Endophenotype Network-based Approach reveals the Pharmacological Mechanism of Osthole against D-Galactose Induced Cognitive Disorder in Rats

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Research Article

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Abstract

Background: Osthole, a natural coumarin, found in many medicinal plants. Previous studies have shown its neuroprotective effects, whereas the effect and fundamental mechanism of Osthole for alleviating AD-associated dysmnesia is still not fully clear.

Purpose: This study aimed to examine the neuroprotection of Osthole against cognitive impairment in the D-galactose-induced rats and its pharmacological mechanism.

Method: The rat was constructed by subcutaneous injection of D-galactose at a dose of 150 mg/kg/day for 56 days as a model. The effect of Osthole on cognitive impairment was evaluated by behavior and biochemical analysis. Subsequently, a combination of in silico prediction and experimental validation was performed to determine the underlying mechanisms of Osthole against Alzheimer's disease, while to verify the network-based predictions, western blot, Nissl staining, and immunofluorescence were applied.

Result: Osthole could improve memory dysfunction induced by D-galactose in Sprague Dawley male rat. Endophenotype-based network approach highlight several AD-related pathological processes that may be regulated by Osthole, including neuronal apoptosis, neuroinflammation and endoplasmic reticulum stress. Among them, the proapoptotic markers (Bax), antiapoptotic protein (Bcl-2), moreover, the microgliosis (Iba-1), Astrocytosis (GFAP), and inflammatory cytokines (TNF-α), levels of ER stress-associated proteins (BIP, p-PERK/PERK, Caspase12, CHOP and XBP1s) were evaluated in both hippocampus and cortex. And the results indicated that Osthole significantly ameliorated neuronal apoptosis, neuroinflammation and ER stress in D-galactose induced rats.

Conclusion: This study explored the pharmacological mechanism of Osthole against D-galactose induced memory impairment and identified Osthole as a potential anti-AD drug candidate targeting multiple signaling pathways by endophenotype network-based.

Introduction

Alzheimer's disease (AD), one of the most common neurodegenerative diseases in the elderly, is one of the main syndromes of senile dementia, with the memory loss and cognitive dysfunction as the main clinical manifestation. According to report, by 2025, the number of people aged 65 and older with Alzheimer's disease is projected to reach 7.2 million (Gaugler et al., 2022). The pathogenesis of AD is relevant to the complex interplay of the disruption of synaptic homeostasis and the malfunction in the highly correlated endosomal/lysosomal clearance pathway (Knopman et al., 2021). Unfortunately, no efficient drugs have been found against this disease until now (Bendlin & Zetterberg, 2022; Cummings et al., 2022).

Osthole, named as osthol, is a naturally occurring coumarin derivative found in a variety of Chinese medicinal herbs such as Cnidium monnieri and Angelica pubescents (Sun et al., 2021b). Previous studies have revealed that Osthole exerts extensive pharmacological activities, such as anti-inflammatory,
neuroprotective activities, antioxidant, and immunity enhancement (Sun et al., 2021a; Zafar et al., 2021).

For instance, Osthole via Wnt/β-catenin signaling to promoted neural stem cells proliferation and neuronal differentiation, and suppressed apoptosis (Yao et al., 2015); Osthole reduced tau phosphorylation in AD through PI3K/AKT/GSK-3 AKT signaling pathway in APP/PS1 mouse (Yao et al., 2019); Osthole could also regulate oxidative stress to reduce glutamate-induced apoptosis and protect neurons in vitro and vivo models of AD (Chu et al., 2020). Nevertheless, the neuroprotective and regulatory mechanisms of Osthole for alleviating AD-associated dysmnesia are not yet fully understood due to the complexity of the pathological mechanisms of AD.

As an emerging interdisciplinary discipline, network medicine provides a comprehensive framework to integrate large-scale, multi-omics data to redefine human disease and therapeutic (Barabási et al., 2011). Due to the inherent complexity of AD, there are common underlying mechanisms and intermediate endophenotypes between AD with other diseases. As an example, six typical endophenotype networks have been proposed in AD: (i) amyloidosis, (ii) tauopathy, (iii) neuroinflammation, (iv) mitochondrial dysfunction, (v) vascular dysfunction, and (vi) lysosomal dysfunction (Fang et al., 2020). Recently, we have applied endophenotype-based approach to repurpose sildenafil as a candidate drug for AD (Fang et al., 2021), and explore the neuroprotective mechanisms of Medicarpin in scopolamine-induced mice (Li et al., 2021). Thus, AD-related endophenotype modules can be effectively employed to characterize pathogenesis and drug therapeutic mechanism for AD.

