

Liensinine and neferine exert neuroprotective effects via the autophagy pathway in transgenic *Caenorhabditis elegans*

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Abstract

Liensinine and neferine are the main bisbenzylisoquinoline alkaloids obtained from the seeds of *Nelumbo nucifera*, which commonly used as edible food and traditional medicine in Asia. It was reported that liensinine and neferine could inhibit the activities of acetylcholinesterase and cross the blood-brain barriers, maybe promising candidates to treat Alzheimer's disease. Here we employed the APPswe transfected SH-SY5Y neural cells and transgenic *Caenorhabditis elegans* to investigate the neuroprotective effects and underlying mechanism of liensinine and neferine. Finally, we found that liensinine and neferine could significantly improve the viability and reduce ROS levels in APPswe cells, inhibit β -amyloid and tau-induced toxicity, and enhance stress resistance in nematodes. Moreover, liensinine and neferine had obviously neuroprotective effects by assaying chemotaxis, 5-hydroxytryptamine sensitivity and the integrity of injured neurons in nematodes. Preliminary mechanism studies revealed that liensinine and neferine could upregulate the expression of autophagy related genes (*Igg-1*, *unc-51*, *pha-4*, *atg-9* and *ced-9*) and reduce the accumulation of β -amyloid induced autophagosomes, which suggested autophagy pathway played a key role in neuroprotective effects of these two alkaloids. Altogether, our findings provided a certain working foundation for the use of liensinine and neferine to treat Alzheimer's disease based on neuroprotective effects.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, affects more than 50 million people worldwide.(Lane, Hardy et al. 2018) With the aggravation of population aging, the incidence of AD is increasing year by year, already inducing tremendous economic and social pressures. However, the pathogenesis remains unclear. There are many reasons for inducing AD, and the cardinal features of Alzheimer's pathology are amyloid plaques and neurofibrillary tangles (NFTs).(Lane, Hardy et al. 2018) Simultaneously, oxidative stress and autophagy dysfunction also participate in the development of AD by promoting A β deposition, tau hyperphosphorylation, and the subsequent loss of synapses and neurons.(Chen and Zhong 2014) A crucial role in the suspension of potential damages is the timely drug delivery of neuroprotective medications before AD turns into mildly symptomatic.(DeKosky and Marek 2003)

Alkaloids are small natural molecules that contain nitrogen with significant biological activities and are widely found in nature. Many alkaloids have been reported to have significant neuroprotective effects.(Kong, Tay et al. 2021) 'Lian Zi Xin' is the seeds of Lotus (*Nelumbo nucifera*), which is a common traditional Chinese medicine to treat neurodegenerative diseases, insomnia, high fever, and cardiovascular diseases.(Jo, Kim et al. 2021) Liensinine and neferine (Fig. 1) are the main bisbenzylisoquinoline alkaloids of 'Lian Zi Xin', and they are known to possess a variety of pharmacological activities including anti-cancer,(Zhou, Li et al. 2015, Manogaran, Beeraka et al. 2019, Sivalingam, Amirthalingam et al. 2019) antioxidation,(Li, Gao et al. 2021, Jia, Liu et al. 2022) anti-inflammation,(Zhao, Wang et al. 2010, Liang, Ye et al. 2022) treatment of neurodegenerative diseases(Jung, Karki et al. 2015, Wong, Wu et al. 2015, Plazas, Avila et al. 2022) and potential neuroprotective effects.(Jo, Kim et al. 2021)

In this paper, we used APPswe cells and *Caenorhabditis elegans* (*C. elegans*) as model to systematically investigate the neuroprotective effects of liensinine and neferine. APPswe cells are ideal A β cell model constructed by transfecting the APP695 *Swedish* mutant gene into SH-SY5Y cells through lentivirus transfection.(Li, He et al. 2011) *C. elegans* is a convenient model in studying neurodegenerative diseases with simple structure, short lifespan and easy cultivation. Although *C. elegans* doesn't have A β and tau genes, it can be easily constructed by gene editing to express human A β and tau protein in their muscle cells and neurons for screening AD-related drugs and clarifying their mechanisms.(Shaye and Greenwald 2011)

Here, our results showed that liensinine and neferine could significantly inhibit A β and tau-induced toxicity by using APPswe cells and *C. elegans* models. In addition, we also reported that liensinine and neferine have significant protective effects against A β -induced neuronal damage via improving oxidative stress tolerance and autophagy activity.

2. Materials and methods

2.1. Preparation of liensinine and neferine

Liensinine and neferine were purchased from Shanghai Standard Technology Company. The solid was dissolved in Dimethyl Sulfoxide (DMSO), diluted to the corresponding 10x stock solution and ensured that the DMSO concentration is below 3%.

2.2. Cell cultures and treatments

SH-SY5Y cells were purchased from American Type Culture Collection (ATCC), cultured in Minimum Essential Medium (MEM) and F12 (1:1) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin Solution (PS) at 37°C, in a 5% CO₂ atmosphere. Human APP *Swedish* mutant gene (APPswe695) was transfected into SH-SY5Y cells by lentiviral transfection to induce the expression of A β protein.

2.3. Cell viability assay

4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Nanjing Jiancheng Biological Engineering Research Institute) assay was used to study the effect of liensinine and neferine on cell viability. Collected adherent cells and adjusted the cell density at 1000–10000 per well, pre-incubated in a 96-well plate with liensinine and neferine for 24h. Added 50 μ L MTT solution into each well, and cultured for 4h. Carefully sucked the culture medium, and added 150 μ L DMSO to fully dissolve the crystals. The absorbance value was measured at OD570 of enzyme-linked immunosorbent assay.

2.4. Determination of ROS levels

The ROS level of APPswe cells was measured using the Human reactive oxygen species (ROS) ELISA Kit (Shanghai Enzyme-linked Biotechnology). Collected samples and removed particulates by centrifugation for 10min at 3000 $\times$ g. Set standard wells and testing wells, added different concentrations of standard

50µL to the standard wells; added testing sample 10µL and then added sample diluent 40µL to testing sample wells. Added 100µL HRP-conjugate reagent to each well and incubated for 60min at 37°C. Added 50µL chromogen solution A and 50µL chromogen solution B to each well, gently mixed, and incubated for 15min at 37°C protected from light. Finally, added 50µL stop solution to each well and read the Optical Density (O.D.) at 450nm.

2.5. Strains and maintenance conditions

Caenorhabditis elegans strains used in this study were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis, MN, USA), and the Sunybiotech company. N2 (wild-type), CL4176: *dvIs27* [*Pmyo-3::A-Beta(1-42)::let-851 3'UTR + rol-6(su1006)*], CL2355: *dvIs50* [*pCL45snb-1::A-Beta(1-42)::3'UTR(long) + mtl-2::GFP*], GMC101: *dvIs100* [*unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP*], BR5706: *bkIs10* [*aex-3p::hTau V337M + myo-2p::GFP*] and VH254: *hdEx81* [*F25B3.3::tau352(PHP) + pha-1(+)*] were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis, MN, USA). PHX3692 [*unc-47p::mcherry + pCL45snb-1::A-Beta(1-42)::3'UTR(long) + mtl-2::GFP*] and PHX3392 [*lgg-1::mCherry::GFP:lgg-1 + unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP*] were constructed by the Sunybiotech company. All strains were cultured at 20°C on solid nematode growth medium (NGM) plates with *Escherichia coli* (*E. coli*) OP50 except CL4176, CL2355, and PHX3692 that were cultured at 16°C.

