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# EXPLORING THE COMPLEMENTARY INFLUENCE OF AN OLEUROPEIN-ENRICHED PHYTOCHEMICAL EXTRACT ON NEURO-BIOCHEMISTRY, BEHAVIOR, AND HISTOPATHOLOGY IN AGING AND DEPRESSED MALE RATS

**Shirin TARBIAT[1]*, Kübra ERGÜN[2], Refika AYDOĞDU[1], Estelle Larissa NDENGOM DJOMALEU[1], Kürşat FiLİKCİ[3]**

*[1]Üsküdar University, Faculty of Engineering and Natural Sciences, Department of Molecular Biology and Genetics, 34662, Istanbul, Türkiye*
*[2]Yıldız Technical University, Graduate School of Science, Department of Molecular Biology and Genetics, 34220, Istanbul, Türkiye*
*[3]Harran University, Faculty of Veterinary Medicine, Department of Pathology, 63510, Şanlıurfa, Türkiye*

**Abstract:** Aging correlates with neurodegenerative diseases such as depression, marked by neuro-biochemical changes triggering neuronal loss. Nutraceuticals exhibit antioxidant and neuroprotective properties and offer potential intervention. Our study examines the preventive effects of an olive leaf extract consisting of 20% pure oleuropein in an animal model of aging and depression. Aging in Wistar rats was induced by intraperitoneal injections of 150 mg of D-galactose/kg body weight for 6 weeks. The depression was caused by forced swimming. We examined Monoamine oxidase and acetylcholinesterase activities, histopathology of the brain, and the serum plasminogen activator inhibitor-1, superoxide dismutase, and monoaldehyde levels of all animal groups. Depressed and aged rats showed increased Monoamine oxidase, acetylcholinesterase, and plasminogen activator inhibitor-1 levels, and a decrease in superoxide dismutase levels. The administration of 200 mg of extract before depression or aging inductions significantly reduced the Monoamine oxidase and acetylcholinesterase activities. Degeneration/necrosis was observed in neurons of all experimental animal groups. We demonstrate that the olive leaf extract with 20% pure oleuropein markedly maintained the neurotransmitter levels in the brain and enhanced the antioxidant defense system in depressed and aged animal models but was unable to yield behavioral benefits.

**Keywords:** Oleuropein, Depression, Aging, Monoaminoxidase, Acetylcholinesterase

***Corresponding author:** Üsküdar University, Faculty of Engineering and Natural Sciences, Department of Molecular Biology and Genetics, 34662, Istanbul, Türkiye
**E mail:** shirin.tarbiat@uskudar.edu.tr (S. TARBIAT)

Shirin TARBIAT https://orcid.org/0000-0001-7931-1546
Kübra ERGÜN https://orcid.org/0000-0002-8479-8317
Refika AYDOĞDU https://orcid.org/0009-0005-3006-1642
Estelle Larissa NDENGOM DJOMALEU https://orcid.org/0000-0001-6884-390X
Kürşat FiLİKCİ https://orcid.org/0000-0001-9710-9480

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## 1. Introduction

Aging involves a gradual deterioration of body functions. As time passes, physiological function is altered and pathophysiological conditions progress more rapidly. The aging brain becomes increasingly vulnerable to neurodegenerative diseases. Moreover, aging is linked to substantial reduction in adult neurogenesis, which can contribute to the development of neurodegenerative conditions like depression, Alzheimer's, and Parkinson's diseases (Campos Costa et al., 2013; Frangos et al., 2023). Neuro-biochemical changes in normal aged brain trigger the loss of neurons especially in subcortical nuclei because of reductions in the brain protein level including enzymes involved in the synthesis of neurotransmitters such as dopamine, norepinephrine, serotonin and acetylcholine. Several classes of antidepressant drugs are used in the treatment of depression. The mechanism of action of most of these drugs is based on targeting particular enzymes such as monoaminoxidase (MAO) and acetylcholinesterase (AChE) (Nimgampalle et al., 2023). MAO is an enzyme that catalyzes the degradation of various neurotransmitters such as serotonin, dopamine and norepinephrine in the central nervous system. MAO inhibitors suppress the breakdown of these neurotransmitters, thereby augmenting their concentrations and enhancing cellular function in instances of depression-associated impairment, consequently playing an important role in the pathophysiology of depression in elderly. According to existing research, compounds known to elevate the concentration of monoamines have been associated with the elicitation of antidepressant effects. Furthermore, a substantial body of evidence suggest that numerous currently available antidepressants operate primarily through the modulation of biogenic amins, particularly serotonin in the brain. Aselegiline, isocarboxazid,

phenelzine, and tranylcypromine are the most commonly prescribed drugs. Way of functioning of these drugs is based on MAO inhibition leading to enhancement of monoamine neurotransmitters and thereby increasing their availability (Van den Eynde et al., 2022).

A wealth of in vivo and in vitro research has consistently demonstrated that the synthesis, release, and choline uptake associated with acetylcholine are notably diminished during the aging process, resulting in reduced levels within the brains of elderly. Studies have specifically revealed an increase in overall brain AChE activity in the context of natural brain aging in rodent models (Lista et al., 2023). Consequently, the maintenance of neurotransmitter levels through the inhibition of cholinesterases and MAOs serves as a crucial mechanism for neuroprotection against conditions such as depression, cognitive decline, and dementia during advanced age.

Recent evidence has demonstrated the significant role of oxidative stress in the progression of aging and related disorders. The deleterious effects of oxidative damage are attributed to the releases of reactive oxygen and reactive nitrogen species, thereby exacerbating the imbalance between endogenous antioxidant compounds (glutathione vitamin E, superoxide dismutase (SOD), catalases) and oxidation processes within the body. The brain, owing to its high oxygen consumption and its rich content of polyunsaturated fatty acids contrasted with a comparatively low concentration of antioxidant enzymes, is particularly susceptible to the pernicious impacts of oxidative stress. Notably, the dopaminergic neurons in the substantia nigra face heightened vulnerability to oxidative damage, as the production of free radicals within these neurons significantly compromise their integrity. Given that the impairment or loss of dopaminergic neurons constitutes a primary factor in the onset of depression, recent investigations underscore the association between heightened levels of oxidative stress and the severity of depression in the elderly (Hajam et al., 2022; Chrzastek et al., 2023).

