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Naringin Attenuates D-galactose-Induced Brain Aging Via Regulation Of Oxidative Stress Markers TNF-α, NF-κβ And Modulation Of The Neurotrophic Markers PGC1-α, NT-3 AGEs, And GFAP *In Vivo*

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Abstract

Aging is a global issue that we all deal with on a daily basis. The level of reliance among elderly persons everywhere increases as a result of brain aging and cognitive dysfunction. Naringin is a natural compound that showed various medicinal applications and is commonly used as an anticancer, antioxidant, and anti-inflammatory properties. The current research evaluated naringin influence on D-galactose (D-gal) stimulated brain aging *in vivo*. Mice were distributed randomly to 4 groups (10 mice/ group): Normal group, D-gal group, Groups 3& 4: Naringin (150&300 mg/kg; orally) concurrent with D-gal for 8 weeks. Behavioural, brain biochemical, and histopathological changes were assessed. Results: Naringin treatment increased rate of discrimination in recognizing of objects, Y Maze percent fluctuation, locomotive activity, and brain levels of AMPK, LKB1, PGC1-α, nerve growth factor (neurotrophin-3; NT-3), dopamine, and serotonin with a significant decrement of brain contents of TNF-α, NF-κβ, AGEs, and GFAP compared to D-gal-treated mice. In addition, naringin ameliorated neuron degeneration. In conclusion, naringin, with its anti-inflammatory effects, has stimulated mitochondrial biogenesis and modulated neurotrophic factors and neurotransmitters regulating nerve regeneration. Yet, naringin could be a promising drug with potential neuroprotective action against brain aging stimulated neurodegeneration.

Keywords: Naringin; Aging; AGEs; GFAP; NT3

1.Introduction

Aging is a complex procedure that causes organ performance to deteriorate gradually, that mostly influences the brain. Brain aging is related to the gradual deficiency of biological performance, cognitive functioning, mental capacity, spatial ability, and memory [1]. Aging of the population is a global phenomenon. The percent of adults 60 or more was 12.3% in 2015 and it is supposed to reach 21.5% of the worldwide population by 2050 [2]. Brain aging could be indicated by measuring stimulated aging indicators like advanced glycation end products (AGE) and Glial fibrillary acidic protein (GFAP). When AGE attaches to its receptor, NADPH oxidase levels increase and oxidative stress occurs, leading to neuronic damage and cognitive malfunction [3]. Moreover, Neurotrophin-3 (NT-3), dopamine, and serotonin are the common decreased aging markers. Neurotrophin-3 (NT-3) levels could increase or decrease in rat's cerebral cortex and cerebellum during normal aging. Neurotrophins (NTs) are essential for maintenance and proper functioning of neural cells. Procedures depending on NTs signalling could impair during aging leading to neurodegenerative disorders [4].

Inflammatory cellular damage is also identified as one of the key signs of aging as ROS may induce nuclear

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factor-κB (NF-κB) transcription factors, which subsequently stimulate the pro-inflammatory cytokines mainly tumour necrosis factor-α (TNF-α) [5,6]. So, the oxidative stress and inflammation are important goals to explore the efficient plans to deal with aging related disorders.

One of the most effective agents regarding aging is the control of energy metabolism due to its significant role in cellular homeostasis. Any troubles in maintaining energy balance induce many diseases and accelerate aging. Effective energy control and normal metabolic homeostasis are potent shield against stress which is an important sign of qualified and extended lifespan. AMP-activated protein kinase (AMPK) pathway is playing an important role in regulating metabolic homeostasis as increased AMPK activity has the ability to extend the lifespan [7,8]. Presently, it is reported that AMPK could be stimulated by Liver Kinase B1 (LKB1). Researchers reported the decline of AMPK during aging. The deactivation of AMPK after oxidative stress led to metabolic dysregulation, stimulated oxidative stress, and declined autophagic clearance. AMPK inhibits the signalling of mTOR, and NF-B. Additionally, dysregulation of NF-B pathway by AMPK represses inflammation [9,10]. Furthermore, Mitochondrial dysfunction has been implicated in the aging process. Normal mitochondrial function is valuable for preventing or delaying aging related disorders. Peroxisome proliferator-activated receptor γ coactivator α (PGC-1α) plays a significant role in regulating mitochondrial biogenesis and is reported to be downregulated during aging [11].

D-galactose (D-gal) is a common and trusted model for aging in vivo research and medication screening taking into account that D-gal could resemble both the morphological and physiological aspects of normal aging process, particularly when combined with brain damage [12]. D-gal is naturally metabolized into glucose. Studies reported that D-gal stimulated apoptosis and inflammation of neural cells in the cerebral cortex and hippocampus along with deteriorated spatial learning and memory. Also, high levels of D-gal could lead to the stimulation of ROS which cause abnormal mitochondrial function specially in brain, more inflammatory responses, and up normal redox homeostasis, leading to neurodegenerative damage [13,14].

Recently, using of natural products for health purposes has grown more appealing throughout the majority of the globe. Naringin is a natural flavonoid found in fruits and vegetables that has many pharmacological properties including antioxidant, anti-inflammatory, anti-hypercholesterolemic, neuroprotective and anticarcinogenic actions [15–17]. But the neuroprotective action of Naringin was not fully studied during brain aging, so it would be interesting to deeply understand naringin effect during brain aging through evaluating changes in different aging-related mechanisms. In order to fulfil this aim, the role of naringin *in vivo* was examined on the D-gal induced aging in mice by different behavioural tests, evaluating oxidative stress and inflammatory markers, measuring brain contents of many aging markers such as AMPK, LKB1, PGC1-α, AGEs, GFAP, neurotransmitters as serotonin and dopamine as well as NT-3.

2.Materials and methods

2.1. Chemicals

D-galactose(D-gal) and naringin were obtained from Sigma Aldrich. (**AMPK**: 5'-Adenosine monophosphate Activated protein Kinase; **LKB1**: Liver Kinase B1; **PGC1-α**: Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; **TNF-α**: Tumour Necrosis Factor-alpha; **NF-κB**: Nuclear Factor Kappa Beta; **AGEs**: Advanced Glycation End Products; **GFAP**: Glial fibrillary acidic protein; **NT-3**: Neurotrophin-3) were obtained from SunLong Biotec Co., LTD, China.

2.2. Animals

40 Male Swiss mice (20-35) g were chosen for the current research. They were kept in clear cages having filter covers in a monitored atmosphere with 12-hour periods of daylight and darkness, 50% humidity at 28˚C. During the trial, mice received a regular pellet meal and were provided with unlimited access to water. The instructions included in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals were followed when conducting the animal research. (NIH No. 85:23 revised 1985).

