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RESEARCH ARTICLE

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Inhibiting mitochondrial inflammation through Drp1/HK1/NLRP3 pathway: A mechanism of alpinetin attenuated aging-associated cognitive impairment

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Abstract

Mitochondrial inflammation triggered by abnormal mitochondrial division and regulated by the Drp1/HK1/NLRP3 pathway is correlated with the progression of aging-associated cognitive impairment (AACI). Alpinetin is a novel flavonoid derived from Zingiberaceae that has many bioactivities such as antiinflammation and anti-oxidation. However, whether alpinetin alleviates AACI by suppressing Drp1/HK1/NLRP3 pathway-inhibited mitochondrial inflammation is still unknown. In the present study, D-galactose (D-gal)-induced aging mice and BV-2 cells were used, and the effects of alpinetin on learning and memory function, neuroprotection and activation of the Drp1/HK1/NLRP3 pathway were investigated. Our data indicated that alpinetin significantly alleviated cognitive dysfunction and neuronal damage in the CA1 and CA3 regions of D-gal-treated mice. Moreover, D-gal-induced microglial activation was markedly reduced by alpinetin by inhibiting the Drp1/HK1/NLRP3 pathway-suppressed mitochondrial inflammation, down-regulating the levels of p-Drp1 (s616), VDAC, NLRP3, ASC, Cleaved-caspase 1, IL-18, and IL-1β, and up-regulating the expression of HK1. Furthermore, after Drp1 inhibition by Mdivi-1 in vitro, the inhibitory effect of alpinetin on Drp1/HK1/NLRP3 pathway was more evident. In summary, the current results implied that alpinetin attenuated aging-related cognitive deficits by inhibiting the Drp1/HK1/NLRP3 pathway and suppressing mitochondrial inflammation, suggesting that the inhibition of the Drp1/HK1/NLRP3 pathway is one of the mechanisms by which alpinetin attenuates AACI.

KEYWORDS
aging-associated cognitive impairment, alpinetin, Drp1/HK1/NLRP3 pathway, mitochondrial inflammation

Abbreviations: AACI, aging-associated cognitive impairment; AP, alpinetin; ASC, apoptotic-associated speck-like protein; Aβ, β-amyloid; CREB, cyclic AMP response element-binding protein; D-gal, D-galactose; Drp1, dynamin-related protein 1; ERK, extracellular-signal-regulated kinase; HK1, hexokinase 1; Iba1, ionizing calcium-binding adapter molecule 1; IL-18, interleukin-18; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; Mdivi-1, mitochondrial division inhibitor 1; MEM, memantine; MWM, Morris water maze; NF-κB, nuclear factor kappa-B; NGF, nerve growth factor; NLRP3, NOD-like receptor protein 3; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; VDAC, voltage-dependent anion channel.

Yuanyuan Chen and Chuan Yang contributed equally to this work.

Animal grouping and specimen selection were carried out according to the principle of randomization in the present study.

1 | INTRODUCTION

Aging is the main high-risk factor for multiple cognitive aging diseases such as aging-associated cognitive impairment (AACI), mild cognitive impairment, Parkinson's disease, and Alzheimer's disease, which have become a global social burden and challenges that have damaged public health as the population ages worldwide (Hou et al., 2019). The pathogenesis of AACI is complex and multifactorial, and involves neuronal damage, oxidative stress, mitochondrial dysfunction, and neuroinflammation. Although some interventions have been reported, such as Lactobacillus plantarum TWK10, methionine restriction, and HL271(Bang et al., 2018; Lee et al., 2021; Ren et al., 2021), unfortunately, there have been no effective means of preventing and treating AACI in clinical practice (Scheltens et al., 2021). Although aging-induced neurological damage is irreversible, it is possible to delay or reduce neurological injury by slowing down the aging process with early intervention. Thus, slowing down the rate of aging through early intervention may be a promising strategy for improving neurodegenerative disorders, such as AACI (Guerrero et al., 2021; West, 2017).

Accumulation of oxidative stress and overproduction of reactive oxygen species (ROS) during aging are considered crucial mechanisms leading to AACI. ROS originating from both oxidative stress and mitochondria can propagate immune activation and damage the mitochondria, thereby leading to mitochondrial dysfunction (Ionescu-Tucker & Cotman, 2021). Damaged mitochondria, such as disrupted mitochondrial dynamics and accumulation of mitochondrial fragmentation, play an important role in the activation of neuroinflammatory signaling pathways associated with aging (Neves & Sousa-Victor, 2020). Therefore, mitochondrial dysfunction is thought to promote the disease progression of AACI (Mota et al., 2021; van Horssen et al., 2019). An increasing number of studies have shown that mitochondrial dynamics disorders are closely associated with the occurrence and development of neuroinflammation related to brain aging, especially mitochondrial inflammation mediated by dynamin-related protein 1 (Drp1) (Grimm & Eckert, 2017; Zhang, Wang, et al., 2020). Briefly, after activation, Drp1 can first be recruited to the outer mitochondrial membrane from the cytoplasm and initiate mitochondrial division (Liu et al., 2021; Medala et al., 2021; Oliver & Reddy, 2019), resulting in disorders and dysfunction of mitochondrial dynamics. In addition, activated Drp1 preferentially combines with the voltage-dependent anion channel (VDAC), leading to the separation of hexokinase 1 (HK1) from VDAC (Elsherbini et al., 2020). HK1 inactivation triggers the NOD-like receptor protein 3 (NLRP3) inflammasome (Moon et al., 2015), which then causes the release and secretion of interleukin-1 beta (IL-1β) and interleukin-18 (IL-18) depending on caspase-1 (Bektas et al., 2018).

Ginger and *Alpinia katsumadai Hayata* (*Zingiber officinale Rosc*.) are the most popular spices worldwide, especially in Asian countries, and have many distinct bioactive compounds with antiinflammatory and antioxidant properties (Ran et al., 2019). Ginger and its bioactive components, such as zerumbone, curcumin, 6-gingerol, and shokol, have been reported to enhance learning and memory in animal models (Adetuyi & Farombi, 2021; Arcusa et al., 2022; Ishaq et al., 2022; Kim et al., 2010, 2018; Liu et al., 2019; Talebi et al., 2021; Zarei et al., 2021). Some reports have suggested that ginger extract can alleviate cognitive deficits by regulating ERK/CREB activation or inhibiting nitric oxide production (Lim et al., 2014; Mustafa et al., 2019). *Alpinia katsumadai Hayata* has been reported to protect neurons from injury by decreasing the level of Mn-SOD and increasing Cu and Zn-SOD immunoreactivity (Li et al., 2013), and to protect neurons from cerebral ischemia by inhibiting the activation of glial cells in the hippocampal CA1 region (Li et al., 2011). Cardamine, an active component of *Alpinia katsumadai Hayata*, has been reported to protect PC12 cells from oxidative damage by activating Nrf2-driven antioxidant enzymes (Peng et al., 2017). Alpinetin (a natural flavonoid compound of ginger) has been proven to ameliorate many inflammatory diseases, such as colitis, allergic asthma, endometritis, and osteoarthritis, owing to its good antiinflammatory and antioxidant activities (Gao et al., 2020; Liang et al., 2018; Lv et al., 2018; Wu et al., 2020). A previous study suggested that alpinetin could protect against hepatic ischemia/reperfusion injury in mice by suppressing the NF-κB/MAPK pathways (Pan et al., 2021). Mitochondrial inflammation plays a crucial role in AACI, whether alpinetin can alleviate AACI by inhibiting the Drp1/HK1/NLRP3 pathway-suppressed mitochondrial inflammation remains unclear. Therefore, the purpose of our study was to determine whether alpinetin could attenuate AACI by inhibiting the Drp1/HK1/NLRP3 pathway and suppressing mitochondrial inflammation.

2 | MATERIALS AND METHODS

2.1 | Experimental drugs

Alpinetin (AP, purity ≥98% by HPLC, batch number HR2880W6, Figure 1a) and D-galactose (D-gal, batch number HR6271W3) were purchased from BaoJi Herbest Bio-Tech Co., Ltd., (BaoJi, China). Memantine (MEM, batch number 918564) was obtained from H. Lundbeck A/S.

