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" *Astaxanthin Mitigates Neurocognitive Deficits in D-Galactose-Induced Brain Aging: Targeting HMGB1/TLR4 / NF-κB signaling pathway*"

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ABSTRACT:

Introduction: D-galactose (D-gal) caused brain aging and cognitive decline. Astaxanthin (ASX) has anti-inflammatory and antioxidant impacts.

Objective: to demonstrate the underlying mechanisms and the neuroprotective impact of ASX in D-gal-induced cognitive impairments

Material & methods: Thirty male Wister albino rats were split into three groups: control, D-gal, and D-gal+ASX. Rats were subjected to the NOR and EPM tests, and after they were killed, the hippocampus gene expression of HMGB1, TLR4, NF-kB, and BDNF was evaluated, along with measurements of MDA, SOD, TNF-α, and IL-6. GFAP and NF-kB hippocampus immunoreaction were performed.

Results: In contrast to the control group, D-gal had higher hippocampal GFAP and NF-kB immunoreaction and a significantly lower discrimination index of the NOR test, time in the open arms of EPM, SOD, and gene expression of BDNF, D-gal also demonstrated cognitive impairment with significantly higher MDA, TNF-α, IL-6, and hippocampal gene expression of HMGB1, TLR4, and NF-kB. D-gal-induced brain alterations were significantly ameliorated by ASX.

Conclusion: By down-regulating the HMGB1/TLR4/NF-kB signaling cascade, improving gliosis, and utilizing neurotrophic, antioxidant, and anti-inflammatory pathways, ASX mitigated D-gal-induced cognitive impairments.

Key words: Astaxanthin, Aging, BDNF, Cognitive impairment, D-galactose, HMGB1, NF-kB, TLR4.

Introduction

Numerous cellular impacts, such as elevated oxidative stress, modifications to cellular metabolism, and damage to nucleic acids, are part of the intricate physiological process of aging. Because of its high oxygen demand, brain tissue is especially vulnerable to age-related redox homeostasis-related degeneration. Anxiety, diminished cognitive performance, and different metabolic and signaling system dysfunctions are part of the behavioral abnormalities that result from such brain degradation **(Sadigh-Eteghad et al., 2017)**.

It is commonly known that oxidative stress, inflammation, and apoptosis are all part of the pathophysiology of aging. The most often used model is D-galactose-induced brain aging (D-gal) **(Younis et al., 2024)**.

The D-gal-induced brain aging model was created recently and has been extensively applied to gain a better understanding of how aging occurs. Its short research period, low side effects, convenience of use, and higher animal survival rates during the experimental period are the reasons for this **(Liu et al., 2020)**.

Aldose and hydrogen peroxide are produced during the breakdown of D-gal. These substances act as potent catalysts for the production of free radicals from oxygen, causing oxidative stress and its associated symptoms, which include protein loss, mitochondrial dysfunction, hippocampal neuronal damage, and cognitive deficit **(Gok et al., 2015; Atef et al., 2022).**

Excess ROS are created when oxidative stress triggers inflammatory signaling pathways, resulting in apoptosis and cellular dysfunction. ROS primarily targets mitochondria. The subsequent generation of ROS causes mitochondrial dysfunction and cell death, which speeds up aging-related changes **(Ma et al., 2024)**.

One of the main chemokines generated by inflammatory cells is HMGB 1, which stimulates the release of cytokines by binding to receptors for AGEs. TLR-4 is the first HMGB1 receptor discovered. This results in the activation and translocation of NF-κB into the nucleus **(Wu et al., 2016; Awad et al., 2024)**

TLR4 plays a critical role in regulating inflammation. NF-κB, the main target gene of TLR4, is responsible for the transcriptional production of inflammatory factors. Since NF-κB signaling is the main cause of pathogenesis in many disease problems, it is one of the principal targets for therapeutic intervention **(Khodir, Bayomy, et al., 2025)**.

NF-κB is found in almost all cell types and has a role in immunology, differentiation, apoptosis, and inflammation. TLR4 stimulates the release of cytokines by activating NF-κB **(Negm et al., 2025)**.

BDNF is essential for synaptic transmission, neuronal growth, and neuronal plasticity. BDNF promotes the survival and growth of cholinergic neurons, which aids in learning and memory. People's BDNF levels sharply decline with age, which may cause cognitive issues **(Khodir et al., 2025)**.