2.6. Aβ induced paralysis assay

Synchronized Aβ transgenic CL4176 nematodes were maintained on 35mm NGM culture plate containing *E. coli* OP50, liensinine and neferine. Cultured at 16°C for 36h and then raised the temperature to 23°C for Aβ expression. Scored the paralyzed nematodes began at 24h after raising the temperature. If the nematodes can't move normally only their heads shake slightly or there is a transparent circular sterile circle around the head, they are considered paralyzed. (Dostal and Link 2010) Recorded the number of paralyzed nematodes per hour until all of them in the culture plate were paralyzed. The assay was repeated at least three times and calculated the PT50 (time duration in which half of nematodes were paralyzed).

2.7. Chemotaxis assay

1M Sodium acetate and 1M Sodium azide (1:1) were blended as attractants. 1M sterile water and 1M Sodium acetate (1:1) were blended as the control odorant. Synchronized L1 CL2355 nematodes were maintained on a 100mm NGM culture plate with *E. coli* OP50, liensinine and neferine. Cultured at 16°C for 42h and then raised temperature to 25°C for 32h. Washed the nematodes with M9 buffer to remove the *E. coli* OP50 and drugs, and transferred the nematodes to the center of an uncoated 100mm NGM plate. Dripped 10µL attractant and 10µL control odorant on both sides of the plate respectively, placed upside down at room temperature for one hour. Calculated the chemotaxis index (CI) = (number of nematodes on the attractant side - number of nematodes on the control side)/total number of nematodes.

2.8. 5-hydroxytryptophan sensitivity assay

Synchronized L1 CL2355 nematodes were maintained on a 35mm NGM culture plate with *E. coli* OP50, liensinine and neferine. Cultured at 16°C for 42h and then raised temperature to 25°C for 32h. Washed the nematodes with M9 buffer to remove the *E. coli* OP50 and drugs, and transferred to a 96-well plate with 150μL 5mg/ml 5-hydroxytryptophan. Continued to incubate at 25°C for 12h and recorded the number of active nematodes.

2.9. Neuronal integrity assay

Under normal conditions, the dorsal nerve cord and abdominal nerve cord and the neurons connecting the middle of the two cords can be observed.(Jorgensen 2005) Due to the expression of Aβ, the neuronal integrity of the PHX3692 nematodes has been seriously affected. Synchronized L1 PHX3692 nematodes were maintained on a 35mm NGM culture plate with *E. coli* OP50, liensinine and neferine. Cultured at 16°C for 42h and then raised the temperature to 25°C for 32h to induce Aβ expression. Dropped 5μL 90% oil onto the prepared 2% agarose pad and fixed the treated nematodes on the agarose pad with a sealing agent. The neurons of nematodes can be observed by a fluorescence microscope (Revolve FL, Echo Laboratories, USA). Counted the number of D-type motor neurons in the abdominal nerve cord.(Waldherr, Strovas et al. 2019)

2.10. Motion ability assay

Synchronized L1 BR5706 nematodes were cultured in a 96-well plate with *E. coli* OP50, 1.08mM FUDR was added in L3 and after 24h liensinine and neferine were added. Washed the nematodes with M9 buffer to remove the *E. coli* OP50 and drugs, transferred to uncoated 35mm NGM plate. Used WormLab software to record the distance of nematodes movement within 10 seconds.

2.11. Whipping rate assay

Synchronized L1 VH254 nematodes were cultured in a 96-well plate with *E. coli* OP50, 1.08mM FUDR was added in L3 and after 24h liensinine and neferine were added. Washed the nematodes with M9 buffer to remove the *E. coli* OP50 and drugs. Calculated the whipping rate in the liquid within 10 seconds after treatment with liensinine and neferine on day 4.

2.12. Stress resistance assay

For the oxidative stress assay *in vitro*, synchronized L1 wild-type nematodes were cultured in a 96-well plate with *E. coli* OP50, 1.08mM FUDR was added in L3 and after 24h liensinine and neferine were added. Washed the nematodes with M9 buffer to remove the *E. coli* OP50 and drugs, transferred to a transparent 96-well plate with 50mM paraquat, and recorded the alive nematodes every 12h until all died.

2.13. Measurement of reactive oxygen species (ROS)

Endogenous reactive oxygen species (ROS) levels were measured using fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA).(Yoon, Lee et al. 2018) The nematodes were treated as in the paralysis assay. Washed the nematodes with M9 buffer to remove the *E. coli* OP50 and drugs, transferred to a black bottom 96-well plate with 1mM fluorescent probe 2',7'-dichlorodihydrofluorescein

diacetate (H₂DCF-DA). Avoided light and incubated at 37°C for 2h, measured with a fluorescence microplate reader (excitation wavelength: 485 nm; emission wavelength: 535 nm).

2.14. Total RNA isolated and RT-PCR

Total RNA was isolated by Trizol (Tiangen, China) and converted to cDNA with an All-in-One cDNA synthesis supermix kit. Quantitative real-time PCR (RT-PCR) was performed using a 2 × SYBR Green qPCR master mix kit (Bimake, China) and a LightCycler96 Real-time PCR system (Roche, Switzerland). Actin was used as a reference gene, and data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

2.15. Fluorescence assay

The autophagosome accumulation PHX3392 model was constructed by A β transgenic GMC101 nematodes to observe the effect of liensinine and neferine on autophagosome accumulation.(Lin, Gao et al. 2022) Synchronized PHX3392 nematodes were maintained on a 35mm NGM culture plate with *E. coli* OP50, liensinine and neferine. Cultured at 20°C for 42h and then raised the temperature to 25°C for 8h. Dropped 5 μ L M9 buffer onto the prepared 2% agarose pad, and fixed the treated nematodes on the agarose pad with a sealing agent. Observed the fluorescence using the fluorescence microscope (Revolve FL, Echo Laboratories, USA).(Chang, Kumsta et al. 2017, Kumsta, Chang et al. 2019)

2.16. Western Blotting

Collected the PHX3392 nematodes after being treated with liensinine and neferine in 1x phosphate buffer solution at -80 for 2h. Total protein was extracted with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 1x protease inhibitor and 1x phosphatase inhibitor cocktail, loading buffer was added after quantification to prevent protein degradation. A 10–180 kDa protein marker (Solarbio, China) was used as an indicator of molecular weight. Protein samples were run at 30V for 40min on a stacking gel, and at 80V for 120min on a separating gel. The mCherry-GFP-LGG-1 levels were detected with rabbit anti-GFP monoclonal antibody (dilution 1:1000, Cell Signaling Technology), β -actin monoclonal antibody (dilution 1:1000, Proteintech) was used as the internal control. Anti-mouse IgG HRP-linked antibody (dilution 1:2000, Cell Signaling Technology) was the secondary antibody. Protein bands were detected using the standard enhanced chemiluminescence (ECL) western blotting Substrate. Mean densities of the mCherry-GFP-LGG-1 bands were analyzed using Image J.

2.17. Statistical analysis

Each assay was repeated at least three times. We used GraphPad Prism 8.0 and Image J to analyze the data. Student's *t*-test was used to compare two groups and One-way analysis of variance (ANOVA) was used to compare multiple groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Liensinine and neferine improved APPswe cell viability

To explore the effects of liensinine and neferine on intracellular A β toxicity, we used APPswe transfected cells constructed by SH-SY5Y neural cells as *in vitro* A β cell model. APPswe cells was treated with liensinine and neferine at concentrations of 5, 10 and 20 μ M respectively. We found that both liensinine and neferine could improve cell viability, and 10 μ M liensinine and neferine showed the most significant effect, increased by 18.90% and 23.91% compared with APPswe blank control (Fig. 2), indicating that liensinine and neferine could significantly inhibit intracellular A β toxicity.