The growing recognition of the health-promoting attributes of nutraceuticals and bioactive compounds, particularly those derived from plant sources, such as polyphenols, transcends the fundamental nutritional advantages of dietary constituents, as they exhibit the capacity to bolster the body's antioxidant defense mechanism, Empirical evidence links adherence to a Mediterranean diet, reach in plant-based foods, fish, and olive oil, to a reduced susceptibility to a broad spectrum of age-related ailments. Notably Oleuropein, a prominent polyphenolic compound found in olive leaves, stands as the pivotal bioactive constituent of the olive tree. In-depth investigation through a series of in vitro and in vivo studies have elucidated the multifaceted potential of olive leaf extracts, unveiling their antidiabetic, anticancer, antioxidant, anti-inflammatory, cardioprotective, blood pressure-lowering, and neuroprotective properties. In an endeavor to uncover novel biological characteristics and delineate the neuroprotective attributes of olive leaf extract, with a specific focus on its role as a beneficial supplement in combating age-associated disease, we conducted an animal study employing a comprehensive methodology. We employed biochemical, behavioral, and histopathological approaches to assess the preventive effects of olive leaf sourced from cultivar's of Kazdaglari/Istanbul/Turkey, containing a 20% oleuropein content (Riolo et al., 2022; Rishmawi et al., 2022; Micheli et al., 2023; Reyes-Goya et al., 2024).

## 2. Materials and Methods

### 2.1. Chemicals, Reagents, and Test Materials

Olive leaves hydro alcoholic extract was collected from Sankara Brain and Biotechnology Research Center (Istanbul University) and identified by Professor. Dr. Ihsan Kara. According to the supplier, the extracts were 20% pure oleuropein and the other 80% consisted of various other phytochemicals of olive leaf extracts. All other chemicals and kits were purchased from Sigma–Aldrich (USA).

### 2.2. Animals, Design and Experimental Procedure

Forty male Wistar albino rats (220-250 g) were provided by animal Lab of Uskudar University, Istanbul, Türkiye, was used for the study. The rats were housed in regulated conditions at 22 °C following a 12-hour light and 12-hour dark cycle, within standard cages, ensuring unrestricted availability of both water and food.

The animals were divided into five groups (n = 8) at random to ensure unbiased allocation: (I) control group that received only saline (5 ml/kg) by subcutaneous injection; (II) depressed group that subjected to a depression-like behavior model using the forced swimming test; (III) aged group that received D-galactose (150 mg/kg/five days a week) for 6 weeks; (IV) oleuropein + depressed group that received oleuropein (200 mg/kg/day intraperitoneal, this dose was selected based on the findings from the study by (Bakir et al., 2018) as supplement for one month and underwent depression; (V) oleuropein + aged group that received oleuropein (200 mg/kg/day intraperitoneal) as supplement for one month and underwent aging by receiving D-galactose (150 mg/kg/five days a week) for duration of 6 weeks.

Depression was induced by forced swimming test. The aging was induced by administration of D-galactose (150 mg/kg/five days a week) for 6 weeks, which is based on oxidative stress. The effective doses were selected in accordance to Ruan et al. (2013). The tail suspension test is applied to detect the general behavior of the experimental animal groups at the end of trial.

Oleuropein and D-galactose were dissolved in water prior to administration. D-galactose was given at 150 mg/kg, five days per week, with a total volume of 0.5 mL containing 3.3 mg of D-galactose per rat. Oleuropein was administered at 200 mg/kg/day in a total volume of 0.5 mL, containing 4.4 mg of oleuropein per rat.

After the completion of the timetable, clusters of rats were terminated via decapitation under the influence of chloroform anesthesia. Subsequently, their brains were extracted and preserved at − 80 °C until the biochemical analyses was performed. Blood samples were collected from animals using a perfusion technique. The brains were treated with a fixative solution (4% paraformaldehyde in 0.1 phosphate buffer, pH 7.4) allowing them to undergo histopathological examination.

Ethical committee guidelines limited the total number of animals to 40 male rats. To meet statistical requirements, each group required eight animals, leaving no capacity for additional positive control groups.

**2.3. Biochemical Analysis**

**2.3.1. Homogenization of rat brains**

The animals were sacrificed by decapitation and their entire brain was blended in ice-cold solution consisting of 50 mM Tris-HCl, pH 7.4-, and 300-mM sucrose, using a glass homogenizing vessel at 900 rpm placed on ice. Afterward, the mixture was centrifuged at 1,000x g for 10 min. to eliminate nuclei and leftover fragments. The protein content in the resulting supernatant was estimated using Lowry method.

**2.3.2. Determination of MAO activity**

The MAO assay was conducted using the spectrophotometric approach as outlined by Zhi et al. (2016) with some minor adjustments. Briefly, brain homogenates from the animals were diluted to the desired concentrations in 100 mM ice-cold potassium phosphate buffer (pH 7.6). A 0.2 ml reaction mixture included 0.01 mg/ml protein and 0.25 mM 4-(Trifluoromethyl) benzylamine, vallinic acid (1mM), 4-aminoantipyrine (500 µM) and peroxidase 4 U/ml) in 0.2 M potassium phosphate (pH 7.6). The method operates on the principle that MAO transforms 4-(Trifluoromethyl) benzylamine into aldehyde, ammonia and hydrogen peroxide. With the presence of peroxidase, the hydrogen peroxide then converts 4-aminoantipyrine into oxidized 4-aminoantipyrine which subsequently reacts with vanillic acid to producing a red quinoneimine dye. This dye was detected at wavelength of 490 nm.

**2.3.3. Determination of AChE activity**

AChE activity was determined by following the hydrolysis of acetylthiocholine according to the method of Ellman et al. as described in detail by Tarbiat et al. (2020). The Acetylcholinesterase (AChE) Inhibition Assay was conducted using Ellman's method, which quantifies AChE activity by measuring the production of a yellow-colored product detectable at 405 nm. In this assay, AChE hydrolyzes acetylthiocholine iodide to produce thiocholine, which subsequently reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), yielding 2-nitro-5-thiobenzoate (TNB). The rate of TNB formation, indicated by the increase in absorbance at 405 nm, reflects the enzyme's activity. For the assay, a 200 µL protein solution (2 mg/mL in 0.1 M phosphate buffer, pH 8.0) serving as the enzyme source was combined with 100 µL of DTNB solution (3.3 mM in 0.1 M phosphate buffer, pH 7.0) containing 6 mM $NaHCO_3$, and 500 µL of phosphate buffer (pH 8.0). After a 3-minute incubation at 25 °C, the change in absorbance at 405 nm was measured using a 96-well microplate reader to determine AChE activity.

