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Investigation of the protective and therapeutic efficacy of *Myrtus communis* extract in aluminum chloride and D-galactose-induced Alzheimer's disease in rats

Kübra YALMAN[1], Ali ŞEN[2], Özge ÇEVIK[3], Beril KADIOĞLU-YAMAN[1], Büşra ERTAŞ[4], Sıla YILDIZ[4], Göksel ŞENER[5]*

1 Department of Pharmacology, Faculty of Pharmacy, Yeditepe University, Istanbul, Turkey.
2 Department of Pharmacognosy, Faculty of Pharmacy, Marmara University, Istanbul, Turkey.
3 Department of Biochemistry, Faculty of Medicine, Aydın Adnan Menderes University, Aydın, Turkey.
4 Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey.
5 Department of Pharmacology, Faculty of Pharmacy, Fenerbahce University, Istanbul, Turkey.
* Corresponding Author. E-mail: goksel.sener@fbu.edu.tr; gokselsener@hotmail.com (G.Ş.); Tel. +90 216 414 29 62.

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ABSTRACT: This study investigated the possible protective and therapeutic effects of *Myrtus communis* subsp. *communis* ethanol extract (MC) in aluminum chloride ($AlCl_3$) and D-galactose (D-Gal) induced Alzheimer's disease in rats. MC was orally given to rats as a protective treatment for 90 days and, in other two groups starting from the 60th day MC (100-200 mg/kg) was administered, concomitantly with $AlCl_3$ and D-Gal. Learning and memory functions were evaluated by the behavioral tests. Biological activities of MC treatment were examined in hippocampal tissues by ELISA tests. D-Gal and $AlCl_3$-treated rats showed increased amyloid beta (Aβ) and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels, acetylcholinesterase activity and decreased neprilysin, Na^+-K^+ATPase and SOD levels in parallel with a decrease in Novel Object Recognition Test, Morris Water Maze and Passive Avoidance Test scores. On the other hand, MC administration reversed the behavioral impairments and improved learning and memory. Moreover, MC treatment decreased Aβ and 8-OHdG levels and acetylcholinesterase activity and increased neprilysin levels, Na^+-K^+ATPase and SOD levels. Our results suggest that MC has beneficial effects on cognitive and neuronal functions through its anticholinesterase and antioxidant properties.

KEYWORDS: Myrtle; antioxidant activity; anticholinesterase activity; cognitive impairment

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease mainly characterized by memory loss and cognitive impairment. In the early stages of the disease, patients may experience mild cognitive impairment. However, since it is a progressive disease, symptoms may deteriorate, patients may lose their ability to continue with their daily activities and their quality of life may worsen over years [1,2]. It is estimated that 75% of people with dementia worldwide are undiagnosed and it is concerned that the number of cases will be reaching 78 million by 2030 [3]. Main neuropathological hallmarks of the disease include the formation of amyloid-beta (Aβ) plaques, hyperphosphorylation of tau protein and formation of neurofibrillary tangles, neuroinflammation and cholinergic neuron degeneration [4]. Aβ oligomers can disrupt membrane bilayers leading to oxidation of intracellular protein and nucleic acids and the production of reactive oxygen species (ROS). The cellular antioxidant defense system generally overcomes these abnormalities but where ROS production exceeds, this condition is considered as oxidative stress. Oxidative stress also plays a role in the early stage of AD pathogenesis and can be used as a biomarker of AD progression. Therefore, controlling oxidative stress is a promising therapeutic strategy that may provide new options for the prevention and treatment of AD [5,6].

Currently, there are limited treatment options for Alzheimer's disease. Acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) are used in mild/moderate stages and glutamate receptor (NMDA) antagonist (memantine) is used in moderate/severe stages for the treatment of AD. In 2021, a monoclonal antibody (aducanumab) that inhibits the formation of Aβ plaques was approved by the FDA for early-stage

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patients as the first "disease-modifing agent" [7]. However, the role of Aβ in the treatment of Alzheimer's disease is controversial, and disagreements regarding the approval of Aducanumab raise doubts for patients, clinicians, and also researchers [8,9]. Therefore, more comprehensive studies are needed for Alzheimer's disease treatment.