3.2. Liensinine and neferine decreased ROS levels in APPswe cells

In neurodegenerative diseases like Alzheimer's and Parkinson's, the brains show oxidative damage, and oxidative stress often seems to be implicated in many of them. Oxidative stress plays a critical role in AD development, and it can even be considered a crucial central factor in the pathogenesis of AD.(Bai, Guo et al. 2022) We used APPswe cells to explore whether liensinine and neferine could enhance oxidative stress resistance. After being treated with 10 μ M liensinine and neferine, the intracellular ROS levels in treated APPswe cells decreased by 15.31% and 20.37% compared with untreated APPswe cells (Fig. 3), which suggested that liensinine and neferine could improve cellular antioxidant capacity.

3.3. Liensinine and neferine delayed A β -induced toxicity in C. elegans

Many lines of evidence support that β -amyloid (A β) peptides play an important role in AD, the most common cause of dementia. Reduction in A β has been the major target of recent experimental therapies against AD.(Gouras, Olsson et al. 2015) To explore the effects of liensinine and neferine on A β toxicity in nematodes, we used the transgenic CL4176 nematodes expressing temperature-induced human A β protein in the muscle cells. With the increase of A β expression, CL4176 showed a gradual paralysis phenotype. CL4176 nematodes were treated with liensinine and neferine at 50, 100 and 200 μ M concentrations respectively. The results showed that nematodes exposed to 100 μ M liensinine and neferine had the most significant effect on prolonging mean paralysis time, which was 44.66% and 60.31% longer than the control (Fig. 4), indicating that liensinine and neferine could inhibit A β -induced toxicity to nematodes. Consequently, we used 100 μ M liensinine and neferine as the treatment concentration for subsequent experiments.

3.4. Liensinine and neferine had protective effects on A β -induced neuronal damage in C. elegans

A characteristic of AD is the extracellular deposition of β -amyloid (A β) plaques, which is thought to provoke neuroinflammation, neuronal dysfunction, and progressive neuronal loss.(Mangalmurti and Lukens 2022) To explore the protective effects of liensinine and neferine on A β -induced neuronal damage, we firstly used the transgenic strain CL2355, which *pan*-neuron expressed A β , to measure two characteristic neuronal controlled behaviors, chemotaxis and 5-HT sensitivity.

The chemotaxis response in *C. elegans* is mediated by the activation of several sensory neurons and interneurons to stimulate the motor neurons.(Wu, Wu et al. 2006) 5-HT is a key neurotransmitter that modulates several behaviors of *C. elegans*, including egg-laying, locomotion, and olfactory learning. (Schafer and Kenyon 1995) When exogenous 5-HT was applied to the nematodes, they become paralyzed as a result of the sensitivity to excessive 5-HT. Compared to the control group, 100μM liensinine and neferine could significantly improve the chemotaxis index (CI) value and vitality of nematodes respectively (Fig. 5), which suggested 100μM liensinine and neferine could effectively protect Aβ-induced neuronal damage in *C. elegans*.

The neurotransmitter γ-aminobutyric acid (GABA) is an inhibitory synapse and is important in *C. elegans* motor neurons. 26 of the 302 neurons present in *C. elegans* express the neurotransmitter GABA. In the ventral nerve cord, 6 DD-like and 13 VD-like cells express GABA,(Jorgensen 2005) with the expression of Aβ, it is commonly to cause neuronal damage and loss. Therefore, we furtherly used the transgenic PHX3692 nematodes, D-type motor neurons marked with mCherry, to assess the neuroprotective effects of liensinine and neferine. Due to the expression of Aβ, the number of D-type neurons of PHX3692 nematodes is seriously decreased. After being treated with 100μM liensinine and neferine respectively, the number of D-type motor neurons in PHX3692 strains was obviously increased compared with control (Fig. 6). According to above experiments, it was strongly suggested that liensinine and neferine showed a significant protective effect on nerve damage induced by Aβ.

3.5. Liensinine and neferine inhibited the expression of hyperphosphorylated tau protein

Accumulation of phosphorylated tau is a key pathological feature of AD. Phosphorylated tau accumulation causes synaptic impairment, neuronal dysfunction, and neurofibrillary tangles formation. (Drummond, Pires et al. 2020) To investigate the inhibitory effects of liensinine and neferine on hyperphosphorylated tau protein, we first used BR5706 nematodes, which have severe movement defects due to excessive phosphorylation of tau protein in the body to detect the motion ability. The results showed that 100μM liensinine and neferine could significantly improve motion vitality of nematodes respectively (Fig. 7A), which suggested that liensinine and neferine had protective effects on motion damage caused by abnormal phosphorylated tau protein expression.

To further investigate the effects of liensinine and neferine on damage caused by abnormal phosphorylated tau protein expression in *C. elegans*, we used VH254 nematodes, which became increasingly uncoordinated movement with age due to hyperphosphorylation of tau protein in the body. After being treated with 100μM liensinine and neferine respectively, the whipping rate was effectively improved (Fig. 7B). It was further confirmed that liensinine and neferine had protective effects on motion damage caused by abnormal phosphorylated tau protein expression.

3.6. Liensinine and neferine improved antioxidative stress resistance in *C. elegans*

Oxidative stress occurs due to a disparity in redox states brought on by either an excessive generation of reactive oxygen species (ROS) or a reduction in antioxidant function.(Sarasiya and Norman 2018) Evidence has suggested that oxidative stress may augment the production and aggregation of A β and facilitate the phosphorylation and polymerization of tau, thus forming a vicious cycle that promotes the initiation and progression of AD.(Zhao and Zhao 2013)

When exogenous 50mM paraquat is applied to wild-type nematodes (N2), it causes acute oxidative stress injury. We used it to explore whether liensinine and neferine can enhance oxidative stress resistance of nematodes. We found that 100 μ M liensinine and neferine respectively prolonged the average lifespan of N2 worms by 6.94% and 13.40% compared to the control (Fig. 8A, 8B). To evaluate the impact of liensinine and neferine to intracellular ROS levels, we measured the ROS accumulation at 32h after up shifting the temperature using H₂DCF-DA (a fluorescent probe that reacts with ROS). It was observed that the intracellular ROS level in nematodes was obviously decreased by 26.23% and 32.87% compared to the control (Fig. 8C). Additionally, both liensinine and neferine could upregulate the mRNA levels of downstream key genes, *sod-3* and *gst-4*, of oxidative stress (Fig. 9). Combined with above experiments, all results demonstrated that liensinine and neferine could significantly enhance oxidative stress resistance of nematodes.