In this work (Fig. 1), firstly, Osthole improved spatial learning and memory via behaviors testing in the D-galactose-induced rats, suggesting the potential anti-AD ability of osthole. Then, the endophenotype network-based approach, which included AD-related endophenotype modules, a human protein-protein interactome and an Osthole drug-target network to study exploring osthole mechanisms of action (MOAs) against AD. Particularly, by network proximity prediction to measure the AD-related endophenotype module's and Osthole target network's network distance. Integrated pathways were secondarily constructed to identify AD-related biological pathways that are regulated by Osthole targets. In the end, we validated the proposed pharmacological mechanism by experimental techniques.

Materials And Methods

Animals

Sprague Dawley male rats (4 ~ 5weeks old) weighing 120 ~ 150g were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China) (SYXXK[YUE]2018-0001) and living in a 12-hour light/dark cycle with plenty of food and water, a temperature of 22 ± 2°C, 40~70% relative humidity. After 2 weeks of adaptive feeding, the test animals were equally randomly divided into five groups (number = 10 rats/group). Control group: rats treated for 56 days with 0.5% (v/v) Tween 80 in 0.9% normal saline as vehicle. D-galactose group: rats treated with D-galactose (150 mg/kg/day; i.h for 56 days). Osthole-L group: rats treated with D-galactose + Osthole (10 mg/kg/day, Solvent as control, for 56 days). Osthole-H group: rats treated with D-galactose + Osthole (20 mg/kg/day, Solvent as control, for 56 days). Donepezil
group: rats treated with D-galactose + Donepezil (3mg/kg/day, Solvent as control, for 56 days). D-galactose (Aladdin Bio-Chem Technology, Shanghai, China), Donepezil (Aladdin Bio-Chem Technology, Shanghai, China), Osthole (> 98% purity, Tauto Biotech, Shanghai, China).

All animal procedures, ethics and animal welfare were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (No. 20210601001) and conducted in accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Behaviors Analysis

Morris water maze test (MWM)

A MWM test assessed spatial learning-memory ability, including a recording system, a hidden platform (10 cm in diameter) 2 cm under the water surfer, and is performed by a circular pool (160 cm diameter, 60 cm height), with water temperature of 23 ± 2°C filled to 30 cm height. Four average virtual quadrants form the pool, with the hidden platform in the heart of one. In the MWM test, rats were given adaptive training on the first day, followed by directed navigation on the 2nd and 6th days. During the experiment, we randomly placed each rat in the water facing the tank wall three times a day, starting from different points. An analysis of escape latency was performed based at the moment each rat found the platform from the starting point. Its escape latency would be 60s, and it would be guided to the platform and stay there for 10 seconds if it could not find the platform within 60s. The navigation in position was carried out on the seventh day of the experiment, the rats can swim freely in 60 seconds without the hidden platform. For assessing spatial memory, time spent in the purpose quadrant and platform crossing times was recorded.

Novel object recognition test (NORT)

It is a test that measures the memory capability based on rodent’s natural ability to touch and explore new things, NORT, a set of equipment for experiment: including square case (50 cm x 50 cm x 50 cm), camera equipment and VisuTrack behavior analysis software (Model: XR-VT, Shanghai Xinsoft). In the NORT test, there were two important phases: training and testing. The rats were taken to the case containing two indistinguishable objects (A and B) in opposite positions for 5 min during the training phase. Each time, the rats were placed in the same position as far as possible, with the back facing the object and the same distance from the two objects. During testing, a fresh object with a different color and shape (object C) was substituted for one of the old objects (A). It was recorded how much time the rats spent exploring the familiar and the novel object in the box within 5 minutes was recorded. (Putting front paws on the object, sniffing the object, licking the object, etc. are exploration objects. Posing or climbing on the object without moving cannot be considered exploration of a new object). As a measure of objective novelty performance, the percentage of time the rat spent exploring the novel object C, out of the exploration time, was calculated as follow: recognition index, RI = novel object/ (novel object + old object)
object) x 100%. A 75% ethanol solution was used to clean the box and items between each test to avoid stimulating rats with odors or excrement.

**Nissl Staining**

Sodium pentobarbital was administered to four rats from each group after the behavioral experiment. Four% paraformaldehyde was applied to perfuse brain tissues and store them for over 24 hours after the perfusion. Following preservation of the brain tissues, they were immersed in 30% sucrose (4% paraformaldehyde) and dehydrated. They were afterward cast in O.C.T. embedded, and coronal sections 20 µm thick were made by Freezing microtome (LEICA CM 1950). After 10-minute Nissl staining (Beyotime Biotechnology, Shanghai, China), the sections were washed with ddH₂O, dehydrated with gradient ethanol, transparent with xylene and sealed with Rhamsan gum, and finally recorded with a Nikon microscope for morphology.