Aluminum is a neurotoxic metal found in food industry, daily used cosmetic products, and environmental sources such as a residue of acid rain which accumulated on vegetables and fruits, and chronic exposure is involved in the pathogenesis of AD. Chronic exposure to Aluminum chloride ($AlCl_3$) leads to cognitive impairment, cholinergic dysfunction and oxidative damage in rat brain [10-12]. Co-administration of $AlCl_3$ and the senescence agent D-galactose (D-Gal), induces neurotoxicity and is used as a good model for investigating AD-like pathologies resulting in cholinergic neuron degeneration and increased oxidative stress [13-15].

In recent years, compounds of natural origin have been frequently the subject of research as potential agents in the treatment of neurodegenerative diseases [16]. 'Myrtle' is the name for *Myrtus communis* L. (Myrtaceae), has a long history as a medicinal plant that is mentioned in Dioscorides' (40-90 AD) Materia Medica in the Mediterranean region and Asia [17]. Experimental studies have shown that it has a wide range of pharmacological and therapeutic effects. The widespread use of *M.communis* in traditional medicine and the introduction of its pharmaceutical forms (such as topical ointments and drops) into the industry necessitate more detailed study, clarification and knowledge of the phytochemical, pharmacological and toxicological properties of the plant. Neuroprotective effects of *M. communis* were first demonstrated by an *in vitro* study of Tumen et al [17], investigating the inhibitory potential of the leaves and berries of *M. communis* L. (myrtle) against acetylcholinesterase (AChE) and butyrylcholinesterase enzymes. Acetylcholine (ACh) and the cholinergic system is the main neurotransmitter system affected in cognitive impairment. Acetylcholinesterase and butyrylcholinesterase are two enzymes responsible for the degradation of ACh, which AChE is present in the hippocampus and the cerebral cortex, and butyrylcholinesterase in the plasma. Inhibition of AChE results in an increase in ACh levels and therefore enhances cholinergic transmission, still being the main target to ameliorate the cognitive impairment in mild to moderate AD [18]. Therefore, we investigated both the protective and therapeutic efficacy of *Myrtus communis* extract (MC) in aluminum chloride and D-galactose induced Alzheimer's disease model in rats.

2. RESULTS

2.1. Novel object recognition test

Results of the NORT showed that the AD group score was significantly lower ($p<0.01$) than control, but all animal performances in MC treatment and donepezil groups were significantly higher ($p<0.001$) when compared to the AD group (Figure 1).

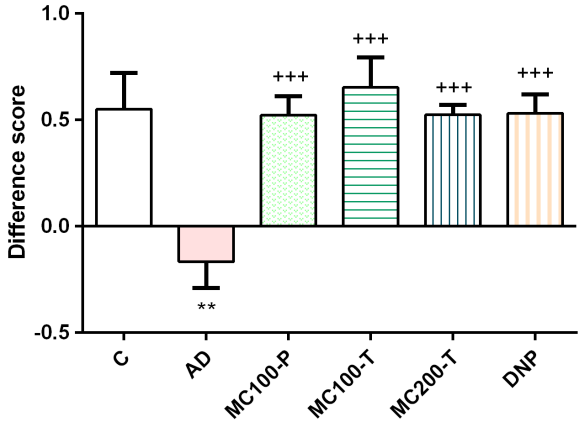

Figure 1. Evaluation of new object recognition test via difference scores.
C: Control, AD: Alzheimer's disease model, MC100-P: *Myrtus communis* extract protective treatment group, MC100-T: *Myrtus communis* treatment group, MC200-T: *Myrtus communis* treatment group, DNP: Donepezil. **p<0.01 vs. control group; +++ p<0.001 vs. AD group.

2.2. Morris water maze test

Figure 2 shows the latency to find the escape platform over four consecutive days. The performances of all groups improved throughout the days. Subsequent comparisons further showed that a statistically significant difference was observed between the control and all other groups, except the MC100-T group. Also, all treatment groups found the platform in a shorter time than the AD group.