3.7. Liensinine and neferine upregulated the expression of autophagy related genes

Autophagy is an essential degradation pathway in clearing abnormal protein aggregates in mammalian cells and is responsible for protein homeostasis and neuronal health.(Li, Liu et al. 2017) Autophagy-lysosome defects occur early in the pathogenesis of AD and have been proposed to be a significant contributor to the disease process.(Zare-Shahabadi, Masliah et al. 2015) The accumulation of A β and tau could inhibit the autophagosome formation, another, abnormal fusion of autophagosomes and lysosomes was observed with accumulation of misfolded proteins, which induced the abnormal accumulation of autophagosomes.(Nixon, Wegiel et al. 2005) The phenomenon of autophagosome accumulation has been observed in brains of AD patient.(Zhang, Yang et al. 2021)

To clarify the underlying mechanism of neuroprotective effects of liensinine and neferine, firstly, we assayed the mRNA levels of autophagy membrane formation key genes, *lgg-1*, *unc-51*, and *atg-9*. The results showed that both liensinine could obviously increase the expression of *lgg-1*, *atg-9* (Fig. 9), which suggested that liensinine and neferine could enhance the autophagosome formation, and active autophagy pathway to exert neuroprotective effects. Furtherly, we assayed the mRNA levels of autophagy process key genes, *pha-4* and *ced-9*. The results showed that liensinine could obviously increase the expression of *pha-4*, while neferine could obviously increase the expression of *ced-9* (Fig. 9).

Moreover, we conducted an autophagosome accumulation assay in PHX3392 nematodes, which autophagosomes were accumulated and visualized as GFP-positive punctate. The results showed that 100 μ M liensinine and neferine could significantly reduce the accumulation of autophagosomes in nematodes (Fig. 10A, 10B). LGG-1- is a membrane protein on the autophagosome of nematodes, and its

protein expression level can indirectly represent the number of autophagosomes. After treatment with 100 μ M liensinine and neferine respectively, we observed that the expression level of LGG-1 protein in PHX3392 nematodes was obviously decreased (Fig. 10C, 10D), furtherly confirmed liensinine and neferine could reduce the accumulation of autophagosomes.

Above experiments suggested that liensinine and neferine promote the formation of autophagosomes while reducing their abnormal accumulation, which may be related to activating the fusion of autophagosomes and lysosomes.

4. Discussion

Natural small molecules of alkaloids extracted from plants have been a long way for human beings to fight against numerous diseases. In the folk, 'Lian Zi Xin', the embryo of *Nelumbo nucifera*, is often used to make functional and healthcare foods, and is considered conducive to the prevention of AD.(Meng, Liu et al. 2022) Liensinine and neferine are its main alkaloid constituents, in this paper, we furtherly demonstrated their neuroprotective effects and clarified their underlying mechanism by using APPswe cells and *C. elegans*.

The occurrence of Alzheimer's disease is related to many factors, the two core pathological hallmarks are amyloid plaques and neurofibrillary tangles formed by hyperphosphorylated tau protein. The amyloid cascade hypothesis suggests that the deposition of β -amyloid ($A\beta$) triggers neuronal dysfunction and death in the brain.(Ballard, Gauthier et al. 2011) According to our results, liensinine and neferine could significantly inhibit intracellular $A\beta$ toxicity (Fig. 2), and obviously ameliorate $A\beta$ toxicity, protect the integrity of neurons and improve the chemotaxis and serotonin sensitivity in nematodes (Fig. 4–6), the effect of neferine is more significant than that of liensinine. The above results furtherly confirmed that liensinine and neferine could inhibit the toxicity of $A\beta$ protein and have a protective effect on neuronal damage induced by $A\beta$ protein.

In tauopathies, tau protein becomes hyperphosphorylated, detaches from microtubules, abnormally localizes to the soma and dendrites, is cleaved by caspases and aggregates into neurofibrillary pathology. These processes disrupt cellular transport and cause synapse loss and, ultimately, many neurons die causing disrupted neural circuits and cognitive decline.(Spires-Jones, Stoothoff et al. 2009) According to our results, liensinine and neferine could significantly improve motion ability and whipping rate of nematodes (Fig. 7), which suggests that liensinine and neferine had protective effects on motion damage caused by abnormal phosphorylated tau protein expression.

Oxidative stress participates in the development of AD by promoting $A\beta$ deposition, tau hyperphosphorylation, and the subsequent loss of synapses and neurons.(Chen and Zhong 2014) We found that liensinine and neferine could decrease intracellular ROS levels, resist paraquat-induced oxidative stress and reduce the accumulation of ROS in nematodes (Fig. 8), which suggests that liensinine and neferine could reduce oxidative damage and resist $A\beta$ -caused damage.

Liensinine and neferine are very similar in structure, with only one methyl difference (Fig. 1). The introduction of methyl groups can effectively improve the pharmacological effects of molecules, including improving the physical and chemical properties of drugs, enhancing the interaction between small molecules and target proteins, and improving the metabolic properties of drugs.(Barreiro, Kummerle et al. 2011) It can also be seen from our experimental results that the effect of neferine is more significant than that of liensinine.

Autophagy is an evolutionarily conserved lysosome-mediated degradation process and is essential for survival, development, and organismal homeostasis. Emerging facts showed that defect in autophagy were likely to contribute to AD. Autophagy-lysosome defects occur early in the pathogenesis of AD and have been proposed to be a significant contributor to the disease process.(Zare-Shahabadi, Masliah et al. 2015) According to the results, we found that both liensinine and neferine could upregulate the mRNA levels of autophagosome formation and autophagy process key genes (Fig. 9), indicating that liensinine and neferine could enhance autophagy activity. Additionally, the impairment in the autophagy-lysosome system disturbs the turnover of other molecules associated with AD, which may also contribute to neuronal dysfunction in AD.(Li, Zhang et al. 2010) Generally, during autophagy, autophagosomes fuse with lysosomes to form autolysosomes to degrade misfolded proteins. Abnormal fusion of autophagosomes and lysosomes can lead to the abnormal accumulation of autophagosomes,(Zhang, Yang et al. 2021) which has been observed in brains of AD patients. Interestingly, we found that liensinine and neferine could obviously reduce the accumulation of autophagosomes in worms (Fig. 10). These findings indicated that liensinine and neferine could enhance autophagy, while reducing the accumulation of autophagosomes due to abnormal fusion to maintain autophagy homeostasis.

Based on the above results, we have explored the neuroprotective effects and molecular mechanisms of liensinine and neferine using in *vitro* and in *vivo* models. Our findings provided a foundation for the use of liensinine and neferine to treat Alzheimer's disease based on neuroprotective effects.

Declarations

Acknowledgment

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Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

All authors contributed to the study conception and design. Experiment was designed by Hongbing Wang and Shan Li. Entire experiment was performed by Mengchen Wu, Yehui Gao, Jinyun Jiang and Mengfan Xue. Data collection and analysis were performed by Mengchen Wu, Hongru Lin, Chen Zhang and Botian Ma. The first draft of the manuscript was written by Hongbing Wang, Shan Li and Mengchen Wu and all authors commented on previous of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

Authors have no competing interests to declare that are relevant to the content of this article.