2.3. Experimental design

The experimental procedures were done according to the method of Liu et al., [18] with slight modifications. Sub-cutaneous (S.c.)injection with D-gal (200 mg /kg/day) continued for 8 weeks till induced aging. The mice were distributed in a random way into 4 groups (10 mice/group): Group 1: Normal group. Group 2: D-gal group, Groups 3& 4: Naringin (150&300 mg/kg; orally) concurrent with D-gal for 8 weeks.

2.3.1. Behavioural tests

2.3.1.1. Object Recognition Test

The following experiment was performed according to Burke et al., [19]. Animals' innate desire to discover unfamiliar items more than they do regular ones is utilized as a measure of stimuli awareness. The experiment includes a model period needed in which an item is faced, after that a test period where the model item is introduced with a novel item. The

discrimination proportion is the rate of tries towards the novel item against the ordinary one. The rise of discrimination ratio means that mice discovered the new item more.

$$\text{Discrimination ratio} = \frac{\text{No. of tries towards the new item.}}{\text{Total no. of tries towards to both items}}$$

2.3.1.2.Y Maze Test

Y maze was carried out in accordance with the study done by Hidaka et al., [20]. An animal must remember which arm it had entered on a previous occasion to enable it to alternate its choice on the following trial. The testing process is conducted in a three-armed (A, B and C), Y-shaped device. The experiment contains 2 stages (training phase and test phase). The animal is free to move through the maze for about 8 mins. After 24hrs,the test was repeated and the animal movement for 8 mins is recorded. Whenever the animal goes through an arm, with all its limbs inside, its letter is recorded. The alternations number is the successful entries into three different arms. The percent of alternation is

$$\frac{\text{Number of alternations}}{(\text{Total arm Entries} - 2)} \times 100$$

2.3.1.3. Locomotor activity test:

It was evaluated by measuring animal movements by the use of grid floor activity cage (Model no. 7430, Ugo-Basile, Italy). Mice were adapted for 1 h before placing the animal in the activity cage (exposure) [21]. Before beginning oral therapy, the mice's activity counts were checked in three consecutive events, each lasting five *min*. This was done to get them accustomed to the device. Then mice were located in the activity cage and the activity counts of the mice were measured for 5 mins [22].

2.3.2. Tissue biochemical analysis

Cervical dislocation was used for mice scarifying. Whole brain from each animal was directly dissected out and washed with PBS, homogenized to get 20 percent homogenate then centrifuged for 5 mins at 5000 x g using a cooling centrifuge and finally kept at –80°C [23].

2.3.2.1. Assessment of brain contents for AMPK, LKB1, PGC1-α, TNF-α, and NF-κB, AGEs, GFAP, and NT-3

The brain contents of several inflammatory and neurotrophic markers (**AMPK**: 5'-Adenosine monophosphate Activated protein Kinase; **LKB1**:Liver Kinase B1; **PGC1-α**:Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; **TNF-α**: Tumour Necrosis Factor-alpha; **NF-κB**: Nuclear Factor Kappa Beta; **AGEs**: Advanced Glycation End Products ; **GFAP**:Glial fibrillary acidic protein; **NT-3**:Neurotrophin-3) were determined using ELISA according to the manufacturer's instructions of SinoGeneClon Biotech Co., Ltd, China ELISA.

2.3.2.2. Real-time PCR quantification of serotonin and dopamine neurotransmitters

Total RNA was extracted from brain tissues by TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

The mRNA expression level of β-actin was also detected as an internal control for each sample. RNA was transcribed using a Prime script RT kit (Takara Bio Inc, China). Housekeeping control gene is GAPDH. RNA was transcribed using Omni script RT kit (Qiagen, Hilden, Germany).

Serotonin Forward, 5' ATGGCTCTGCTGGTGATAA 3'
Reverse, 5' TCAGTCTTGTTGGCTTGG 3'.
Dopamine Forward, 5' CGCGTAGACTCTGAGATTCTGAATT 3'
Reverse, 5' GAGTTAAGGAGCCACCACATCAGT 3'.

2.4. Statistical analysis

One-way ANOVA was used for comparing groups followed by Tukey test for multiple comparisons using GraphPad Prism software, version 5 (Inc., USA). The difference was considered significant when $p < 0.05$, where a: Significantly different from normal control, b: Significantly different from D-gal control and c: No significant difference.

2.5. Histopathological examination:

Histopathology for the brain tissue of all groups were performed. Brain tissues were fixed in 10% formalin and processed for paraffin sections of 4 cm thick, then stained by haematoxylin and eosin (H & E).

3.Results

3.1. Effect of naringin on object recognition, Y Maze, and locomotor activity

Obtained data revealed that D-gal decreased discrimination rate with 30%, Y Maze percent alternation with 56%, and locomotor activity with 37%, in comparison with control (P<0.05). Naringin at 100 and 300 mg/kg improved the decline in the parameters by 19% and 28% for discrimination, 64% and 110% regarding Y Maze, and locomotor activity by 36% and 57%, in comparison with D-gal group (P<0.05). Furthermore, 300 mg/kg of naringin reverted all three parameters to the normal levels (Fig. 1).

