted by current medical treatments. Alzheimer's disease (AD) is one of the most common NDs with a significant increase in the number of AD patients by 117 % from 1990 to 2016 and is projected to increase by 648 % by 2050 as many countries enter the aging society [1]. Multiple etiological factors have been postulated as potential contributors to the development of AD including (i) activation of the amyloid pathway [2], (ii) endoplasmic reticulum stress (ER stress) and unfolded protein response [3,4], and (iii) neuroinflammatory response [5]. For activation of the amyloid pathway, the amyloid precursor proteins (APPs) are cleaved by β-secretase (BACE-1) leading to the formation of cytotoxic amyloid beta peptides, especially amyloid beta peptides (1–42) ($A\beta_{1\text{-}42}$), which can easily undergo oligomerization and aggregation in neurons [6]. Thus, inhibition of BACE-1 is of great interest as a cure for AD [7]. The accumulation of misfolded or aggregated amyloid peptides results in (i) increased cellular reactive oxygen species (ROS), (ii) ER stress response through three cascades including inositol-requiring protein-1 (IRE-1), protein kinase RNA-like ER kinase (PERK or PEK), and activating transcription factor-6 (ATF-6) which employ immunoglobulin heavy chain binding protein (Bip) as a stress sensor [3], and (iii) neuroinflammation [8], which partly originates from the activation of the mitogen-activated protein kinases (MAPK pathway). Three distinct protein cascades including extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinases (p38), and stress-activated protein kinases/Jun amino-terminal kinases (JNK) participate in MAPK signaling, leading to enhanced levels of pro-inflammatory cytokines such as interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in inflamed microglia cells [9]. Unfortunately, ROS, inflammation, and ER stress contribute to the up-regulation of BACE-1 [10–13], resulting in a vicious cycle. Prolonged activation of ER stress, ROS production, and neuroinflammation is capable of triggering neuron and microglia death, which is another feature of NDs, including AD. Therefore, considerable interest now focuses on compounds that act on several relevant and safe targets instead of just focusing on a particular mechanism associated with neurodegenerative disorders to promote the discovery of effective drugs [14,15].

Phikud Navakot (PN) is a Thai traditional medicine combining nine herbs (in equal weights) including the root of *Angelica dahurica*, the root of *Angelica sinensis*, the aerial part of *Artemisia pallens*, the rhizome of *Atractylodes lancea*, rhizome of *Ligusticum chuanxiong*, root and rhizome of *Nardostachys jatamansi*, rhizome of *Picrorhiza kurrooa*, the rhizome of *Saussurea costus*, and gall of *Terminalia chebula* [16,17]. Locals utilize PN to treat circulatory disorders, dizziness, and syncope [16,17]. However, PN has also shown other

promising health-promoting activities including acting as a cholesterol-lowering agent by up-regulation of low-density lipoprotein receptor (LDL-R) or down-regulation of 3-hydroxyl-3-methylglutaryl-CoA reductase (HMGCR) [18], cardioprotective effect by up-regulation of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase (HO)-1 and reduced pro-inflammatory cytokines in rats with acute myocardial infarction [19], antioxidant activities by preventing hydrogen peroxide (H_2O_2)-mediated oxidative stress in liver cells, which could be due to up-regulation of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [20]. Recently, our group revealed that PN extract and its abundant bioactive compound, gallic acid, possessed anti-neuroinflammation against lipopolysaccharide (LPS)-mediated neuroinflammation in neurons, microglia, and co-culture between them via inhibition of ERK1/2 [17]. Gallic acid has also been shown to reduce BACE-1 activity and neuroinflammation in rat models of AD [21], while gallic acid also reduced ER stress hippocampus of adult-onset hypothyroid rats via increased endoplasmic reticulum-associated degradation (ERAD) through IRE-1 and ATF-6 [22]. PN exhibits high safety. The administration of high-dose PN (1000 mg/kg) in rats for 90 days resulted in no treatment-related mortality or clinical signs of toxicity [23]. PN is also devoid of DNA-damaging chemicals (genome-safe), as shown by the comet assay in human cells [16]. These features suggest the possible role of PN extract as a multi-target plant extract for the prevention of ND including AD through inhibition of the amyloid pathway, oxidative stress, neuroinflammation, and ER stress with high safety. Aluminum chloride ($AlCl_3$) and thapsigargin (TG) have been employed as triggers of the distinctive characteristics of AD to explore potential compounds that might attenuate the development of AD [24–26]. $AlCl_3$ is commonly used to induce AD pathogenesis in both cell and animal models [27], while TG is an ER stress activator [28]. Consequently, this study assessed the neuroprotective function of PN extract in countering neuroinflammation and ER stress induced by $AlCl_3$ or TG. Neuro2a cells, BV2 microglial cells, and AD flies were used as experimental models. Our data showed that the PN extract exhibited neuroprotective features that can attenuate the development of AD. Such characteristics included the suppression of BACE-1, anti-inflammatory effects, reduction of ER stress, and antioxidant activity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals, reagents and antibodies

Cell culture medium and supplements, such as minimum essential medium (MEM), Roswell Park Memorial Institute medium 1640 (RPMI-1640), glutamine, penicillin, streptomycin, fungizone, trypsin and TRIZol were acquired from Invitrogen (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Human amyloid beta (1–42) ELISA kit was purchased from BioLegend (San Diego, CA, USA). 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2HTetrazolium-5-Carboxanilide (XTT), phenazine methosulfate (PMS), aluminum chloride ($AlCl_3$), thapsigargin (TG), and dichlorofluorescein diacetate (DCFDA) BAPTA-AM, apocynin and BACE-1 activity detection kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies; BACE-1, βIII-tubulin, Bip, ERK1/2, phosphorylated ERK1/2 (*p*-ERK1/2), p38, phosphorylated p38 (*p*-p38), and actin were purchased from Cell Signaling (Danvers, MA, USA).

2.2. Plant samples and phikud navakot extract preparation

The compositions of Phikud Navakot (PN) extract, plant names, and voucher specimens were previously reported [17]. In brief, plants, including *Angelica dahurica* (Hoffm.) Benth. and Hook. f. ex Franch. and Sav., *Angelica sinensis* (Oliv.) Diels, *Artemisia vulgaris* L., *Atractylodes lancea* (Thunb.) DC., *Ligusticum sinense* Oliv., *Nardostachys jatamansi* (D.Don) DC, *Picrorrhiza kurroa* Benth, *Terminalia chebula* Retz., and *Saussurea lappa* Clarke were verified by Dr. Uthai Sotanaphun, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand. The voucher specimens (MUS1122-MUS1130) were deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok. Available plant names were checked with http://www.theplantlist.org on March 30, 2022. PN extract was also prepared and characterized as described by our previous work without modification [17]. Aluminum chloride ($AlCl_3$), thapsigargin (TG), BAPTA-AM, apocynin, and PN extract were dissolved in DMSO at concentrations of 10 mM, 40 μM, 200 μM, 200 μM, and 4 mg/mL, respectively, then diluted with the medium to reach the final concentrations of interest, which had a DMSO concentration of less than 0.05 %. An equivalent quantity of 0.05 % DMSO was used as a vehicle control in each experiment.

2.3. Cell culture

Mouse microglial cells (BV-2 cells, ATL033001, Interlab Cell Line Collection, Genova, Italy) were cultured in RPMI 1640 medium containing 10 % (*v/v*) heat-inactivated FBS, 100 unit/mL penicillin, 100 μg/mL streptomycin, 5 % (*v/v*) sodium bicarbonate, and fungizone. Cells were cultured at 37 °C in a 5 % CO_2 incubator. BV-2 cells at the density of 1×10^5, 5×10^4 and 1×10^4 cells/well were seeded in six-well plates, 12-well plates, and 96-well plates, respectively.

Mouse neuroblastoma cells (Neuro-2a, ATCC CCL-131TM, American Type Culture Collection, Manassas, VA, USA) were cultured and differentiated in DMEM medium with 2 % FBS and retinoic acid (20 μM) at 37 °C for six days. During differentiation, the medium was changed every two days. Cells were seeded on a six-well plate, 12-well plate, and 96-well plate with an initial seeding density of 1.5×10^5, 1×10^5 and 1.5×10^4 cells/well for further experiments.

2.4. Determination of cytotoxicity

The cytotoxicity effects of $AlCl_3$ and TG on BV-2 and Neuro-2a were investigated as follows: Cells were treated with either $AlCl_3$ (50, 100, 200, 300, 400, 500, and 1000 μM) or TG (15.62, 31.25, 62.5, 125, 250, 500, and 1000 nM) or vehicle control. After 24–48 h of incubation at 37 °C in a CO_2 incubator, the cells were rinsed and analyzed for XTT reduction assay and intracellular ATP levels as described previously [17].

2.5. Effects of $AlCl_3$ and TG on Cytokine Release in BV-2 cells

BV-2 cells were exposed to either $AlCl_3$ (50, 100, 200, 300, and 500 μM) or TG (1, 2.5, 5, 10, 25, 50, 100, 125, and 250 nM) for 24 h. After the treatment, the culture medium was harvested and determined for cytokine levels, including IL-6, TNF-α, IL-1β, and IFN-γ using cytokine MULTIPLEX assay kit (Cat. MCYTOMAG-70K-05, Millipore) following the manufacturer's protocol and detected by Multiplex MAGPIX system (Luminex, Austin, TX, USA). Data were analyzed using xPONENT4.2 and Belysa Immunoassay Curve Fitting data analysis software.

2.6. Effects of $AlCl_3$ and TG on oxidative stress parameters

The intracellular reactive oxygen species (ROS) levels and malondialdehyde (MDA), which is a marker for lipid peroxidation were used to measure the oxidative stress parameters. For determination of intracellular ROS, cells were treated with either $AlCl_3$ (100, 300, and 500 μM) or TG (1, 2.5, 5, 10, 25, 50, 100, and 200 nM) for 30 min to 24 h. The formation of intracellular ROS, specifically hydrogen peroxide, was measured using dichlorofluorescein diacetate (DCFDA), as previously described [29].

Malondialdehyde (MDA) was evaluated using a thiobarbituric acid reactive substances (TBARS) assay with minor changes to the previously described [30]. The concentration of the chromophore was calculated from a calibration curve prepared with fresh 1,1–3, 3-tetramethoxypropane solutions. The results are then expressed as MDA equivalents, normalized to total cellular protein determined by Bradford assay (Bio-Rad, Hercules, CA, USA).