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Figures

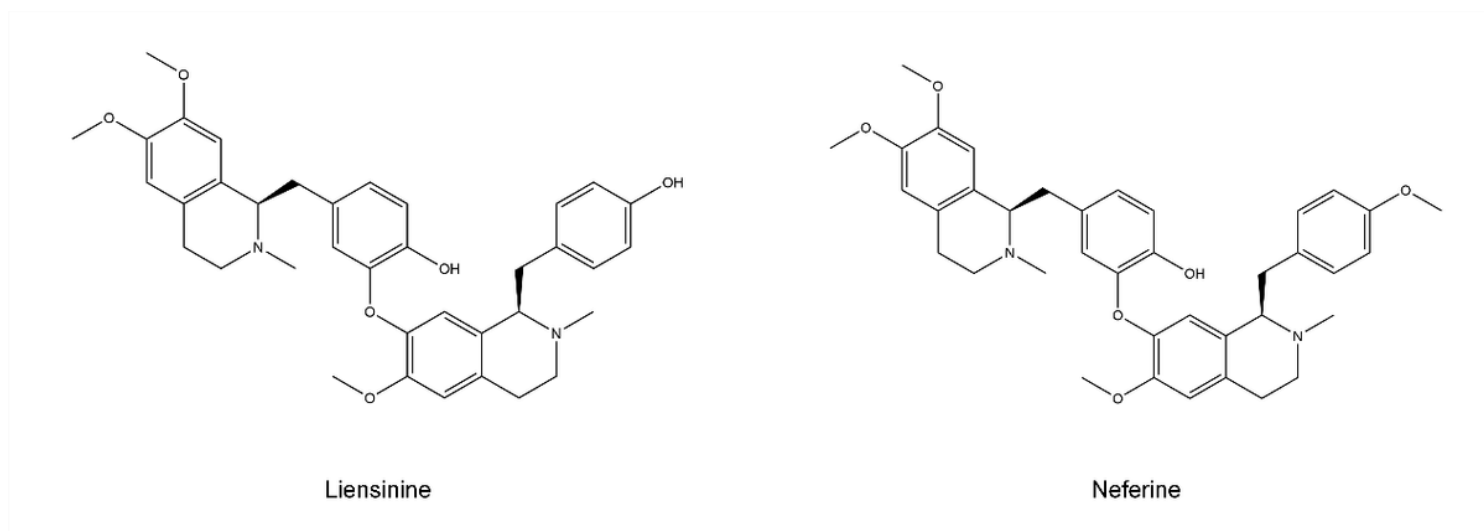


Figure 1

Structure of liensinine and neferine

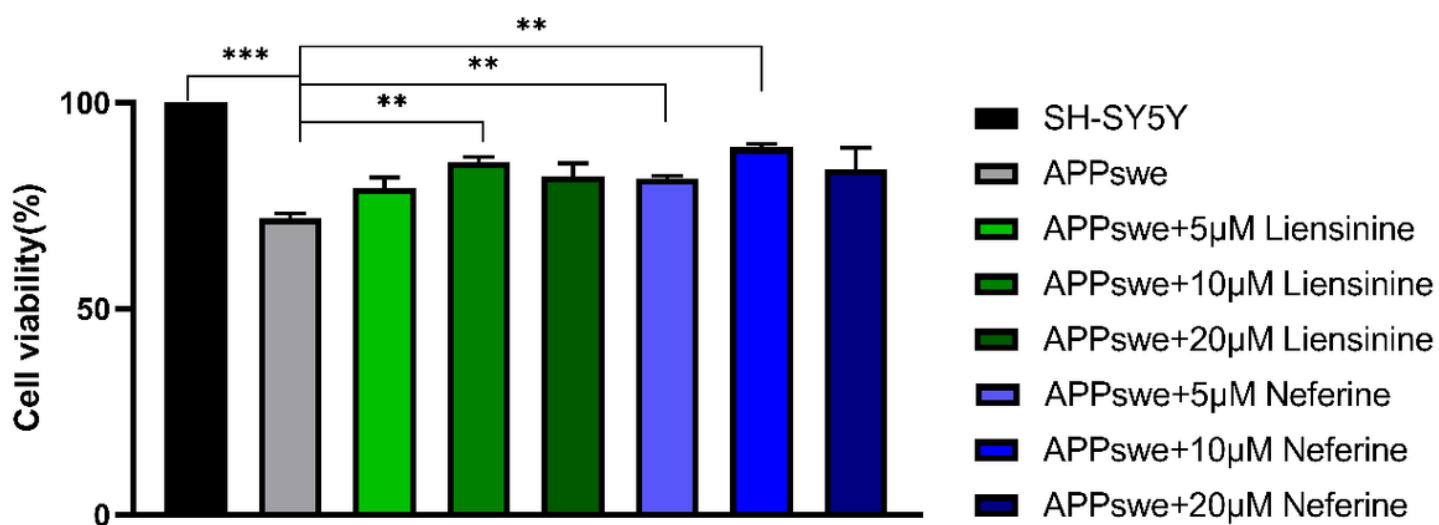


Figure 2

Liensinine and neferine improved APPswe cell viability. Cell viability of APPswe at various liensinine and neferine concentrations (5, 10, 20μM). (n=3, ** $p < 0.01$; *** $p < 0.001$)

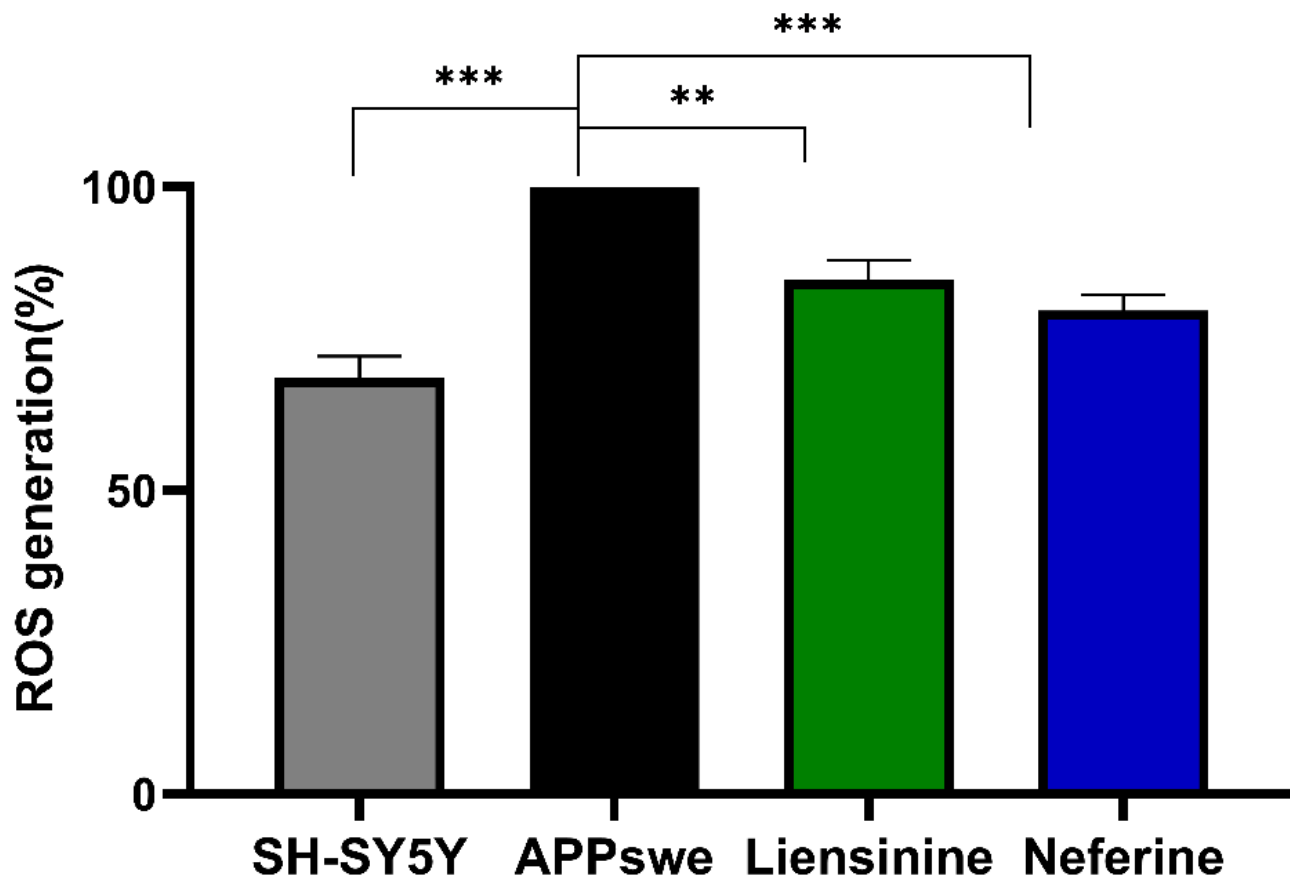


Figure 3

Liensinine and neferine reduced intracellular levels of ROS. Liensinine and neferine reduced intracellular ROS levels in APPswe cells. (n=3, ** $p < 0.01$; *** $p < 0.001$)