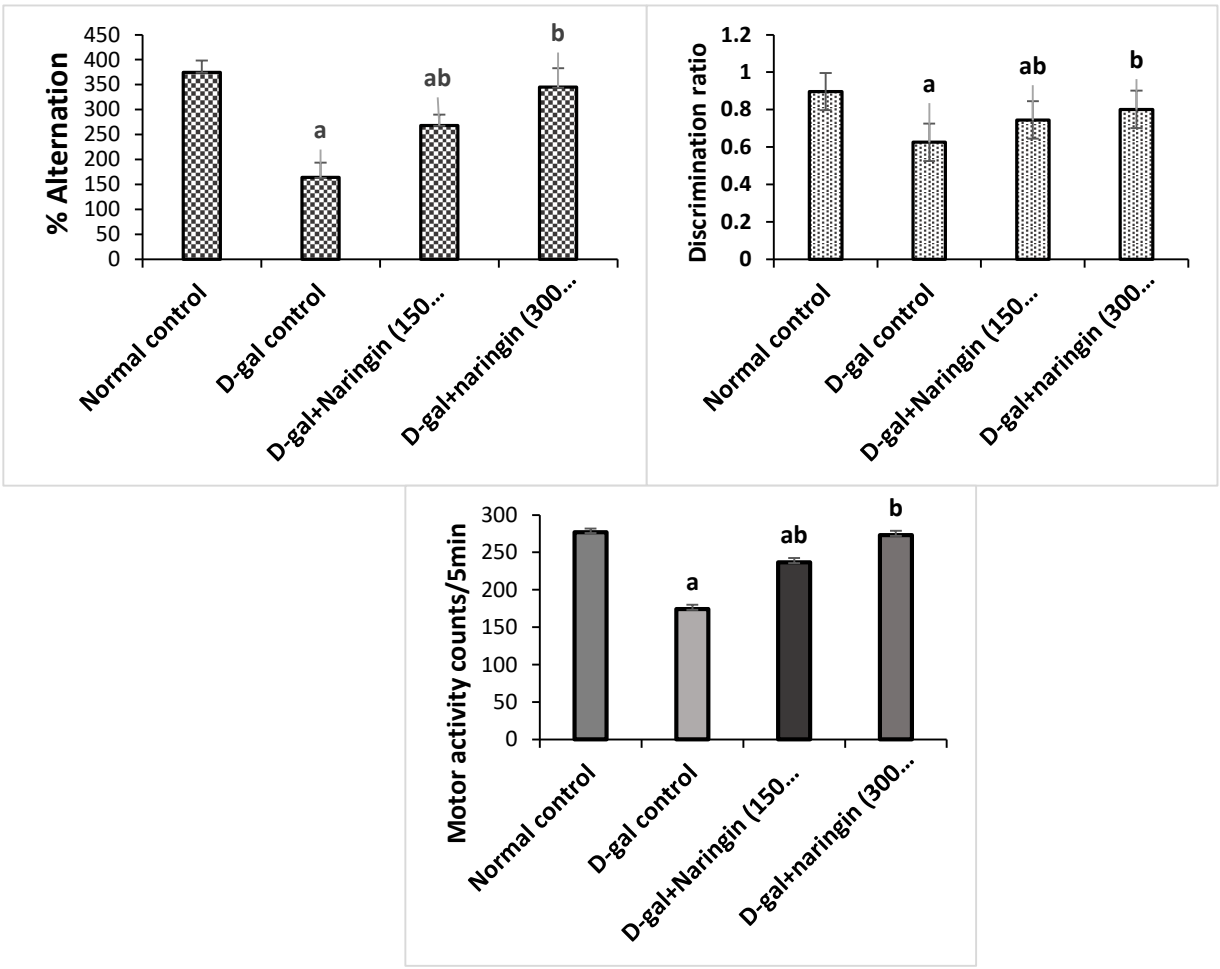


Figure 1: Effect of naringin on percent alternation (Y Maze), Discrimination ratio (object recognition) and motor counts (locomotor activity) in D-gal induced brain aging in rats. Data are represented as mean ± SE (*n* = 6). a: Significant difference at P < 0.05 in comparison with normal control group; b: significant difference at P < 0.05 in comparison with D-gal group.

3.2. Effect of naringin on TNF-α and NF-Kβ levels

TNF-α and NF-Kβ levels in brain were increased in D-gal group by 4 and 7.5 folds respectively, (P<0.05). 150 and 300 mg/kg of naringin dramatically declined TNF-α by 40% and 56%, NF-Kβ by 57% and 83%, (P<0.05) in comparison with D-gal group. While the high dose of naringin retained NF-Kβ only to its normal (Table 1).

Table 1: Effect of naringin on TNF-α and NF-Kβ brain content

	Normal control	D-gal control	D-gal+Naringin (150 mg/kg)	D-gal+naringin (300 mg/kg)
TNF-α (ng/L)	51.30±5.03	262.90±58.32 [a]	157.20±5.44 [ab]	116.20±20.92 [ab]
NF-Kβ (pg/ml)	331.00±50.30	2815.00±147.48 [a]	1207.00±122.30 [ab]	477.00±70.41 [b]

*Data were expressed as mean ± SE.

3.3. Effect of naringin on AGEs, GFAP, and NT-3 brain contents

D-gal elevated both AGEs and GFAP by 1.7-fold but decreased NT-3 by 35% when compared to control (P<0.05). The low dose of naringin decreased AGEs by 20%, GFAP by 27%, with no change in NT-3 if compared to D-gal group. On the other hand, 300 mg/kg of naringin decreased AGEs by 25% and GFAP by 57%, and elevated NT-3 by 40% (P<0.05) if compared with D-gal group (Fig. 2).