2.7. Effects of $AlCl_3$ and TG on protein expression

BV-2 and Neuro-2a cells were incubated with either $AlCl_3$ (100, 300, and 500 μM) or TG (5, 10, and 50 nM) for 24 and 48 h. Neuro-2a cells were harvested for the determination of BACE-1, βIII-tubulin, and Bip, while BV-2 cells were harvested for BACE-1, Bip, ERK1/2, *p*- ERK1/2, p38, and *p*-p38. Immunoblot was performed as described previously with minor modifications [17,31]. The cells were lysed by adding RIPA buffer, supplemented with a protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). The protein lysate at 30 μg was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were subsequently blocked blocking solution at room temperature (RT) for 1 h, followed by overnight incubation at 4 °C with one of the following rabbit primary antibodies: anti-βIII tubulin (1:1000 dilution), β-actin (1:5000 dilution), BACE-1 (1:1000), Bip (1:1000 dilution), ERK1/2 (1:1000 dilution), phosphorylated ERK1/2 at Thr202/Tyr204 (1:1000 dilution), p38 at Thr180/Tyr182(1:1000 dilution), and phosphorylated p38 (1:1000 dilution) in 0.5 % BSA in TBS-T overnight at 4 °C. Immunoreactive signals were detected using an HRP-conjugated anti-rabbit IgG secondary antibody (1:5000) and Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). Gel images were captured using the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA), and the immunoreactive bands were quantified using Image J software.

2.8. Effects of calcium chelator (BAPTA-AM), ER stress inhibitor (apocynin), and PN extract on $AlCl_3$ and TG-induced cytokine release, oxidative stress, and protein expression

BV-2 and Neuro-2a cells were pre-incubated with either PN extract (10 and 20 μg/mL), BAPTA-AM (1 μM), or apocynin (1 μM) for 12 h, followed by the addition of $AlCl_3$ (300 μM) or TG (50 nM) for 24 h. After that, the conditioned medium and cells were collected, prepared, and subjected to the above-described analyses.

2.9. Drosophila culture and treatment

Flies carrying human APPs and BACE-1 (BDSC 56756) were received from the Bloomington Stock Center at Indiana University and cultured on Formula 4–24 blue® medium (Carolina, Burlington, NC, USA). To specifically induce the expression of APP and BACE-1 in the fly brain (AD-mimicking flies): 20 μg/mL of RU486 was added to the fly medium on day one of treatment. For the treatment scheme, newly eclosed flies were divided into four groups as follows:

Gr. 1: fly food + RU486 (control group)
Gr. 2: fly food + RU486 + 200 μg/mL PN extract.
Gr. 3: fly food + RU486 + 400 μg/mL PN extract.
Gr. 4: fly food + RU486 + 10 μM donepezil (AD drug)

Flies were cultured at 28 °C for 28 days. To keep the food fresh, it was changed every three days.

2.10. Determination of climbing index in flies

After 7, 14, 21, and 28 days of the treatment, the flies in each experiment were divided into four groups and placed in a transparent tube without anesthesia. They were then allowed to rest for 15 min at room temperature. The tube was then tapped to bring all of the flies to the bottom. Following tapping, the climbing rate was measured and analyzed as previously stated [32,33]. Three experiments were independently performed.

2.11. Quantification of BACE-1 activity and amyloid beta levels in fly brains

Quantification of BACE-1 activity and amyloid beta peptides (1–42) ($A\beta_{1\text{-}42}$) were determined as previously described with some modifications when flies were treated for 28 days [32,33]. Thirty fly heads were homogenized in T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Then, the lysate was subjected to either testing for BACE-1 activity using a BACE-1 activity detection kit or testing for $A\beta_{1\text{-}42}$ using a human $A\beta_{1\text{-}42}$ ELISA kit following the manufacturer's protocol.

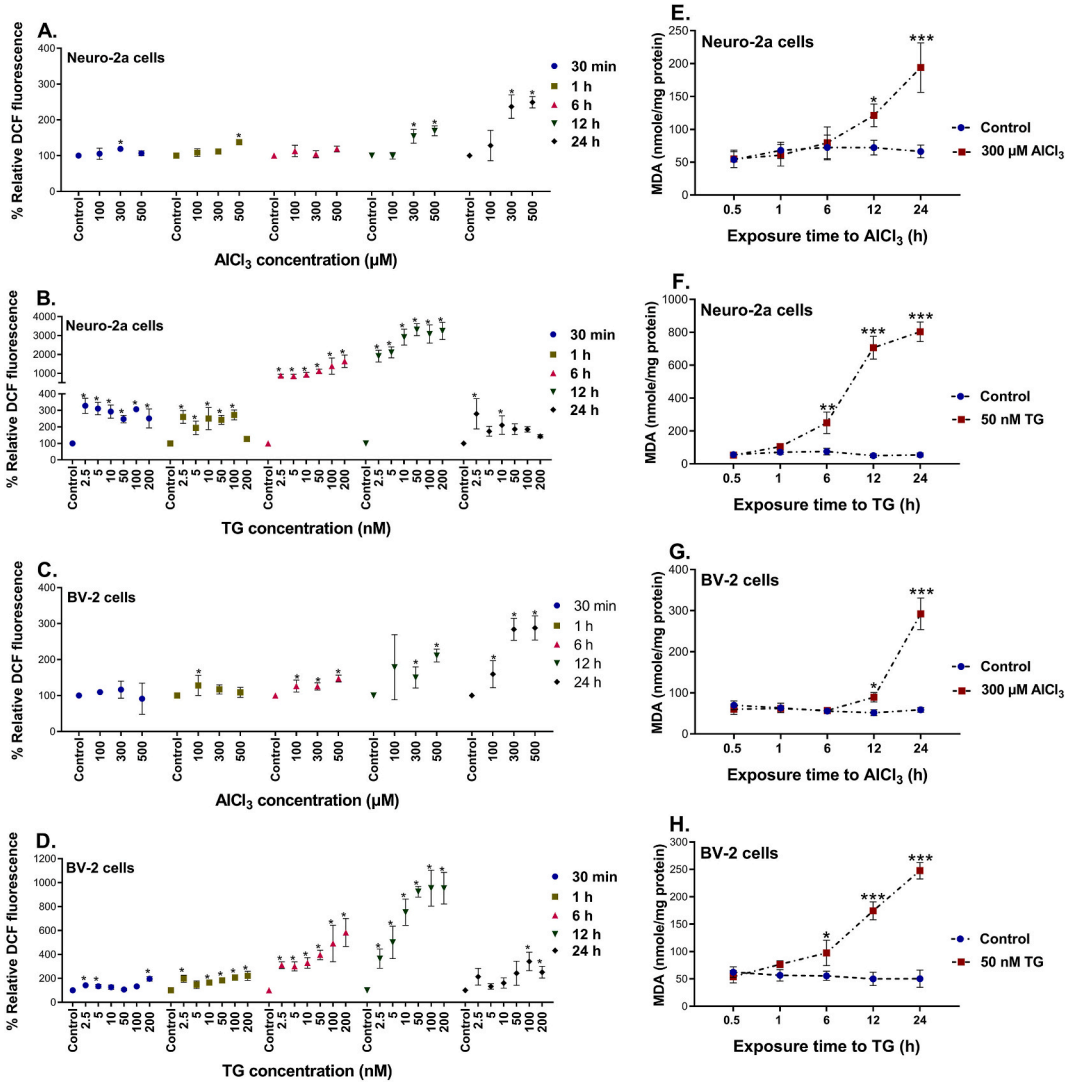

Fig. 1. Effects of $AlCl_3$ and TG on the Production of Oxidative Stress and MDA in Neuro-2a and BV-2 Cells. (A.) % relative DCF fluorescence in Neuro-2a cells during $AlCl_3$ treatment, (B.) % relative DCF fluorescence in Neuro-2a cells during TG treatment, (C.) % relative DCF fluorescence in BV-2 cells during $AlCl_3$ treatment, (D.) % relative DCF fluorescence in BV-2 cells during TG treatment (E.) MDA levels in Neuro-2a cells during $AlCl_3$ treatment, (F.) MDA levels in Neuro-2a cells during TG treatment, (G.) MDA levels in BV-2 cells during $AlCl_3$ treatment and (H.) MDA levels in BV-2 cells during TG treatment. The values are mean ± SD of three independent experiments and statistical significance was analyzed against untreated control by one-way ANOVA followed by Tukey's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$.

2.12. Determination of gene expression in Drosophila

Drosophila gene expression was quantified using reverse transcription–quantitative polymerase chain reaction (RT–qPCR). Fly heads at day 28 of treatment were collected and extracted for total RNA using a TRIZol reagent. The cDNA was synthesized using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The PCR reaction was further performed using a synthesized cDNA with THUNDERBIRD™ SYBR qPCR Mix (Toyobo, Osaka, Japan). The PCR conditions were denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. All primers, except RpL32 [34], were designed using the NCBI primer designing tool. The primers were listed in the Supplementary Table S1. The expression levels of each transcript were normalized to a housekeeping gene (RpL32) and then calculated fold change compared to the Gr.1.