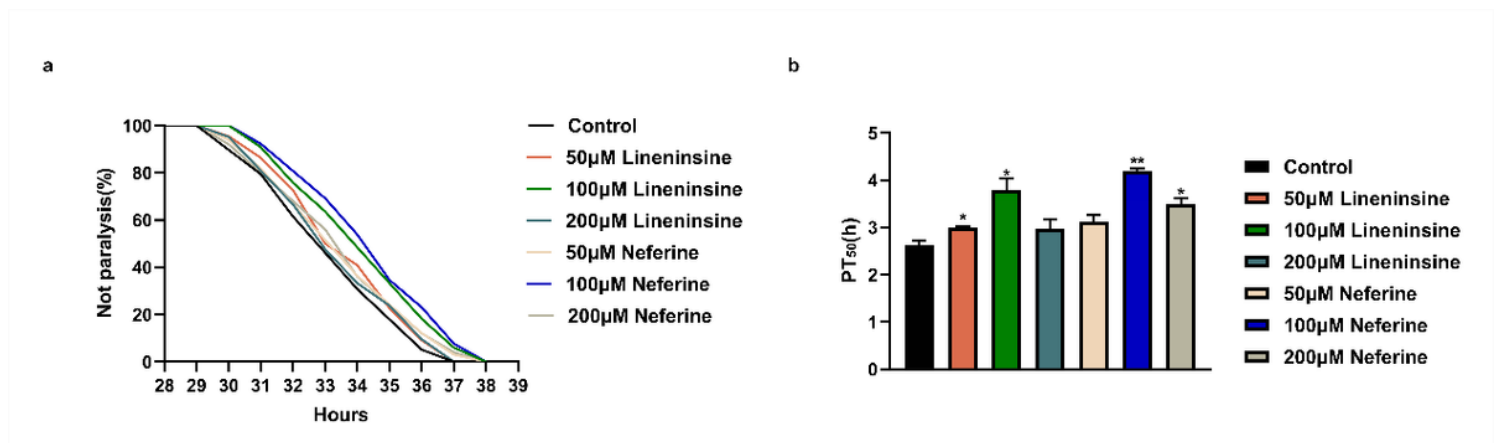


Figure 4

Liensinine and neferine delayed A β -induced toxicity in *C. elegans*. (a) Percentage of nematodes not paralyzed at various liensinine and neferine concentrations (50,100,200μM). (b) The PT50 was used for

quantitative analysis, which represents the time interval from the beginning of paralysis to 50% of nematode paralysis. (n=3, * $p<0.05$; ** $p<0.01$) Each group contains more than 30 nematodes

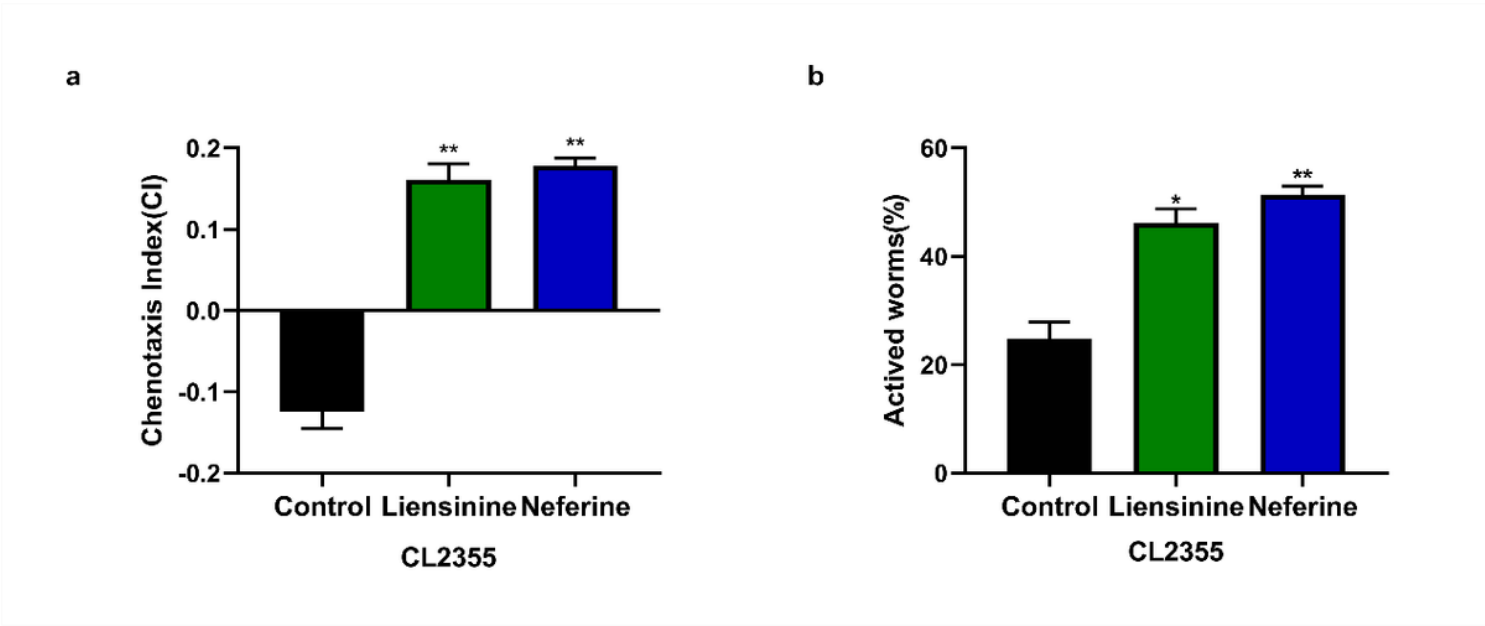


Figure 5

Liensinine and neferine enhanced chemotaxis and serotonin sensitivity in *C. elegans*. (a) Liensinine and neferine increased the Chemotaxis Index (CI) of CL2355 nematodes expressing neuronal A β . (b) The proportion of active CL2355 nematodes after being treated with liensinine and neferine. (n=3, * $p<0.05$; ** $p<0.01$) Each group contains more than 30 nematodes