2.2 | Animals

SPF Kunming mice (male, 6 weeks old, 18–22 g) were provided by Hunan SJA Laboratory Animal Co., Ltd., Changsha, China (Certificate of Conformity: 430727210100021832). All animals were raised in a standard specific pathogen-free (SPF) facility (humidity: 65% ± 5%; temperature: 21 ± 2°C) at the School of Pharmacy, Chengdu University of Traditional Chinese Medicine, and provided free chow and water. After being adaptively acclimated for 1 week, mice were randomly separated into the Cont group (0.5% CMC-Na solution, i.g.), D-gal group (0.5% CMC-Na solution, i.g.), D-gal + MEM group (Memantine, 2.5 mg/kg/day, dissolved in 0.5% CMC-Na solution, i.g.), D-gal + APL group (Alpinetin, 50 mg/kg/day, dissolved in 0.5% CMC-Na solution, i.g.) and, D-gal + APH group (Alpinetin, 100 mg/kg/day, dissolved in 0.5% CMC-Na solution, i.g.). Mice in the Cont group were

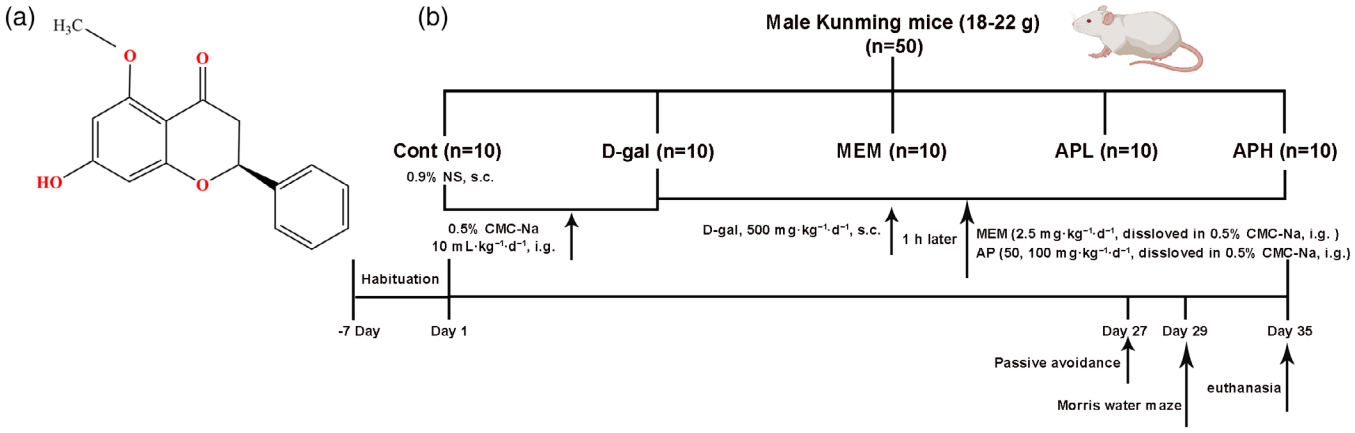

FIGURE 1 The structural formula of alpinetin (a) and experimental flow chart of study (b).

subcutaneously (s.c.) injected with normal saline, and all mice in the other groups were injected subcutaneously (s.c.) with 500 mg/kg D-gal (dissolved normal saline) to establish the AACI model (once a day, 35 days) (Zhao et al., 2020). Simultaneously, except for the Cont and D-gal groups with an equal volume of 0.5% CMC-Na solution, other mice were orally administered (i.g.) the corresponding medicaments. The experimental flow chart is shown in Figure 1b. The protocol was approved by the Animal Experiments Ethics Committee of Chengdu University of Traditional Chinese Medicine (Institute of Material Medica Integration and Transformation for Brain Disorders) (No. IBD2020008).

2.3 | Cell culture

BV-2 cells were purchased from the Cell Resource Center of the Institute of Basic Medical Sciences (IBMS), CAMS/PUMC, and incubated in DMEM (Gibco, USA) with 1% penicillin/streptomycin (Gibco, USA) and 10% fetal bovine serum (CellMax, Beijing, China) at 37°C with 5% CO_2. After treatment with the corresponding medicaments, cell viability was assessed using a commercial CCK-8 Kit (#08123013; Guangzhou Cellcook Biotech Co., Ltd., China), in strict accordance with the manufacturer's instructions. All cell experiments were repeated at least thrice.

2.4 | Passive avoidance test

A PAT-8 video analysis system (TechMan Soft, Sichuan, China) was employed to measure the effect of alpinetin on passive avoidance, according to a previously reported protocol (Shimoyoshi et al., 2019). Briefly, animals were placed in the illuminated field to adapt to their surroundings for 5 min on the 27th day of drug administration. During training, when the mice entered the black side, they received a foot shock (0.7 mA) immediately. Testing was conducted after 24 h, and the step-through latency and number of step-through of the mouse were recorded within 5 min by the system.

2.5 | Morris water maze test

The Morris water maze (MWM) was employed to assess the effect of alpinetin on the spatial cognitive function of mice as described previously (Wei et al., 2021; Zhao et al., 2020). Briefly, all animals were placed in the MWM system and trained for 5 days. The training time was set at 1 min. If the mouse could find the platform within 1 min of the training period every day, the time was considered as its escape latency. Otherwise, the mouse was compulsorily guided to the platform and stayed there for 10 s, and its escape latency was recorded as 1 min. After the visible platform was removed on the sixth day, the number of crossing platforms and time in the target quadrant of the mouse were separately recorded using an MWM video imaging analysis system (Noldus, Netherlands).

2.6 | Preparation of samples

After MWM tests, all mice were anesthetized with pentobarbital (40 mg/ kg, i.p., dissolved in sterilized normal saline, #69020100, MERCK, i.p.), transcardially perfused with cold normal saline, sacrificed by decapitation, and the brain tissues were collected. The right hemisphere of the brain was fixed with 4% paraformaldehyde (PFA, #BL539A, Biosharp, Anhui, China) for morphological analysis, whereas the left hemisphere was used for western blot analysis.

2.7 | Hematoxylin and eosin (H&E) staining

H&E staining was performed to evaluate the neuronal damage in the hippocampus. Dehydration and paraffin embedding were performed according to the standard protocols. After 4 μm-thick slices of brain tissue were cut and dewaxed with xylene (#2022012101, KESHI, Chengdu, China), they were dehydrated in a descending ethanol series at room temperature. H&E staining was conducted as a routine protocol, and each stained slice was observed using optical microscope (#HS6, Sunny Optical Technology [group]co., Ltd., Zhejiang, China).

2.8 | Nissl staining

A Nissl staining kit containing cresyl violet (#C0117, Beyotime, Shanghai, China) was used to stain the Nissl bodies according to the manufacturer's instructions. The slices were conventionally dewaxed into water, and Nissl staining solution was added dropwise, then rinsed quickly, dehydrated, and sealed when transparent. All slices were evaluated and images were captured using an optical microscope (#HS6, Sunny Optical Technology [group]co., Ltd., Zhejiang, China).

2.9 | Immunohistochemical analyses

Immunohistochemical (IHC) analyses were performed using standard procedures. In brief, after baking, dewaxing, and rehydrating, 4-μm paraffin embedded slices were subjected to antigen retrieval and peroxide blocking. All slices were incubated with anti-Iba1 (1:500, #GB11105, Servicebio, Wuhan, China) and an HRP-conjugated secondary antibody (anti-mouse). Subsequently, the slices were developed using a DAB kit (#AR1022, BOSTER, Wuhan, China) and counterstained with nuclear hematoxylin. The photographs were captured and the target protein was quantitatively analyzed at high magnifications.

2.10 | Immunofluorescence

To visualize the proteins, standard staining protocols were performed on the brain slices and cells. Briefly, the sample of the brain or BV-2 cells was prepared and separately incubated with the primary antibodies, rabbit anti-p-Drp1(s616) (1:300, #3455 S, CST, Shanghai, China), mouse anti-Tom20 (1:200, #sc-17764, Santa Cruz, Shanghai, China), and rabbit anti-NLRP3 (1:300, #bs-10021R, Bioss, Hangzhou, China) and conjugated secondary antibodies Cy3 (1:200, #GB21303, Servicebio, Wuhan, China) or 488 (1:300, #GB25301, Servicebio, Wuhan, China) and nuclei were counterstained with Hoechst 33258 (1 μg/mL, #G1011, Servicebio, Wuhan, China). Immunofluorescence (IF) images were captured using a fluorescent microscope and quantified using ImageJ software.

2.11 | Western blotting

The levels of total protein derived from the brain tissues or lysed cells were determined with a BCA kit (#G2026, Servicebio, Wuhan, China), then mixed with SDS loading buffer for boiling. Western blotting was used to quantify the level of the target protein, following a standard protocol (Pasetto et al., 2021). Briefly, the target protein was separated using SDS-PAGE, transferred to PVDF membranes, and blocked in TBST containing 5% skimmed milk (#G5002, Servicebio, Wuhan, China). After incubation with primary rabbit antibodies against the target protein, anti-rabbit HRP-conjugated secondary antibody was added. The primary antibodies used were rabbit anti-Drp1 (#8570 S), rabbit anti-p-Drp1 (Ser616, #3455 S), rabbit anti-VDAC (#4661 S), rabbit anti-HK1 (#2024 S), rabbit anti-Cleaved-caspase 1 (#89332 S) (1:1000, CST, Shanghai, China), rabbit anti-NLPR3 (1:1000, #bs-10021R, Bioss, Hangzhou, China), rabbit anti-ASC (1:1000, #A22046, ABclonal, Wuhan, China), rabbit anti-IL-1β (1:1000, #bs-0812R, Bioss, Hangzhou, China), rabbit anti-IL-18 (1:1000, #GR3285548-4, Abcam, Shanghai, China), and rabbit anti-β-actin (1:5000, #GB11001, Servicebio, Wuhan, China). Finally, the protein bands were visualized with a chemiluminescence reagent (#4AW011-100, 4A BIOTECH, Beijing, China) and quantified using a Chemiluminescent/Fluorescent/Gel Imaging and Analysis System (ChampChemi 610 Plus, Beijing Sage Creation Science Co., LTD).