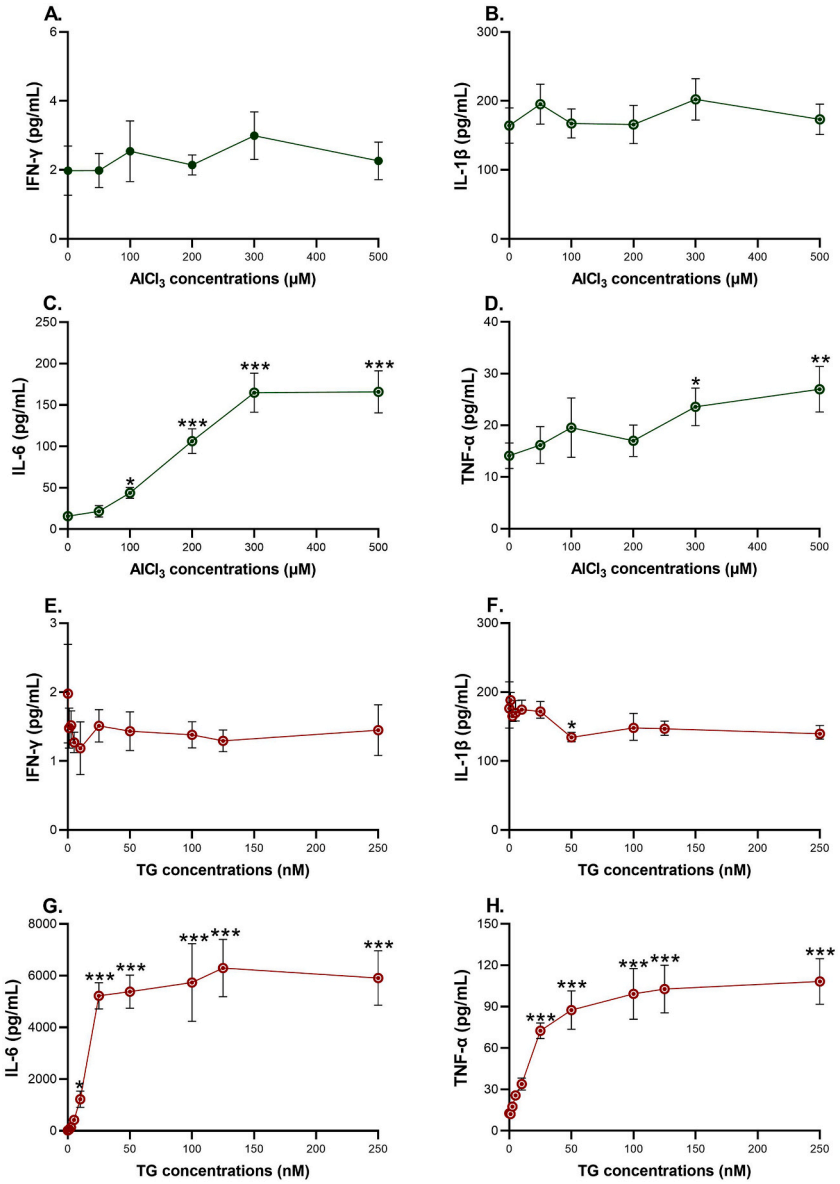

Fig. 2. Effects of $AlCl_3$ and TG on the Production of Pro-inflammatory Cytokines in BV-2 Cells. (A.) IFN-γ levels during $AlCl_3$ treatment, (B.) IL-1β levels during $AlCl_3$ treatment, (C.) IL-6 levels during $AlCl_3$ treatment, (D.) TNF-α levels during $AlCl_3$ treatment, (E.) IFN-γ levels during TG treatment, (F.) IL-1β levels during TG treatment, (G.) IL-6 levels during TG treatment and (H.) TNF-α levels during TG treatment. The values are mean ± SD of three independent experiments and statistical significance was analyzed against untreated control by one-way ANOVA followed by Tukey's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$. and ***, $p < 0.00$.

2.13. Statistical analysis

Unless indicated, values are displayed as the mean ± standard deviation (SD) from at least three independent experiments. Statistical significance between groups was determined using a one-way ANOVA followed by Tukey's multiple comparisons test (Prism 9.4.1 software, Boston, MA). The p-value of 0.05 or less ($p \leq 0.05$) was regarded as statistically significant.

3. Results

3.1. $AlCl_3$ and TG induce oxidative stress in Neuro-2a cells and BV-2 cells

Before beginning the experiments, we evaluated the cytotoxic effects of $AlCl_3$ and thapsigargin (TG) on Neuro-2a cells incubated with varying concentrations of TG and $AlCl_3$ for 24 and 48 h, respectively. $AlCl_3$ is commonly used as an agent to induce AD pathogenesis in both cell and animal models, while TG is an ER stress activator [27,28]. The data are shown in the Supplementary Fig. S1. Furthermore, we utilized $AlCl_3$ and TG concentrations at up to 500 μM and 250 nM, respectively, in the subsequent studies, as these dosages covered both non-toxic and sub-toxic levels of $AlCl_3$ and TG.

Both neuronal cells (Neuro-2a) and microglia cells (BV-2 cells) declined in cell viability when exposed to $AlCl_3$ and TG, as shown in the Supplementary Fig. S1. Inflammation and oxidative stress may be two primary processes underlying this observation. Thus, we first examined oxidative stress levels when cells were treated with $AlCl_3$ or TG using DCFDA and TBARS assay, which are used to quantify intracellular ROS and MDA levels, respectively. Neuro-2a and BV-2 cells were treated with non-toxic or sub-toxic concentrations of $AlCl_3$ (up to 500 μM) or TG (up to 250 nM) for 24 h. Fig. 1A demonstrates that $AlCl_3$-exposed Neuro-2a cells (300 and 500 μM $AlCl_3$) exhibited significant intracellular ROS starting at 12 h after exposure compared to the control, with further ROS induction occurring

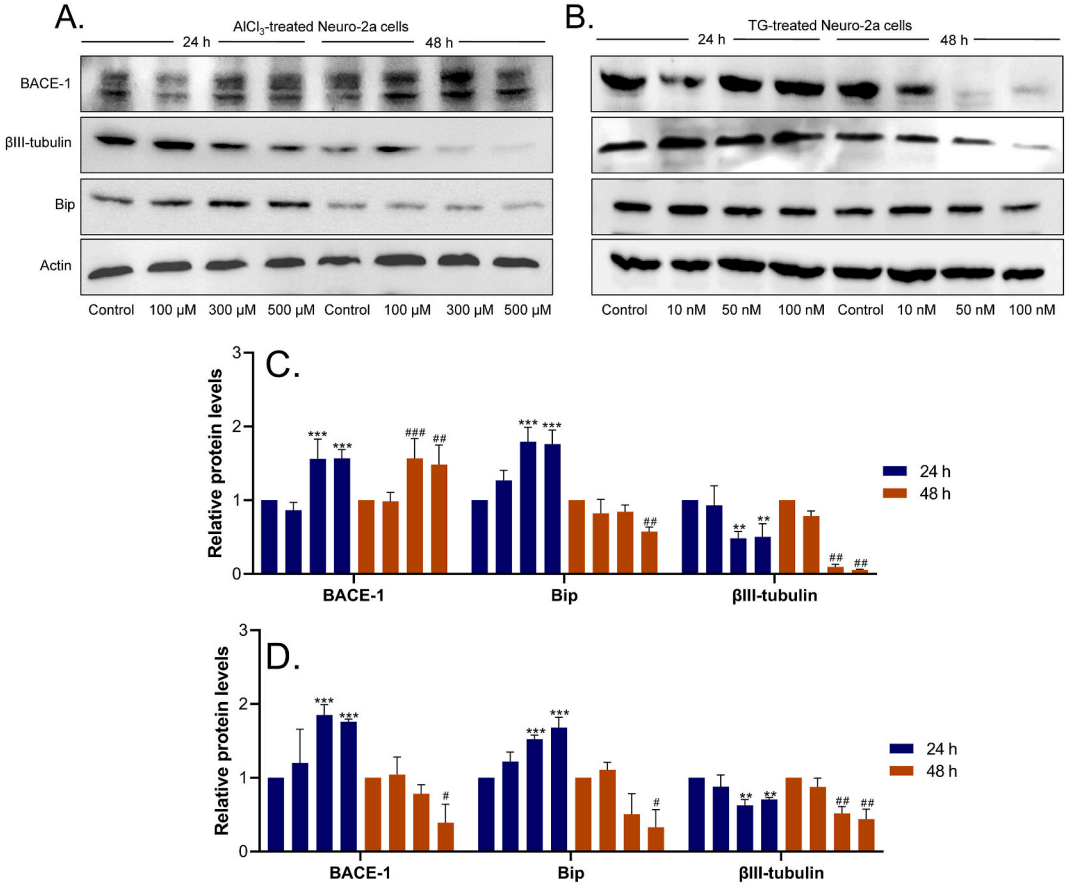

Fig. 3. Effects of $AlCl_3$ and TG on BACE-1, βIII-tubulin, and ER Stress Protein in Neuro-2a Cells. (A.) Immunoblot figure of Neuro-2a cells treated with $AlCl_3$ for 24 and 48 h, (B.) Immunoblot figure of Neuro-2a cells treated with TG for 24 and 48 h, (C.) Quantification of band intensity of each protein (relative to actin) of Neuro-2a cells treated with $AlCl_3$ for 24 and 48 h, and (D.) Quantification of band intensity of each protein (relative to actin) of Neuro-2a cells treated with TG for 24 and 48 h. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. **, $p < 0.01$, ***, $p < 0.001$ (compared to control at 24 h), #, $p < 0.05$, ##, $p < 0.01$, and ###, $p < 0.001$ (compared to control at 48 h).

24 h after exposure. Both non-toxic (300 μM $AlCl_3$) and sub-toxic concentrations (500 μM $AlCl_3$) showed no significant differences. Interestingly, the ER stress activator (TG) rapidly and strongly induced intracellular ROS (compared with $AlCl_3$) in Neuro-2a cells starting at 6 h, but intracellular ROS were quenched 24 h after treatment (Fig. 1B). This was also observed in $AlCl_3$ and TG exposed to BV-2 (Fig. 1C and D).