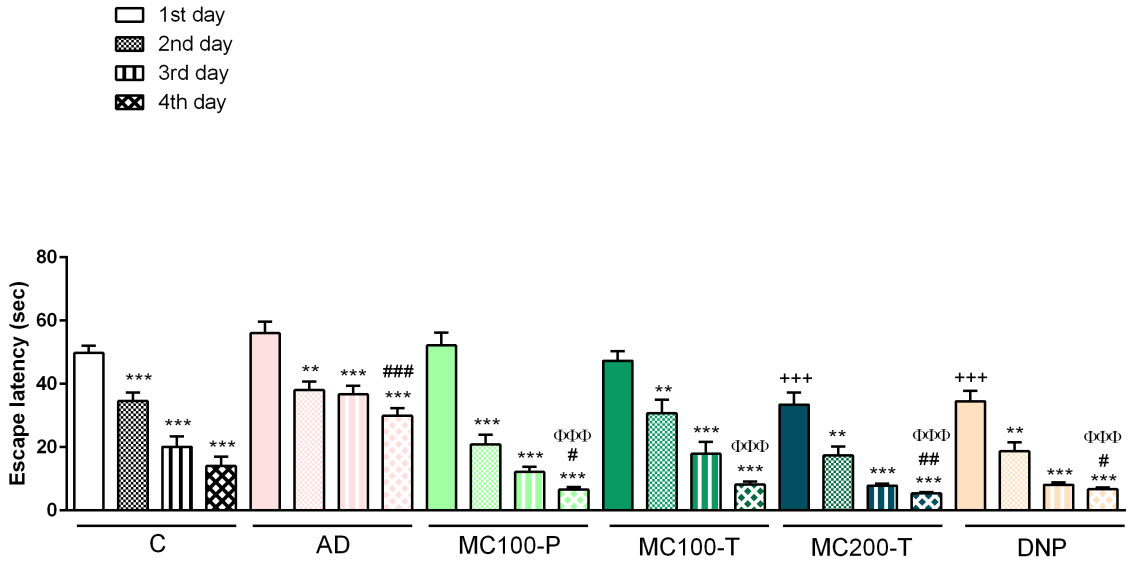

Figure 2. Escape latency to reach the platform during acquisition trials.
C: Control, AD: Alzheimer's disease model, MC100-P: *Myrtus communis* extract protective treatment group, MC100-T: *Myrtus communis* treatment group, MC200-T: *Myrtus communis* treatment group, DNP: Donepezil. ***p<0.001, **p<0.01 vs. each group's own 1st day; ### p<0.001, ## p<0.01, # p<0.05 vs. the 4th day of the control group; +++ p<0.001 vs. the 1st day of the AD group; ϕ ϕ ϕ p<0.001 vs. the 4th day of the AD group.

In the probe trial test (Figure 3), there was a statistically significant decrease in time spent in the target quadrant in the AD group compared to the control group. Moreover, the time spent by MC groups in the target quadrant was significantly longer compared to the AD group.

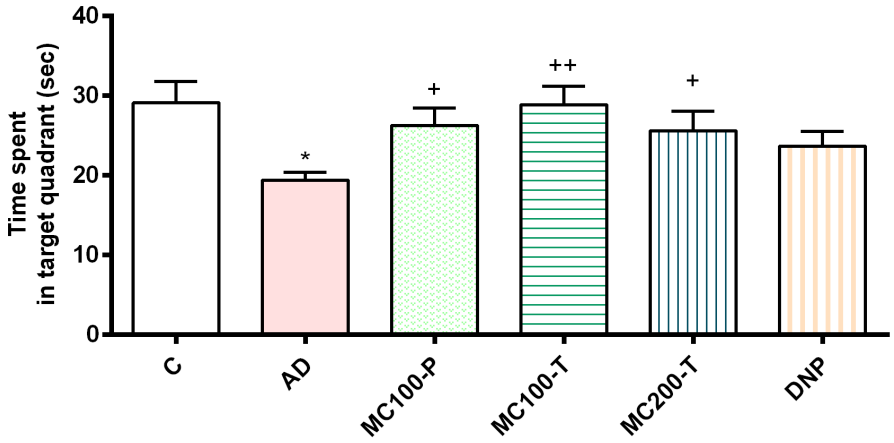

Figure 3. Time spent in the hidden platform quadrant in the probe trial.
C: Control, AD: Alzheimer's disease model, MC100-P: *Myrtus communis* extract protective treatment group, MC100-T: *Myrtus communis* treatment group, MC200-T: *Myrtus communis* treatment group, DNP: Donepezil. *p<0.05 vs. control group; ++ p<0.01, + p<0.05 vs. AD group.

2.3. Passive avoidance test

The retention time of an unpleasant stimulus (electric shock) was found to decline significantly in the AD group compared to the control group. The analysis showed a significant increase in latency time of passive avoidance task score in the treatment groups when compared with the AD group ($p < 0.001$, Figure 4).