Astaxanthin (ASX), a xanthophyll carotenoid found in salmon, crabs, and algae such as Haematococcus pluvialis, has been suggested to offer protection against oxidative stress. ASX is safe and has no side effects when taken with meals. It builds up in rats, is fat soluble, and doesn't seem to have any negative effects **(Zhuge et al., 2021)**. Furthermore, ASX protects patients from apoptosis, inflammation, and oxidative stress **(Hong et al., 2024)**.

Because ASX can lessen oxidative stress, it has been employed in numerous animal models and has demonstrated anti-aging effects **(Liu et al., 2018)**. In this study, the neuro-protective effect of ASX in D-gal-induced cognitive impairments was

demonstrated, along with the underlying mechanisms with referral to HMGB1/TLR4/NF-κB pathway.

Materials and methods

Animals. Thirty Wister albino male rats weighing between 200 and 250 grams were used after approved from the Faculty of Medicine's Research Ethical Committee at Menoufia University in Egypt (registration No. 8/2025BIO15). The rats were housed in 80 x 40 x 30 cm wire-mesh cages. Following two weeks of constant environmental conditions, all animals were granted unrestricted access to food and water during the study period.

Experimental design. (10/group):

1. **Control group**: For ten consecutive weeks, rats were given a daily oral gavage of 2 mL olive oil and a single daily SC injection of 1ml isotonic saline for 10 consecutive weeks.
2. **D-galactose (D-gal) group**: rats received a daily oral gavage of 2 mL olive oil along with SC injection D-gal (Sigma-Aldrich St. Louis, USA) at a dose of 150 mg/kg/day dissolved in isotonic saline once a day for 10 weeks **(Younis et al., 2024)**.
3. **D-galactose + Astaxanthin (D-gal+ASX) group**: Rats received a single daily 25 mg/kg body weight ASX (Sigma, St Louis, MO, USA) orally by gavage dissolved in 2 mL olive oil **(Xue et al., 2017); Baeissa et al., 2024)**, and daily administration of D-gal (150 mg/kg/day) by SC injection daily for 10 weeks.

After ten weeks, a neurobehavioral examination was performed on each rat. After being anesthetized, the rats' cervical vertebrae were dislocated and lengthened to scarify them. The brain was extracted. The left hemisphere was divided in half, with one half being used for biochemical analysis and the other half for RT-PCR study. The right hippocampal tissues was fixed in formalin saline for histological analysis.

Neurobehavioral tests

Novel Object Recognition (NOR): Each rat underwent a three-day testing process that included training, testing, and habituation. During the habituation phase, rats were placed in an open-field apparatus that measured 50 cm by 50 cm by 40 cm. They were allowed to adjust to their environment for 10 minutes. For the first five minutes of

training, we put two identical items in each rat's room. Each rat was kept in the chamber for five minutes following the item exchange, which occurred twenty-four hours later, during the testing phase. We put the stopwatch to work. [= (familial object exploration time–novel object exploration time)/total exploration time×100%] is one way to calculate the discriminating index **(Zou et al., 2017).**

Elevated Plus Maze (EPM) Test: As previously described **(Tchantchou et al., 2018)**, In this experiment, we used an apparatus to have the rats identify a plus sign in order to assess their anxiety-like behavior. Each rat was placed in the middle of the apparatus and allowed ten minutes to explore the labyrinth. The animals' movements were monitored by an overhead security camera. The duration of time spent in the open arms mazes was meticulously documented. The duration and the intensity of anxiety-like behavior were inversely correlated.

Hippocampal homogenate preparation

Each hippocampal specimen was weighted and homogenized, the centrifuged for 14 minutes at 11,000 rpm. The supernatant was stored at -80°C for additional biochemical analysis.

Biochemical Analysis

However, in accordance with the manufacturer's instructions, rat ELISA kits (TNF-α: ERT2010 1, Assaypro LLC, Saint Charles, Missouri, USA, IL-6: ab100772, Abcam, Cambridge, UK) were utilized to assess the quantities of IL-6 and TNF-α in the hippocampus. Hippocampal MDA and SOD were measured using colorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt) in accordance with the manufacturer's instructions.

Quantitative assay of hippocampal HMGB1, TLR4, NF-κB and BDNF genes expression using RT‑PCR.