**Immunofluorescence**

Frozen sections were prepared the same as Nissl staining. Coronal sections 20µm thick were stained according to the steps of Iba-1 fluorescent staining instructions. The sections were fixed with 4% paraformaldehyde for 20min, secondarily cleaned, sealed with 10% goat serum for 30min. Afterward, sections were permeabilized and blocked 48 hours at 4°C with Iba-1 antibody (1:100, Abcam). A fluorophore-conjugated secondary antibody (1:200, ZSGB-BIO) was applied to the sections for 2 hours, followed by Dapi (Beyotime Biotechnology, Shanghai, China) staining for additional 10 min. ImageJ software (National Institutes of Health) was used to quantify images acquired with a fluorescence microscope Model DMi8 (Leica, Germany).

**Western blot**

A phosphatase inhibitors and protease inhibitors (Millipore) were added to SDS lysis buffers to homogenize the hippocampus and cortex. With the Bradford method (Bio-Rad), the protein supernatants of the lysates were obtained after centrifugation at 15,000r and 4°C for 15 min. Proteins were transferred to PVDF membranes (Millipore) after isolated on SDS-PAGE gels, and sealed with skim milk and detected overnight with primary antibody at 4°C. Overnight incubation of endoplasmic reticulum stress related antibodies, against apoptotic antibodies and neuroinflammation antibodies : PSD95 antibody #ab12093(abcam); Synaptophysin antibody #ab52636(abcam); BIP antibody #3177(CST); CHOP antibody #2895 (CST); PERK antibody #5683 (CST); p-PERK (Thr980) antibody #3179(CST); XBP1s antibody #12782(CST) ; Caspase12 antibody # A0217 (ABclonal); GFAP antibody #95717(CST); Anti-TNF Receptor I antibody #ab19139(abcam); Bax antibody #AF0120 (Affinity Biosciences); Bcl-2 antibody #AF6139 (Affinity Biosciences); β-actin antibody # A1978(Sigma); Anti-rabbit IgG (CST); Immunoreaction of anti-mouse IgG antibody (CST) was completed, ECL luminescent solution was 1:1, and the immunoblotting was recorded by ChemiDocXRS+ (Bio-RAD, USA), a comprehensive gel imager.
Endophenotype network approach

Collection of interacted protein targets of Osthole

The drug-target interactions (DTIs) of Osthole were first extracted from our previous integrated natural product-target database (Huang et al., 2019), which includes 38,220 high-quality experimentally validated DTIs linking 3,882 natural products to 5,643 human proteins. Succeeding, we further supplemented the targets by querying Herbal Ingredient Target Database (HIT)(Yan et al., 2021), a comprehensive database with all the herbal ingredients and target information based on literary evidence. After eliminating duplicates and non-Homo sapiens targets, a total of 46 high-quality human protein targets for Osthole was receive (Supplementary material Table S1).

Construction of endophenotype network for AD

The endophenotype can be involved in the pathogenesis of multiple diseases at the same time, which characterizes the functional intermediate genetic characteristics of an independent biological system. Characterizing the pathogenesis of AD, building predictive models, and revealing the molecular mechanisms of AD drugs are based on constructing AD-related endophenotypic networks. In this work, 25 sets of genes related to several pathological AD pathological mechanisms in the Quick GO database (https://www.ebi.ac.uk/QuickGO/). Based on previous studies, these endophenotype modules can be grouped into seven major classifications, including amyloidosis, neuron inflammation, neuron apoptosis, endoplasmic reticulum stress, tauopathy, and autophagy (Li et al., 2021). The detailed gene catalog for these endophenotype modules can be found in Supplemental Table material S2.

Construction of the human protein-protein interactome

The human protein-protein interactome was served as the background in which to measure the network distance among different protein sets. Based on 15 authoritative databases, six diverse sources of protein-protein interactions (PPIs) with experimental evidence were collected: (1) protein three-dimensional (3D) interactome; (2) high-throughput Y2H binary; (3) kinase-substrate interactions; (4) signaling interactions; (5) literature protein; and (6) complexes. A whole of 351,444 PPIs covering 17,706 unique proteins constitute the comprehensive high-quality human protein-protein interactome. More detailed data concerning the integration process can be found in the prior study (Cheng et al., 2019).