2.12 | Statistical analysis

Quantitative analysis of HE, IHC, and IF images was conducted using the ImageJ software. Western blot bands were quantified using Quantity One software. All data are displayed as the mean ± SEM and were analyzed using the R language. The escape latency of the MWM test was analyzed using a double-factor variance analysis. Differences among the groups were analyzed using one-way analysis of variance followed by Dunnett's posttest with multiple comparison tests. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Alpinetin ameliorated D-gal-treated aging-related cognitive disorder in mice

Passive avoidance and MWM tests were used to measure the effect of alpinetin on the learning and memory function of animals. Compared to the Cont group, the step-through latency of passive avoidance was significantly shortened, while the number of step-through increased in the D-gal group, whereas, the above cognitive function changes were markedly reversed after treatment with alpinetin or MEM (Figure 2a,b, $p < 0.05$, $p < 0.01$). In the MWM test, the escape latency of all animals was increasingly shortened, while the path length was prolonged (F [4, 60] = 3.427, $p < 0.05$) with increasing training time, however, the changes in escape latency and path length were significantly reversed after treatment with alpinetin (F [4, 60] = 6.061, $p < 0.01$) and (F [4, 60] = 6.533, $p < 0.01$) and MEM (F [4, 60] = 4.5259, $p < 0.01$) (Figure 2c,d). As expected, alpinetin and MEM improved spatial learning and memory disorders caused by D-gal, showing an increase in the number of crossing platforms and a prolonged time in the target quadrant (Figure 2e–g, $p < 0.05$). These results imply that alpinetin n ameliorated the cognitive disorders induced by D-gal in mice.

3.2 | Alpinetin reduced D-gal-treated neuronal damage of mice hippocampus

H&E and Nissl staining were used to measure the morphological changes in the hippocampus. Obvious features of neurogenic injury in

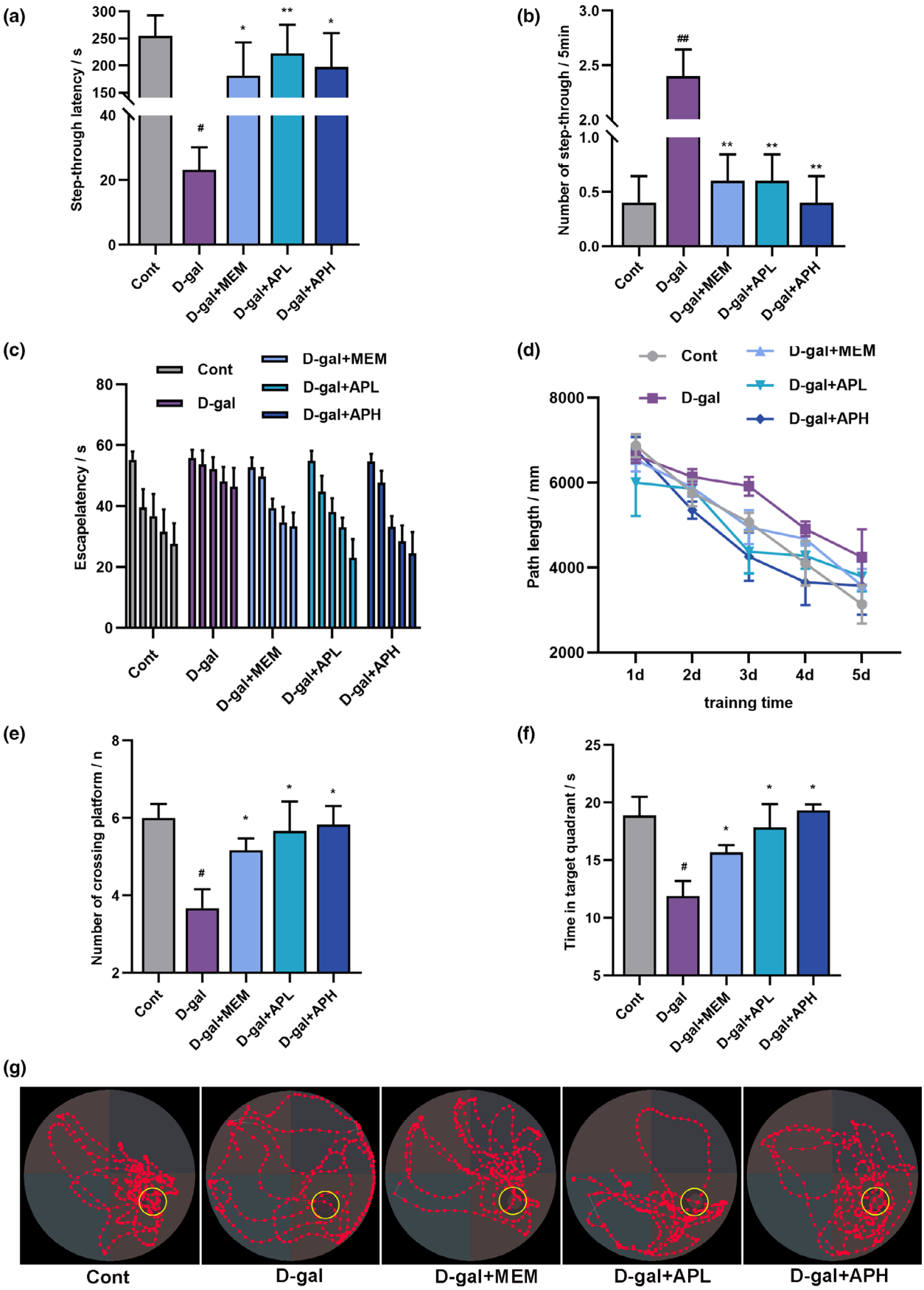

FIGURE 2 Legend on next page.

the CA1 and CA3 regions were found in the D-gal group, such as cortex thinning, neuronal atrophy, and neuron reduction, and loss (Figure 3a–c, $p < 0.05$). However, nerve cell damage in the CA1 and CA3 regions of mice was markedly alleviated after treatment with alpinetin and MEM (Figure 3a–c, $p < 0.05$). As expected, the Nissl staining result was similar to that of H&E staining, showing that Nissl bodies counts of CA1 and CA3 regions in the alpinetin and MEM groups were significantly higher than those of the D-gal group (Figure 3d–f, $p < 0.05$). These results indicate that alpinetin protects against neuronal damage induced by D-gal in mice.

3.3 | Alpinetin attenuated neuroinflammation via inhibiting NLRP3 inflammasome

To evaluate the inhibitory effect of alpinetin on the activation of microglia, Iba1 (microglia marker) staining was performed on brain sections. Compared to the Cont group, the number of Iba1-labeled microglia in the cortex and hippocampus was markedly enhanced in the D-gal group (Figure 4a–c, $p < 0.01$), but significantly decreased when treated with alpinetin and MEM (Figure 4a–c, $p < 0.05$ or $p < 0.01$). Immunofluorescence was used to measure the NLRP3 levels. Compared with the Cont group, the fluorescence intensity of NLRP3 in the CA1 and CA3 regions in the D-gal group was markedly enhanced, which was reduced after treatment with alpinetin and MEM (Figure 4d–f, $p < 0.01$). Furthermore, the protein expression of NLRP3 and its downstream related proteins (such as ASC, Cleaved-caspase 1, IL-1β, and IL-18) in the D-gal group was significantly up-regulated compared with that in the Cont group (Figure 4g–p, $p < 0.05$, or $p < 0.01$), indicating that the NLRP3 inflammasome was activated by D-gal in mice. Compared to the D-gal group, the above mentioned up-regulated proteins were markedly down-regulated in the alpinetin and MEM groups, implying that alpinetin inhibited the activation of the NLRP3 inflammasome induced by D-gal (Figure 4g–p, $p < 0.05$ or $p < 0.01$). In addition, our previous studies found that 10^{-9} mol/L is the optimum protective concentration of alpinetin for cell viability (Figure 5a,b, $p < 0.05$ or $p < 0.01$). Then, BV-2 cells treated with D-gal were utilized to further evaluate the inhibitory effect of alpinetin on NLRP3 inflammasome. After BV-2 cells were exposed to D-gal for 6 h and incubated with alpinetin for 12 h, western blotting and immunofluorescence analysis were performed. The fluorescence intensity and protein expression of NLRP3 were dramatically enhanced in D-gal cells compared with those in Cont cells, whereas the levels of NLRP3 were significantly decreased by alpinetin (Figure 5c–f, $p < 0.05$ or $p < 0.01$). As expected, the over-expression of NLRP3 inflammasome-related proteins (such as ASC, Cleaved-caspase 1, IL-1β, and IL-18) induced by D-gal was also markedly down-regulated by alpinetin (Figure 5g–n, $p < 0.05$ or $p < 0.01$). These findings imply that alpinetin alleviated neuroinflammation induced by D-gal by inhibiting the activation of the NLRP3 inflammasome.