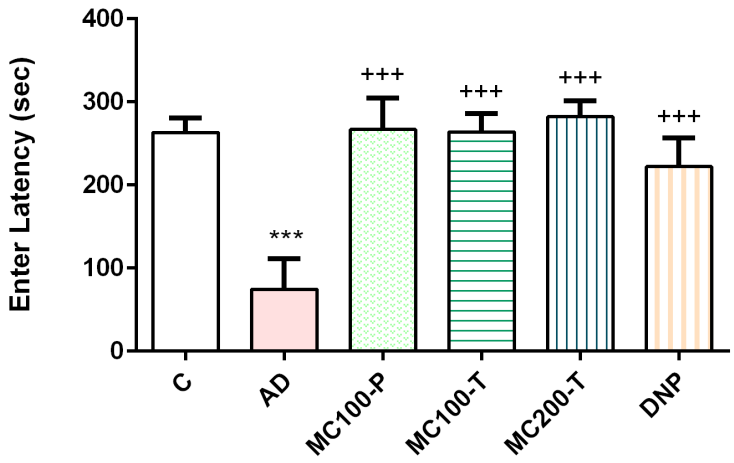

Figure 4. Evaluation of passive avoidance test results
C: Control, AD: Alzheimer's disease model, MC100-P: *Myrtus communis* extract protective treatment group, MC100-T: *Myrtus communis* treatment group, MC200-T: *Myrtus communis* treatment group, DNP: Donepezil. ***p<0.001 vs. control group; +++ p<0.001 vs. AD group

2.4. Biochemical analysis

The rats treated with $AlCl_3$ and D-Gal showed significant increase in the amyloid β levels ($p < 0.001$) and AChE activity ($p < 0.01$). The AChE activity were significantly decreased in the rats treated with MC ($p < 0.01$) and donepezil (DNP) ($p < 0.01$ Figure 5). The amyloid β level was significantly decrease in the protective group (MC100-P) ($p < 0.001$) and the group treated with 100 mg/kg MC for 30 days (MC100-T) ($p < 0.05$) compared to the AD group (Figure 5).

When the control group and the AD group were compared, a significant decrease was observed in the levels of Neprilysin in the AD group ($p < 0.01$, Figure 5). When the MC100-P, MC200-T and DNP groups were examined, it was found that there was a significant increase in Neprilysin levels compared to the AD group.

8-OHdG level was significantly increased in the AD group compared to the control group ($p < 0.01$). It was found that there was a significant decrease in 8-OHdG levels in all groups (Figure 5).

The Na^+-K^+ATPase and SOD levels were lower in the AD group compared to the control group ($p < 0.01$, Figure 5). When the DNP group was compared with the AD group, it was observed that there was a significant increase in Na^+-K^+ATPase levels of the DNP group ($p < 0.05$, Figure 5). Although there are no significant differences in MC treatment groups, MC treatment increased the Na^+-K^+ATPase and SOD levels (Figure 5).