High intracellular ROS attack lipid molecules, resulting in reactive lipid peroxidation products, such as MDA [30]. We then

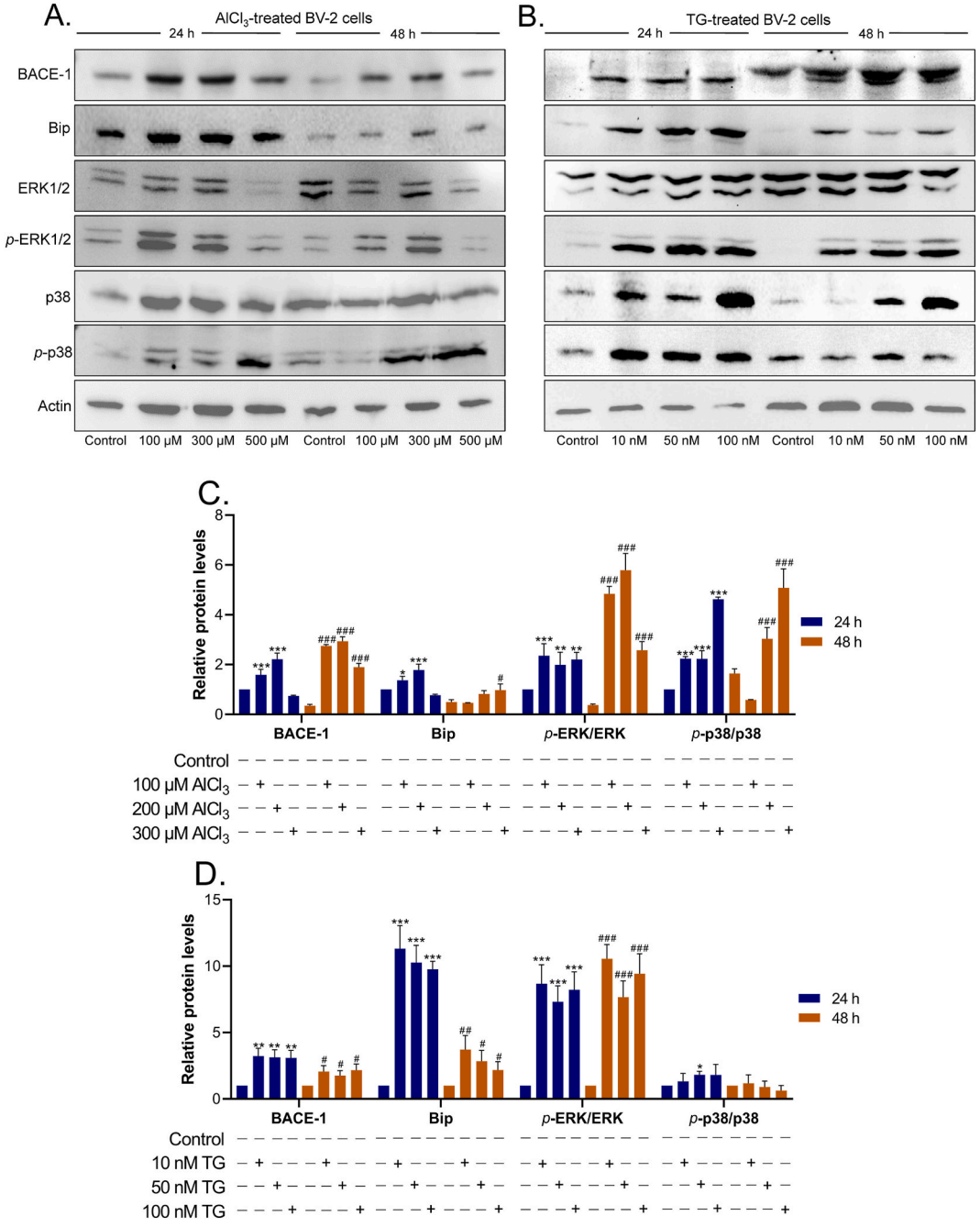

Fig. 4. Effects of $AlCl_3$ and TG on BACE-1, MAPKs, and ER Stress Protein in BV-2 Cells. (A.) Immunoblot figure of BV-2 cells treated with $AlCl_3$ for 24 and 48 h, (B.) Immunoblot figure of BV-2 cells treated with TG for 24 and 48 h, (C.) Quantification of band intensity of each protein (relative to actin or its total form) of BV-2 cells treated with $AlCl_3$ for 24 and 48 h, and (D.) Quantification of band intensity of each protein (relative to actin or its total form) of BV-2 cells treated with TG for 24 and 48 h. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ (compared to control at 24 h), #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$ (compared to control at 48 h).

determined the MDA levels in both cells. As shown in Fig. 1E–H, the levels of MDA were incrementally elevated in $AlCl_3$ (300 μM) and TG (50 nM) exposed to Neuro-2a and BV-2 cells, confirming oxidative stress formation in the cells (Fig. 1A–D).

3.2. $AlCl_3$ and TG and induce cytokine production in BV-2 cells

Oxidative stress and lipid peroxidation were induced by $AlCl_3$ and TG in Neuro-2a and BV-2 cells, as indicated by Fig. 1. The inflammatory responses were then investigated for only BV-2 cells because Neuro-2a cells are neurons and BV-2 inflammatory response can be mimicked to isolate primary microglia and microglia *in vivo*. BV-2 cells were treated with $AlCl_3$ or TG at non-toxic concentration and sub-toxic concentration (Supplementary Fig. S1) for 24 h and the pro-inflammatory cytokines were quantified (Fig. 2). Fig. 2C and D shows that $AlCl_3$ induced IL-6 and TNF-α levels in a dose-dependent manner but failed to induce IFN-γ and IL-1β and (at 500 μM) (Fig. 2A and B). Similar to $AlCl_3$-treated BV2 cells, Fig. 2G and H shows that TG induced IL-6 and TNF-α production in a dose-dependent manner before reaching a steady state at 100 nM. However, TG did not induce both IFN-γ and IL-1β even at the high

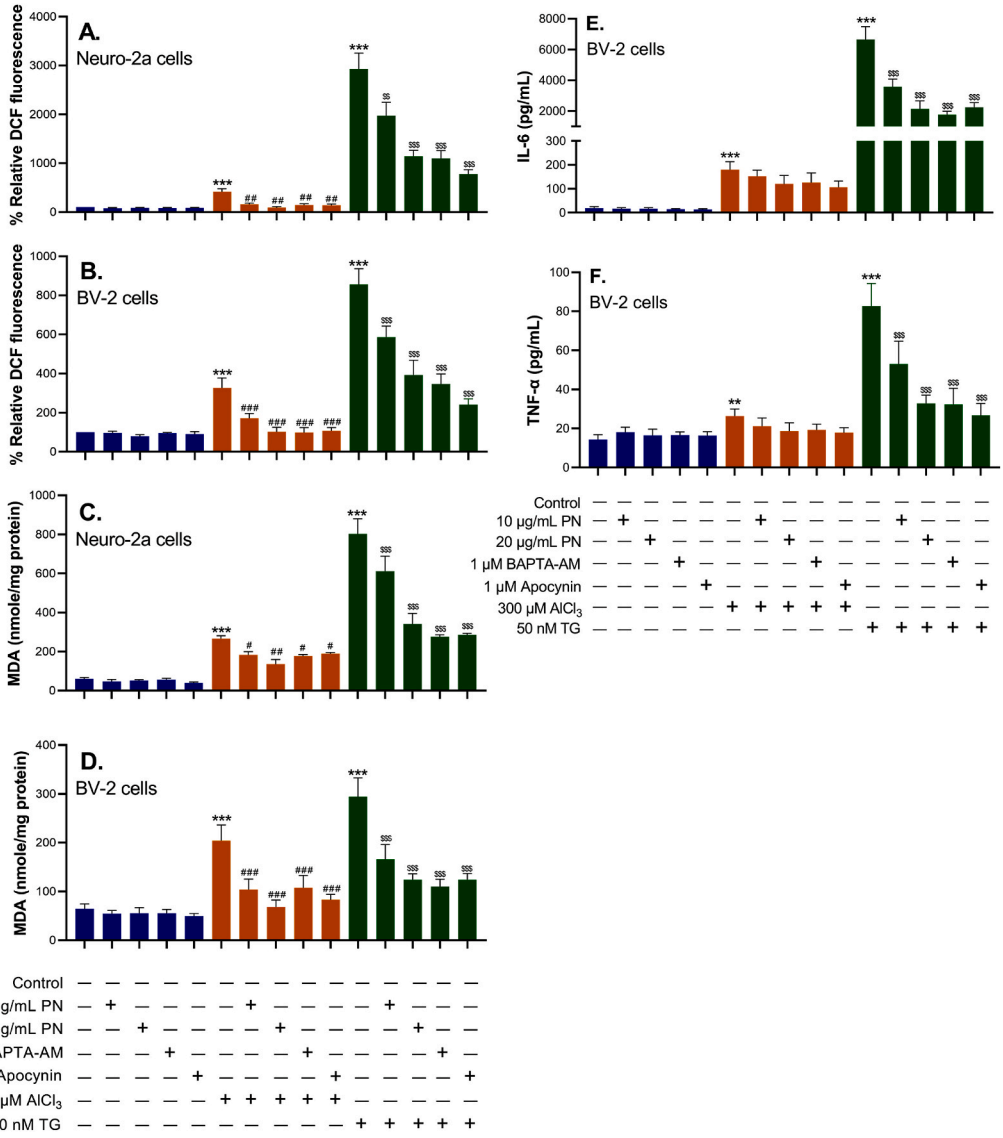

Fig. 5. PN Extract Reduced $AlCl_3$ and TG-mediated Oxidative Stress in both Neuro-2a Cells and BV-2 cells and Reduced $AlCl_3$ and TG-mediated Cytokine Release in BV-2 Cells. Neuro-2a cells and BV-2 cells were pre-treated with PN extract, BAPTA-AM, or apocynin for 12 h before treatment with $AlCl_3$ or TG for another 24 h. (A.) % relative DCF fluorescence in Neuro-2a cells, (B.) % relative DCF fluorescence in BV-2 cells, (C.) MDA levels in Neuro-2a cells, (D) MDA levels in BV-2 cells, (E.), IL-6 levels in BV-2 cells and (F.) TNF-α levels in BV-2 cells. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. **, $p < 0.01$ and ***, $p < 0.001$ (compared to control), $^{\#}$, $p < 0.05$, $^{\#\#}$, $p < 0.01$ and $^{\#\#\#}$, $p < 0.001$ (compared to $AlCl_3$-treated cells), and $^{\$\$}$, $p < 0.01$ and $^{\$\$\$}$, $p < 0.001$ (compared to TG-treated cells).

dose (Fig. 2E and F).

In summary, $AlCl_3$ and TG promoted inflammation in BV-2 cells, possibly contributing to the reduction in cell viability under $AlCl_3$ and TG treatment. Interestingly, our data suggested that $AlCl_3$ and TG may provoke neuroinflammation in the same axis.