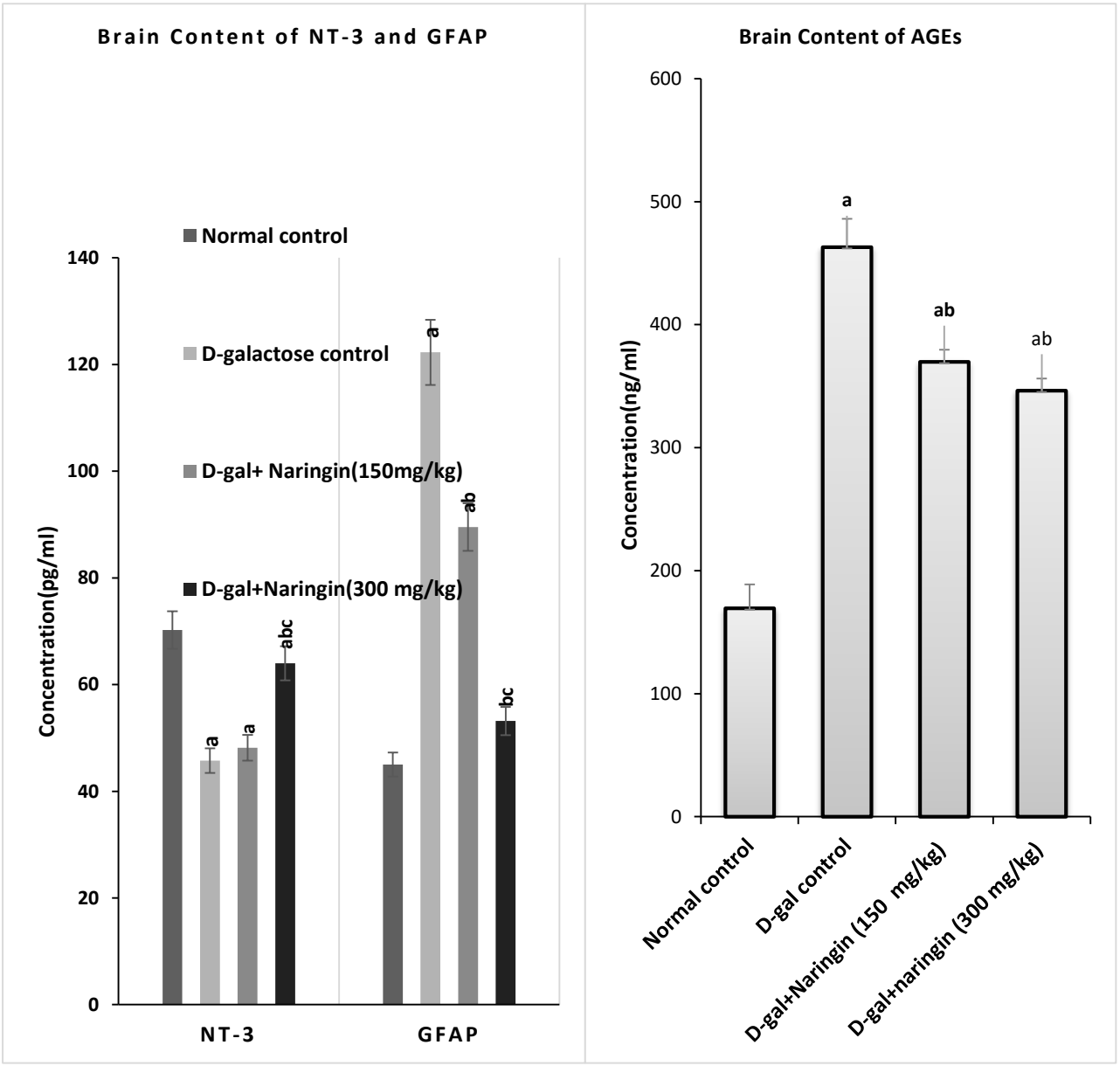


Figure 2: Effect of naringin on AGEs, GFAP, and NT-3 brain content in D-gal-induced brain aging in rats. Data are represented as mean ± SD (n = 6). a: Significant change at P < 0.05 in comparison with normal control group; b: significant change at P < 0.05 in comparison with D-gal group

3.4. Effect of naringin on AMPK/LKB1/ PGC1α brain contents

AMPK, LKB1, and PGC1α protein levels was significantly declined in D-gal group by 66%, 72%, and 71%, respectively (P<0.05). The administration of low dose naringin did not improve brain contents of AMPK while increased LKB1 by 90% and PGC1α by 51% (P<0.05). The high dose of naringin upregulated

protein levels of AMPK by 47%, LKB1 by 141%, and PGC1α by 122%, respectively (Fig. 3).

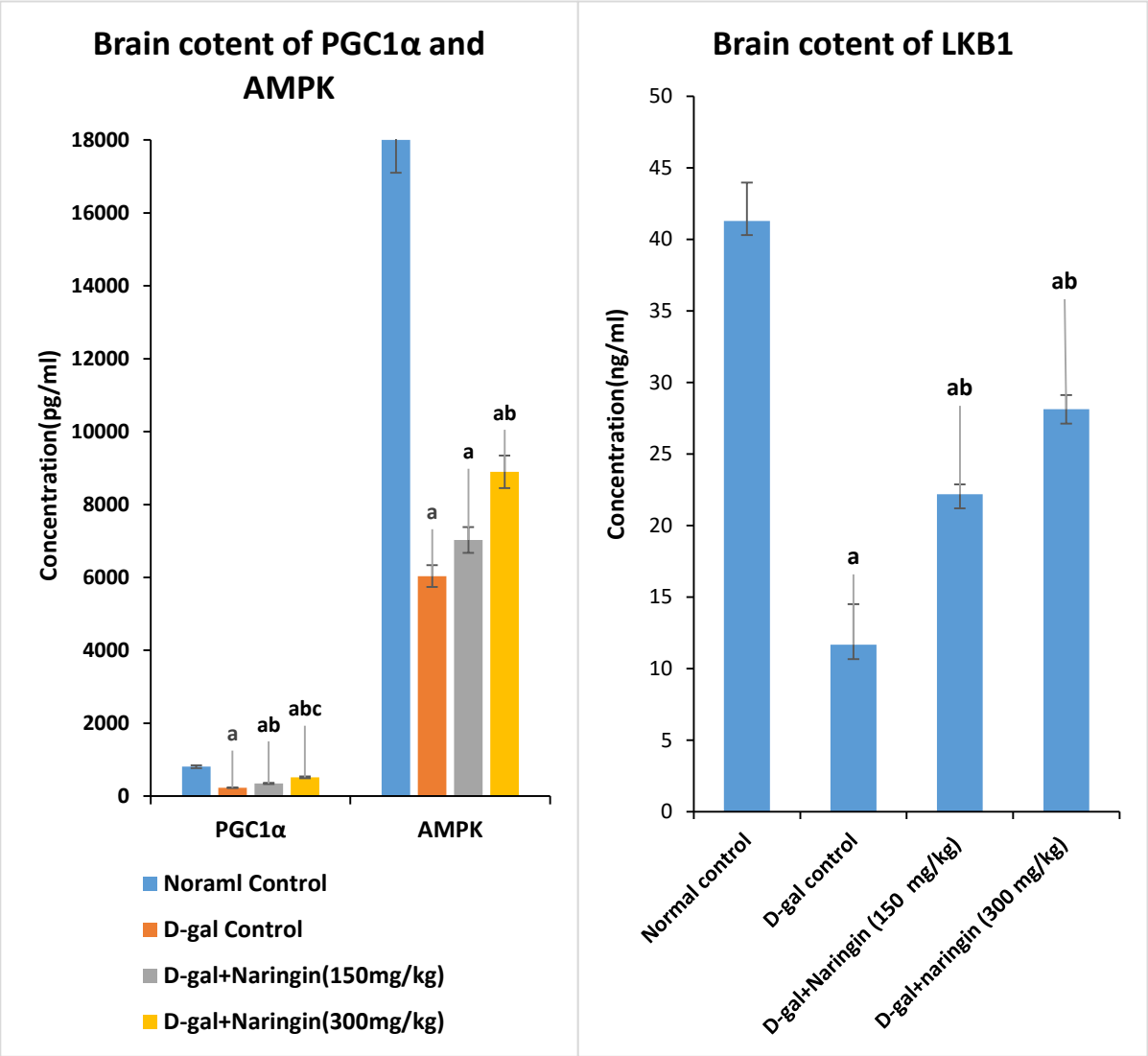


Figure 3: Effect of naringin on AMPK/LKB1/ PGC1α brain content in D-gal-induced brain aging in rats. Data are represented as mean ± SD (n = 6). a: Significant change at P < 0.05 in comparison with normal control group; b: significant change at P < 0.05 in comparison with D-gal group

3.5. Effect of naringin on serotonin and dopamine brain contents

D-gal reduced brain contents of serotonin and dopamine by 52% and 36%, respectively (P<0.05). Naringin (150 & 300 mg/kg) elevated serotonin levels by 65% and 88% and dopamine levels by 26% and 36%, respectively (Fig. 4).