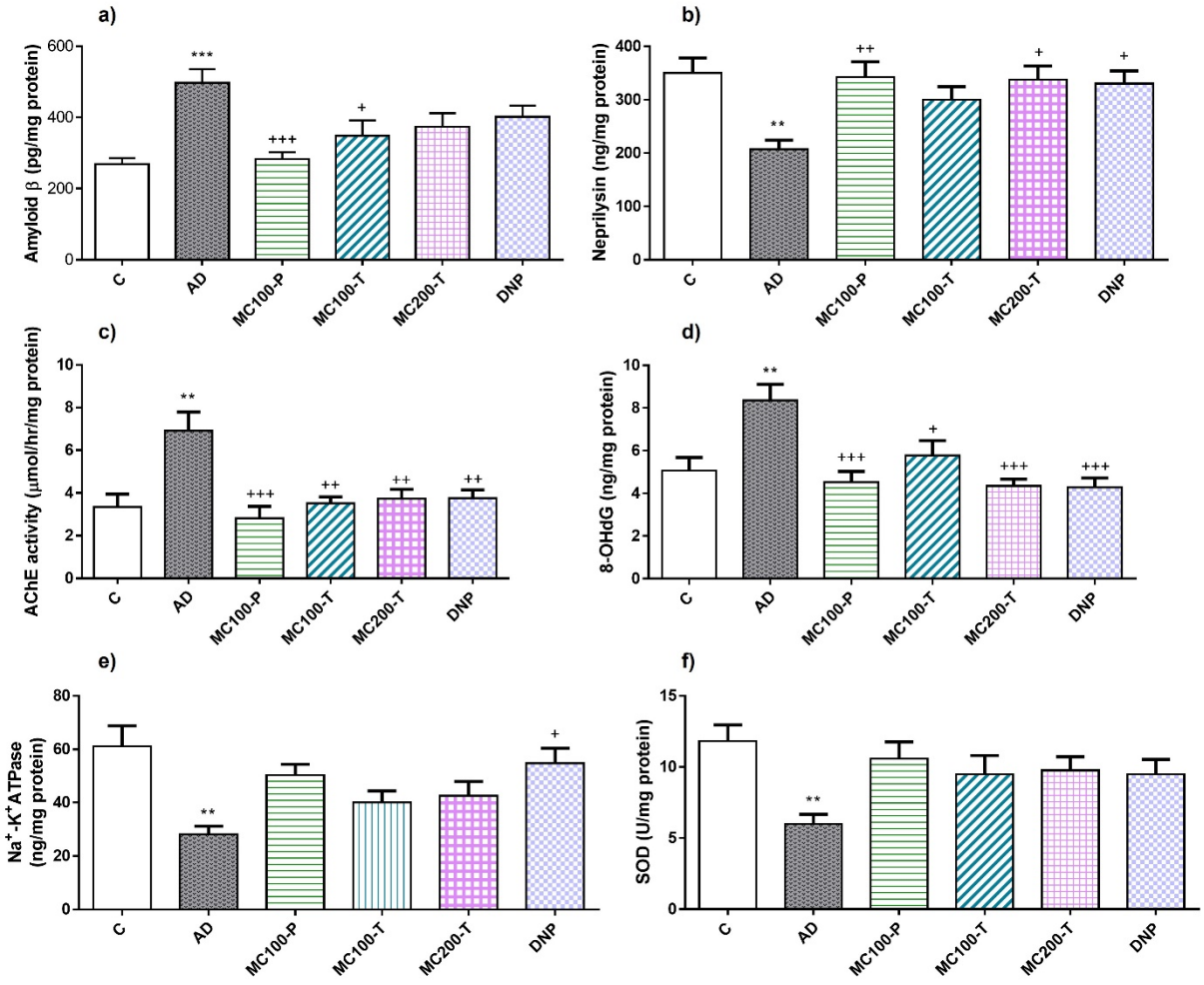

Figure 5. a) Amyloid beta (Aβ), b) Neprilysin, c) Acetylcholine esterase (AChE) activity, d) 8OHdG, e) Na+-K+ATPase, and f) SOD levels in hippocampal tissue of rats.
C: Control, AD: Alzheimer's disease model, MC100-P: *Myrtus communis* extract protective treatment group, MC100-T: *Myrtus communis* treatment group, MC200-T: *Myrtus communis* treatment group, DNP: Donepezil. * p<0.05, **p<0.01, ***p<0.001 vs. control group; + p<0.05, ++ p<0.01, +++ p<0.001 vs. AD group.

3. DISCUSSION

AD is a multifactorial disorder with cognitive decline and dysregulation of neuronal signaling [19]. A growing number of studies showed the application of D-gal or aluminum mimics the pathophysiological changes of AD [6,12,15,20,21,22]. Since aluminum accumulated in different brain areas including the cortex and hippocampus which is important for learning and memory induces neurotoxicity and the generation of free radicals. Therefore, excessive intake of aluminum leads to learning and memory impairment and cognitive dysfunction and can be a major risk factor for AD. Besides $AlCl_3$, D-gal induces oxidative stress by triggering reactive oxygen species (ROS) formation in the brain [23,24]. Our study indicated that exposure to D-Gal and $AlCl_3$ combination leads to cognitive impairment and induces Aβ pathology and oxidative stress. The results show that performances in both spatial and non-spatial memory tests are worse in AD model rats in parallel with biochemical parameters.