3.3. Effects of $AlCl_3$ and TG on BACE-1, βIII-tubulin and ER Stress Protein in Neuro-2a cells

$AlCl_3$ and TG are known to participate in neurological disorders, including AD. To explore this, we studied the effects of $AlCl_3$ and TG on the important protein biomarkers for AD (BACE-1), neurogenesis (βIII-tubulin), and ER stress protein (Bip). Neuro-2a cells were treated with $AlCl_3$ (100, 300, and 500 μM) or TG (10, 50 and 100 nM) for 24 h and 48 h. Then, cell lysate was subjected to Western blot analysis. Fig. 3 demonstrates that after 24 h of treatment, $AlCl_3$ (200 and 300 μM) increased the protein expression of BACE-1 and Bip while decreasing βIII-tubulin, suggesting that the amyloid and ER stress pathways were activated while neurogenesis was inhibited. At 48 h of $AlCl_3$ treatment (200 and 300 μM), continued activation of BACE-1 and inhibition of neurogenesis were observed, whereas the ER stress pathway (Bip) was quenched (Fig. 3A and C), suggesting transient ER stress activation by $AlCl_3$. Almost similar responses were observed in TG-treated Neuro-2a cells. Within 24 h of TG treatment (50 and 100 nM), both BACE-1 and Bip significantly increased while βIII-tubulin decreased (Fig. 3B and D), with all protein markers decreasing after 48 h of TG treatment. These results indicated that $AlCl_3$ and TG affected the amyloid pathway, ER stress, and neurogenesis in a similar manner within 24 h of treatment; however, neurons compensated for the toxicity of $AlCl_3$ and TG in distinct ways during further incubation. Thus, we treated cells for only 24 h in further experiments.

3.4. Effects of $AlCl_3$ and TG on BACE-1, MAPKs, and ER stress protein in BV-2 cells

The effects of $AlCl_3$ and TG on BACE-1, ER stress protein (Bip), and mitogen-activated protein kinases (MAPKs; ERK and p38) in BV-2 cells were further assayed. In this experiment, we studied MAPKs instead of βIII-tubulin which is a neuron-specific protein, and activation of MAPKs contributed to the release of proinflammatory cytokines, as observed in Fig. 2. BV-2 cells were treated with $AlCl_3$ (100, 300, and 500 μM) or TG (10, 50, and 100 nM) for 24 h and 48 h. Protein levels of BACE-1 and Bip were increased when treated with 100 and 300 μM $AlCl_3$ for 24 h, but not at 500 μM $AlCl_3$. At 48 h, $AlCl_3$-mediated BACE-1 level was significantly increased at all tested concentrations, whereas $AlCl_3$-mediated Bip level was significantly increased only at 500 μM of $AlCl_3$ (Fig. 4A and C). Moreover, at 24 h of $AlCl_3$ exposure, both phosphorylated protein levels of ERK and p38 were significantly induced at all tested concentrations compared to control. At 48 h of exposure, the phosphorylated ERK and p38 levels were significantly induced by $AlCl_3$ at 100–500 μM and 300–500 μM, respectively (Fig. 4A and C). The same trend was observed in TG-treated BV-2 cells (Fig. 4B and D), albeit with different degrees of activation compared to $AlCl_3$-treated cells. At 24 and 48 h of TG treatment (10, 50, and 100 nM), BACE-1, Bip, and phosphorylated ERK levels were significantly increased in all indicated concentrations. As noted, TG-induced Bip expression was higher within 24 h of exposure compared to 48 h after exposure. We observed that phosphorylated p38 level seemed to increase when treated with 50 and 100 nM at 48 h, but significantly increased only at 50 nM after 24 h of exposure. These results suggested that the enhanced cytokine production observed in Fig. 2 may be due to the activation of MAPKs (ERK and p38) in BV-2 cells.

3.5. PN extract reduces $AlCl_3$ and TG-induced oxidative stress in Neuro-2a and BV-2 cells, and $AlCl_3$ and TG-induced Cytokine Release in BV-2 cells

As demonstrated in Figs. 1–4, $AlCl_3$ and TG induced oxidative stress in both Neuro-2a and BV-2 cells and inflammatory responses in BV-2 cells. $AlCl_3$ and TG also activated ER stress and MAPK signaling. PN extract has been reported to act as an anti-neuroinflammatory by inhibiting proinflammatory cytokines and ERK [17]. Therefore, we studied the ameliorating effects of PN extract on $AlCl_3$ and TG-induced oxidative stress and inflammation. TG is an ER stress activator by specifically inhibiting the endoplasmic reticulum Ca^{2+-}ATPase leading to increasing cytosolic calcium concentrations, ER calcium depletion, and activation of unfolded protein response [25]. Thus, as a control for inhibiting ER stress, we introduced two compounds, BAPTA-AM, and apocynin, as calcium chelators and ER stress inhibitors, respectively [35,36].

Neuro-2a cells and BV-2 cells were pre-treated with PN extract (10 and 20 μg/mL), BAPTA-AM (1 μM), or apocynin (1 μM) for 12 h before treatment with $AlCl_3$ (300 μM) or TG (50 nM) for another 24 h. Fig. 5A to D reveal that a single treatment of PN extract, BAPTA-AM, and apocynin did not affect intracellular ROS and MDA levels, while $AlCl_3$ and TG increased intracellular ROS and MDA levels compared to the control in both cell lines. Interestingly, PN extract (10 and 20 μg/mL), BAPTA-AM, and apocynin prevented $AlCl_3$-and TG-mediated intracellular ROS and MDA formation in both Neuro-2a cells and BV-2 cells. Fig. 5E and F shows the effects of PN extract, BAPTA-AM, and apocynin on the proinflammatory cytokines of $AlCl_3$-or TG-treated BV-2 cells. All agents (PN extract, BAPTA-AM, and apocynin) did not affect $AlCl_3$-mediated neuroinflammation in BV-2 cells because the IL-6 and TNF-α were not reduced compared to $AlCl_3$-exposed BV-2 cells. By contrast to $AlCl_3$, PN extract, BAPTA-AM, and apocynin substantially suppressed IL-6 and TNF-α production (Fig. 5E and F). In conclusion, PN extract attenuated intracellular ROS, lipid peroxidation, and inflammatory responses caused by $AlCl_3$ and TG with almost comparable efficiency to BAPTA-AM and apocynin, thereby shedding insights on the anti-ER stress properties of PN extract for the first time.

3.6. Protective effects of PN extract on $AlCl_3$ or TG-exposed Neuro-2a cells

Results in Fig. 5 implied that PN extract may exert properties as an anti-ER stress agent. ER stress is known to contribute to

neuroinflammation and neurological disorders [37]. Thus, we investigated the neuroprotective role of PN extract on $AlCl_3$-or TG-exposed neurons. The neuro-2a cells were pre-treated with PN extract, followed by the addition of $AlCl_3$ or TG. The three protein levels participating in the amyloid pathway (BACE-1), ER stress (Bip), and neurogenesis (βIII-tubulin) were then evaluated. Single treatment of PN extract (20 μg/mL), BAPTA-AM (1 μM), and apocynin (1 μM) did not affect these three proteins, while both $AlCl_3$ (300 μM) and TG (50 nM) led to the elevated expression of BACE-1 and Bip, while decreasing βIII-tubulin expression (Fig. 6). The neuroprotective benefits of PN extract and apocynin, except BAPTA-AM, against $AlCl_3$ were demonstrated by their ability to prevent the induction of BACE-1 and Bip while restoring βIII-tubulin with comparable effectiveness (Fig. 6A and B).

Consistent with Fig. 6, the same results were obtained in TG-treated neurons. PN extract and apocynin significantly reduced the expression of BACE-1, and Bip and increased βIII-tubulin (Fig. 7A and B). In summary, PN extract exerted protective effects against $AlCl_3$ and TG, possibly via its anti-ER stress properties in neurons.

3.7. Protective effects of PN extract on $AlCl_3$ or TG-exposed BV-2 cells

We further elucidated the neuroprotective effects of PN extract using BV-2 cells as a model. The same experimental procedure as section 3.6 was followed. $AlCl_3$-or TG-treated BV-2 cells showed clear induction of protein markers, including BACE-1, Bip, and phosphorylation of p38 and ERK. Fig. 8A and B and 9A-B show that PN extract (20 μg/mL), and apocynin (1 μM) significantly decreased protein levels of Bip and phosphorylation of p38 and ERK in $AlCl_3$-or TG-treated BV-2 cells. For BACE-1, PN extract significantly decreased the protein level in $AlCl_3$-or TG-treated BV-2 cells, while apocynin had the effect only in TG-treated cells. These results indicated that PN extract appears to inhibit ER stress in microglia cells as in neurons.

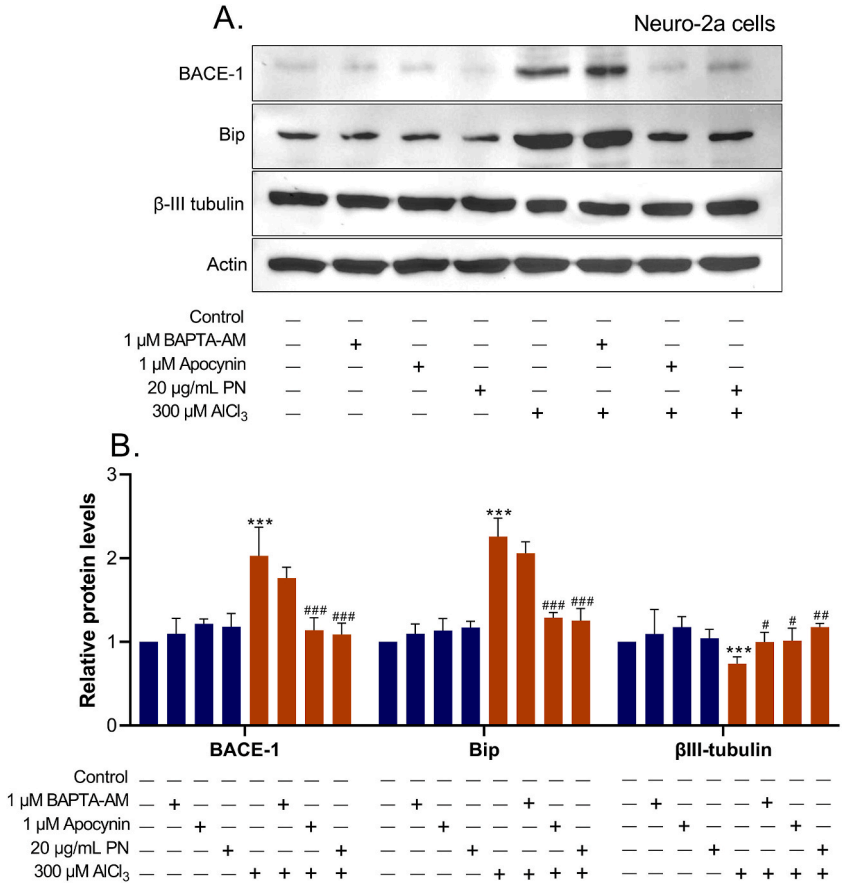

Fig. 6. Protective Effects of PN Extract on $AlCl_3$-exposed Neuro-2a Cells. Neuro-2a cells were pre-treated with PN extract, BAPTA-AM, or apocynin for 12 h before treatment with $AlCl_3$ for another 24 h. (A.) Immunoblot figure of Neuro-2a cells treated with $AlCl_3$ and indicated agents, and (B.) Quantification of band intensity of each protein (relative to actin) of Neuro-2a cells treated with $AlCl_3$ and indicated agents. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. ***, $p < 0.001$ (compared to control), #, $p < 0.05$, ##, $p < 0.01$, and ###, $p < 0.001$ (compared to $AlCl_3$-treated cells).