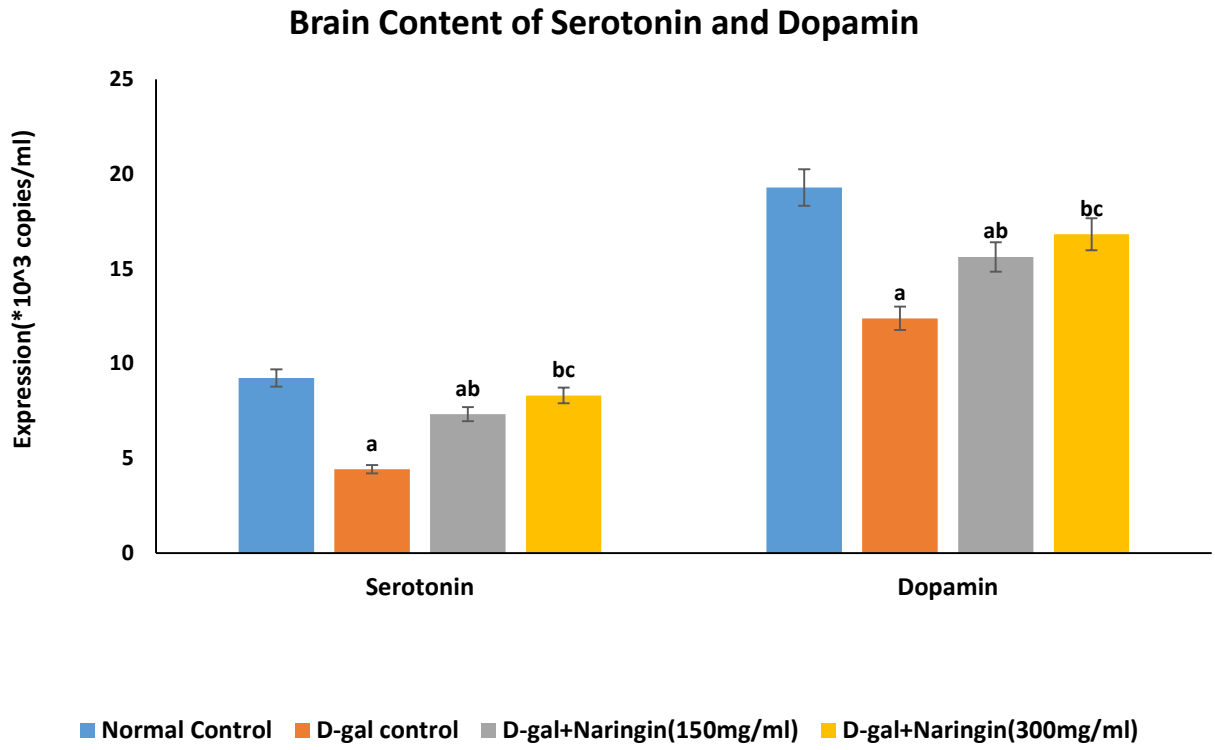


Figure 4: Effect of naringin on brain neurotransmitters ,serotonin and dopamine, expression level in D-gal-induced brain aging in rats. . Data are represented as mean ± SD (n = 6). a: Significant change at $P < 0.05$ in comparison with normal control group; b: significant change at $P < 0.05$ in comparison with D-gal group.

3.6. Histopathological results

3.6.1. Impact of naringin on cerebral cortex

Group 1: displayed moderate (meninges, meningeal, intra-cerebral blood vessels, cerebral cortex, moderate neurons and glial cells), and white matter showed average neurons and glial cells in fibrillary background, while group 2; D-gal showed meninges with marked inflammatory infiltrate, cerebral cortex with many deteriorated neurons and obvious astrogliosis, and white matter showed dispersed degenerated neurons, slight edema, and regular glial cells. Group 3; Naringin (150 mg/kg) showed average meninges, cerebral cortex with mild hypercellularity, scattered degenerated neurons and average blood vessels, and white matter displayed dispersed damaged neurons and moderate glial cells in the fibrillary background. Group 4; Naringin (300 mg/kg): brain showed average meninges, cerebral cortex with moderate cellularity, distributed deteriorated neurons, normal blood vessels, and eosinophilic plaque-like areas, and white matter displayed dispersed damaged neurons and regular glial cells in the fibrillary background (Fig. 5).