3.4 | Alpinetin inhibited inflammasome activation through Drp1/HK1/NLRP3 pathway

As the Drp1/HK1/NLRP3 pathway is one of the key mechanisms underlying development and exacerbation of neuroinflammation related to brain aging (Grimm & Eckert, 2017; Zhang, Wang, et al., 2020), the Drp1/HK1/NLRP3 pathway was evaluated using immunofluorescence and western blotting. Compared to the Cont group, the immunofluorescence intensity and protein expression of p-Drp1 (s616) in the CA1 and CA3 regions were significantly up-regulated in the D-gal group; however, the levels of p-Drp1 (s616) were markedly down-regulated after treatment with alpinetin and MEM (Figure 6a–e, $p < 0.05$ or $p < 0.01$). Moreover, the level of VDAC was markedly enhanced in the D-gal group, while HK1 was markedly reduced compared with that in the Cont group; nevertheless, these changes were significantly reversed after alpinetin and MEM treatment (Figure 6f–i, $p < 0.05$, or $p < 0.01$). Furthermore, D-gal-induced BV-2 cells were used to confirm the inhibitory effect of alpinetin on Drp1/HK1/NLRP3 pathway. As expected, the Drp1/HK1/NLRP3 pathway was activated by D-gal, showing a high expression of p-Drp1 (s616) and VDAC, and lower expression of HK1 (Figure 7a–h, $p < 0.05$ or $p < 0.01$), whereas the above changes in protein expression were also reversed after treatment with alpinetin (Figure 7a–h, $p < 0.05$). These results suggest that alpinetin inhibits inflammasome activation by suppressing the Drp1/HK1/NLRP3 pathway.

3.5 | Alpinetin alleviated mitochondrial inflammation through Drp1-mediated pathway

As Drp1 is a key upstream mediator of the Drp1/HK1/NLRP3 pathway, Mdivi-1 (a selective inhibitor of Drp1) (Chen et al., 2016) was used to further evaluate the inhibitory effect of alpinetin on Drp1/HK1/NLRP3 pathway. BV-2 cells were separately pretreated with 10 μM Mdivi-1 or 10 μM Mdivi-1 + 10^{-9} M alpinetin for 1 h, and then exposed to D-gal (40 mg/mL) for 6 h. Compared to the D-gal group, the high immunofluorescence intensity and protein expression of p-Drp1 (s616) induced by D-gal were significantly suppressed by

FIGURE 2 Alpinetin ameliorated D-gal-treated aging-related cognitive disorder in mice ($n = 7–9$). The step-through latency (a) and the number of step-through (b) of each group mouse within 5 min in passive avoidance test. (c) Escape latency within training 5 days in the Morris water maze test. (d) Path length plotted against the training 5 days. (e) The number of crossing platforms during a 60 s in the probe test. (f) The time spent in the target quadrant during a 60 s. (g) The pattern representative swimming trace of different groups of mice during the memory test in 60 s after the platform is removed. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus D-gal group.

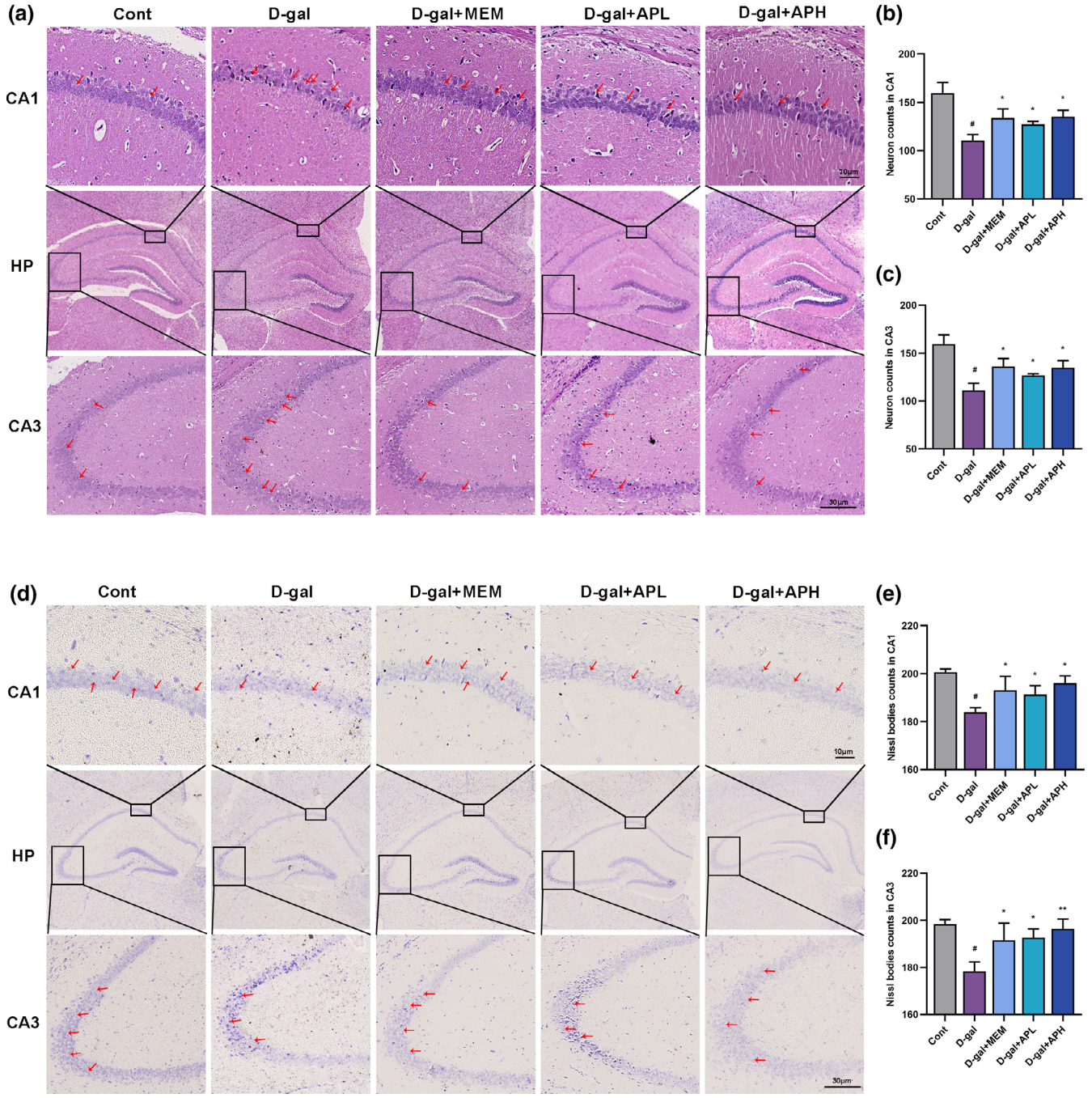

FIGURE 3 Alpinetin reduced D-gal-treated neuronal damage of mice hippocampus ($n = 5$). H&E staining (a), and Nissl staining (d), magnification: 100× and 200×. (b, c) The grade of neuronal damage in the CA1 and CA3 regions of each group. (e, f) The Nissl bodies counts in the CA1 and CA3 regions of each group. Red arrows indicate atrophic neurons and Nissl bodies. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus D-gal group.

Mdivi-1, alpinetin, and Mdivi-1+ alpinetin, respectively (Figure 8a–d, $p < 0.05$ or $p < 0.01$). Moreover, there was an evident increase in inhibition in the Mdivi-1 + alpinetin group compared to that in the alpinetin group (Figure 8a–d, $p < 0.05$). Furthermore, the expression of VDAC was markedly enhanced and HK1 decreased in the D-gal group compared with the Cont group (Figure 8e–h, $p < 0.05$ or $p < 0.01$), while it was significantly reversed by Mdivi-1, alpinetin and Mdivi-1+ alpinetin respectively (Figure 8e–h, $p < 0.05$ or $p < 0.01$). Compared to the alpinetin group, these reversed effects of VDAC and HK1 were clearly observed in the Mdivi-1 + alpinetin group (Figure 8e–h, $p < 0.05$). In addition, as expected, NLRP3 activation by D-gal was markedly inhibited by Mdivi-1, alpinetin and Mdivi-1 + alpinetin (Figure 9a–d, $p < 0.05$ or $p < 0.01$). Simultaneously, the inhibitory effect on NLRP3 was also markedly increased in the Mdivi-1 + alpinetin group compared with the alpinetin group (Figure 9a–d, $p < 0.05$ or $p < 0.01$). As expected, the high expression of NLRP3