Various neurotransmitters have a crucial role in neuroregulation and pathological changes of these neurotransmitters may lead to behavioral deficits. The cholinergic hypothesis suggests that hypofunction of cholinergic neurons is the main reason for cognitive impairment. Therefore, increasing the concentrations of ACh is expected to improve cognitive functions in AD [25]. Since AChE enzyme is responsible for metabolizing ACh, increased AChE enzyme activity is found to be related to cognitive decline in patients with AD [26]. In our study, AChE activity was increased in D-Gal and $AlCl_3$ treated rats. Administration of MC (both protective and treatment) decreased AChE activity as well as DNP. MC protective group is most effective

in decreasing AChE activity. The deposition and accumulation of Aβ result in the formation of amyloid plaques, resulting in neuronal signaling and synaptic toxicity in the brain. Cognitive impairment is considered as a result of neuronal damage caused by an imbalance between the formation and degradation of Aβ [27].

Neprilysin is an enzyme responsible for the degradation of Aβ. It regulates the levels of amyloid peptides and prevents Aβ accumulation and plaque formation in the brain [28]. In our study, the rats in the AD group showed increased levels of Aβ and decreased levels of neprilysin. The protective MC administration significantly increased Neprilysin levels and reduced Aβ levels. In our previous study, we showed that treatment with MC extract beside an inhibitory effect on AChE activity, it increased neprilysin levels and decreased Aβ levels in the ovariectomized diabetic rats [29].

In the light of the previous studies, there is an important link between oxidative stress and Alzheimer's pathologies. Indeed, it induces amyloid beta accumulation and neurodegeneration. The 8-OHdG is the most common marker of oxidative DNA damage in AD patients' brains [5,30,31]. In the present study, the 8-OHdG level was significantly increased in the AD group. On the other hand, MC protective, MC treatment and DNP groups showed a significant reduction in 8-OHdG levels.

Increasing number of studies established the role of activated Na^+-K^+ATPase signaling in learning and memory by affecting different mechanisms [32,33]. In addition, there are antioxidant defense systems such as superoxide dismutase (SOD) enzyme to catalyze the dismutation of superoxide radicals and terminate the excessive ROS. The decreased activity of SOD was reported in late AD brain [34]. We observed that MC treatment increased D-gal and $AlCl_3$ induced decreases in levels of Na^+-K^+ATPase and SOD in rats. The antioxidant effect of MC was shown in several studies which is consistent with our results [35-37].

In the present study, we evaluated memory which is expected to impair in AD using behavioral tests including the novel object recognition test for recognition memory, the Morris water maze test for spatial memory, and the passive avoidance test for long-term memory [38-40]. Our behavioral study results showed that MC treated rats found the hidden platform faster during the acquisition trials and also spent more time in the target quadrant during the probe trials in MWM. Moreover, MC treatment significantly improved recognition memory in the NORT and significantly increased the latency in the retention trial of the passive avoidance test. According to the results of the behavioral tests, it can be concluded that MC significantly ameliorates recognition memory, spatial memory and long-term memory impairments induced by D-Gal and $AlCl_3$.

Our study revealed that long-term exposure to $AlCl_3$ and D-Gal causes cognitive impairment by inducing oxidative stress and reducing cholinergic activity. This is the first study that indicates preventive effect of *Myrtus communis* on D-Gal and $AlCl_3$ induced Alzheimer's disease model. Our results showed that administration of MC exerts improvement in D-Gal and $AlCl_3$ induced AD in rats by its anticholinesterase and antioxidant effects. Our previous study demonstrated that myrtle leaves have high phenolic compound content (472.7±2.36 mg/g extract as gallic acid equivalent) [41]. Also, the study of Arslan et al. demonstrated that myrtle leaves contained phenolic compounds including myricetin rhamnoside, myricetin hexoside, quercetin rhamnoside, myricetin, ellagic acid, cafeic acid derivative, sinapinic acid derivative and trihydroxy cinnamic acid derivative with LC-MS/MS analysis [42]. The phenolic compounds were found to prevent Alzheimer's disease pathology development by modulating different Aβ aggregation pathways in vivo study [43]. Moreover, previous studies have suggested that myricetin [43], ellagic acid [44], quercetin rhamnoside [45], cinnamic acid derivatives [46], caffeic acid derivatives [47] have anti-alzheimer and/or neuroprotective activity. Therefore, the phenolic compounds in MC may be responsible for the protective and therapeutic efficacy of the MC in the aluminum chloride and D-galactose-induced Alzheimer's disease model in rats.