Network proximity analysis

The network proximity method was applied to analyze the network distance and relevance between AD-related endophenotype module and Osthole’s drug targets. Performed a gene set of endophenotype module (A), a series of the drug targets (B), the nearest distance $d_{AB}$ measured by the average shortest path length of all nodes in module A to module B in the human protein-protein interactome is defined as:

$$
\langle d_{AB} \rangle = \frac{1}{\|A\| + \|B\|} \left( \sum_{a \in A} \min_{b \in B} d(a, b) + \sum_{b \in B} \min_{a \in A} d(a, b) \right)
$$
Where \( d(a, b) \) refers to gene \( a \) and drug target \( b \) shortest path length.

The significance of network distance between osthole and AD endophenotypic modules was calculated by permutation tests with 10,000 replicates. The mean and standard deviation (\( \sigma_d \)) of the reference distribution were used to calculate a \( z \)-score (\( z_d \)) by normalizing the observed (non-Euclidean) distance. More information on the network proximity measure is presented in earlier studies (Fang et al., 2022; Wu et al., 2020).

**Statistical analysis**

Statistical analysis of this study was performed by Python (V3.2, http://www.python.org/). Data was analyzed using GraphPad Prism 8 (LA Jolla, CA, USA) and presented as mean ± SEM. ImageJ was applied to calculate the strip gray value. Statistical comparisons were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni or Dunnett post hoc test. \( P \)-value < 0.05 was considered as statistically significant.

**Results**

**Osthole alleviates D-galactose-induced memory and synaptic dysfunction**

Cognitive dysfunction and memory deficits are the primary early symptoms of AD. Previous studies have shown that, long-term high-dose injection of D-galactose can lead to neuroinflammation, neuron damage and cognitive dysfunction in rats (Azman & Zakaria, 2019; Chiroma et al., 2018). MWM test and NORT were applied for assessment whether Osthole could improve spatial learning and memory impairment. Eight rats in each experimental group were randomly selected for behavioral testing. The representative swimming trajectory and the time of escape (seconds) to the hidden platform during the training period (5 days) is shown in Fig. 2a and 2b. The D-galactose group from the second day of swimming training was significantly longer than that of control group \( (P<0.05) \), while escape latency is significantly reduced in the Osthole treatment group and Donepezil group \( (P<0.05) \). Similarly, after training, when the hidden platform was removed and the last day of testing was performed, showing that the Osthole group and Donepezil group remained in the target quadrant longer than the D-galactose group rats in Fig. 2c \( (P<0.05) \). In addition, the number of platform crossings was significantly increased in the Osthole and Donepezil groups compared with the D-galactose group in Fig. 2d \( (P<0.05) \), showing that Osthole can improve spatial learning disabilities induced by D-galactose. The results of the NORT were shown in Fig. 2e \( (P<0.05) \). Compared with the control group, the level of discrimination index of the D-galactose treatment group showed significantly decreased, whereas Osthole-treated group or Donepezil-treated group significantly improved performance in recognition index.

Western blot analysis of synaptophysin (Syp) and postsynaptic density protein (PSD95), the presynaptic protein associated with memory, was significantly reduced in the brains of D-galactose-treated rats compared to control rats. Interestingly, Osthole and Donepezil significantly up-regulated the protein of
PSD95 and Syp in the brains of D-galactose induced rats Fig. 2f-h \( (P < 0.05) \). All of them indicating that Osthole exerted beneficial effects on D-galactose-induced learning and memory disorders.

**Endophenotype network identified potential AD pathological mechanisms regulated by Osthole**

Osthole significantly attenuated spatial learning and memory deficits in rats with D-galactose-induced memory impairment. Next, by measuring the network distance between phenotype modules in AD and the target network of Osthole the therapeutic mechanism of Osthole on AD was discovered using the method of network proximity approach. It hypothesized that it was within or near a specific disease endophenotype module in the human interactome network that the target network of Osthole should be, and the therapeutic effect of Osthole on AD is contributed by targeting multiple AD-related endophenotype mechanisms. As shown in Fig. 3, after setting \( p < 0.05 \) and \( Z < -1.8 \) as the network proximity scoring thresholds, 10 AD-related pathways were identified to be significantly correlated with Osthole. Neuroinflammation is an vital pathological factor in AD(Zhou et al., 2021), the network prediction revealed that Osthole might regulate the inflammatory response \( (Z = -2.714) \). Meanwhile, neuronal apoptosis occurs extensively during development and pathology including AD(Fricker et al., 2018), and we predicted three apoptosis-related pathways, including positive regulation of neuron death \( (Z = -2.364) \), MAPK cascade \( (Z = -2.451) \) and neuron apoptotic process \( (Z = -2.880) \). Besides, ER stress including responses to ER stress \( (Z = -1.940) \) and ER unfolded protein response \( (Z = -1.846) \) also emerge significant. Increasing evidences demonstrated that ER stress was involved in several brain pathological processes observed in AD, such as \( \beta \)-amyloid production, tau phosphorylation, inflammation and the cell death, and thus plays an essential role in the pathogenesis of AD(Li et al., 2015). Our prediction suggested that neuroinflammation, endoplasmic reticulum stress and apoptosis could be modulated by Osthole to exert anti-AD outcomes, warranting further analysis and validation.