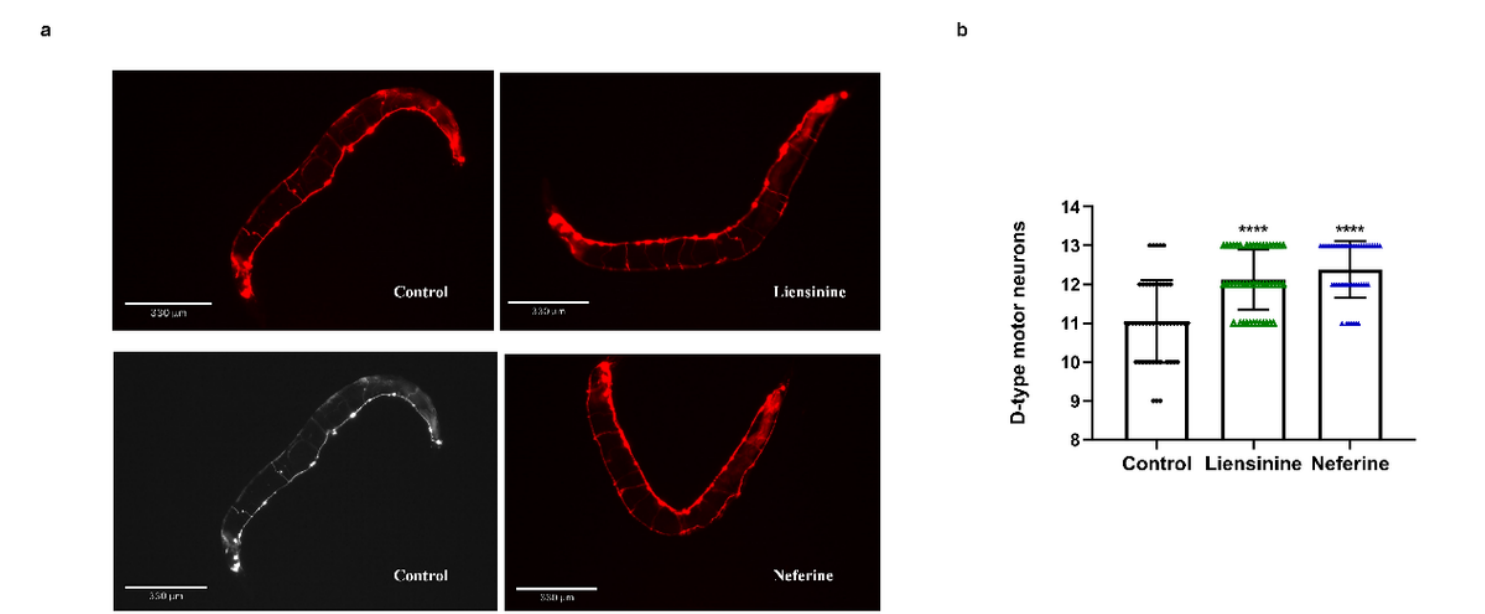


Figure 6

Liensinine and neferine protected neuronal integrity in *C. elegans*. (a) D-type motor neurons of PHX3692 nematodes shown by fluorescence confocal microscope. (b) The number of D-type motor neurons after being treated with liensinine and neferine. (n=3, ****p<0.0001) Each group contains more than 30 nematodes

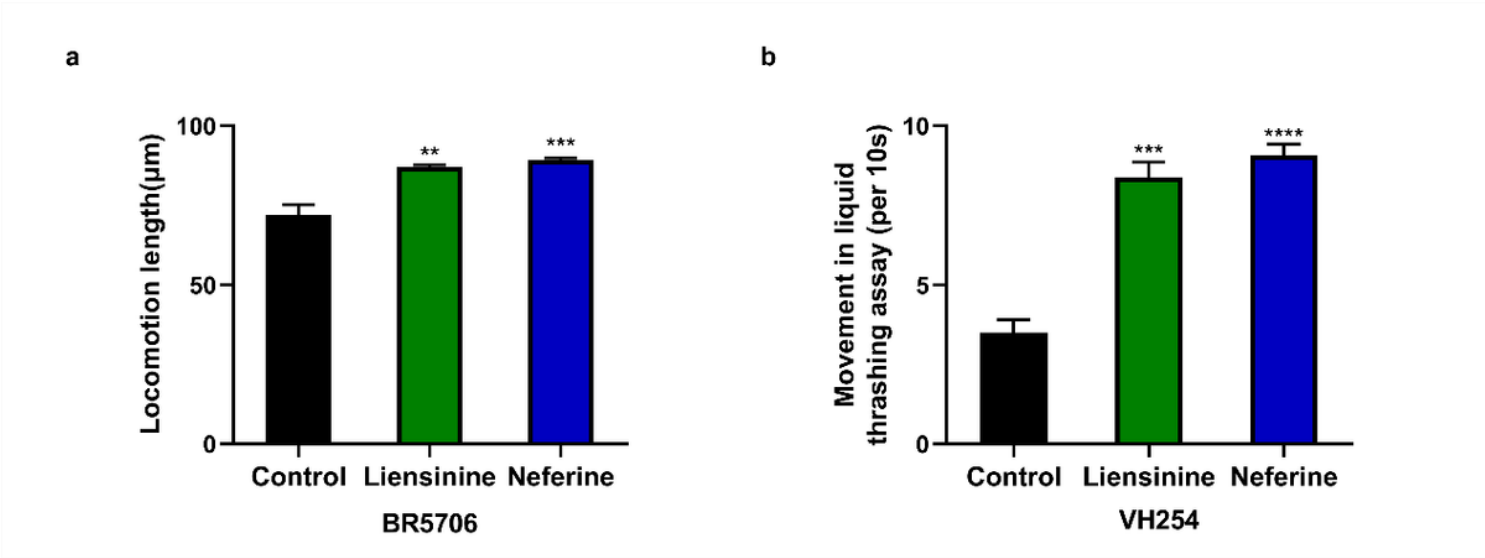


Figure 7

Liensinine and neferine inhibited the expression of hyperphosphorylated tau protein in *C. elegans*. (a) Liensinine and neferine improved motion ability of BR5706 nematodes. (b) Liensinine and neferine improved the whipping rate of VH254 nematodes. (n=3, **p<0.01; ***p<0.001; ****p<0.0001) Each group contains more than 30 nematodes

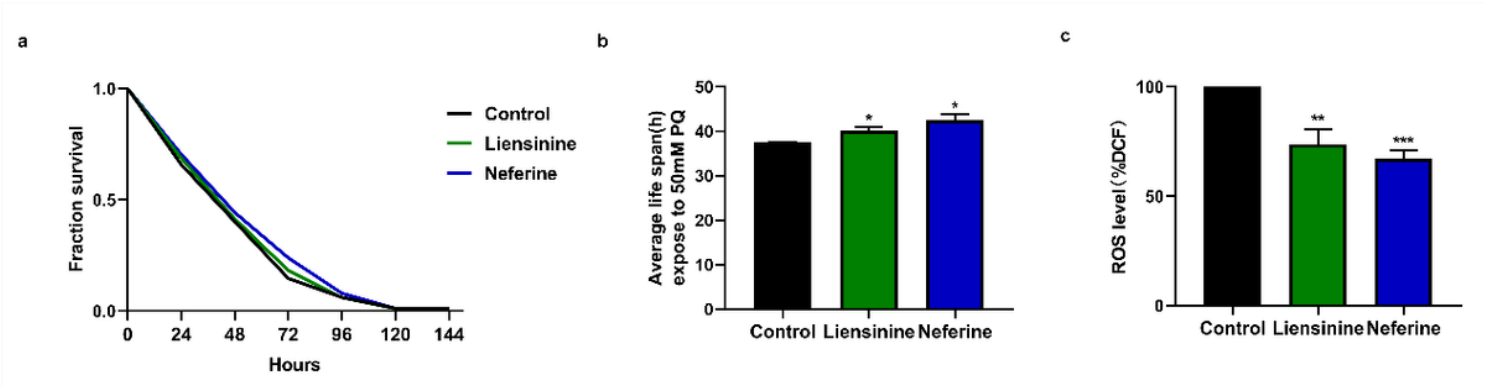


Figure 8

Liensinine and neferine enhanced oxidative stress resistance and decreased the accumulation of ROS in *C. elegans*. (a-b) Liensinine and neferine enhanced the number of surviving nematodes exposed to 50mM paraquat. (c) Liensinine and neferine decreased intracellular ROS levels measured by fluorescent prob H₂DCF-DA. (n=3, *p<0.05; **p<0.01; ***p<0.001) Each group contains more than 30 nematode