The QiagenRN easy plus Universal Kit from the USA was used to prepare hippocampal samples for total RNA isolation. The quality and purity of the RNA were then assured. Until it was required, RNA was stored at -80 °C. The first step therefore was to create cDNA in a single cycle on an Applied Biosystems 2720 heat cycler in Singapore using the QuantiTect Reverse Transcription Kit, which is produced by Qiagen in the USA. In RT-PCR processes, GAPDH primers were used as an RNA loading control. cDNA

amplification was the second step. SensiFASTTMSYBR Lo-ROX Kit, USA, used cDNA in SYBR green-based quantitative real-time PCR for Relative Quantification (RQ) of HMGB1, TLR4, NF κB, and BDNF gene expression using the following primers (Midland, Texas): the forward primer for HMGB 1 was (TGAGGGACAAAAGCCACTC), and the reverse primer was (TTGGGAGGGCGGAGAATC), The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC). The reverse primer was (GAGCAGTCATGTCCTTGGGT). The forward primer for TLR4 was (TCAGCTTTGGTCAGTTGGCT), and the reverse was (GTCCTTGACCCACTGCAAGA)

The forward primer for BDNF was GCTGCCTTGATGTTTACTTTG and reverse ATGGGATTACACTTGGTCTCGT.

Finally, the 2.0.1 version of the Applied Biosystems 7500 software was used to finish the data analysis. The RQ of HMGB1, TLR4, NF κB, and BDNF gene expression was conducted using a comparative ΔΔCt technique, which normalizes the amount of target gene (HMGB1, TLR4, NF-κB & BDNF) mRNA to an endogenous reference gene (GAPDH) and compares it to a control. **(Khodir, Bayomy, et al., 2025)**

Histological study:

at get at the hippocampal location, each cerebral hemisphere was divided coronally into two sections, which were subsequently preserved in 10% formalin. After being cleansed and dehydrated, the samples were embedded in paraffin blocks. For routine histological analysis, serial coronal slices were stained with Hx. & E.

Immunohistochemical studies:

Blocks of paraffin were deparaffinized and then rehydrated using decreasing alcohol grades. Following the use of 3% H2O2 in methanol to inhibit endogenous peroxidase activity and the use of a protein blocker to block nonspecific binding sites, the primary antibodies against GFAP [1:300, rat monoclonal, Lab vision MS-1376-R7] and NF-kB (monoclonal, dilution 1:200, Abcam) were added and incubated overnight. A 2% concentration of biotinylated goat polyvalent secondary antibody was added for 10 minutes, and then the avidin-biotin-peroxidase complex was added.

Hippocampal morphometric assessment

In H&E-stained slices, the number of pyramidal cells was counted using three non-overlapping fields per section. For quantitative immunohistochemical analysis, the area percentage of GFAP and NF-kB immunopositive cells was calculated using three non-overlapping fields/sections.

Statistical analysis

SPSS version 23 was used to process the data (SPSS Inc., USA). To make sure all of the data sets were normally distributed, the Shapiro-Wilk test was used. The mean ± SD was used to express the data. A post-hoc Tukey test and a one-way ANOVA were used to determine the significance of group differences. For statistical significance, P values below 0.05 were used.

Results

While the discrimination index of the NOR test, time in the open arms of EPM, hippocampal SOD, and hippocampal gene expression of BDNF values of the D-gal were significantly lower than those of the control, the D-gal group's hippocampal MDA, hippocampal TNF-α, hippocampal IL-6, and hippocampal gene expression of HMGB1, TLR4, and NF κB were substantially higher than those of the control. The D-gal +ASX group's hippocampal MDA, hippocampal TNF-α, hippocampal IL-6, and hippocampal gene expression of HMGB1, TLR4, and NF κB were substantially lower than D-gal but still substantially higher than control; in contrast, the discrimination index of the NOR test, time in the open arms of EPM, hippocampal SOD, and hippocampal gene expression BDNF values were substantially higher than D-gal. (**Table 1)**

Table (1): The measured discrimination index of NOR test, time in the open arms of EPM, hippocampal MDA, SOD, TNF-α, IL-6, hippocampal HMGB1 gene expression, hippocampal TLR4 gene expression, hippocampal NF-κB gene expression and hippocampal BDNF gene expression in all studied groups