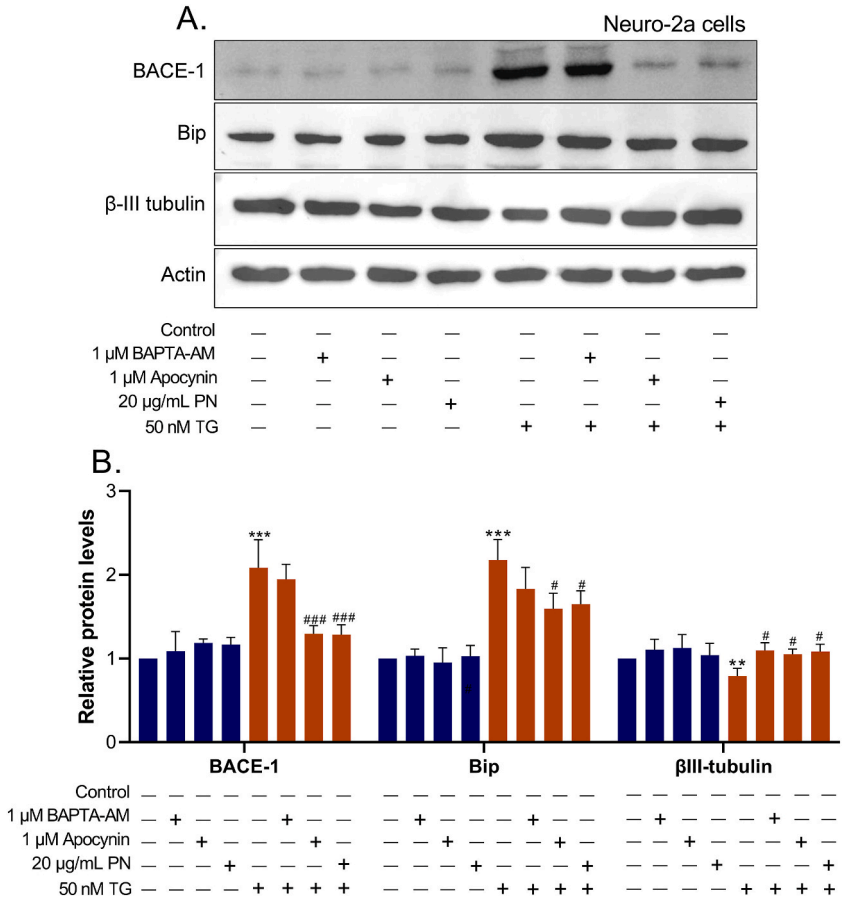

Fig. 7. Protective Effects of PN Extract on TG-exposed Neuro-2a Cells. Neuro-2a cells were pre-treated with PN extract, BAPTA-AM, or apocynin for 12 h before treatment with TG for another 24 h. (A.) Immunoblot figure of Neuro-2a cells treated with TG and indicated agents, and (B.) Quantification of band intensity of each protein (relative to actin) of Neuro-2a cells treated with TG and indicated agents. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. **, $p < 0.01$ and ***, $p < 0.001$ (compared to control), $^{\#}$, $p < 0.05$, and $^{\#\#\#}$, $p < 0.001$ (compared to TG-treated cells).

3.8. PN extract ameliorates AD pathogenesis in flies

As previously stated, the amyloid pathway, ER stress, neuroinflammation, and oxidative stress have been reported to participate in AD pathogenesis. We previously reported the anti-neuroinflammation activity of PN extract in the co-culture between neurons and microglia [17]. Thus, PN extract could be used as an anti-AD agent by inhibiting the above-mentioned mechanisms. To prove this hypothesis, we employed *Drosophila* harboring human amyloid precursor protein (APP) and β-secretase 1 (BACE-1) as an *in vivo* model. Administering RU486 to the fly medium induces the specific expression of APP and BACE-1 in the fly brains, which ultimately leads to the formation of the amyloid peptides (Aβ, AD biomarker), thus representing the Aβ pathway of AD.

As stated in materials and methods, the flies were divided into four groups and cultured at indicated times. On days 7, 14, 21, and 28 of treatment, flies were tested for climbing ability which can be used to determine the locomotor function [38]. Fig. 10A to D demonstrate that the climbing index of only RU486-treated flies (dark blue) decreased progressively (from 3.3 to 1.0) from day 7 to day 28, indicating that AD flies had impaired locomotor function, as previously reported [32,33]. This climbing ability was rescued through the addition of donepezil (AD drug). Interestingly, PN extracts at 200 and 400 μg/mL also significantly rescued climbing ability (Fig. 10D) in AD flies. We further examined the activity of BACE-1, which is a rate-limiting step enzyme in amyloid peptide production. Hence, BACE-1 inhibitors are promising therapeutic targets to treat AD [7,39]. Fig. 10E shows that high BACE-1 activity was observed in only RU486-treated flies (dark blue), while a reduction in BACE-1 activity was observed in PN- and donepezil-treated flies. To confirm this finding, we measured the amount of $A\beta_{1\text{-}42}$ (hallmarks of AD), which is the cleavage consequence of BACE-1 on APP. The high amount of $A\beta_{1\text{-}42}$ was clearly shown in only RU486-treated flies (Fig. 10F, dark blue), while reductions in $A\beta_{1\text{-}42}$ levels were obtained in PN- and donepezil-treated flies.

Fly heads at day 28 of treatment were also subjected to the gene expression of ER stress- and antioxidant-related genes by RT-qPCR. Confirming the cell study, PN extract acted as anti-ER stress by inhibiting Bip, ATF-6, IRE-1, and PEK expression (Fig. 10G–J). PN extract also inhibited SOD1 and GPx expression. These two enzymes control oxidative stress and lipid peroxidation (Fig. 10K to L),

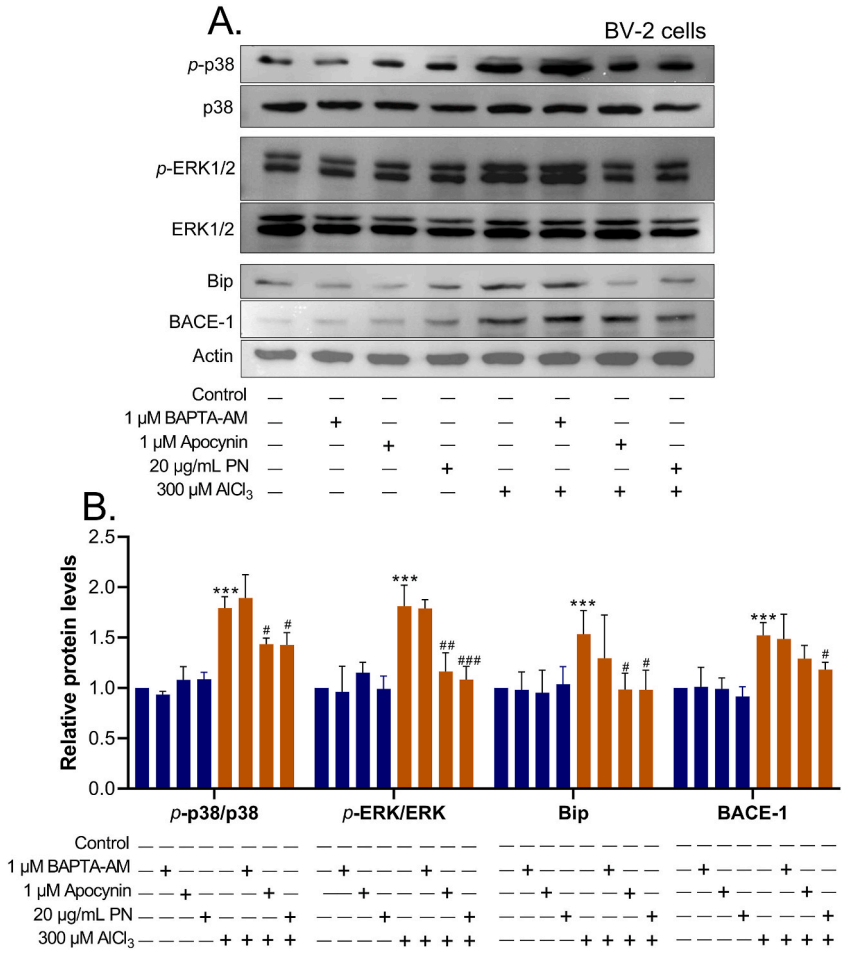

Fig. 8. Protective Effects of PN Extract on $AlCl_3$-exposed BV-2 Cells. BV-2 cells were pre-treated with PN extract, BAPTA-AM, or apocynin for 12 h before treatment with $AlCl_3$ for another 24 h. (A.) Immunoblot figure of BV-2 cells treated with $AlCl_3$ and indicated agents, and (B) Quantification of band intensity of each protein (relative to actin or its total form) of BV-2 cells treated with $AlCl_3$ and indicated agents. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. ***, $p < 0.001$ (compared to control), $^{\#}$, $p < 0.05$, $^{\#\#}$, $p < 0.01$, and $^{\#\#\#}$, $p < 0.001$ (compared to $AlCl_3$-treated cells).

indicating low oxidative stress and lipid peroxidation in PN- and donepezil-treated flies. In summary, PN extract exhibited a dose-dependent manner against amyloid pathway (BACE-1 and $A\beta_{1\text{-}42}$ levels), ER stress response (Bip, ATF-6, and IRE-1 and PEK), and oxidative stress control (SOD1 and GPx). These results confirmed the role of PN extract as an anti-ER stress inhibitor, concurring with the cell study (Figs. 6–9).