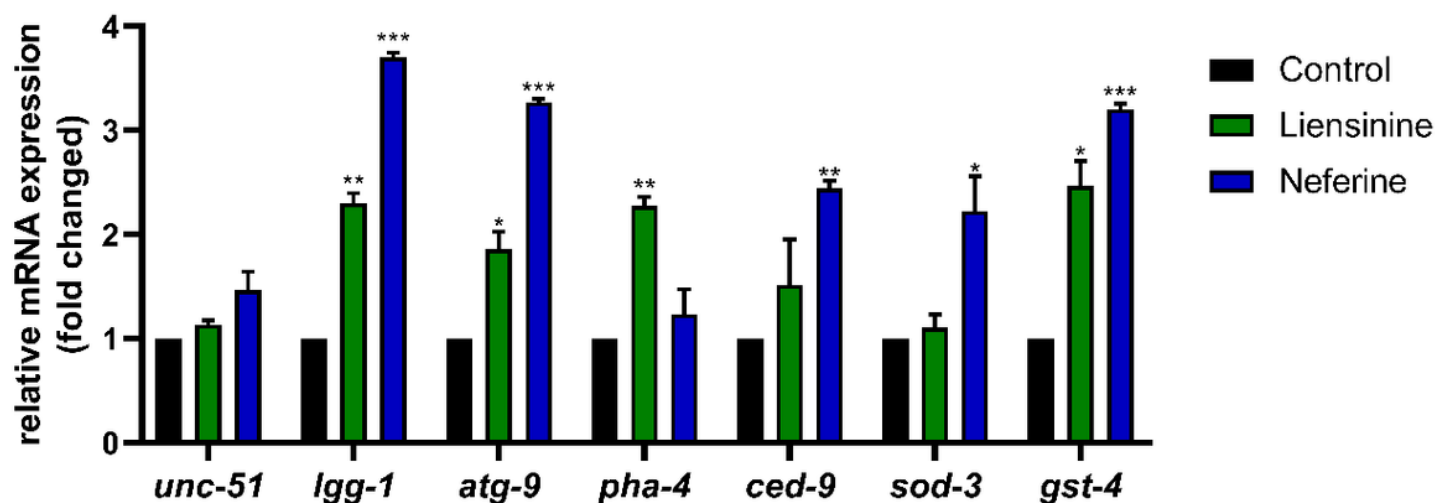


Figure 9

Liensinine and neferine exerted a neuroprotective effect through the autophagy pathway. Gene expression levels after being treated with liensinine and neferine. (n=3, * $p<0.05$; ** $p<0.01$; *** $p<0.001$) Each group contains more than 2000 nematodes

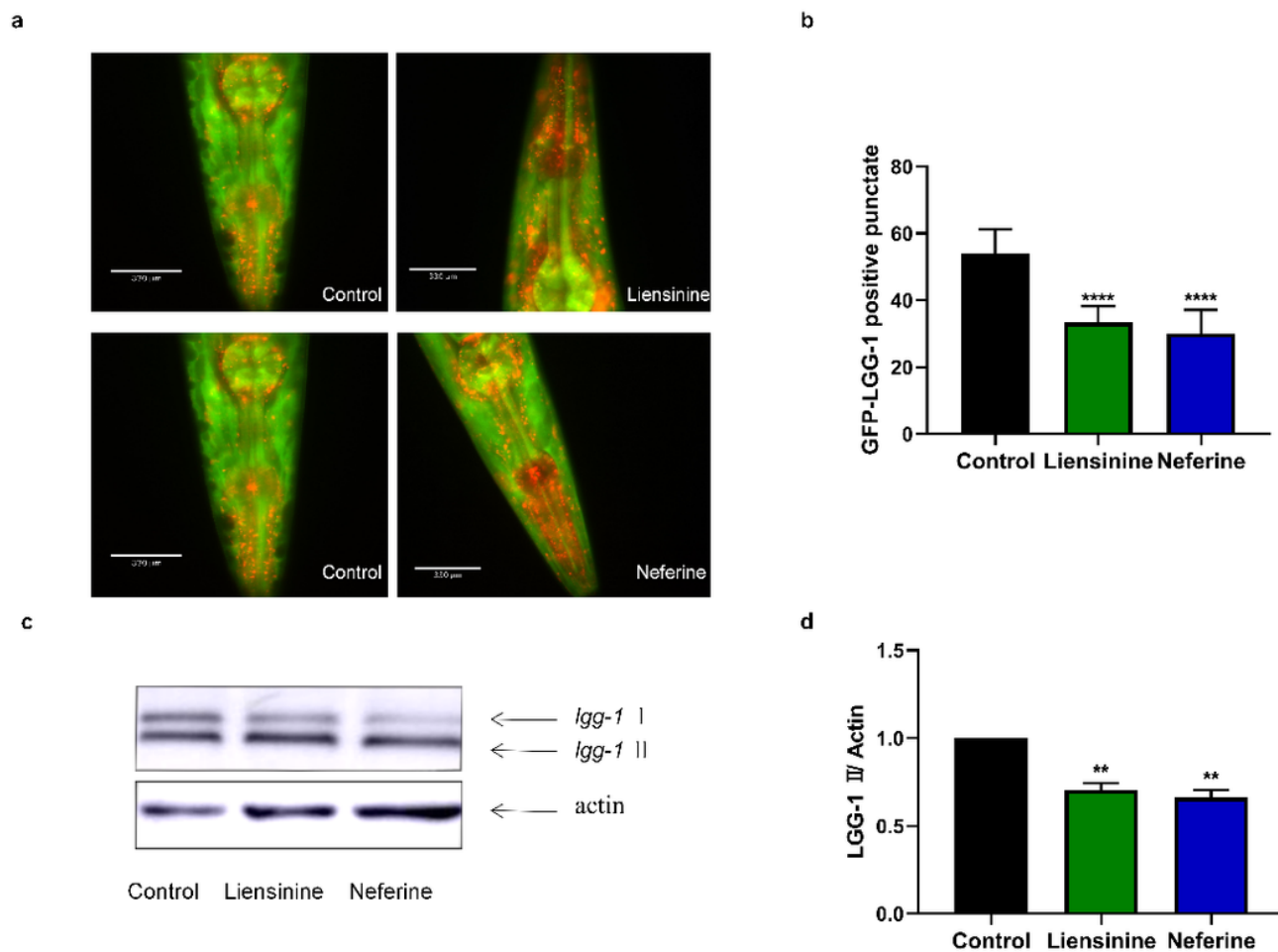


Figure 10

Liensinine and neferine reduced accumulation of autophagosomes. (a) The fluorescence diagram of autophagosomes and autolysosomes after being treated with liensinine and neferine. (b) The number of autophagosomes after being treated with liensinine and neferine. (c) The western blot of LGG-1- / . (d) Quantified western blot of gel densities by Image J. ($n=3$; $**p<0.01$; $****p<0.0001$) Each group contains the same number of nematodes