**2.3.4. Determination of plasma monoaldehyde (MDA) and SOD activity**

Plasma MDA level which is the sign of lipid peroxidation in blood was determined as described by Ohkawa et al. (1979). The reaction of lipid peroxides in animal tissues with thiobarbituric acid (TBA) is pH-dependent, with optimal reactivity observed at pH 3.5. Based on this finding, a standardized procedure for assessing lipid peroxide levels in animal tissues was developed as follows: Sample Preparation: Mix 10% tissue homogenate with sodium dodecyl sulfate, acetate buffer (pH 3.5), and an aqueous solution of TBA. Incubation: Heat the mixture at 95 °C for 60 minutes. Extraction: After cooling, extract the red pigment produced using a mixture of n-butanol and pyridine. Measurement: Measure the absorbance of the organic layer at 532 nm. Quantification: Use tetramethoxypropane as an external standard to express lipid peroxide levels in terms of nanomoles of malondialdehyde.

Plasma SOD activity was measured using the method described by Sun et al. (2019). Superoxide dismutase (SOD) activity was assessed using the method developed by Kakkar et al. (1984). Briefly, the reaction mixture comprised 0.1 mL of hippocampal supernatant, 1.2 mL of 52 mM sodium pyrophosphate buffer (pH 8.3), 0.1 mL of 186 µM phenazine methosulfate, and 0.3 mL of 300 µM nitroblue tetrazolium. The reaction was initiated by adding 0.2 mL of 750 µM NADH solution. After a 90-second incubation at 30 °C, the reaction was terminated by introducing 0.1 mL of glacial acetic acid. The mixture was then vigorously stirred, combined with 2.0 mL of n-butanol, and centrifuged at 4,000 g for 10 minutes. The absorbance of the organic layer was measured spectrophotometrically at 560 nm. SOD activity is expressed in units per milligram of protein.

**2.3.5. Determination of plasminogen activator inhibitor-1 (PAI-I)**

To evaluate the antiaging and anti-depressant effect of olive leaf extract serum PAI-1 activity was determined by a chromogenic plasminogen-coupled assay described by de Vries et al. (1994) using Elisa kit.

**2.4. Animal Behavior Experiments**

**2.4.1. Tail suspension test (TST)**

The (TST) experiment was performed based on the method described by Cryan et al. (2005). Animals were suspended 50 cm above the table using adhesive tape affixed approximately 1 cm from the tip of their tails. The duration of immobility was recorded during a 6-minute test period. Mice were considered immobile when they hung passively and remained completely motionless.

**2.4.2. Forced swimming test (FST)**

The (FST) experiment used according to the method described by Sallinen et al. (1999) with slight

modification. This test was also performed to induce depressed behavior in rats. This test was used to evaluate despair-like behavior in mice.

On the 20th day of the experiment, a pretest session was conducted where mice were forced to swim for 15 minutes in transparent glass cylinders (25 cm in height, 10 cm in diameter) containing 10 cm of water maintained at 24±1 °C. At this depth, mice could not touch the bottom of the cylinders with their tails or hind limbs. After the pretest, mice were removed, dried, and returned to their cages.

Twenty-four hours later, the test session was conducted. Mice were returned to the same cylinders for a 5-minute session during which immobility time was recorded for each mouse. Two blinded observers quantified immobility time, and video recordings were made to confirm scoring manually. Mice were considered immobile when they floated motionless in the water, with only occasional minimal movements of the paws or tail to keep their heads above water.

**2.5. Histopathological Studies**

Histopathological analysis was performed at the pathology department of Ankara University. Brain samples which have been fixed in formaldehyde trimmed and then placed in cassettes. Subsequently, the samples underwent to a 12-hour wash in running water. The tissues were later processed using a routine tissue tracking device (Leica TP1020) and embedded in paraffin (Thermos Electron Corp. Shandon Histocentre 3). Thin sections 5 μm in thickness (Leica RM2255), were produced from each block; after deparaffinization and dehydration steps in an automatic staining apparatus (Leica Autostainer XL), the sections were stained according the Harris's Hematoxylin-Eosin (HE) method. Additionally, Congo Red staining was employed. Post-staining, the preparations were covered with a coverslip using an automated sealing machine (Leica CV5030). The findings were analyzed under a light microscope (Olympus BX51), leading to a diagnosis and subsequent grading. Moreover, relevant areas were photographed (Olympus DP71). The findings were semi-quantitatively graded by two Pathologists in 10 different fields based on their intensities under 4x, 10x and 40x lenses. This evaluation system categorized the results as (-) negative, (+) mild, (++) moderate and (+++) severe.

**2.6. Statistical Analysis**

Data analyses were performed using graph-pad one-way analysis of variance (ANOVA). Data are expressed as the mean ± SD. $P < 0.05$ was considered significant.

## 3. Results

**3.1. Biochemical Analysis**

**3.1.1. Effect of oleuropein on MAO and AChE activity from rats' brain homogenates**

In the present study we found that the brain MAO activity was significantly increased in the depressed and aged groups compared to the control group. Pretreatment with oleuropein significantly reduced MAO activity in groups (IV) and (V) compared to the depressed and aged groups. No significant difference in MAO activity was observed between oleuropein pretreated groups (Figure 1a).

The results presented in Figure 1b depicts AChE activity in brain homogenates of groups. In the aged and depressed groups, AChE activity increased significantly compared to the control group. Pretreatment with oleuropein significantly decreased the AChE activity in both pretreated groups compared to the depressed group but there was no significant difference between untreated and pretreated aged groups.