**Integrated pathway map analysis**

According to the network-based analysis, an integrated pathway map was drawn via mapping drug targets of Osthole into the pathways that was directly related to the pathological process of AD. As depicted in Fig. 4, this map was consisted of four pathways, including the PI3K/ AKT signaling pathway, ER stress-related signaling pathways, TNF signaling pathway and NF-\( \kappa \)B signaling pathway. Here, three representative functional modules involved in the map are selected to illustrate the potential mechanism of Osthole in the treatment of AD.

**Apoptosis module**

Apoptosis is a programmed cell death that occurs when damaged or affected by various factors(Maino et al., 2017; Obulesu & Lakshmi, 2014). In the brains of AD patients, the majority of neuronal death is mediated by apoptosis, and apoptotic neuronal death, which were likely to an important characteristic of AD(Fricker et al., 2018). PI3K-AKT is a major cell survival pathway that regulates apoptotic mechanisms
and proteins associated with anti-apoptotic factors via phosphorylation (Kumar & Bansal, 2022; Saahene et al., 2021). As shown in Fig. 4, Osthole could act on multiple targets on PI3K-AKT signaling pathway as well as proteins highly associated with apoptosis (e.g., BCI-2, CASP3), suggesting its potential anti-AD mechanism mediated by apoptosis.

**Neuroinflammation regulation module**

Neuroinflammation is prone to be a chronic process that fails to resolve by itself and is recognized to be one of the driving forces for neurodegeneration (Leng & Edison, 2021). The interaction of Aβ with microglia and astrocyte promotes the production of inflammatory cytokines, such as, TNF-α, IL-1 and IL-6, which further increases the level of activated microglia and Aβ, thereby promoting the inflammatory progression of AD (Dhapola et al., 2021; Fakhoury, 2018). In addition, activation of NF-κB promotes the expression of inflammatory cytokines such as Cyclooxygenase-2 (COX-2), TNF-α and IL-1β (Ju Hwang et al., 2019; Shi et al., 2016). Figure 4 shows that Osthole may exert potential anti-inflammatory effects in AD treatment by affecting key proteins in the TNF signaling pathway and NF-κB signaling pathway.

**Endoplasmic reticulum stress regulation module**

Several studies have reported that ER stress plays an integral role in the pathogenesis of AD (Uddin et al., 2021). Accumulation of misfolded proteins and perturbation of intracellular Ca$^{2+}$ balance are believed to be the basis for triggering ER stress, leading to neurological dysfunction and the cell death (Hashimoto & Saïdo, 2018). Accumulating evidence suggests that Aβ oligomer disrupt Ca$^{2+}$ homeostasis and increase ROS production, leading to oxidative stress and caspase-3-related cell death (Uddin et al., 2020). Figure 4 shows that Osthole targets several proteins implicated in the Aβ-associated ER stress signal pathway, including BACE1 (β-secretase 1), APP, JNK, CAPS3, indicating its underlying anti-AD mechanism might be related to the regulation of ER stress-induced apoptosis.

**Osthole inhibited D-galactose-induced neuron apoptosis**

Our network analysis suggested that Osthole alleviated D-Gal-induced learning and memory disorders, probably though regulation of neuron apoptosis. Here, we verify whether the improvement of learning and memory in rats is associated with neuronal apoptosis in the hippocampus and cerebral cortex. Figure 5a shows the loss of neurons in the hippocampal regions CA1 and CA3 of D-galactose-treated rats in a representative Nissl-stained photomicrograph. However, treatment with Osthole or Donepezil markedly reduced neuronal loss induced by D-galactose.