4. CONCLUSION

Our study revealed that long-term exposure to $AlCl_3$ and D-Gal causes cognitive impairment via oxidative stress and cholinergic activity decline. This is the first study that indicates preventive effect of MC on D-Gal and $AlCl_3$ induced-AD model in rats. Our results showed that MC exerted improvement via reducing AChE activity, Aβ level and oxidative stress in AD model in rats. Therefore, it might be suggested that MC may act as a potent therapeutic agent for the prevention and treatment of AD, and further studies need to be carried out to explore the responsible pathways for the actions of MC.

5. MATERIALS AND METHODS

5.1. Preparation of MC extract

M. communis leaves identified by Dr. Gizem Bulut, a botanist in School of Pharmacy, Marmara University collected from Turgutlu region of Manisa in 2010. Voucher specimens were deposited in the Herbarium of School of Pharmacy, Marmara University (Herbarium protocol no: 13006). 100 g of MC leaves dried and powdered in shade were extracted with 96% ethanol using a Soxhlet apparatus. The extraction was continued until the solvent becomes colorless in the soxhlet loop. The resulting liquid extract was filtered and was dried at 40°C under vacuum. The resulting dry extract was stored at +4 °C in an airtight container until use.

5.2. Animal groups and treatment

Female/Male Wistar rats (200–250 gram) were provided by Marmara University Experimental Animal Implementation and Research Center, Turkey. All experimental protocols were approved and performed according to the guideline of Laboratory Animal Experiments Local Ethics Committee of Marmara University (Approval number: 79.2018.mar).

Animals were randomly divided into six groups of 10 each as follows:

Group I (Control group): Animals received normal saline given i.p and distilled water orally for 90 days.

Group II (Alzheimer Disease group): Animals received D-gal 60 mg/kg/day (i.p) and $AlCl_3$ 40 mg/kg/day (i.g) for 90 days.

Group III (MC protective treatment): Animals received D-gal 60 mg/kg/day (i.p) and $AlCl_3$ 40 mg/kg/day (i.g) along with MC (100 mg/kg/day, i.g) for 90 days.

Group IV (MC treatment low dose): Animals received D-gal 60 mg/kg/day (i.p) and $AlCl_3$ 40 mg/kg/day (i.g) for 90 days. MC (100 mg/kg/day, i.g) was administered for the last 30 days.

Group V (MC treatment high dose): Animals received D-gal 60 mg/kg/day (i.p) and $AlCl_3$ 40 mg/kg/day (i.g) for 90 days. MC (200 mg/kg/day, i.g) was administered for the last 30days.

Group VI (Positive control): Animals received D-gal 60 mg/kg/day (i.p) and $AlCl_3$ 40 mg/kg/day (i.g) for 90 days. Donepezil (3 mg/kg/day, orally) was administered for the last 30 days.

D-gal and $AlCl_3$ (dissolved in normal saline) were administered according to previous research [24]. Moreover, MC extracts were given 1 hour before D-gal and $AlCl_3$ administration. The experiment timeline shown in Figure 6.

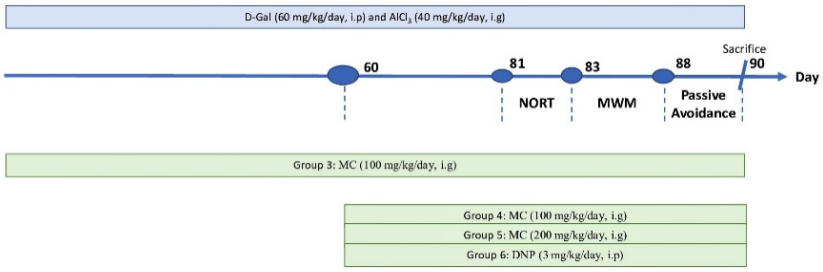

Figure 6. Depiction of the experimental timeline.

5.3. Behavioral tests

5.3.1. Novel object recognition test

The Novel Object Recognition Test is a widely used behavioral assay that evaluates the ability to recognize a previously presented stimulus for the observation of memory alteration. The NORT procedure consists of three phases: habituation, familiarization and test phase. The test was carried out in an isolated room and an open box (65 × 45 × 45 cm) was used as apparatus. In the habituation phase, animals were allowed to freely explore for 10 mins in absence of objects. After 24 h, animals were placed in the box with two identical objects (A+A) during the familiarization phase. The test phase was performed after 1 h of the familiarization phase. Animals were put back in the box with two objects: one was a familiar object and the other one was a novel object (A+B) for 3 mins. Animals' behaviors were videotaped and evaluated by the difference in discrimination time for novel versus familiar objects with the formula below. The discrimination index was calculated as given in our previous study [21]. During the phases, all objects were located in opposite and symmetrical corners of the box and animals were placed in the opposite direction to the object at the beginning of each phase.