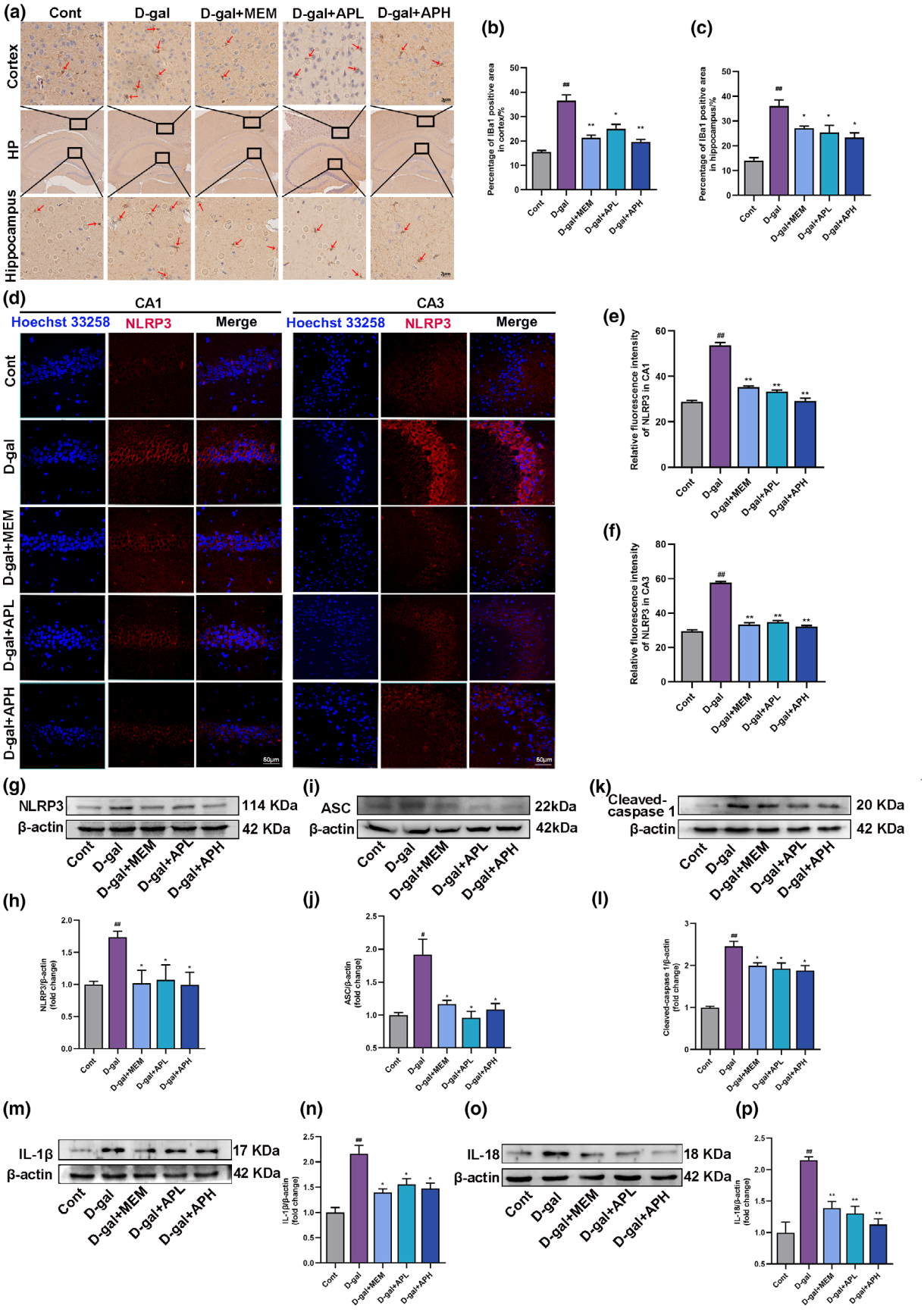

FIGURE 4 Alpinetin attenuated neuroinflammation via inhibiting NLRP3 inflammasome in vivo ($n = 3$). Iba-1 immunohistochemistry staining and quantification (a–c), magnification: 400×. Immunofluorescence analysis of NLRP3 in the CA1 and CA3 regions of each group (d–f), magnification: 400×. (g–p) The western blot results of NLRP3, ASC, Cleaved-caspase 1, IL-1β, and IL-18. Red arrows indicate activated microglia. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus D-gal group.

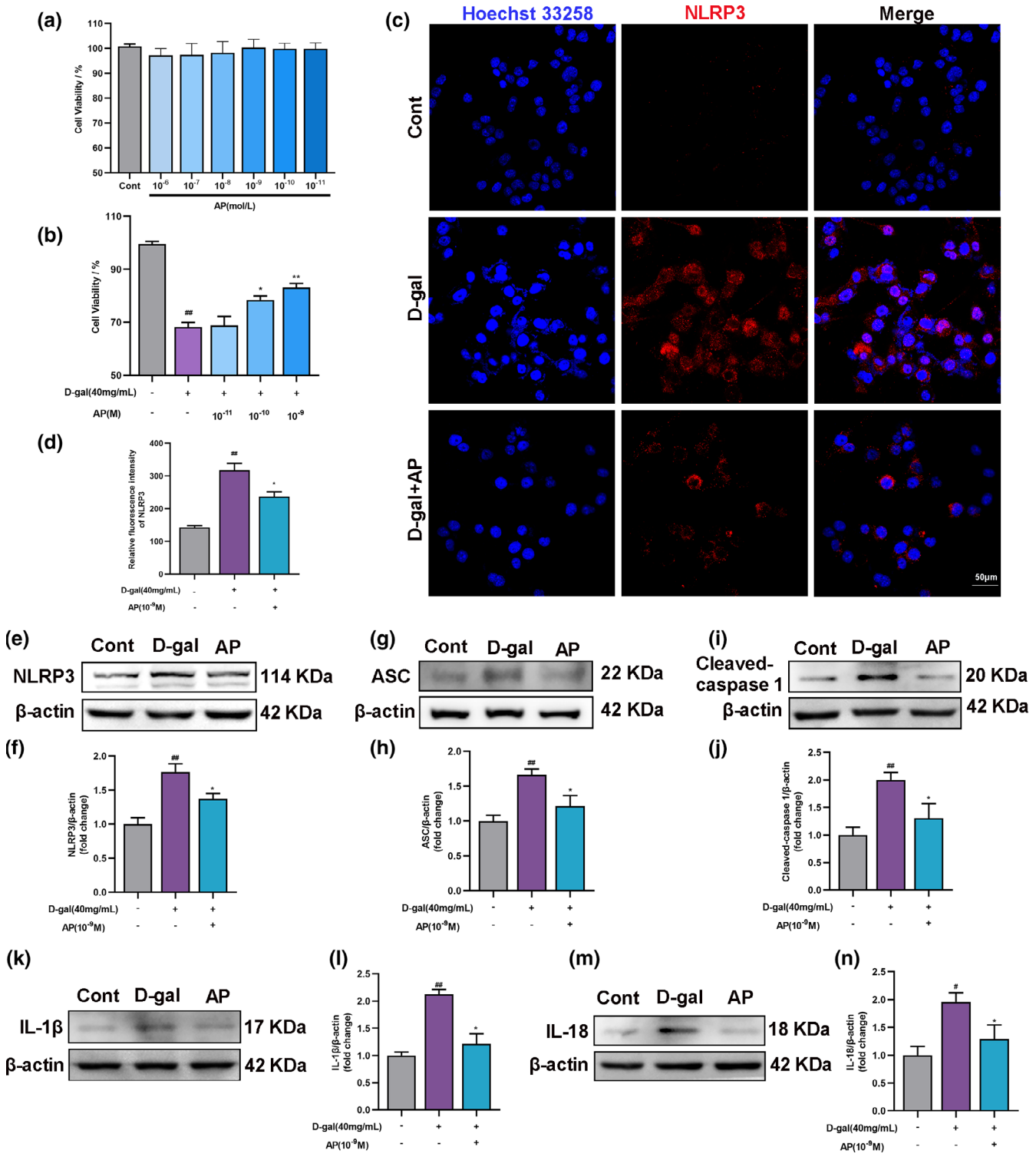

FIGURE 5 Alpinetin attenuated neuroinflammation via inhibiting NLRP3 inflammasome in vitro ($n = 3$). (a) Non-toxic concentration of Alpinetin, (b) the protective effect of alpinetin. (c, d) Immunofluorescence analysis and quantification of NLRP3 in BV-2 cells, magnification: 400×. (e–n) The western blotting results of NLRP3, ASC, Cleaved-caspase 1, IL-1β, and IL-18. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus D-gal group.

downstream related proteins (such as ASC, Cleaved-caspase 1, IL-1β, and IL-18) induced by D-gal was markedly down-regulated by Mdivi-1, alpinetin and Mdivi-1 + alpinetin treatment (Figure 9e–l, $p < 0.05$ or $p < 0.01$). These inhibitory effects were also observed in the Mdivi-1 + alpinetin group compared with the Mdivi-1 + alpinetin group (Figure 9e–l, $p < 0.05$ or $p < 0.01$). The above results indicated that alpinetin inhibited mitochondrial inflammation in a Drp1-dependent manner.