Proapoptotic and antiapoptotic markers were also performed by Western blotting to investigate expression. The results reliably demonstrated that the expression of Bax protein was significantly up-regulated in the hippocampus and cortex of D-galactose-treated rats, while the expression level of Bcl-2 protein was significantly down-regulated. In contrast, treatment with Osthole or Donepezil significantly reduced the increased expression of the proapoptotic protein Bax, while the expression of the anti-apoptotic protein Bcl-2 was up-regulated in both regions of rat brain Fig. 5b-e ($P<0.05$). The results demonstrated that Osthole could reduce the neuronal apoptosis induced by D-galactose in rats.
Osthole inhibited D-galactose-induced activation neuroinflammation

According to our network analysis, the anti-AD mechanism by Osthole may be also related to the regulation of neuroinflammation. To analyze the implication of Osthole on the expression of neuroinflammation, we performed immunoblotting and immunofluorescence to observe the levels of Iba-1 (activated microglia), GFAP (astrocyte activation), and TNFα (Tumor necrosis factor receptor 1). As shown in Fig. 6a the Iba-1 expression in the hippocampus and cortex of rats in the D-galactose group increased significantly compared to the control group. Interestingly, compared to D-galactose alone, Osthole-H group and Donepezil group significantly reduced Iba1 immunofluorescence reactivity in both hippocampus and cortex. Figure 6b-d ($P < 0.05$), illustrate that GFAP and TNFα1 expressions were significantly increased in D-galactose group compared with the control group, and down-regulated in Osthole or Donepezil groups in the hippocampus and cortex, which means that Osthole could regulate D-galactose induced neuroinflammation.

Osthole relieves D-galactose-induced endoplasmic reticulum stress

ER stress pathway-related proteins are also detected according to network proximity prediction. Then, we evaluated the expression of ER stress-associated proteins including BIP (an important chaperone of the endoplasmic reticulum (ER) and is believed to act as a primary sensor in the activation of the unfolded protein response (UPR)) (Hetz & Papa, 2018), PERK (protein kinase R-like ER kinase) (Hughes & Mallucci, 2019), IRE1α (inositol-requiring transmembrane kinase/endonuclease 1α), XBP1s (X-box binding protein 1 spliced), Caspase 12 (cysteinyl aspartate specific proteinase 12, located in the endoplasmic reticulum (ER), responsible for ER stress-induced apoptosis) (Nakagawa et al., 2000), CHOP (C/EBP homologous protein) (Hu et al., 2018) in both hippocampus and cortex by western blot. Encouragingly, as indicated by the reduction in the concentration of BIP ($P < 0.05$) Fig. 7c, (p-PERK)/PERK Fig. 7d ($P < 0.05$), p-IRE1/IRE1 ($P > 0.05$) Fig. 7e, XBP1s Fig. 7f ($P < 0.05$), Caspase12 Fig. 7g ($P < 0.05$), CHOP Fig. 7h (hippocampus, $P < 0.05$) in the brains of D-galactose-induced rats, Osthole was effective in relieving ER stress.

Discussion

Alzheimer's disease is a progressive disease with cognitive dysfunction and memory loss, and this disease progresses and leads to neuronal loss, changes in glial cells and dementia. Characterized by extracellular amyloid-beta (Aβ) deposits and intracellular neurofibrillary tangles (NFT) composed of highly phosphorylated tau proteins, AD is a type of disease with complex pathophysiological processes. D-galactose is an aldohexose, a reducing sugar that occurs naturally in the body and in many foods (Acosta & Gross, 1995). Evidence is accumulating that chronic systemic administration of D-galactose in animals may induce brain aging similar to human brain ageing in many ways, including
cognitive deficits, mitochondrial dysfunction, neuronal degeneration and apoptosis (Banji et al., 2014; Cao et al., 2019). Currently, it is a well-recognized model for studying AD-related memory impairment.

Our study found that Osthole, a natural coumarin could alleviate memory and synaptic dysfunction induced by D-galactose in SD rats. Endophenotype network-based approach was utilized that assembled AD-related endophenotype modules and target information of Osthole in human protein interactome to comprehensively identify the underlying neuroprotective mechanism of Osthole against AD. We then applied network analysis, including network proximity analysis and integrated pathway analysis, to decipher the potential MOAs of Osthole. Our prediction results by the network distance method indicate that Osthole could regulate multiple signaling pathways to against AD, mainly related to the regulation of neuronal apoptosis, neuroinflammation and ER stress.

According to the guidance of the network-based prediction, further in vivo experiments were conducted to explore the neuroprotective pharmacological mechanism of Osthole. We discovered that Osthole inhibited D-galactose-induced neuronal loss with the Nissl staining. Bcl-2 family is one of the most essential factors in regulating apoptosis, while Bax gene is a Bcl-2 family pro-apoptotic members. The ratio of Bax to Bcl-2 determines the direction of apoptosis (Chi et al., 2018; Moujalled et al., 2021). In our study, the ratio of Bax to Bcl-2 increased significantly in the D-galactose group. However, Osthole reduced this ratio, suggesting that Osthole's neuroprotective effect is related to its interaction with Bcl-2 and Bax.