5.3.2. The Morris water maze test

The Morris water maze (MWM) was performed to evaluate spatial learning and memory in an open circular swimming tank with opaque water (to eliminate the visual image of the platform) [48]. The tank was divided into 4 fixed points (N, E, S, and W) on its perimeter to 4 quadrants. A circular escape platform (10×10×10 cm) was submerged 1.5 cm below the water surface in the one of the quadrants (target quadrant). The test included acquisition and probe trial. The acquisition trial was performed in the first 4 days, where each rat was placed gently facing the wall at the edge of a quadrant with no escape area from different start points (N, E, S or W). If the rat failed to find the escape platform within 75 s, it was gently guided to the platform and allowed to stay on it for 20 s. On probe trial on the 5th day of the experiment, the platform was removed. The rats were allowed to swim in the pool for 60 seconds. The probe trial was recorded with a video camera and the time each rat spent in the target quadrant and the time latency to reach it were evaluated [22,49,50].

5.3.3. The Passive Avoidance test

The Passive Avoidance test apparatus (Northel Passive Avoidance System, Istanbul) consists of a two-compartment dark/light shuttle box (20×20×20 cm) which is separated by a guillotine door. The guillotine door opening (6 cm×6 cm) was made on the floor in the center of the partition between the two compartments. The floor of both chambers consisted of stainless-steel rods to produce foot shock. In the acquisition trial, each animal was gently placed in the light compartment (with a 100W bulb); after 5 s, the guillotine door was opened and the animal was allowed to enter the dark compartment. The door was closed and a foot shock (5 mA) was immediately delivered to the grid floor of the dark compartment for 3s as soon as the animal entered the dark compartment. After 20 s, the rat was removed and returned to its home cage. The test trial was performed 24 h after the acquisition trial. Each animal was placed in the light compartment and then, allowed to enter the dark compartment without foot shock (cut off time: 300 s). The latency to enter the dark section during retention was recorded to indicate long-term memory [51].

5.4. Biochemical analysis

The biochemical analysis was performed to examine cholinergic activity and oxidative stress markers. After the completion of behavioral tests, animals were sacrificed by rapid decapitation. Brains were removed, and the hippocampus dissected out and was stored at 80 °C. In hippocampal tissues, Aβ, neprilysin, AChE activity (Bioassay Technology Laboratory, E0093Ra, E1226Ra, E0724Ra, Shanghai, China), 8-OHdG, Na^+-K^+ATPase and SOD (Abbkine, KTE100312, KTE101085, KTE101023, China) were performed according to guidelines using enzyme-linked immunosorbent assay (ELISA) kits and following the manufacturer's instructions.

5.5. Statistical analysis

Analysis of results was performed using Graphpad Prism 6.05 (Graphpad Software, USA). All data obtained were expressed as mean ± SEM. NORT and MWM test results were analyzed with Mann Whitney U nonparametric test. Passive avoidance test results were analyzed with ANOVA followed by Bonferroni multiple comparison post hoc tests. Biochemical data were analyzed with one-way analysis of variance (one-way ANOVA) followed by Tukey multiple comparison tests. $p < 0.05$ was considered statistically significant.

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Author contributions: Concept – K.Y., B.E., S.Y., G. Ş.; Design – K.Y., B.E., S.Y., G. Ş.; Supervision – K.Y., A.Ş., B.K.Y., G.Ş.; Resources – K.Y., A.Ş., Ö.Ç., G.Ş; Materials – K.Y., A.Ş., Ö.Ç., G.Ş.; Data Collection and/or Processing – K.Y., A.Ş., Ö.Ç., B.K.Y., B.E., S.Y., G.Ş; Analysis and/or Interpretation – K.Y., Ö.Ç., G.Ş.; Literature Search – K.Y., B.K.Y., G.Ş.; Writing – K.Y., A.Ş., B.K.Y., G.Ş.; Critical Reviews – K.Y., A.Ş., Ö.Ç., B.K.Y., B.E., S.Y., G.Ş.

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