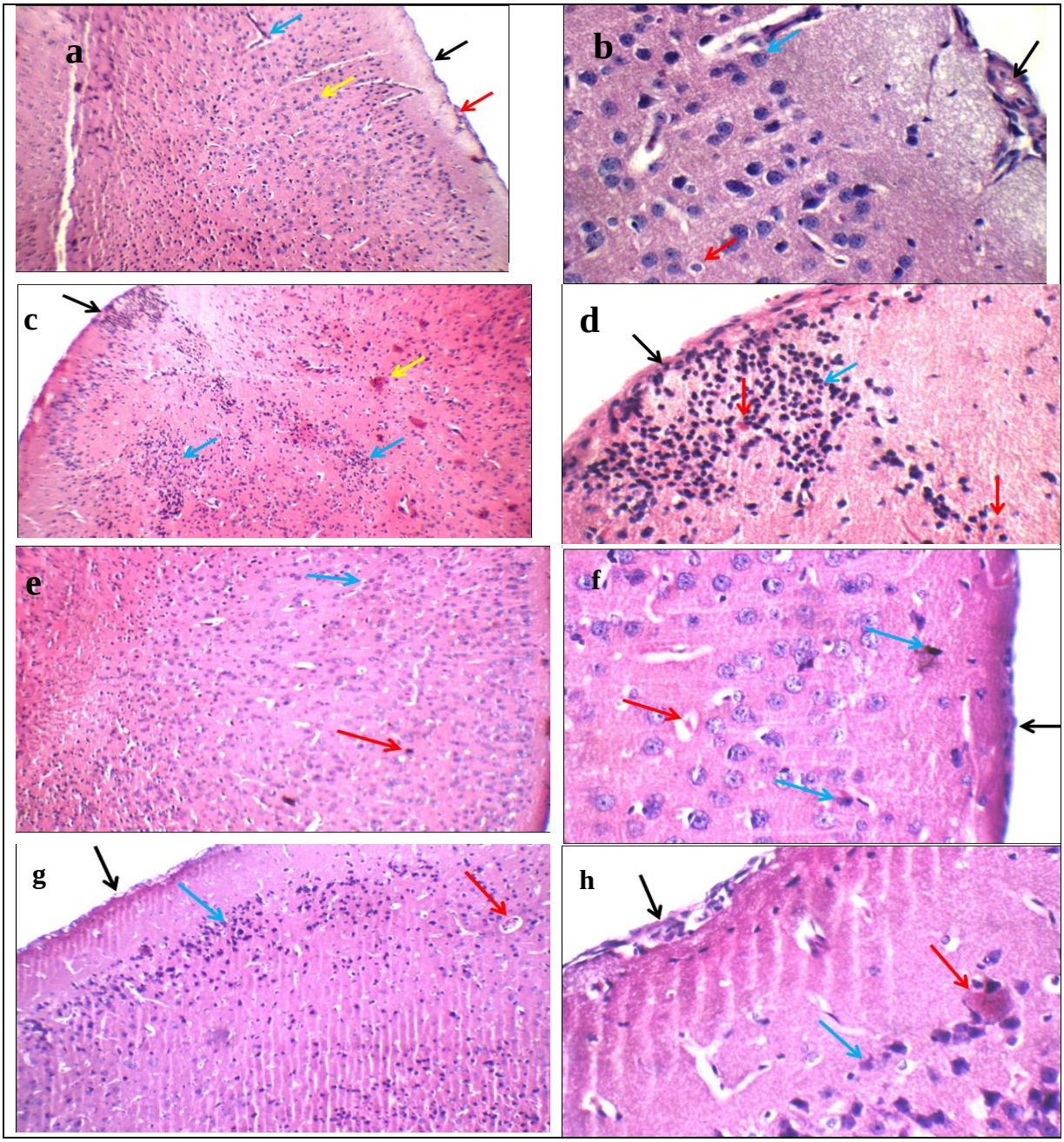


Figure 5: Effect of naringin on the cerebral cortex

a: Normal brain image showing average meninges (black arrow), average meningeal (red arrow) and intra-cerebral blood vessels (blue arrow), and average cerebral cortex with average neurons (yellow arrow) (H&E X 200), b: Normal brain image with high power view showing average meningeal blood vessels (black arrow), average cortex with average neurons (blue arrow) and average glial cells (red arrow) (H&E X 400).

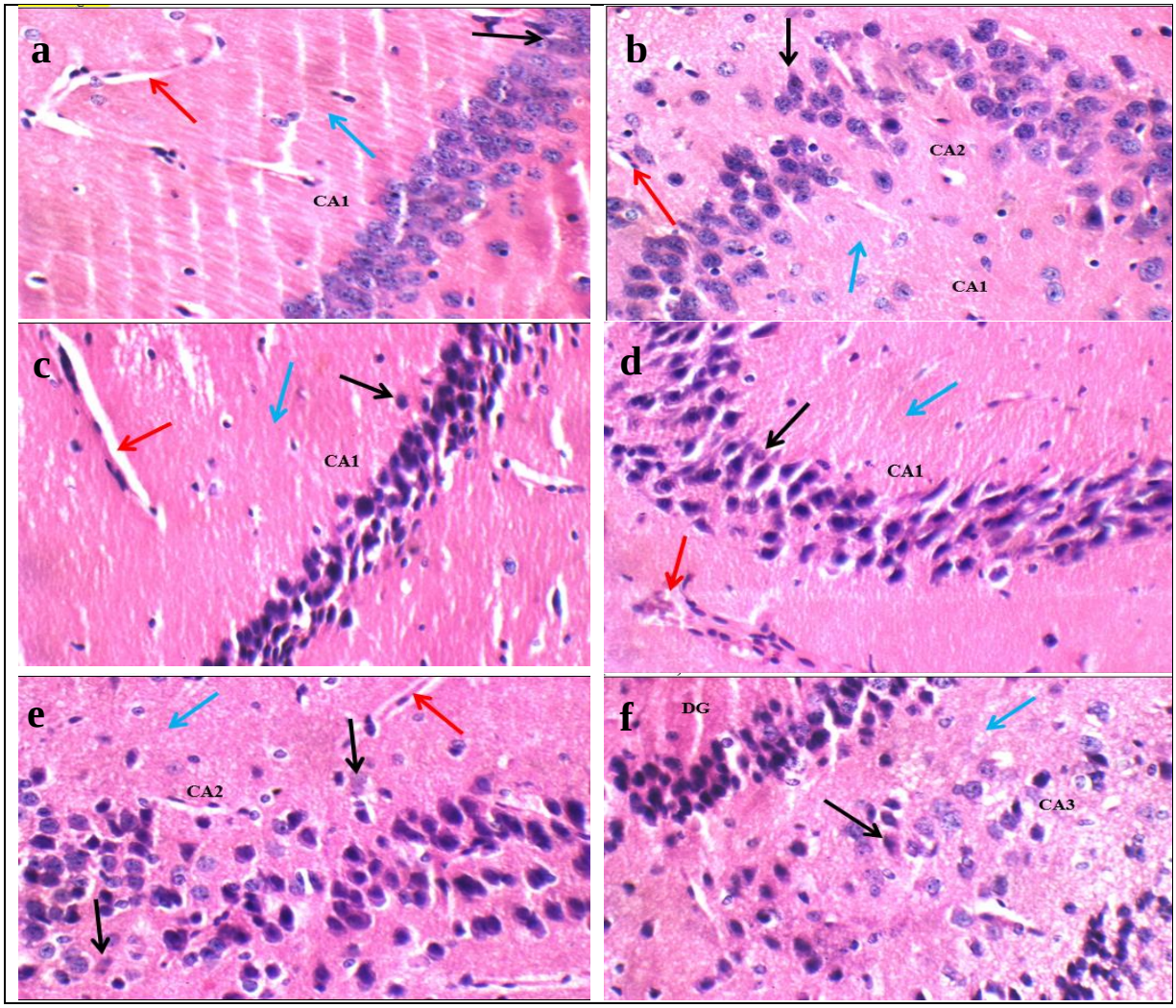
c: D-galactose treated rat's brain showing meninges with marked inflammatory infiltrate (black arrow), and cerebral cortex with marked astrogliosis (blue arrow) and eosinophilic plaque-like areas (yellow arrow) (H&E X 200) d: high power view showing meninges with marked inflammatory infiltrate (black arrow), and cerebral cortex with scattered degenerated neurons (red arrow), and marked astrogliosis (blue arrow) (H&E X 400).

e-Naringin(150mg/kg) treated rats: brain showing average meninges (black arrow), and cerebral cortex with mild hypercellularity (blue arrow) and scattered degenerated neurons (red arrow) (H&E X 200), f: high power view showing average meninges (black arrow), and cerebral cortex with scattered degenerated neurons (blue arrow) and average blood vessels (red arrow) (H&E X 400).

g: Naringin(300mg/kg.) treated rats: brain showing average meninges (black arrow), and cerebral cortex with average cellularity (blue arrow) and average blood vessels (red arrow) (H&E X 200), h: high power view showing average meninges (black arrow), and cerebral cortex with scattered degenerated neurons (blue arrow) and eosinophilic plaque-like areas (red arrow) (H&E X 400**).**

3.6.2. Effect of naringin on Hippocampus

Group 1; Control: displayed regular Cornu Amonis (CA), dentate gyrus (DG), pyramidal neurons, inter-neuron areas, and normal blood vessels, while group 2 showed thin abnormal CA with distributed damaged pyramidal neurons, average DG, eosinophilic plaque-like areas in CA1, and mildly congested blood vessels in CA2. Group 3; Naringin (150 mg/kg): displayed distributed damaged pyramidal neurons in CA, average DG and inter-neuron areas, and also normal blood vessels. Group 4; Naringin (300 mg/kg): displayed dispersed deteriorated neurons in CA, and eosinophilic plaque-like areas in CA1 and in DG (Fig. 6).