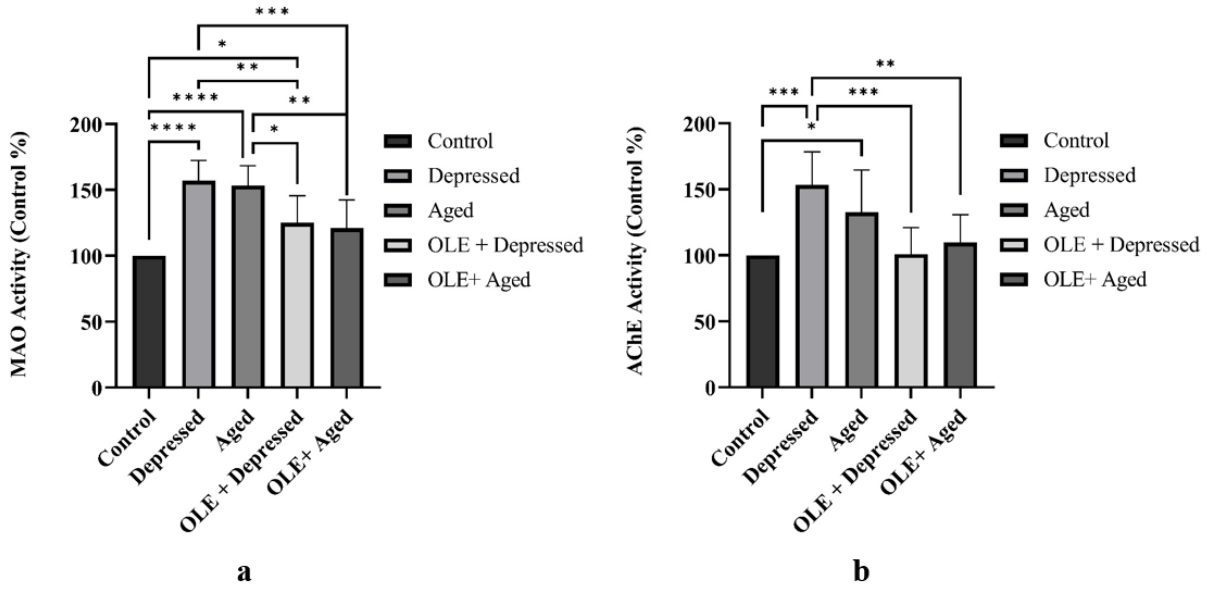


**Figure 1.** The MAO activity (a) and AChE activity (b) of all animal groups. Supplemented animal groups received (200 mg/kg/day) (20 %) Oleuropein (OLE). Each bar represents mean ± SD (n=8). Difference between pairs marked with * (P<0.05), ** (P<0.01) and *** (P<0.001) were significant.

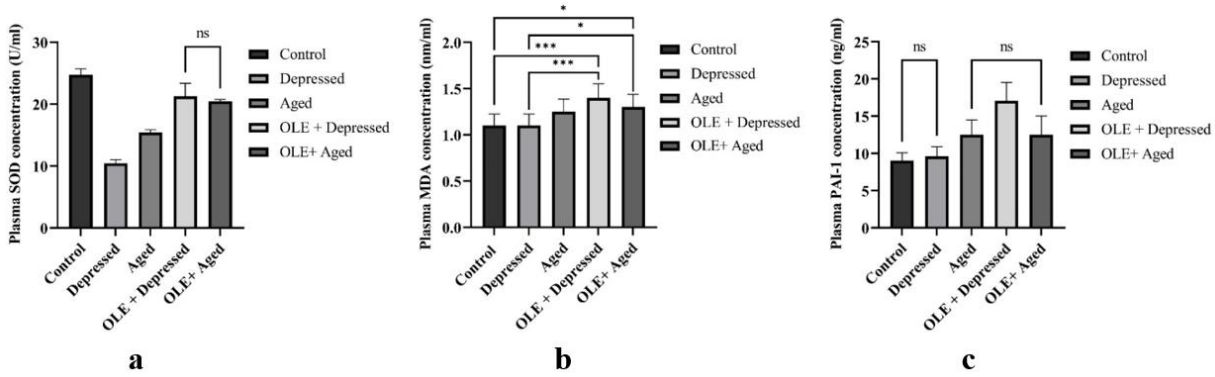


**Figure 2.** Plasma SOD (a), MDA (b) and PAI-1 (c) results of all groups. Each bar represents mean ± SD (n=8). Bar pairs marked with (ns) were not significantly different at $p < 0.05$ in Figure 2a and 2c whereas in Figure 2b bar pairs marked with * ($P<0.05$) and *** ($P<0.001$) were significantly different.

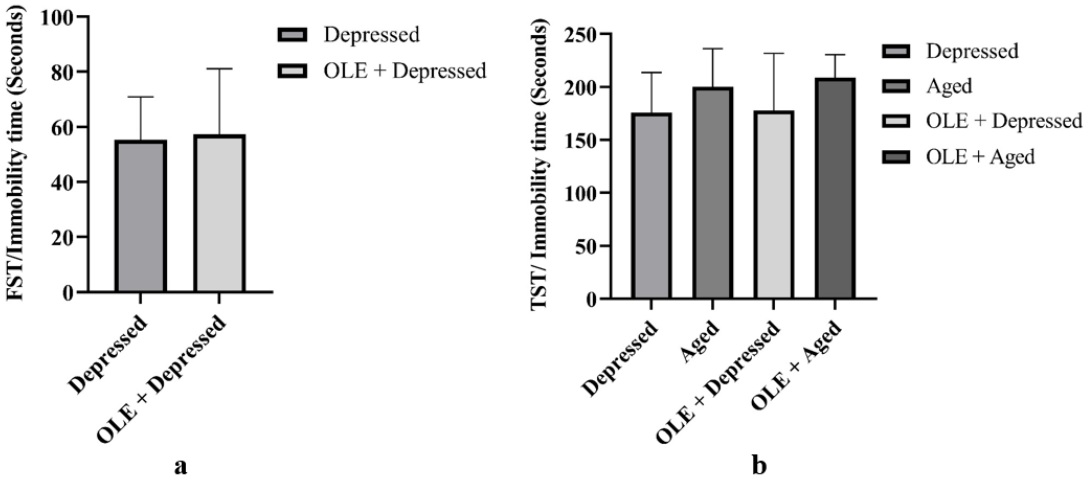


**Figure 3.** Behavior test results using FST method (a) performed on depressed and oleuropein (OLE) pretreated depressed groups and TST method (b) preformed on all groups. Each bar represents mean ± SD (n=8). Difference between bar pairs in each graph were not significant at $P<0.05$.