4. Discussion

Alzheimer's has now become a primary global disease, and the study of naturally occurring compounds focuses mainly on slowing disease progression and improving patients' quality of life [40,41]. Multiple biomolecular targets in medicinal products have the potential to attenuate the development of Alzheimer's disease (AD). These targets include the inhibition of neurotoxic β-amyloid generating enzyme, namely BACE-1 [2,7,39], the reduction of oxidative stress and neuroinflammation [42,43], as well as the inhibition of ER stress [4,44]. Natural compounds have recently garnered significant interest due to their particular benefits in responding to many targets associated with AD. Consequently, they hold promise for development as multi-target substances [41]. Phikud Navakot (PN) is a Thai natural remedy that has been included on the national list of essential medicines. PN comprises a combination of nine plants, each of which is included in equal weight ratios [16,45]. PN extract demonstrated neuroprotective benefits through the suppression of neuroinflammatory reactions produced by lipopolysaccharides [17]. In this investigation, we provided evidence to support the concept that PN extract effectively reduced the phenotypic manifestations of AD. This was achieved by a reduction in the levels of BACE-1 and $A\beta_{1\text{-}42}$ peptides in a *Drosophila* model of AD. We also showed that PN extract-protected aluminum chloride ($AlCl_3$) and thapsigargin (TG) promoted neuroinflammation and endoplasmic reticulum stress in cell cultures of neuronal cells and microglia cells.

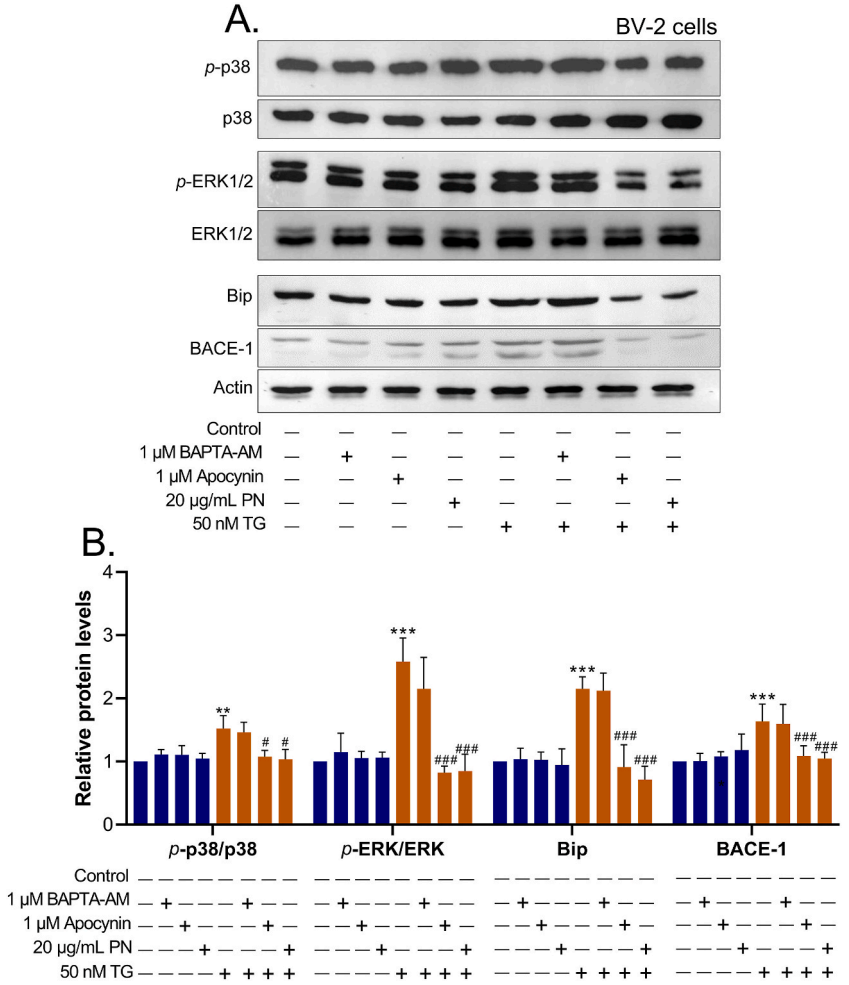

Fig. 9. Protective Effects of PN Extract on TG-exposed BV-2 Cells. BV-2 cells were pre-treated with PN extract, BAPTA-AM, or apocynin for 12 h before treatment with TG for another 24 h. (A.) Immunoblot figure of BV-2 cells treated with TG and indicated agents, and (D.) Quantification of band intensity of each protein (relative to actin or its total form) of BV-2 cells treated with TG and indicated agents. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control by one-way ANOVA followed by Tukey's multiple comparisons test. **, $p < 0.01$, ***, $p < 0.001$ (compared to control), $^{\#}$, $p < 0.05$ and $^{\#\#\#}p < 0.001$ (compared to TG-treated cells).

To assess the possible neuroprotective effects of PN extract, the study employed Neuro-2a cells as a representative model for neuronal cells, and BV-2 cells as a representative model for microglia cells. PN extract was pre-incubated before stimulation with AlCl3 and TG to determine its protective potential and to provide insights into its preventive mechanisms. $AlCl_3$ was employed to produce BACE-1, while TG was utilized to create ER stress in Neuro-2a and BV-2 cells [25,46–48]. TG is a potent inhibitor of the sarco/endoplasmic reticulum Ca2^{+}-ATPase (SERCA) pump. By suppressing SERCA, TG depletes Ca^{2+} stores inside the ER lumen. The resulting deterioration of Ca^{2+} homeostasis activates the unfolded protein response (UPR), a signaling cascade that occurs in response to ER stress. The UPR aims to restore ER function by reducing the load of misfolded proteins and enhancing the folding capacity of the ER. However, if ER stress persists or overwhelms cellular capacity to cope with it, the UPR can also induce inflammation-related cascades, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), ROS formation, and MAPKs, leading to increased production of pro-inflammatory cytokines [49,50]. $AlCl_3$ has been implicated as a potential environmental factor contributing to the pathogenesis of AD [24]. While the exact mechanisms underlying its involvement in AD are not fully understood, several hypotheses have been proposed, including ability to (i) induce neuroinflammation, which involves the production of pro-inflammatory cytokines [51], (ii) cause oxidative stress in the brain by increasing the production of ROS, which can damage neuronal cells and contribute to the progress of AD [52], (iii) activate microglia, causing them to generate pro-inflammatory cytokines that contribute to neuroinflammation [53,54] and (iv) stimulate ER stress, and subsequent induction of pro-inflammatory cytokine production [55]. The administration of $AlCl_3$ resulted in an elevation of BACE-1 level and ER stress protein, Bip. TG was also shown to enhance Bip levels while increasing the BACE-1 level. The effects of $AlCl_3$ and TG on the elevated level of BACE-1 in neuronal and microglia cells were attenuated when the cells were pretreated with apocynin, an ER-stress inhibitor, and BAPTA-AM, a calcium chelator. Administration of apocynin and BAPTA-AM displayed the ability to ameliorate the impact of $AlCl_3$ and TG on the generation of oxidative stress, as

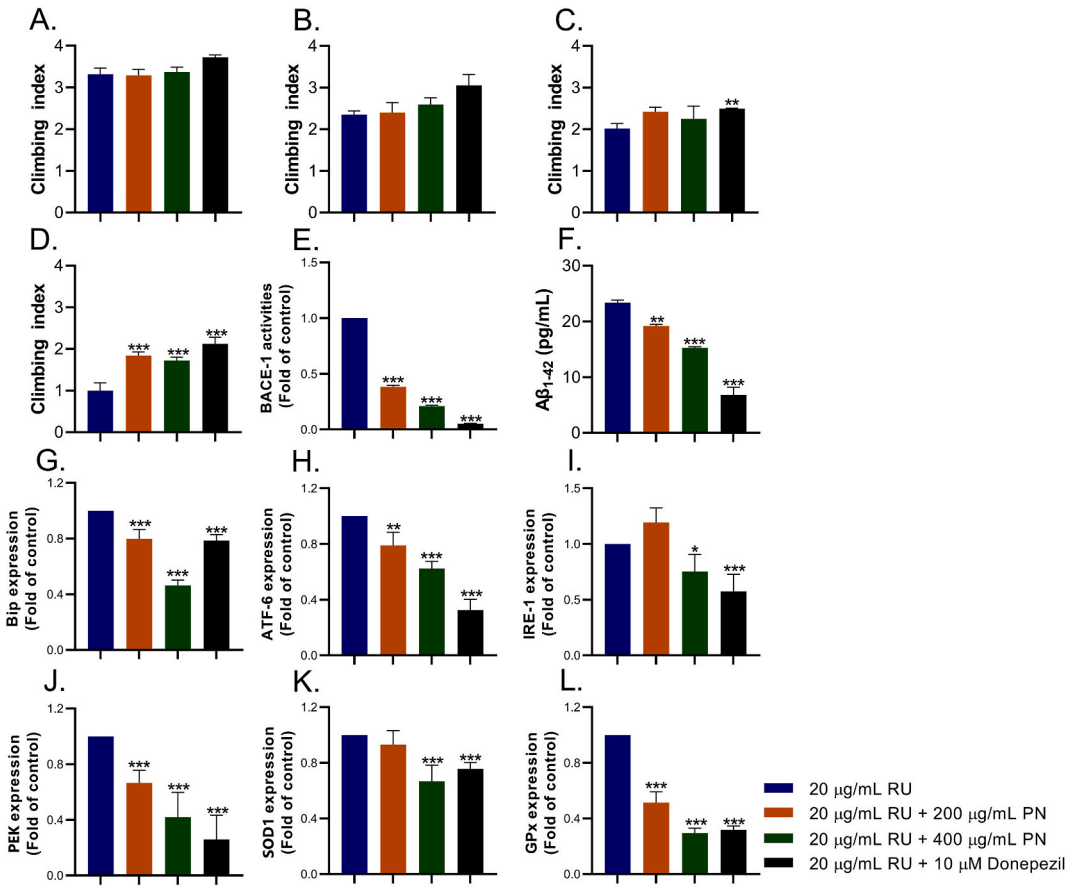

Fig. 10. Anti-AD properties of PN extract in the *Drosophila* model of AD. (A.) climbing index on day 7 of treatment, (B) climbing index on day 14 of treatment, (C.) climbing index on day 21 of treatment, (D.) climbing index on day 28 of treatment, (E.) BACE-1 activities at day 28 of treatment, (F.) amyloid peptide ($A\beta_{1\text{-}42}$) levels at day 28 of treatment, (G.) Bip expression at day 28 of treatment, (H.) ATF-6 expression at day 28 of treatment, (I.) IRE-1 at day 28 of treatment, (J.) PEK expression at day 28 of treatment (K.) SOD1 expression at day 28 of treatment and (L.). GPx expression at day 28 of treatment. One-day-old flies expressing human APP and BACE-1 were treated with 20 μg/mL of RU486, PN extract (200 and 400) μg/mL, and 10 μM donepezil. At the indicated time, flies were collected and subjected to climbing index, BACE-1, $A\beta_{1\text{–}42}$ levels, or RT-qPCR. The values are mean ± SD of three independent experiments and statistical significance was analyzed against control (RU-treated flies, dark blue) by one-way ANOVA followed by Tukey's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$.