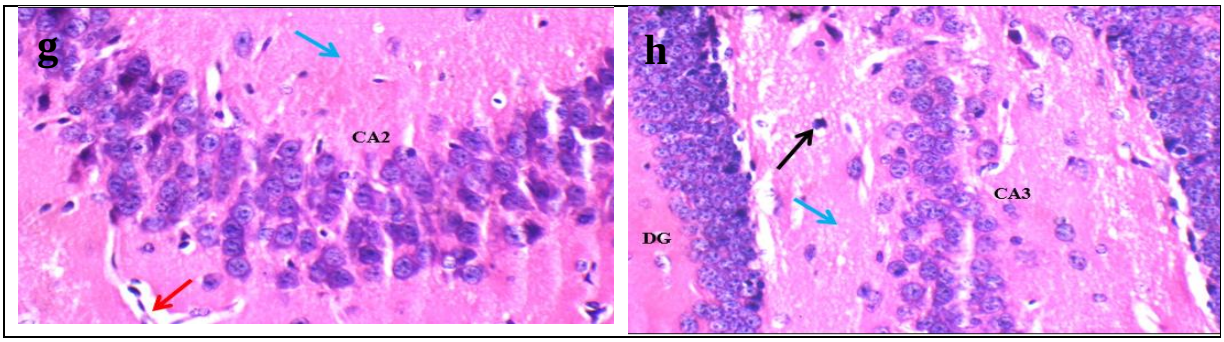


Figure 6: Effect of naringin on the hippocampus

a- **: Control: slide N:** Normal brain image showing higher power view in (CA1) showing average pyramidal neurons (black arrow), average inter-neuron area (blue arrow), and average blood vessels (red arrow) (H&E X 400), **b:** another view in (CA2) showing average pyramidal neurons (black arrow), average inter-neuron area (blue arrow), and average blood vessels (red arrow) (H&E X 400).

c: D-galactose treated rat's brain with high power view showing thin irregular Cornu Amonis (CA1) with scattered degenerated pyramidal neurons (black arrow), average inter-neuron areas (blue arrow), and average blood vessels (red arrow) (H&E X 400), **d:** another view in Cornu Amonis (CA2) showing scattered degenerated pyramidal neurons (black arrow), average inter-neuron areas (blue arrow), and mildly congested blood vessels (red arrow) (H&E X 400).

e: Naringin(150mg/kg) treated rats: another view in Cornu Amonis (CA2) showing scattered degenerated pyramidal neurons (black arrow), average inter-neuron areas (blue arrow), and average blood vessels (red arrow) (H&E X 400), **f:** another view showing Cornu Amonis (CA3) with scattered degenerated pyramidal neurons (black arrow), average inter-neuron areas (blue arrow), and average dentate gyrus (DG) (H&E X 400).

g: Naringin(300mg/kg) treated rats: another view in Cornu Amonis (CA2) showing average pyramidal neurons (black arrow), average inter-neuron areas (blue arrow), and average blood vessels (red arrow) (H&E X 400), **h:** another view showing Cornu Amonis (CA3) with few scattered degenerated pyramidal neurons (black arrow), average inter-neuron areas (blue arrow), and average dentate gyrus (DG) (H&E X 400)

4.Discussion

Aging process is a gradual decrease in all body biological functions. Prominently, brain is among the main organs which are highly susceptible to damage resulted from aging, consequently initiating physiological and biological alterations. It was mentioned by many studies that the long-lasting exposure to D-gal could be a reliable model for normal aging research. It was reported that D-gal injection stimulated natural aging symptoms through inflammatory actions, ROS induction and apoptotic pathways [24–26].

Nutritional and medicinal herbs are continuously and commonly used to prevent and /or protect against neurological damage in a safe and effective manner and are of great value in treatment of aging-associated disorders [27] . Of these medicinal herbs, naringin, which has a profound role regarding anti-inflammation and antioxidant actions, that could recommend its use as a prospective therapeutic remedy to alleviate aging accompanied damage [28].

Brain aging is related to a gradual deterioration in physical activity, cognitive ability, mental capacity, spatial ability. Many research studies reported that persistent administration of D-gal mediate the worsening in cognitive function in correlation with aging signs. These impairments were due to mitochondrial disfunction, induction of oxidative stress, enhancing of apoptosis, inflammation, and aging. In the current work, D-gal induced meaningful alterations in animals' memory and physical activity. These results were further ensured by Y maze, object recognition, and locomotor activity tests as the percent of changes reduced in a significant way in comparison with control. Our results were in the same line with other findings which reported that the D-gal caused a disrupting action on mice capability for learning Y maze, recognize objects, and perform normal physical activities [29,30].

To investigate the potential of naringin on D-gal induced brain behaviour decline symptoms, three parameters associated with the behaviour (object recognition, Y Maze, and locomotor activity) were measured. The object recognition is a common behavioural test for investigating of many properties of learning and memory in mice. Y-maze test is one of the simplest methods that has been used extensively in learning and memory paradigms for mice, while the locomotor activity test is used to measure

physical activity. The two doses of naringin ameliorated the reduction of object recognition, Y Maze percentage alternation, and locomotor activity. In addition, administration of the high dose of naringin returned the discrimination ratio in the three aforementioned parameters to their normal value. These results suggest the highly protective potential of naringin against behavioural and physical disorders associated with D-gal brain aging. Our results were in the same line with Long et al., [17] and Ben-Azu et al., [31] who reported the effective role of naringin in attenuating memory and object recognition functions in mice. Depending on the previous results, naringin has an effective neuroprotective effect supporting its potential use in neurodegenerative diseases.