Microglia is macrophages of the central nervous system and play an important role in neuroinflammation (Vandenbark et al., 2021). In pathological conditions, activated microglia produce many inflammatory cytokines or act directly on nervous tissues to aggravate inflammatory reactions, reactive astrocyte (Pereira et al., 2019; Young et al., 2021). TNF-α is a component of neuropathological changes, one of the up-regulated immune genes in AD. Chronic exposure to inflammatory stimulation of macrophages can induce neurotoxic progression and induce neurotoxic astrocyte (Pereira et al., 2019). In addition, reactive microglia stimulate tau pathology in a cell-autonomous manner. And TNF-α1 belongs to the superfamily of tumor necrosis factors and is one of the most important TNF-alpha receptors. The results of immunofluorescence and immunoblotting of Iba1, GFAP and TNF-α1 suggested that Osthole could down-regulate D-galactose-induced inflammatory response.

Moreover, our endophenotype network approach showed that Osthole (drug target network) had significant network proximity with ER stress-related endophenotype modules. On the other hand, excessive accumulation of Aβ and Tau protein can lead to abnormal ER, which in turn promotes the formation of AD (Frakes & Dillin, 2017). Molecular chaperone BIP regulates protein homeostasis in the ER and ensure protein quality control. BIP synthesis is increased when protein folding in the ER membrane is disturbed. Subsequently, BIP binds to the misfolded protein, preventing it from forming a polymer and helping it properly refold (Hetz & Papa, 2018). Phosphorylation of PERK and its downstream eukaryotic translation initiation factor 2α subunit (eIF2α) directly inhibit protein synthesis after ER stress activation, reducing ER load and maintaining intracellular environmental balance (Hughes & Mallucci, 2019). IRE1α can cleaves many ER-targeted mRNAs through the intermingling of the regulated IRE1-dependent decay
(RIDD) pathway (Bae et al., 2019; Li et al., 2020). XBP1 mRNA, as an IRE1α mediated irregular splicing fragment, is translated into a functional transcription factor XBP1s, which activates a series of gene transcription after entering the nucleus (Song et al., 2018). CHOP is an Endoplasmic reticulum stress-specific proapoptotic factor, widely expressed at low levels in various normal cells (Hu et al., 2018). Caspase12 is a predeath protease located on the surface of the ER membrane and is responsible for ER stress-induced apoptosis (Nakagawa et al., 2000). In this study, Osthole down-regulated BIP, p-PERK /PERK, XBP1s, CHOP, and Capsase12 induced by D-galactose. Thus, the anti-AD mechanism may be connected to the regulation of ER stress-induced apoptosis.

Collectively, according to the network-based prediction and in vivo experiments, we found that Osthole might improve cognitive and memory disorders by modulating neuronal apoptosis, neuroinflammation and ER stress Fig. 1c. Indeed, these mechanisms are intrinsically connected. The UPR, a signaling response triggered by ER stress, that diminishes the synthesis of the wrong protein. But Chronic ER stress not only results in neuronal loss but also trigger inflammation. Composition of neuroinflammation that AD participates in has been recorded for several decades. In addition, there is a feedback loop between neuroinflammation and ER stress, and both can lead to neuronal apoptosis. This evidence suggests that ER stress, neuroinflammation and neuronal apoptosis are closely associated with the pathological process, and probably the multiple modulated functional modules of Osthole against AD.

**Conclusion**

In the combination of endophenotype network-based prediction and in vivo validation, this study demonstrated that Osthole could improve D-galactose induced cognitive and memory dysfunction in rats via affecting neuroinflammation, ER stress and apoptosis, which could serve as a promising candidate for AD therapeutic.