### 3.1.2. Effect of oleuropein on plasma SOD, MDA, and PAI-I levels

The results presented in Figure 2a show plasma SOD activity levels in plasma of all animal groups. The decrease in enzyme activities was significant in depressed and aged groups compared to the control group. Both pretreated groups exhibited higher SOD enzyme activity in comparison with the aged and depressed groups but no significant difference among themselves.

Based on results depicted in Figure 2b, MDA levels were elevated in depressed and aged groups compared to the control group. We obtained conflicting results regarding the level of MDA in oleuropein pretreated animal groups. Difference between MDA levels in oleuropein pretreated groups was not significant.

Studies have indicated that PAI-1, is an inhibitor of fibrinolysis and an important marker of cardiovascular disease, plays an important role in hemostasis. That is also an indicator of the progress of many other disorders such as diabetics, obesity and cancer. PAI-1 is implicated in neurodegenerative disease due to its role in promoting neuroinflammation and contributing to the accumulation of abnormal protein aggregates, such as beta-amyloid plaques in Alzheimer's disease. Elevated levels of PAI-1 are associated with impaired fibrinolysis and increased neurotoxicity, potentially exacerbating the progression of neurodegenerative conditions (Godinez et al., 2022; Sharma et al., 2022; Gumede and Khathi, 2023; Jiang et al., 2023).

Herein, we analyzed PAI-1 protein levels in plasma of all under study animal groups. The results indicated the increase in plasma PAI-1 level in the depressed group was not significant compared to the control group. The pretreated aged group exhibited no significant reduction compared to the untreated aged group. On the other hand, the pretreated depressed group presented conflicting results with a significant increase in PAI-1 level compared to all groups (Figure 2c).

### 3.2. Behavioral Assessments

### 3.2.1. FST Test; assessments of oleuropein effect on immobility time

FST was applied as a model of depressive-like behavior in this study. We induced depression-like behavior for

the depressed and pretreated depressed groups observed depression in their behaviors (supplementary videos). The difference in immobility times between these two groups was not significant (Figure 3a).

**3.2.2. TST test; assessment of oleuropein effect on immobility time**

Depression induced by FST in the depressed group caused rats to become motionless in this group. When we scored the motion times in Oleuropein pretreated group of animals there was no significant change in the immobility time of rats in compared with the Depressed group (Figure 3b). In the TST behavioral assessment test which was performed for all experimental animal groups there was also no significant difference in immobility time among any of the groups. Oleuropein pretreating was ineffective on animals' immobility times.

**3.3. Histopathological Results**

In the histopathological examination of the control group, it was observed that the cerebrum and cerebellum tissues generally preserved their normal histological structures (Figure 4; Table 1). In the depressed group, moderate degree of neuronal degeneration was observed in the cerebrums, characterized by a reduction of Nissl bodies, resulting in more eosinophilic, occasionally oval/round-shaped neurons, along with instances of necrosis (eosinophilic neurons without nuclei and Nissl bodies) was observed (n=6). Additionally, two animals exhibited gliosis. The cerebellum of depressed animals displayed moderate necrosis and degeneration In Purkinje cells, akin to the observations in cerebral neurons (Figure 4). In the aged group, three of the eight specimens revealed severe neuronal degeneration, two displayed mild degeneration and one displayed moderate degeneration and mild gliosis in the cerebrum. In the Purkinje cells of the cerebellums, 4 cases of moderate degeneration/necrosis, one mild and two severe cases of degeneration/necrosis was observed.

Two cases of moderate to severe cases and one mild case of degeneration/necrosis were observed in the cerebrums, and 4 mild cases of degeneration/necrosis were observed in Purkinje cells of the cerebellums of the pretreated depressed group. In the pretreated aged group, five severe and one mild degree of degeneration/necrosis was observed in neurons of the cerebrums and one moderate and two mild degree degeneration/necrosis was observed in Purkinje cells of the cerebellums. Amyloid deposits were not found with Congo Red staining in any of the groups.

In our study, D-galactose was used as a functional and reliable agent to establish an aging model due to its well-documented ability to induce oxidative stress and mimic aging-related biochemical and physiological changes. The administration of 150 mg/kg body weight of D-galactose intraperitoneally for 6 weeks led to significant alterations in key biomarkers, including increased monoamine oxidase (MAO) and acetylcholinesterase (AChE) activities, elevated plasminogen activator inhibitor-1 (PAI-1) levels, and reduced superoxide dismutase (SOD) activity. These findings are consistent with the literature and confirm the functionality of D-galactose as a robust marker of aging.