indicated by levels of ROS and MDA, as well as the release of cytokines. The present study showed that the administration of $AlCl_3$ and TG resulted in elevated levels of TNF-α and IL-6 while not affecting IL-1β and IFN-γ. The production of various cytokines is controlled by different transcription factors such as nuclear factor kappa B (NF-κB), signal transducers and activators of transcriptions (STATs), activator protein-1(AP-1), and nuclear factor erythroid 2-related factor-2 (Nrf-2) [56,57]. The activation of BACE-1 in AD is triggered by several situations including oxidative stress, inflammation, and change in calcium homeostasis [10–13]. The potential involvement of BACE-1 activation in the pathogenesis of AD lies in its ability to enhance the generation of $A\beta_{1\text{-}42}$, hence potentially contributing to the progression and acceleration of AD pathology [2,7,39]. The activation of BACE-1 by $A\beta_{1\text{-}42}$ establishes a positive regulatory loop, hence establishing a toxic cycle [2]. These findings suggested that ER stress might potentially trigger the activation of BACE-1, leading to the development of AD, corresponding with previous research conducted both *in vitro* and *in vivo* [37,58,59].

In neuronal and microglia cells, PN extract reduced the increase in BACE-1, Bip, ROS, and MDA levels caused by $AlCl_3$ and TG. Furthermore, PN extract decreased both the effect of TG and $AlCl_3$ on the production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in microglia cells, as well as neurodegeneration in Neuro-2a cells. Our findings demonstrated that the effects seen were comparable to those of certain phytochemicals, particularly morin, thymol, thymoquinone, quercetin, and baicalein. These phytochemicals were found to decrease the expression of BACE1 and ameliorate the aggregation and toxicity of amyloid proteins [47,60,61]. PN extract inhibited the ER stress induced by $AlCl_3$ and TG in both neuronal and microglial cells. This effect was similar to the observed reduction in $A\beta_{1\text{-}42}$ accumulation and alleviation of ER stress caused by $AlCl_3$ administration in rat brains when melatonin was administered [46]. Based on the findings that ER stress inhibitor and calcium chelator attenuate the effects of $AlCl_3$ and TG on BACE-1 expression, oxidative stress, and inflammation, our results suggested that PN extract may possess neuroprotective properties through the inhibition of BACE-1 expression, oxidative stress, and inflammation via inhibition of ER stress, or may affect a variety of

targets.

The pathways implicated in the mechanism of action of PN extract on its neuroprotective properties were also examined. PN extract inhibited mitogen-activated protein kinases (MAPKs) activation including p38 and ERK1/2 induced by $AlCl_3$ and TG in microglia cells. A previous study also showed that the extract of PN successfully inhibited the activation of microglia induced by LPS by suppressing the activation of ERK1/2 [17]. Hence, it is plausible that $AlCl_3$, TG, and LPS might potentially interact with distinct upstream molecules of MAPKs, thereby influencing the varied effectiveness of PN in preventing MAPK activation. The recognition of multiple points of intercommunication between the ER stress and MAPK signaling pathways might potentially play a role in the development of inflammation and oxidative stress, eventually resulting in neurodegenerative diseases [25,62,63]. The p38 may contribute to the reduction of BACE-1 levels by promoting the degradation of BACE-1 [64], while the up-regulation of BACE1 in glial cells, caused by interferon-γ, is mediated through activation of the ERK1/2 signaling pathways [65]. Cytokines and oxidative stress produced in microglia via MAPK activation resulted in the up-regulation of BACE-1 in AD pathogenesis, accordingly, PN lowered the functioning of ERK1/2 and p38, resulting in a reduction of BACE-1, which may reduce AD development [66]. There is currently limited evidence that ER stress has an actual impact on the induction of BACE-1 in microglia. Nonetheless, ER stress promotes neuroinflammation via unfolded protein response (UPR) signaling, including MAPKs, which may raise BACE-1 expression [3,8]. As a result, the effects of PN on ER stress reduction might decrease BACE-1 levels and neuroinflammation in AD.

Subsequently, an examination was conducted to confirm the capacity of PN extract to induce neuroprotective impacts *in vivo*. *Drosophila* models have been established to evaluate the development and therapeutic strategies of AD, exhibiting particular features of AD including behavioral changes and the generation of proteins such as BACE-1, Aβ, and inflammatory mediators [67,68]. In this study, the administration of PN extract resulted in an elevation in the climbing index and a decrease in the levels of BACE-1 and $A\beta_{1\text{-}42}$.

Administration of PN also resulted in the down-regulation of the mRNA expression of the ER stress markers including, Bip, ATF6, IRE1, and PERK which increased in AD [69,70]. The presence of increased levels of $A\beta_{1\text{-}42}$ and ER stress leads to the occurrence of intracellular oxidative stress. In response, cells try to counteract the effects of this stress by upregulating antioxidant enzymes, superoxide dismutase 1 (SOD1), and glutathione peroxidase (GPx) [71,72]. However, when the levels of ER stress and $A\beta_{1\text{-}42}$ are reduced by the administration of PN, the antioxidant enzyme levels return to their normal state. Elevated activity of antioxidant enzymes has been implicated as a potential factor in the protective response against heightened superoxide generation associated with neurodegenerative disorders including AD [73,74]. The positive impacts of PN on neuroprotection, as revealed in cell culture experiments, were supported by their ability to considerably reduce raised BACE-1 activity and $A\beta_{1\text{-}42}$ levels, as well as ameliorate ER stress in flies that were treated with PN. This characteristic may also contribute to the capacity of PN to improve locomotor function that is reduced in AD flies. Our findings confirmed the potential *in vivo* neuroprotective benefits of PN extract on AD through the suppression of BACE-1 and ER stress, which is known to contribute to the reduction of $A\beta_{1\text{-}42}$ and other key pathological features of AD.

The primary phytochemical constituents found in PN extracts are gallic acid, vanillic acid, caffeic acid, rutin, and ferulic acid [16,17]. The primary bioactive compounds responsible for the decrease of neuroinflammation were recognized as gallic acid and vanillic acid which can pass the blood-brain barrier [17,75–77]. The ability of gallic acid to reduce the progression of AD has been demonstrated through its capacity to lower ER stress, downregulate BACE-1 expression, attenuate oxidative damage, and suppress inflammation [76]. Nevertheless, the evidence currently available establishing the protective properties of vanillic acid against ER stress and the inhibition of BACE-1 remains limited. The potential of vanillic acid in reducing neurodegeneration has been demonstrated by its ability to attenuate oxidative stress and suppress neuroinflammatory responses [78,79]. Similarly, the primary mechanism through which caffeic acid, rutin, and ferulic acid reduce the development of AD is through the reduction of oxidative stress and inflammation. However, little is known about the impact of these substances on reducing ER stress and BACE-1 production. The expression of BACE-1 and the generation of $A\beta_{1\text{-}42}$ are stimulated by ER stress, oxidative stress, and cytokines. Conversely, these factors can also be triggered by $A\beta_{1\text{-}42}$. These actions create a vicious cycle that accelerates the progression of AD [10,80]. Our findings indicated that PN extract has the potential to limit the activity of p38 and ERK1/2, resulting in a reduction of ER stress, BACE-1 expression, release of cytokines, and oxidative damage. These effects ultimately contribute to reducing neuroinflammation and the development of Aβ plaque in the brain.

5. Conclusion

This study demonstrated the neuroprotective properties of Phikud Navakot (PN) extract, a Thai natural remedy to reduce the pathogenesis of AD in the *in vivo* and *in vitro* models. PN extract reduced the elevated levels of BACE-1, ER stress, ROS, and MDA caused by $AlCl_3$ and TG in neuronal and microglia cells. PN extract was also shown to inhibit ER stress and MAPK activation including p38 and ERK1/2, which are linked to inflammation and oxidative stress in microglia cells. In a *Drosophila* model of AD, PN extract effectively reduced levels of BACE-1 and $A\beta_{1\text{-}42}$. as well as ER-stress-related mRNA expression. PN also returned the expression of elevated antioxidant enzymes to their regular levels. These effects may contribute to the neuroprotective properties of PN extracts that appear to act on multiple targets to reduce neuroinflammation and Aβ plaque development in the brain. However, further research is needed to confirm and better understand these potential therapeutic effects and mechanisms of action.

CRediT authorship contribution statement

Piya Temviriyanukul: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Anchana Chansawhang:** Validation, Methodology, Investigation, Formal analysis. **Woorawee Inthachat:** Visualization, Methodology, Investigation. **Punchaya Supasawat:** Investigation, Formal analysis. **Sataporn Phochantachinda:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Pornsiri Pitchakarn:** Writing – review & editing, Writing –

original draft, Formal analysis, Data curation, Conceptualization. **Boonrat Chantong:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Institutional review board statement

The *Drosophila* study was approved by the Institute of Nutrition-Mahidol University Institutional Animal Care and Use Committee (INMU-IACUC) (COA. No. INMU-IACUC: 2022/02).

Data availability

Data included in article/supp. Material/referenced in the article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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