**Abbreviations**

AD, Alzheimer's disease; D-Gal, D-galactose; ER stress, Endoplasmic reticulum stress; NORT, New object recognition test; WMW, The Morris water maze test; DTIs, Drug-target interactions; HIT, Herbal Ingredient Target Database; GO, Gene ontology; PPIs, Protein-protein interactions; MOAs, Mechanisms of action; TCM, Traditional Chinese medicines; SEM, Standard error of the mean; PSD95 postsynaptic density protein 95; Syp, Synaptophysin; UPR, Unfolded protein response; RIDD, Regulated IRE1-dependent decay; P-PERK, phosphorylate PERK; PERK, PKR-like ER; IRE-1α, inositol-requiring enzyme; P- IRE-1α, phosphorylate IRE-1α; BIP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; XBP1s, X-box binding protein 1 spliced; GFAP, glial brillary acidic protein; TNFα1, Tumor necrosis factor receptor 1; Iba-1, ionized calcium binding adapter molecule 1; Bax, BCL2-Associated X; Bcl-2, B-cell lymphoma-2; ACTB, β-actin; PVDF, polyvinylide fluoride sheets; ECL, enhanced chemiluminescence reagent.

**Declarations**
Availability of data and materials

Data availability

All data of the present study are available from the corresponding author upon reasonable requests.

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All authors contributed to the study conception and design. Jiansong Fang and Shuhuan Fang designed the study. Xiaomei Fu, Xue Wang, Yiyi Lai, and Yanfang Liao performed the experiments and analyzed the data. Xiaomei Fu wrote the manuscript. Chuipu Cai, Zhao Dai and Huilin Xu reviewed the final manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

All animal care and experimental procedures were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (No. 202106010001) and conducted in accordance with the
principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Human ethics**

Not applicable.

**Competing interests**

The authors declare no competing interests.

**Reference**


Figures
Figure 1

Endophenotype network-based mechanism exploration framework for Osthole versus AD

(a) The anti-AD effects of Osthole in enhancing spatial learning and memory and modulating ER stress in D-galactose-induced rats by biochemical analysis and behavioral test. (b) In silico identification of anti-
AD mechanisms for osthole by network proximity prediction and drug-target network analysis. (c) Experimental validation the findings of network-based prediction.

Figure 2

Osthole ameliorate D-galactose-induced memory and spatial cognition in rats

(a) representative trajectories; (b) escape latency in seconds to reach the hidden platform during training (5 days) along with; (c) the time spent in the target quadrant; (d) numbers of target crossing; (e) Object preference ratio; (for behavioral studies, N=8; (c), (d) and (e) anlyised by ANOVA with Dunnetts multiple comparisons test; (b) multivariate ANOVA with Bonferroni). Expression of PSD95 and Syp in brains of rats by Western blotting. (ANOVA with Dunnetts multiple comparisons test, N=4). *P<0.05, **P<0.01 vs Controls. *P< 0.05, **P< 0.01, ***P< 0.01 vs D-galactose-treated rats.
Figure 3

A global view of 25 AD endophenotype modules identified and the correlation among the Osthole-target network by network proximity

AD endophenotype modules with a $P$ value < 0.05 and $Z < -1.8$ were regarded as significant (highlighted in red font).
Figure 4

AD-integrated pathway and functional modules

The pathway integration was based on the experimentally validated targets of Osthole and relative pathways in KEGG database.
Figure 5

Osthole decrease neuron apoptosis induced by D-galactose

(a) Nissl staining images of hippocampal CA1 and CA3 regions are shown, number of experiments = 3. Scale bar: 100μm. Effect of Osthole and Donepezil on Bax, Bcl-2 level in cortex (d, e) and hippocampus (b, c) of rats with or without D-galactose. (Data represent mean values ± SEM and were analysed by
Dunnet's test after one-way analysis of variance, $N=4$). $### P<0.01$ vs Controls. $** P< 0.01$, $*** P< 0.001$ vs D-galactose-treated rats.

**Figure 6**

Osthole inhibited D-galactose-induced activation of neuroinflammation
The immunofluorescence images represent the immunoreactivity of Iba-1 in cortex and hippocampus (CA-3), N = 3. Scale bar: 200μm. Western blot analysis of GFAP, TNF-α in cortex (d, e) and hippocampus (b, c) from D-galactose-induced rats. (Data represent mean values ± SEM and were analysed by Dunnet's test after one-way analysis of variance, N=4). *P<0.05, **P<0.01 vs Controls. *P< 0.05, **P< 0.01 vs D-galactose-treated rats.
Osthole relieves D-galactose-induced endoplasmic reticulum stress

Western blotting detection of endoplasmic reticulum stress-related proteins (BIP, p-LERK/PERK, p-IRE1α/IRE1α, XBP1s, Caspase12, CHOP) Fig.7 (a-h). (ANOVA with Dunnett’s multiple comparisons test, N=4). *P<0.05, **P<0.01, ###P<0.001 vs Controls. *P< 0.05, **P< 0.01, ***P< 0.01 vs D-galactose-treated rats.

Supplementary Files

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- TableS1.xlsx
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