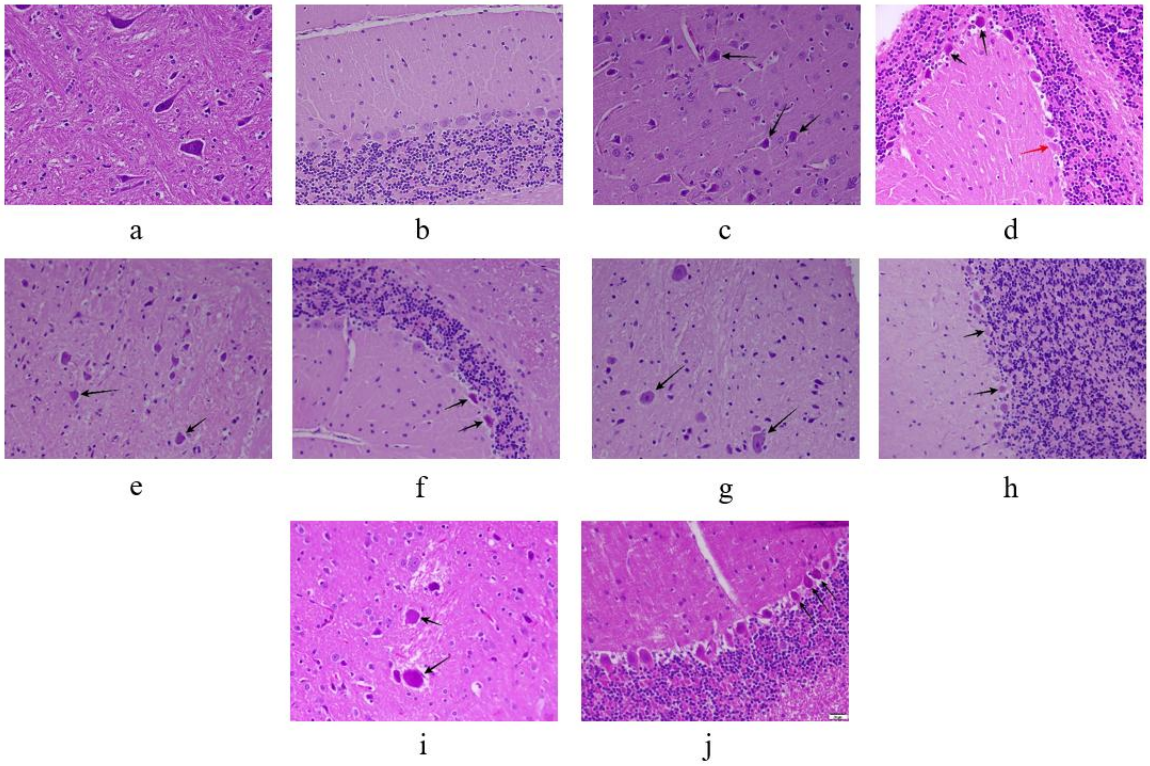


**Figure 4.** Histopathological study and microscopic sections of cerebrum and cerebellum in control rats (a: cerebrum, b: cerebellum), depressed rats (c: cerebrum, d: cerebellum), aged rats (e: cerebrum, f: cerebellum), oleuropein +

depressed rats (g: cerebrum, h: cerebellum), and oleuropein + aged rats (i: cerebrum, j: cerebellum), HandE staining, 40X. Black and red arrows represent degeneration and necrosis respectively.

**Table 1.** Lesions observed in the cerebrum and cerebellum of animals

| Groups | Animal No. | Cerebrum | Cerebellum | | |
|---|---|---|---|---|---|
| | | Bleeding | Neuronal Degeneration | Gliosis | Necrosis in Purkinje Cells |
| Control | 1 | - | - | - | - |
| | 2 | - | - | - | - |
| | 3 | - | - | - | - |
| | 4 | ++ | - | - | - |
| | 5 | - | - | - | - |
| | 6 | - | - | - | - |
| | 7 | - | - | - | - |
| | 8 | - | - | - | - |
| Depressed rats | 1 | - | + | - | - |
| | 2 | - | + | - | - |
| | 3 | - | + | - | - |
| | 4 | - | ++ | - | - |
| | 5 | ++ | + | - | - |
| | 6 | + | + | - | - |
| | 7 | - | ++ | - | - |
| | 8 | - | + | - | - |
| Aged rats | 1 | ++ | ++ | - | - |
| | 2 | + | + | - | - |
| | 3 | - | + | - | - |
| | 4 | + | - | - | - |
| | 5 | ++ | + | - | - |
| | 6 | - | - | - | - |
| | 7 | - | - | - | - |
| | 8 | - | - | - | - |
| Oleuropein + aged rats | 1 | - | +++ | - | + |
| | 2 | - | - | - | - |
| | 3 | - | - | - | - |
| | 4 | - | - | - | - |
| | 5 | +++ | - | - | - |
| | 6 | +++ | - | - | - |
| | 7 | - | - | - | - |
| | 8 | - | - | - | - |
| Oleuropein + Depressed rats | 1 | - | - | - | + |
| | 2 | - | - | - | - |
| | 3 | - | - | - | - |
| | 4 | - | - | - | - |
| | 5 | + | - | - | - |
| | 6 | +++ | - | - | - |

"**-**"= no observation, "**+**"= mild, "**++**"= moderate, "**+++**"= severe.

Additionally, histopathological analyses of the brain demonstrated neuronal degeneration and necrosis, further substantiating the aging phenotype induced by D-galactose. These results reinforce the methodological foundation of the study and validate the use of D-galactose as an effective tool for replicating aging-related biochemical and behavioral changes in experimental models.

## 4. Discussion

The role of neurotransmitters as regulators of mood have been discussed in many animal and human models. It is evident that these compounds are involved in brain biochemical and physiological functions (Rodríguez-Landa and Contreras, 2003). During cognitive decline associated with aging and in the depression state, the levels of neurotransmitters, including serotonin, dopamine, norepinephrine, tyramine, tryptamine, and acetylcholine, are depleted in the brain. Phytonutrients as dietary supplements play a role in the prevention of some neurodegenerative disorders including depression, dementia and Alzheimer's disease. Their direct role

based on the antioxidant ability of phytochemical constituents have been discussed (Barua et al., 2023). Inhibition of AChE and MAO through *Syzygium cumini* leaves extract studied by Borba et al. (2022) revealed that the phytochemical composition of the ethanolic extract of *S. cumini* leaves inhibited the in vitro AChE and MAO activities with IC50 values of 44.54 and 432.7 µg/mL, respectively. They found that extract inhibited AChE activity in vitro but not ex vivo in mice's frontal cortex.

In our study, we assessed the potential of dietary supplementation with olive leaf extract containing 20% oleuropein in maintaining the neurotransmitters levels in the brains of aged and depressed animal models. The enzyme MAO metabolizes neurotransmitters such as serotonin, dopamine, norepinephrine, tyramine, tryptamine, while the enzyme AChE metabolizes acetylcholine. These enzymatic processes play a pivotal role in both the central nervous system and peripheral organs, particularly during the aging process (Mathew et al., 2019). MAO plays a role in the breakdown of neurotransmitters, and its activity has been associated with increased production of reactive oxygen species (ROS), contributing to oxidative stress. Elevated oxidative stress is implicated in the aging process. As the body ages, there is often an increase in MAO activity, leading to higher levels of ROS. Oxidative stress can damage cellular components, contributing to age-related decline and the development of various age-related disease such as depression. According to our literature review, no prior in vitro and in vivo studies are reported regarding assessing MAO enzyme activity with oleuropein treatment as an inhibitor whereas Miceli et al. (2018) explored the effect of oleuropein aglycone as a nutraceutical on MAO-A (the isomeric form particularly significant in the context of depression)-induced autophagy impairment and cardiomyocyte death. They induced overexpressed MAO, resulting in elevated serotonin degradation and increased hydrogen peroxide production, leading to oxidative damage and cellular necrosis. Treatment with oleuropein aglycone countered the cytotoxic effects of MAO-A by activating autophagy and demonstrated potential for cardioprotection through nuclear translocation and the activation of the transcriptional factor EB.