	Control group	D-gal group	D-gal +ASX group
Discrimination index of NOR Test	70.8±2.33	20.9±2.16 *	**51.7±3.14*#**
Time in the open arms of EPM	100.1±3.9	52.8± 5.47*	**74.8± 3.5*#**
Hippocampal MDA (nmol/ gm. Tissue)	7.8 ±0.99	24.56± 2.22*	**14.8± 1.62*#**
Hippocampal SOD (U/gm. Tissue)	9.87±0.98	3.78±0.72*	**7.22±0.81*#**
Hippocampal TNF-α (ng/ml)	19.5±0.81	56.8±7.59*	**37.8±5.6*#**
Hippocampal IL-6 (pg/mL)	101.6±7.23	205.9±8.97*	**151.5±3.99*#**
Hippocampal HMGB1 gene expression	1	3.32±0.07*	**2.33±0.05*#**
Hippocampal TLR4 gene expression	1	2.99±0.04*	**1.98±0.07*#**
Hippocampal NF-κB gene expression	1	2.66±0.01*	**1.87±0.03*#**
Hippocampal BDNF gene expression	1	0.34±0.06*	**0.66±0.03*#**

* Significant compared with control, # Significant compared with D-gal

Histological results in H&E-stained sections: The hippocampus of the control group was formed of normal histological pictures of the three layers. In the D-gal, the hippocampus showed a loss of hippocampal tissue integrity. The hippocampus showed a marked decrease in the number of cells of the pyramidal layer. The D-gal+ ASX, there was an apparent increase in the number of cells of the pyramidal layer. Many pyramidal cells appeared normal with basophilic cytoplasm. **(Fig. 1 A-C)**

In D-gal group, there were substantially fewer pyramidal cells ($p<0.05$) than in the control (30.42±0.52 vs. 87.76±0.76). Though it was still lower than the control, the D-gal+ ASX demonstrated a substantial increase ($p<0.05$) in comparison to the D-gal (57.33±0.33 vs. 30.42±0.52). **(Fig. 1 D)**

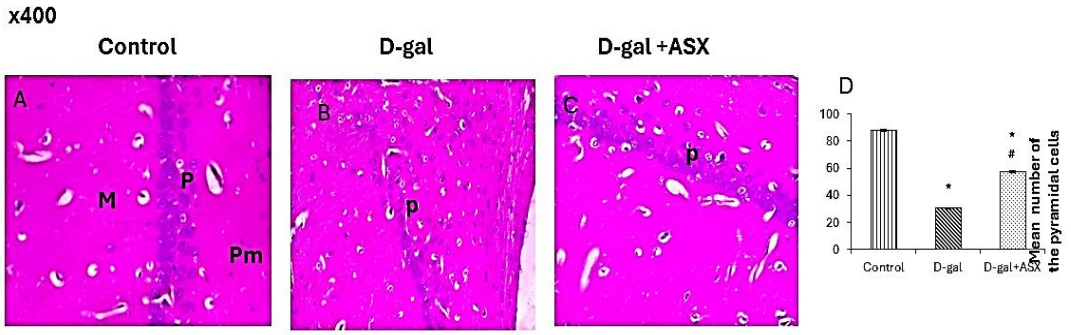

Fig. (1): photomicrographs of the hippocampus of the different groups showing: (A) The hippocampus of the control group was formed of normal histological pictures of the three layers; molecular (M), pyramidal (P) and polymorphic (Pm) layers. (B) In the D-gal group, the hippocampus showed a loss of hippocampal tissue integrity. The hippocampus showed a marked decrease in the number of cells of the pyramidal layer (P). The D-gal+ ASX, there was an apparent increase in the number of cells of the pyramidal layer (P). Many pyramidal cells appeared normal with basophilic cytoplasm, large vesicular nuclei and prominent nucleoli. (X400).

Immunohistochemical results:

The number of astrocytes in GFAP-stained sections was significantly higher ($p<0.05$) in the D-gal group than in the control group (83.5±0.54 vs. 5.7 ±0.6). Astrocytes were significantly lower in the D-gal+ ASX group than in the D-gal group (19.4±0.6 vs. 83.5±0.54), but they were still higher than in the control group. (Fig. (A-D)).