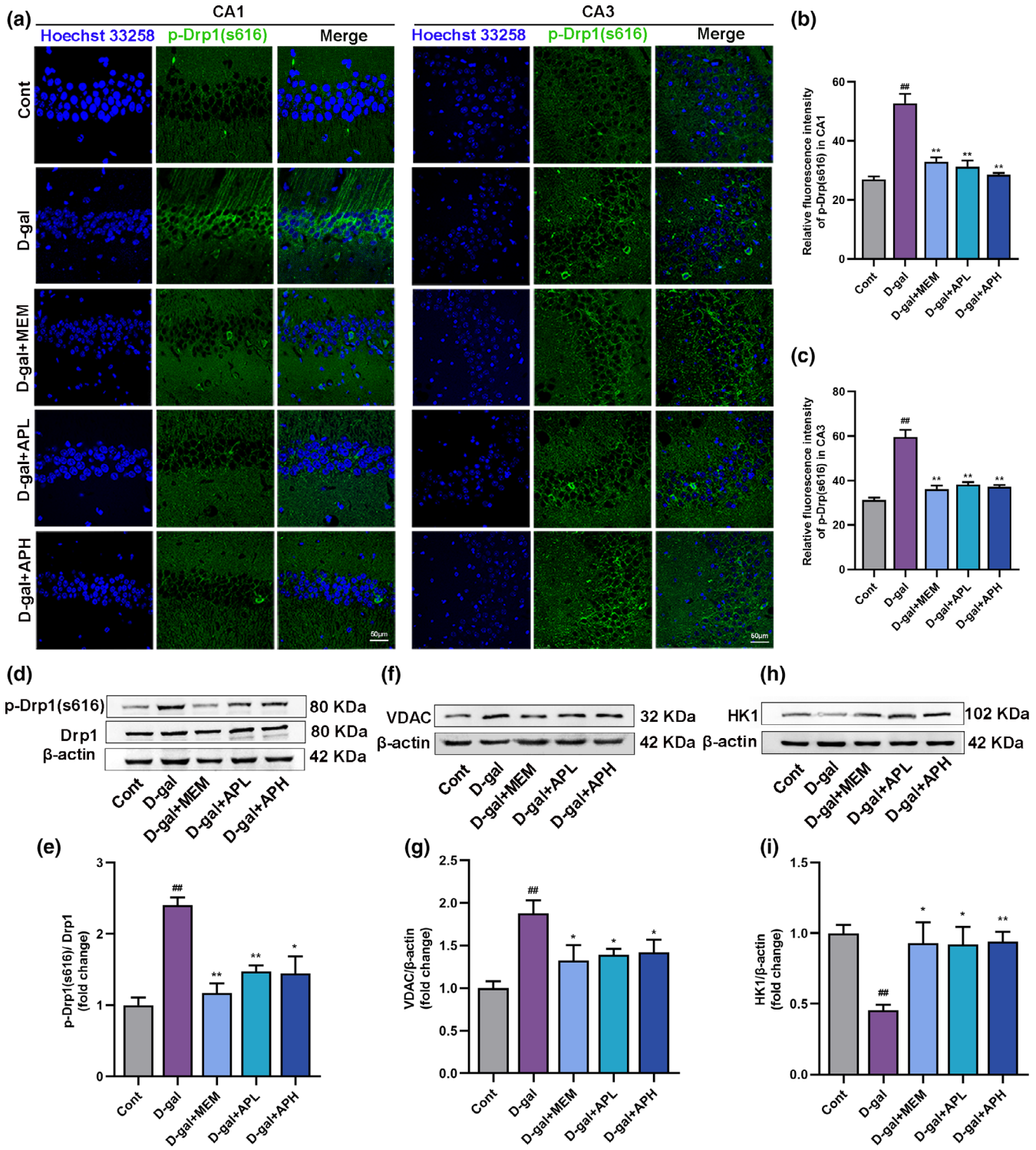

FIGURE 6 Alpinetin inhibited inflammasome activation through Drp1/HK1/NLRP3 pathway in vivo ($n = 3$). (a–c) Immunofluorescence analysis and quantification of p-Drp1 (s616) in the CA1 and CA3 regions, magnification: 400×. (d–i) The western blotting results of p-Drp1 (s616), Drp1, VDAC, and HK1. The data are presented as the mean ± SEM. $^{\#\#}p < 0.05$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus D-gal group.

4 | DISCUSSION

Our findings demonstrated that alpinetin ameliorated the cognitive disorder induced by D-gal in mice by suppressing the Drp1-mediated mitochondrial inflammation pathway, implying that inhibition of the Drp1/HK1/NLRP3 pathway may be one of the mechanisms by which alpinetin attenuates AACI.

Aging and age-related diseases (such as AACI) are commonly associated with oxidative DNA and protein damage. Long-term neuroinflammation, mitochondrial dysfunction, and subsequent cognitive

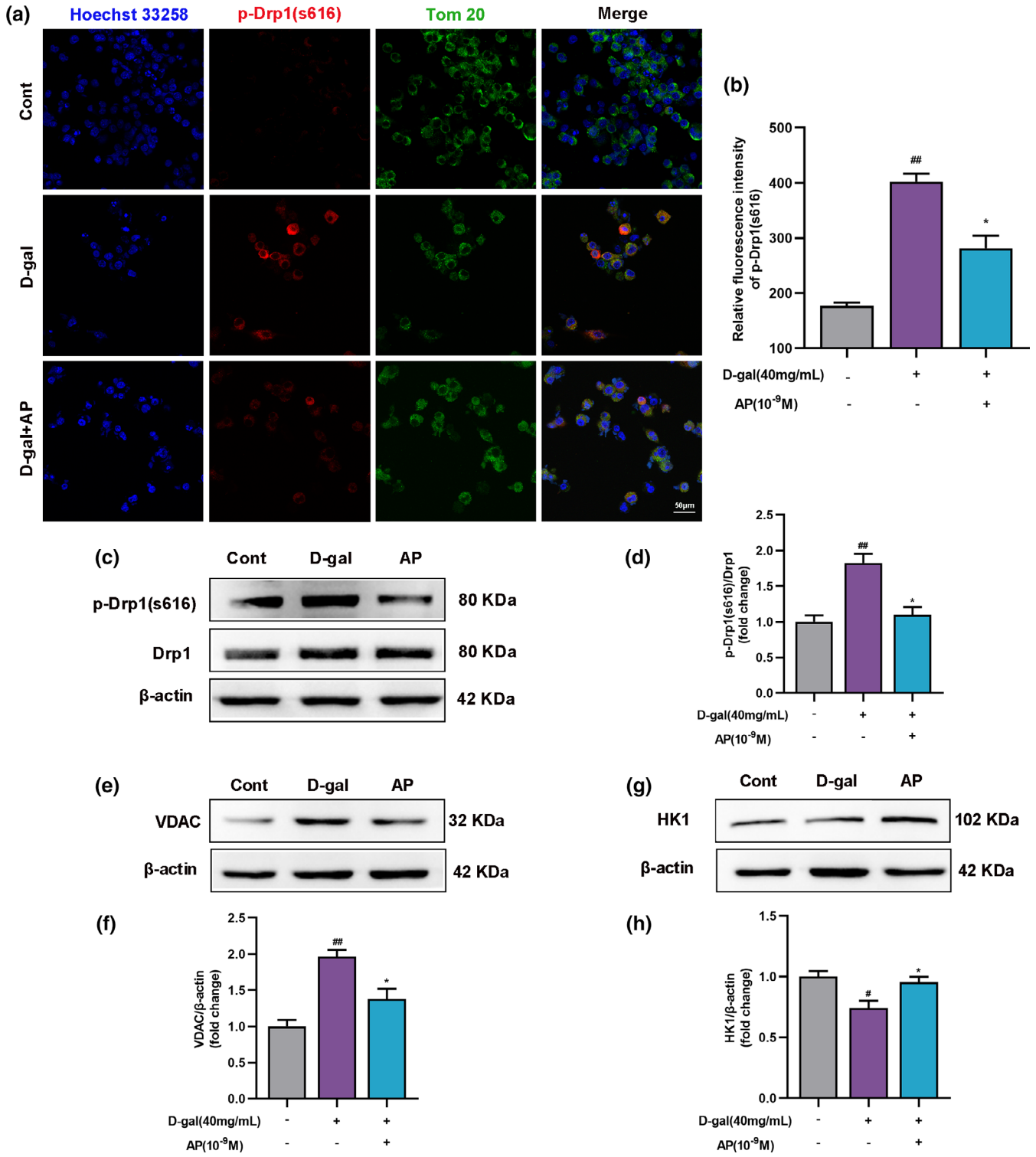

FIGURE 7 Alpinetin inhibited inflammasome activation through Drp1/HK1/NLRP3 pathway in vitro ($n = 3$). (a, b) Immunofluorescence analysis and quantification of p-Drp1 (s616) in BV-2 cells, magnification: 400×. (c–h) The western blot results of p-Drp1 (s616), Drp1, VDAC, and HK1. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus Cont group; $^{*}p < 0.05$, versus D-gal group.

impairment are the principal features of AACI (Olesen et al., 2020; Towner et al., 2019). Although no therapeutic drugs have been approved to treat AACI, some bioactive agents have been reported to alleviate cognitive injury of AACI in aging models, such as 3,4,5-tricaffeoylquinic acid, geraniol, and Trillium tschonoskii maxim saponin (Atef et al., 2022; Sasaki et al., 2019; Wang et al., 2018). Memantine is a non-competitive N-methyl-D-aspartate receptor antagonist that has been shown to the alleviate the spatial learning