Inhibition of AChE, the key enzyme in the breakdown of acetylcholine, is considered one of the treatment strategies against several neurological disorders such as Alzheimer's, dementia, aging, anxiety, and depression. Omar et al. (2018) evidenced the AChE inhibitory effect of olive bio-phenols with IC50 value of 55.44 µM in vitro. They suggested that olive bio-phenols could be promising natural inhibitors, which may reduce the toxicity associated with the oxidative stress involved in the progression of Alzheimer's disease which is consistent with our findings.

Gouvinhas et al. (2022) evaluated the anti-neurodegenerative potential of olive seed extracts from three cultivars in vitro (Cobrançosa Galega Vulgar and Picual). They illustrated that ultrasound-assisted extracts from olive seeds have potent antioxidant capacity and also the ability to inhibit AChE with IC50 values of different cultivars against AChE as; 105.82 ± 17.84, 30.68 ± 12.20, 186.37 ± 18.61 µg/ml and compared their results with the IC50 value of Galantamine as a reference compound (25.03 ± 3.01µg/ml). In our current study we observed elevation in AChE activity in brain homogenates of the depressed and aged groups, potentially associated with changes in AChE synthesis, release, and choline uptake in these rat models. Notably, the groups receiving 20% oleuropein pretreatment exhibited a significant decrease in AChE activity. This effect of oleuropein could be attributed to its possible direct enzyme activity modulation as well as its neuroprotective effects which could involve preserving neuronal integrity (for example through its antioxidant effects) and influencing the turnover of AChE (Gouvinhas et al., 2022; Romero-Márquez et al., 2023). These encouraging results obtained from both MAO and AChE enzyme studies clearly demonstrate the potency of in vivo intraperitoneal application of the 20% oleuropein extract in inhibiting the increase of both enzyme levels in both aged and depressed settings.

Neuronal defense mechanisms against oxidative stress are exerted via antioxidant systems (Olufunmilayo et al., 2023). Therefore, we assessed plasma antioxidant systems using two methods: the determination of plasma SOD and MDA levels. Our results revealed a significant decrease in SOD activity in aged and depressed animal groups, indicative of heightened oxidative stress compared to the control group. Pretreatment with 20% oleuropein extract increased the SOD activity in both pretreated groups compared to the depressed and aged groups, implying a reduction in oxidative stress commonly associated with depression and aging. In our study, pretreatment with a 20% oleuropein extract not only did not result in decreased MDA levels but also yielded a conflicting increase in the pretreated depressed group compared to its untreated counterpart. A similar trend was observed with PAI-I activity. These findings suggest that the percentage of oleuropein in the assessed supplementary extract together with the other phytochemical constituents might be sufficient in exerting positive SOD effects but inadequate to exhibit desired MDA levels in plasma and furthermore, the conflicting increase in MDA levels could also be linked to the possible undesirable effects of other components of the extract in the lipid oxidation pathway. Moreover, this could also suggest that oleuropein favors the pathway involving SOD rather than lipid peroxidation (MDA) pathway since antioxidants often have specific targets in the oxidative stress pathways. A similar argument can also be made regarding PAI-1 results in which no desirable effects were observed.

Sarbishegi et al. (2014) explored the impact of oleuropein, on substantia nigra protection in elderly rats.

Their findings demonstrated that oleuropein effectively mitigated the oxidative damage in substantia nigra by enhancing key antioxidant enzyme activities. The animals were administrated a daily oral dose of 50 mg/kg for a duration of 6 months, resulting in increased antioxidant enzyme levels (SOD, catalase and glutathione peroxidase) and reduced lipid peroxidation within the midbrain compared to the control group ($P<0.05$). Shibani et al (2019) delved into the impacts of oleuropein in counteracting the neurotoxic effects on the hippocampus and memory caused by morphine in rats. Their research focused on the molecular underpinnings involving the suppression of neuronal apoptosis and oxidative stress within the hippocampus region of rats subjected to morphine treatment. Through their investigation of MDA levels, as well as SOD and glutathione peroxidase, they demonstrated that oleuropein administration effectively enhances special learning and memory deficits.

Substantial evidence suggests that the biochemical alterations linked to the aging process can significantly elevate the likelihood of an individual developing depression. In the present study while most of the biochemical assessments demonstrate significant anti-depressant and anti-aging effects, the lack of significant effect in behavioral tests could be due to various factors. Possible reasons might include the specific mechanisms involved in the behavioral tests, which may not fully capture the nuanced effects of the olive leaf extract. Additionally, individual variations in the animals' response to the extract, the dosage use, and the duration of the study could have influenced the outcomes. Badr et al. (2020) investigated the effects of various oleuropein concentrations on corticosterone-induced depression in mice over a 21-day period. The study assessed oleuropein's impact through various biochemical analyses, including lipid peroxidation, reduced Glutathione, and the analysis of biogenic amines (serotonin, dopamine, and nor-epinephrine levels in brain homogenates). Additionally, three different behavioral tests (TST, open-field test, and FST) were conducted through which they demonstrated that oleuropein administration mitigated the adverse effects of corticosterone on both biochemical and behavioral parameters. While our biochemical analyses align with the mentioned study, our observations in the TST and FST tests did not indicate a noticeable effect of the 20% oleuropein pretreatment on animal behavior. This observation might again be linked to the concentration of oleuropein utilized in our study or perhaps even the difference in the induction method employed by both studies.

Ayoub et al. (2016), in their study on Swiss albino mice, stated that neurons in animals with chronic unpredictable mild depression were small and with narrow cytoplasm and dark dense nuclei, and based on this, they suggested that depression may be associated with accelerated aging. In the article published by Sibille (2013), it is stated that depression affects the brain through methods such as changes in monoaminergic neurotransmitter substances, disruption of stress hormone balance, metabolic deterioration, immune reaction, increase in inflammation, oxidative stress and mitochondrial dysfunction, and that aging also affects the brain with similar mechanisms. This suggests that major depression may be related to accelerated aging.

Our findings observed in the brains of the depressed group, although not exactly the same but similar with the results, reported by Ayoub et al. (2016), is similar to chronic unpredictable mild stress findings. This can be considered as evidence that depression and stress can produce similar lesions in the brain. In addition, considering that animals with gliosis are also animals with degenerative/necrotic changes, this suggests that glial cells are there to remove necrotic neuron residues from the area. When the lesions in the cerebellum were examined, generally similar and severe changes were detected in the Purkinje cells in the brain and cerebellum. This finding indicates that depression may have similar negative effects on the brain and cerebellum.