Brain aging is also characterized by the initiation of microglia cells and astrocytes which have crucial roles in neurodegenerative diseases. GFAP is a specific marker for activated microglia cells and astrocytes, respectively. Nichols et al., [32] reported that GFAP mRNA increased in humans and mice with age. Previous studies revealed that D-gal administration to mice activated microglial cells and astrocytes in the brain [33,34]. Additionally, D-gal is metabolized to glucose naturally which lead to overexpression of Advanced Glycation End Product Receptor (RAGE) that causes glycation of adjacent proteins and formation of AGE. Meanwhile, AGE binds with its receptor RAGE initiating NADPH oxidase and ROS production happens, causing neuronic damage and cognitive disfunction [3]. It is known that AGE normally rises during aging and is associated with different age-associated disorders like diabetes, arteriosclerosis, nephropathy and Alzheimer's disease. In the same line with literature, our results confirmed the significant elevation of brain contents of AGE and GFAP in mice after D-gal administration when compared to controls while, the two doses of naringin reduced brain contents of AGEs and GFAP significantly compared to the D-gal group ensuring the possible neuroprotective effect of naringin. The present study is the first to record decreased brain content of GFAP after treatment with naringin. Based on the literature, decreased levels of GFAP after treatment with naringin was observed in diabetic retinopathy by Liu et al., [35] not brain tissues.

NT-3 is a member of the neurotrophic gene family which supports the survival, differentiation, maintenance, and repair of vertebrate neurons. NT-3 normally prevents death of central noradrenergic neurons. Also, NT-3 initiates survival of ventral mesencephalic dopaminergic neurons, cerebellar granule neurons, and Purkinje cells, and acts on sensory or sympathetic neurons of the dorsal root, nodose and sympathetic ganglia. Dysfunction of neurotrophic systems in adults' brain may be associated with changes in neurotrophins levels indicating its diagnostic value. Mechanisms underlying NT-3 pathway could impair because of neuronal repair and aging, leading to neurodegenerative disorders [36]. Therefore, levels of NT-3 in mice were quantified after administration of D-gal and/or naringin then compared to controls. It was estimated that levels of NT-3 were decreased in treated D-gal group and the treatment with the high dose of naringin only elevated NT-3 levels as compared to the D-gal group. The reduction of NT-3 mRNA and protein levels in the cerebral cortex and hippocampus of mouse brain after administration of D-gal was reported before by Gao et al., [37] with other drugs than naringin. The present study is the first one to evaluate the effect of naringin on the protein levels of NT-3. The upregulation of NT-3 protein levels by naringin is another indicative evidence of the possible neuroprotective effect of naringin.

During nonpathological aging, quantitative changes occur in the pre-and post-synaptic elements of both dopamine and serotonin systems that affect cognitive function. The level of dopamine and the density of serotonin receptors declines in humans and rodent brains with age [38]. It was reported that D-gal significantly impaired cognitive performance in rats and resulted in severe alterations in levels of neurotransmitters contents (serotonin, dopamine) by decreasing their concentrations [39]. These results were confirmed in our study as D-gal reduced brain contents of serotonin and dopamine significantly while the administration of naringin elevated brain contents of serotonin and dopamine significantly compared to the D-gal group. The neuroprotective effect of naringin through upregulating serotonin levels was reported by [40]. All previous results ensure the neuroprotective effect of naringin against D-gal induced brain aging in mice through attenuating memory, object recognition functions, and improving physical performance. Also, by reducing brain contents of AGEs and GFAP and upregulation of NT-3 and neurotransmitters serotonin and dopamine.

Our data also evaluated the D-gal induced inflammation through NF-kB activation followed by the activation of neuroinflammatory marker TNF-α. Fifteen years ago, Helenius [41] found that NF-kB was markedly enhanced with aging process in mice and rats different organs. It is well documented that NF-kB high levels are related to inflammation that acts as a significant feature of many age-associated diseases. Many researchers proposed that the upregulation of many inflammatory markers and initiated neuroinflammation by D-gal through the upregulation of NF-kB. Inflammation is a key pathogenic factor in the aging process. Elevated concentration of NF-kB

and TNF-α in brain and cerebrospinal fluid of AD patients has been reported [42]. Treatment with naringin decreased the inflammation initiated after D-gal administration and declined NF-kB and TNF-α protein levels significantly. The anti-inflammatory action of naringin in our study was shown by inhibition of NF-kB associated neuroinflammation. Through blocking NF-kB, naringin suppressed the downstream TNF-α protein. In this regard, other studies reported the inhibition of TNF-α by naringin by inhibiting its upstream NF-kB Signalling cascade [43,44]**.**

One of the pathways which decline during aging is LKB1/AMPK pathway. AMPK is a central molecular target because recent AMPK enhancers are vital for treating metabolic and neurodegenerative disorders. The activation of AMPK reduces inflammatory disorders and the natural products which have anti-inflammatory properties are good candidates for activation of AMPK which can inhibit NF-kB Signalling through activation of Fox Signalling [8]. Besides, PGC-1α, a main goal of AMPK pathway, can bind to the p65 subunit of NF-kB. The activation of NF-kB subsequently reduced the expression of PGC-1 α. On the other hand, high levels of PGC-1 α repressed NF-kB and inflammatory responses in aortic smooth muscle and endothelial cells [45]. These findings highlight that AMPK could mediate NF-kB Signalling and so the deficiency in AMPK levels during aging not only interrupts energy metabolism but also aggravates inflammation.

D-gal group inhibited LKB1/AMPK pathway and increased the number of early apoptotic mitochondria Cai et al., [46]. In the current study, D-gal decreased AMPK, LKB1, and PGC1α levels in mice brain significantly as compared to the control group while the high dose of naringin improved brain contents of AMPK, LKB1, and PGC1α. These results indicated for the first time that naringin activated the AMPK/ LKB1/ PGC1α Signalling pathway as it deactivated the NF-kB cascade.

5.Conclusion

Regarding the aforementioned data, our study is the first to report a decreased in brain content of GFAP and upregulation of NT-3 protein levels after naringin treatment suggesting neuroprotective effect during brain aging. Also, the anti-inflammatory effect was evidenced through the inhibition of NF-kB and TNF-α. The neuroprotective effect of naringin was augmented by the upregulation of neurotrophic factors (AMPK/ LKB1/ PGC1α and NT-3/serotonin/dopamine) as well as downregulation of neurotoxic substances (AGEs/GFAP) that were produced during D-gal stimulation. Naringin is a natural citrus flavonoid with various biological activities that is explained by its anti-inflammatory and antioxidant potential. It is to conclude that naringin is a potential therapeutic candidate against neurodegeneration and brain aging.

6. List of Abbreviations:

D-gal: D-galactose; **AMPK**: 5'-Adenosine monophosphate Activated protein Kinase; **LKB1**:Liver Kinase B1; **PGC1-α**:Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; **TNF-α**: Tumour Necrosis Factor-alpha; **NF-κB**: Nuclear Factor Kappa Beta; **AGEs**: Advanced Glycation End Products ; **GFAP**: Glial fibrillary acidic protein; **NT-3**:Neurotrophin-3; **RAGE**: Receptor of Advanced Glycation End product; **S.C. injection:** Subcutaneous injection; **mRNA**: messanger-Riboneuclic Acid.

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