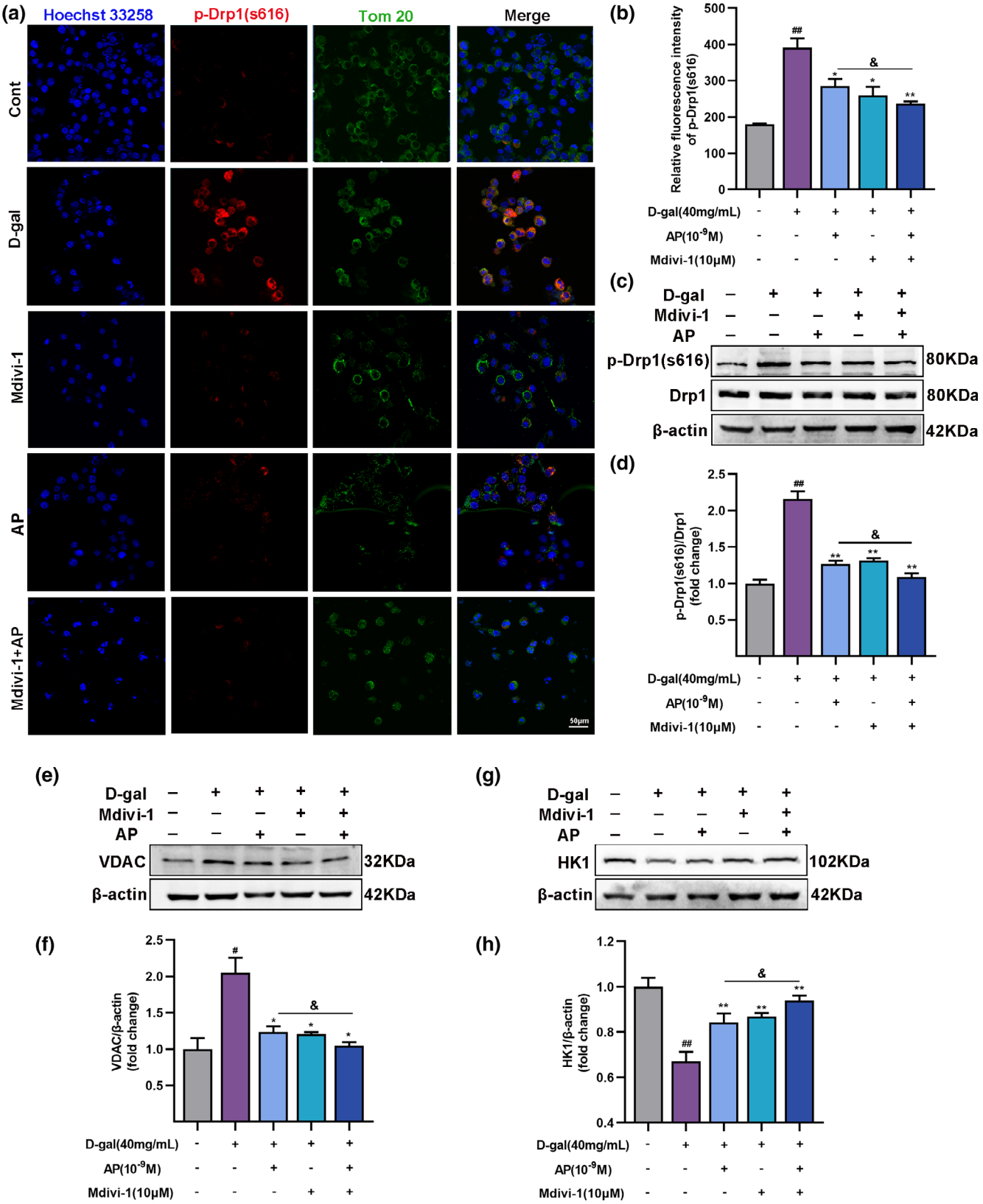

FIGURE 8 Alpinetin alleviated mitochondrial inflammation through Drp1-mediated pathway in vitro ($n = 3$). (a, b) Immunofluorescence analysis and quantification of p-Drp1 (s616) in BV-2 cells, magnification: 400×. (c–h) The western blotting results of p-Drp1 (s616), Drp1, VDAC, and HK1. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus D-gal group; $^{\&}p < 0.05$, versus alpinetin group.

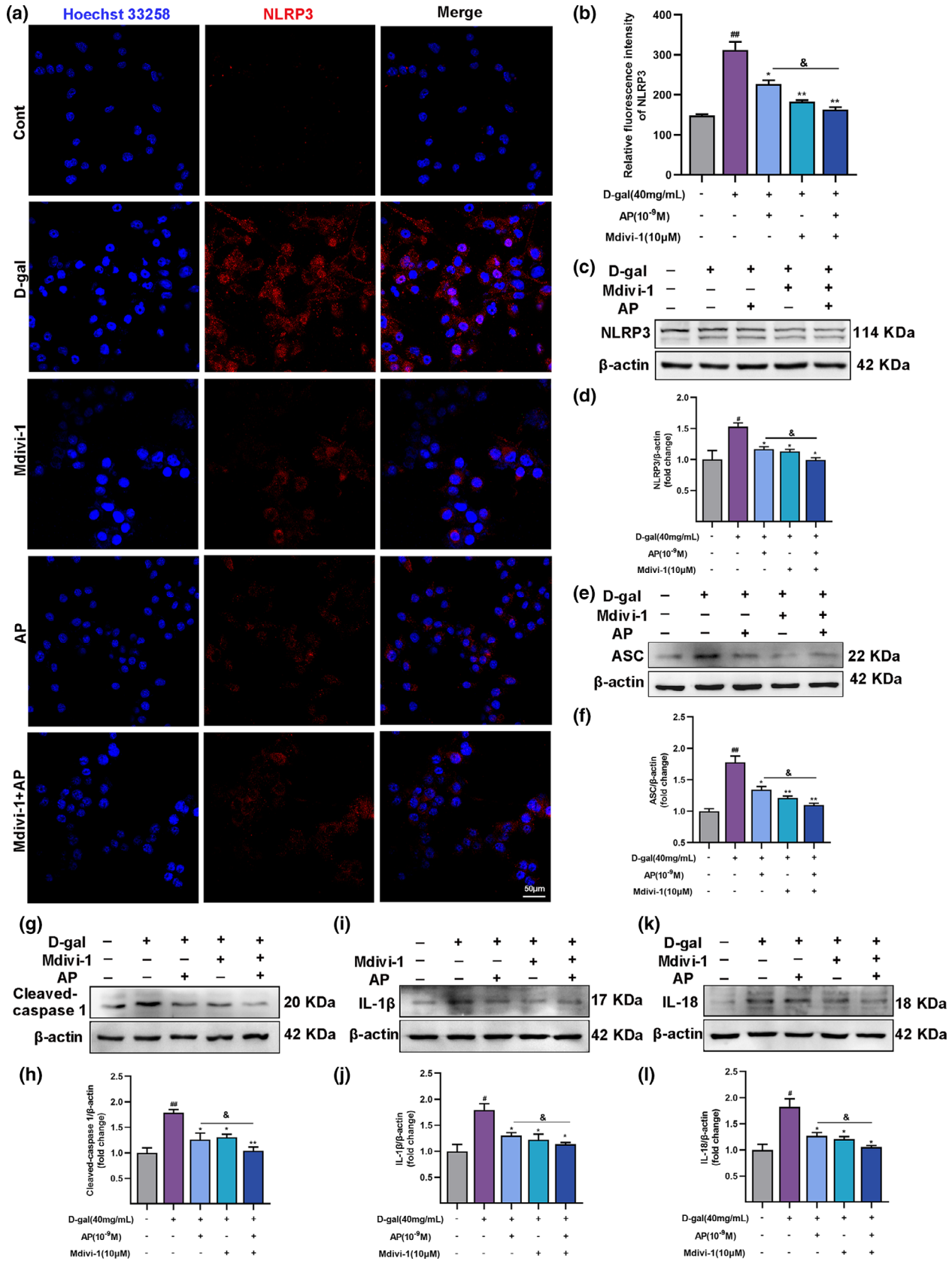

FIGURE 9 Alpinetin alleviated mitochondrial inflammation through Drp1-mediated pathway in vitro ($n = 3$). (a, b) Immunofluorescence analysis and quantification of NLRP3 in BV-2 cells, magnification: 400×. (c–l) The western blotting results of NLRP3, ASC, Cleaved-caspase 1, IL-1β, and IL-18. The data are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus Cont group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus D-gal group; $^{\&}p < 0.05$, versus alpinetin group.