When the histopathological changes in the aging group are examined, the data obtained are the same as the control depression group. This can be interpreted as depression may have effects similar to aging on the brain or may lead to premature aging in the brain. This situation is consistent with the information put forward by Sibille (2013). When the lesions in the cerebellum are examined, lesions of similar shape and severity as in the control depression group are noted. Aging, as with depression, can have negative effects on the cerebellum similar to those on the brain.

It is noteworthy that the pretreated depression group yielded a relative decrease in the number of animals with lesions in the cerebrum compared to the control depression group, while no significant change was observed in cerebellar lesions. This suggests that while oleuropein can partially prevent cerebrum lesions, it does not have a serious effect on the cerebellum. Lesions in the brain in the pretreated aged group were compared to the untreated aged group. While there is no decrease in the number of animals with lesions, there is a relative increase in their severity. This situation occurred similarly in the cerebellum, and a significant increase was observed in both the number of animals with lesions and the severity of the lesions. This suggests that oleuropein is insufficient to stop the symptoms caused by aging and may even increase them relatively. This situation is not compatible with the data in the literature (Özcan and Matthäus, 2017; Lee et al., 2018; Talhaoui et al., 2018; Romero-Márquez et al., 2023).

The loss of Nissl bodies is often associated with reduced protein synthesis capacity and increased metabolic stress. This phenomenon is linked to processes such as oxidative stress, mitochondrial dysfunction, and excitotoxicity. In neurodegenerative conditions like aging and depression, the loss of Nissl bodies serves as a hallmark feature. Studies suggest that this loss is related

to mitochondrial stress, leading to decreased neurotransmitter levels, which in turn impact cognitive and motor functions (Sibille, 2013). Notably, the loss of Nissl bodies is more pronounced in regions with high oxidative stress, such as the hippocampus and frontal cortex (Miceli et al., 2018).

Gliosis, characterized by the activation of astrocytes and microglia in response to neuronal damage, plays a critical role in repairing damaged tissue and clearing dead cells. However, chronic gliosis can exacerbate neuroinflammation and accelerate neurodegeneration. Conditions such as aging and depression are marked by increased microglial activation, especially in the hippocampus, which has been associated with depressive behaviors (Jiang et al., 2023). Furthermore, gliosis has been shown to reduce serotonin and dopamine levels in the brain, contributing to behavioral outcomes (Shibani et al., 2019).

Microhemorrhages in brain tissue are another histological change observed in neurodegenerative conditions like aging and depression. These hemorrhages are triggered by endothelial dysfunction caused by oxidative stress and inflammation. Resulting local hypoxia adversely impacts neuronal metabolism (Godinez et al., 2022). Additionally, microhemorrhages disrupt energy metabolism in the brain and, when combined with gliosis, lead to more extensive neuronal damage (Reyes-Goya et al., 2024).

Neuronal degeneration, particularly in the frontal cortex and hippocampus, is closely linked to cognitive and emotional dysfunction in conditions such as depression and aging. The loss of Nissl bodies and neuronal necrosis in these regions results in neurotransmitter deficiencies, leading to motor dysfunction and emotional instability (Ayuob et al., 2016). Several studies have demonstrated a direct relationship between these histological changes and reduced motor activity, as well as depressive behaviors (Miceli et al., 2018).

Gliosis and the associated neuroinflammation have significant behavioral implications. Increased gliosis, especially in the hippocampus, has been shown to intensify depressive behaviors (Sibille, 2013). Neuroinflammation disrupts serotonergic and dopaminergic signaling, leading to emotional and motor impairments (Shibani et al., 2019).

Similarly, microhemorrhages impair oxygen delivery and energy metabolism in the brain, resulting in behavioral changes. The effects of hemorrhages in the frontal cortex and hippocampus have been linked to depression-like behaviors (Jiang et al., 2023). These changes manifest as reduced motor function and impaired emotional regulation (Reyes-Goya et al., 2024).

## 5. Conclusion

This investigation into the neuroprotective effects of a 20% oleuropein-containing olive leaf extract in our animals yielded noteworthy outcomes. The inhibition of MAO and AChE enzymes demonstrated significant promise, indicative of a potential therapeutic impact on neurotransmitter homeostasis. Concurrently, divergent results emerged from the MDA antioxidant assay and PAI-1 levels, presenting conflicting data. These intricate findings suggest an exact relationship between the olive leaf extract and neurobiological processes. While the positive results from MAO, AChE and SOD activities align with neuroprotective potential, the discordant results in other parameters underscore the complexity of the supplement's impact. It is imperative to recognize the multifactorial nature of these outcomes, acknowledging the possibility of dualistic effects on oxidative stress and pathological markers.

Considering these intricacies, our study advocates for a comprehensive and cautious interpretation of data. Further investigations are indispensable to unravelling the underlying molecular mechanisms governing the observed effects and to delineate the precise dynamics of the olive leaf extracts' influence on neurobiological processes. This research provides a foundation for future inquiries, considering a spectrum of parameters in assessing the holistic impact of neuroprotective interventions.

**Author Contributions**

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

| | S.T. | K.E. | R.A. | E.L.N.D. | K.F. |
|---|---|---|---|---|---|
| C | 30 | 30 | 20 | 10 | 10 |
| D | 70 | 30 | | | |
| S | 100 | | | | |
| DCP | | 30 | 30 | 20 | 20 |
| DAI | 50 | 30 | | | 20 |
| L | 20 | 20 | 20 | 20 | 20 |
| W | 50 | 20 | 10 | 10 | 10 |
| CR | 20 | 20 | 20 | 20 | 20 |
| SR | 50 | 20 | 10 | 10 | 10 |
| PM | 20 | 20 | 20 | 20 | 20 |
| FA | 20 | 20 | 20 | 20 | 20 |

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

**Conflict of Interest**

The authors declared that there is no conflict of interest.

**Ethical Consideration**

The Animal laboratory Ethic Committee of Üsküdar University, İstanbul, Türkiye (UU-HADYEK), approved all the experimental procedures (approval date: 12 June 2020, protocol code: 2020-06).

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