The D-gal 's percentage of NF-kB stain in NF-kB-stained sections was dramatically higher ($p<0.05$) than control (81.6±0.33 vs. 6±0.22). The D-gal+ ASX had a dramatically drop in the percentage of NF-kB stain when compared to the D-gal (30.1±.65 vs. 81.6±0.33). (Fig. (E-H))

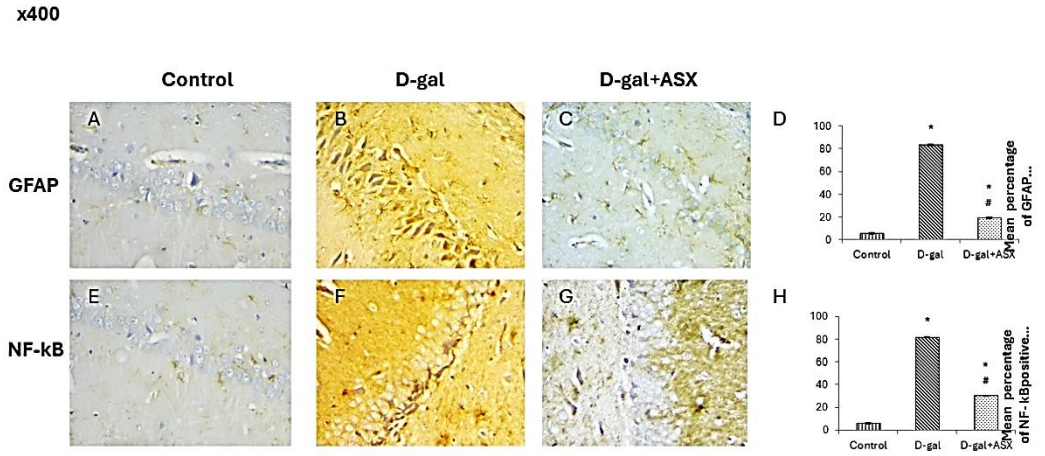

Fig. (2): Representative micrographs showing a substantially upregulation of the GFAP (A-D) and NF-kB (E-H) immunoreaction in the D-gal and a dramatically downregulation in the D-gal+ ASX group.

Discussion

A number of clinical conditions, including anxiety and memory loss, are associated with aging. Animals' brains can age in a manner remarkably similar to human brain aging when they are given D-gal for six to ten weeks (Younis et al., 2024). According to the interpretation of the EPM and NOR tests, our results showed clear cognitive impairments between the D-gal and the control. This is consistent with Younis et al.'s (2024) findings. When D-gal reacts with free amines in amino acids, it creates AGES, which cause aging-like symptoms **(Younis et al., 2024; El-Far et al., 2024)**.

In line with earlier research, ASX supplementation reduced the cognitive deficits caused by D-gal in the D-gal+ASX group **(Liu et al., 2020)**. **Ji et al., (2017)** demonstrated the value of astaxanthin in preventing inflammation and oxidation in a variety of illness models.

Initially, oxidative stress brought on by mitochondrial damage, or increased production of ROS, is a major factor in the accelerated aging process. Therefore, the fundamental strategy to avoid age-related illnesses was to use mitochondria-targeted rechargeable antioxidants to suppress the formation of ROS **(Ni et al., 2018)**.

The activity of antioxidant enzymes in brain tissue is intimately linked to aging. The most significant antioxidant enzyme is SOD. MDA is known to be the most significant byproduct of membrane lipid peroxidation, which indirectly reduces levels of oxidative damage in cells, as well as a signal of aging **(Liu et al., 2020)**.

OS has a major impact on aging, as evidenced by our study's greater MDA level and decreased D-gal SOD in comparison to the control. These results align with the findings of **Atef et al., (2022)**. By raising galactitol synthesis and aldose reductase activity, D-gal damages cells oxidatively by generating AGEs, which in turn generates too many ROS and reduces antioxidant capacity. ROS causes oxidative damage to DNA inside cells, which results in apoptosis and cellular dysfunction **Shwe et al., (2018)**.

ASX considerably improved oxidative stress indicators in D-gal+ASX, which is consistent with earlier studies **(Ni et al., 2018)**. The ability of ASX to neutralize free radicals is high **(Fassett & Coombes, 2011)**. Additionally, ASX enables the molecule to remove free radicals from the cell's inner **(Kidd, 2011)**. By boosting the expression of its antioxidant genes and activating Nrf2, ASX can have an indirect antioxidant impact **(Cui et al., 2020)**.