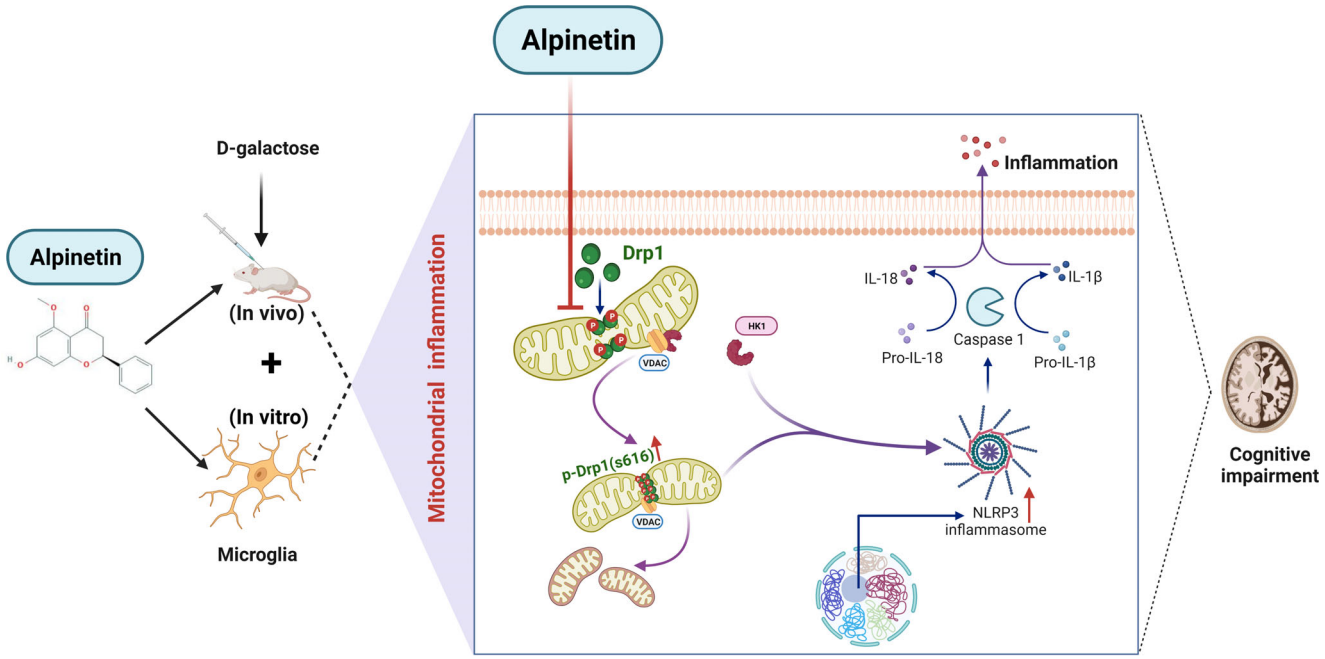

FIGURE 10 Schematic illustration of the alpinetin's mechanism to attenuate AACI by inhibiting the Drp1/HK1/NLRP3 pathway and suppressing mitochondrial inflammation.

disorders and reduce the levels of TNF-α and IL-1β in aged senescence-accelerated mouse resistant 1 (Zeng et al., 2020). Therefore, memantine was used as a positive control in this study. In recent years, flavonoids have received increasing attention because of their potential as neuroprotectants (Ayaz et al., 2019; Calis et al., 2020; Hu et al., 2022; Li et al., 2021; Nie & Stürzenbaum, 2019). The main flavonoid extracted from ginger and *Alpinia katsumadai Hayata* (*Zingiber officinale Rosc.*), alpinetin, is widely known to have antiinflammatory and antioxidant activities and low systemic toxicity (He et al., 2005; Qiu et al., 2019). Alpinetin has been reported to attenuate many inflammatory diseases by inhibiting the NF-κB and MAPK pathways (Lv et al., 2018), such as osteoarthritis, allergic asthma, chronic obstructive pulmonary disease, and endometritis (Liang et al., 2018). As a well-accepted experimental model of aging-related cognitive dysfunction in physiological aging (Hakimizadeh et al., 2021), D-gal-induced aging mice were used to assess the effect of alpinetin on cognitive function and neuroprotection. Our data indicated that D-gal induced cognitive disorders and neuropathological injury in mice, similar to the results of previous studies (Shwe et al., 2020; Zhao et al., 2020; Zhong et al., 2019). The current investigation suggests that alpinetin significantly alleviated cognitive dysfunction and pathological changes in the hippocampus of D-gal-induced mice. The dose of alpinetin (50–100 mg/kg/day, i.g.) in the present study was used in vivo according to previous reports (He et al., 2016; Tan & Zheng, 2018; Wu et al., 2020; Yu et al., 2020). Based on the literature (Nair & Jacob, 2016), the dose range of alpinetin in the human body is approximately 243–486 mg/day. Alpinetin is a natural flavonoid component present in many Zingiberaceae plants such as ginger, *Alpinia katsumadai Hayata*, and galangal, which are the most popular spices worldwide, especially in Asian countries. It has been reported that the content of Alpinetin in *Alpinia katsumadai Hayata* is up to 172 mg/g (Wu et al., 2016). As a spice and medicine, *Alpinia katsumadai Hayata* is used in an amount of 3–6 g per day, and it is relatively easy to obtain sufficient amounts of alpinetin daily.

As tissue-resident macrophage-like immune cells of the brain, microglia are susceptible to ROS generated by aging and facilitate persistent neuroinflammation. Long-term neuroinflammation is considered a hallmark of AACI and plays a vital role in aging and age-related diseases (Simpson & Oliver, 2020; Zhou et al., 2022). It is well-known that activated microglia are the basis of NLRP3 inflammasome excitation and subsequent neuroinflammation. Iba1 is typically used as an acellular marker of microglia (Lituma et al., 2021). NLRP3/Caspase-1 pathway is the classical activation pathway of the NLRP3 inflammasome. When the NLRP3 inflammasome is triggered, the activated NLRP3 inflammasome is assembled inside the microglia (Ising et al., 2019) and pro-caspase-1 is cleaved to active caspase-1 by an induced polyprotein complex consisting of NLRP3 and ASC of active caspase-1, resulting in the release of IL-1β and IL-18 (Cade et al., 2019; Elsherbini et al., 2020; Hanslik & Ulland, 2020; Zhang, Huang, et al., 2020). Activated NLRP3/Caspase-1 pathway has been proven to be one of the key mechanisms of cognitive deficit caused by aging (Wang et al., 2022). The current investigation indicated that the number of $Iba1^{+}$ microglia was evidently increased and the NLRP3/Caspase-1 pathway was markedly activated in the D-gal group, while these changes were significantly antagonized after treatment with alpinetin, implying that alpinetin alleviates neuroinflammation-related aging by inhibiting the NLRP3/Caspase-1 pathway.

The persistent presence of damaged and dysfunctional microglial mitochondria in the aging brain plays a key role in the activation of

inflammatory signaling (Joshi et al., 2019; Neves & Sousa-Victor, 2020). As a regulatory protein of mitochondrial dynamics, Drp1 plays an important role in the promotion of mitochondrial fragmentation via phosphorylation at S616 (Yang et al., 2017). Several studies have suggested that Drp1-mediated mitochondrial inflammation is involved in AACI, especially in the Drp1/HK/NLRP3 pathway (Grimm & Eckert, 2017; Xu et al., 2020; Zhang, Wang, et al., 2020). After Drp1 is phosphorylated at S616, overactivated Drp1 is recruited to the mitochondria and preferentially combined with VDAC, thereby separating HK1 from VDAC and causing HK1 inactivation. Activated VDAC not only causes mitochondrial dysfunction (Simões-Alves et al., 2019), but also cooperates with inactivation of HK1 to trigger NLRP3 inflammasome activation (Elsherbini et al., 2020), thereby resulting in the release of IL-1β (Cade et al., 2019; Elsherbini et al., 2020; Hanslik & Ulland, 2020; Zhang, Huang, et al., 2020). Our data suggest that alpinetin inhibits the activation of the Drp1/HK/NLRP3 mitochondrial inflammation pathway in aging models by inhibiting the high expression of Drp1 and VDAC, and enhancing the low expression of HK1 in vivo and in vitro. Since damaged mitochondrial dynamics can cause the release of inflammatory cytokines by activating the NLRP3 inflammasome (Bektas et al., 2018), Drp1 knockout or blocking with a specific inhibitor such as Mdivi-1 can improve mitochondrial fusion activity and reduce cell injury by suppressing Drp1 levels (Bayne et al., 2020; Liu et al., 2020; Manczak et al., 2019; Park et al., 2015; Zhang et al., 2021). As expected, the inhibitory effects of alpinetin on the Drp1/HK/NLRP3 pathway were markedly enhanced after application of the combination Mdivi-1. These results imply that the inhibition of the Drp1/HK/NLRP3 pathway to reduce mitochondrial inflammation is one of the mechanisms by which alpinetin alleviates AACI.

5 | CONCLUSIONS

According to our findings, alpinetin can attenuate AACI by inhibiting the Drp1/HK1/NLRP3 pathway and reducing mitochondrial inflammation (Figure 10), suggesting that inhibition of the Drp1/HK1/NLRP3 pathway is a key mechanism of alpinetin to improve AACI and Alpinetin may be a potential natural flavonoid in relieving AACI.

AUTHOR CONTRIBUTIONS

Yuanyuan Chen, designed, performed the research, and wrote the original manuscript. Chuan Yang and Mi Zou projected animal administration. Dan Wang, Ruilin Sheng and Qi Chen performed the methodology. Meng Zhan and Wenqin Yang performed the data analysis. Xiao Liu critically revised the manuscript. Shijun Xu conceived the study and critically reviewed, edited, and revised the manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declared that no potential conflicts of interest with respect to the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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