Chronic inflammation was also observed in the aging brain. As people age, their microglia may become more susceptible to inflammatory stimuli, and dementia is brought on by elevated levels of inflammatory cytokines **(Corlier et al., 2018)**. ROS can trigger inflammatory reactions by triggering the activation of NF-kB, which promotes the release of TNF-α and IL-6, among other inflammatory mediators. Moreover, chronic inflammation most likely started a vicious cycle of rising ROS production **(Khodir et al., 2025)**.

This explains the d-gal-induced inflammatory response, which is in line with Atef et al., 2022 and is evidenced by the substantial increase in TNF-α and IL-6 as well as the significant up-regulation of NF-kB gene expression in the D-gal group's hippocampus regions **(Atef et al., 2022)**.

The inflammatory state caused by D-gal was significantly reduced by ASX. ASX has a potent anti-inflammatory impact, which may be linked to its antioxidant action, and it helps to improve organ function through physiological changes **(Visioli & Artaria, 2017)**. By inhibiting NF-KB, ASX has been demonstrated to have anti-inflammatory effects **(Chen et al., 2020)**. Prior research has connected ASX's medicinal uses to its function in suppressing the expression of inflammatory genes and cytokine production **(Priyadarshini & Aggarwal, 2018)**.

BDNF is a crucial neurotrophic factor that controls a number of brain functions. Furthermore, by managing the NF-kB signaling cascade, BDNF controls the

inflammatory state. The relationship between neuroinflammation and cognitive decline is thus significantly mediated by BDNF **(Atef et al., 2022)**. The current investigation showed that D-gal dramatically decreased the amount of BDNF, which are consistent with earlier research by **Atef et al., (2022)**.

Following treatment with ASX, BDNF was significantly up-regulated in comparison to the D-gal group. This is consistent with a prior study that found that ASX reduced brain aging by restoring BDNF levels in rats' brains and hippocampal regions **(Wu et al., 2014)**. In addition ASX slows brain aging by increasing levels of BDNF in the rat hippocampus **(Wu et al., 2014).**

By attaching itself to TLR-4, HMGB 1 promotes the release of cytokines. NF-κB activity was linked to TLR4 signaling activation, which increased the production of pro-inflammatory cytokines, leading to leukocyte buildup and localized inflammation. NF-κB functions is a TLR4 signaling pathway downstream effector **(Khodir et al., 2025)**. In line with earlier research, D-gal significantly increased HMGB1/TLR4/NF-kB hippocampus gene expression and NF-kB hippocampal immunoreaction when compared to control **(Atef et al., 2022)**.

By downregulating hippocampus HMGB1/TLR4/NF-kB and NF-kB immunoreaction in comparison to D-gal, our findings provides evidence that ASX can improve cognitive deficits in an aging model caused by D-gal, which is consistent with earlier research **(Khodir et al., 2024**).

In the hippocampal CA1, D-gal caused chaotic pyramidal cell loss. Previous research showing that D-gal produced neurodegenerative changes supported our histopathological findings **(Samad et al., 2022)**. ASX dramatically improved these changes.

Rat astrocytes are implicated in D-gal-induced brain aging, according to numerous studies. Astrocytes may become activated as a result of brain damage. Overactive astrocytes can impede normal neuronal functioning and result in cognitive impairment by exacerbating oxidative stress, decreasing the number of neurons, blocking synaptic transmission, and releasing neurotoxic or cytotoxic chemicals **(Liu et al., 2020)**. According to our findings, D-gal increased the quantity and activity of GFAP-positive astrocytes in the rat hippocampal region, which is consistent with a prior study by **Kou et al., (2016)**.

In the hippocampus of the D-gal+ASX, we found that ASX dramatically reduced GFAP immunoreactivity, improving gliosis. This finding may be related to ASX's anti-inflammatory effect and is consistent with other research **(Liu et al., 2020)**.

Conclusion

By down-regulating the HMGB1/TLR4/NF-kB signaling cascade, improving gliosis, and utilizing neurotrophic, antioxidant, and anti-inflammatory pathways, ASX mitigated D-gal-induced cognitive impairments. ASX is a viable target for altering the aging process because of these effects.

Conflicts of interest

The authors declare that they have no conflict of interest

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