Clavulanic Acid: A Novel Potential Agent in Prevention and Treatment of Scopolamine-Induced Alzheimer’s Disease

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Cite This: ACS Omega 2022, 7, 13861−13869

ABSTRACT: Background and Aim: Alzheimer’s disease (AD) is the most common form of dementia in the elderly. It is characterized as a multifaced disorder with a greater genetic contribution. The contribution of many genes such as BDNF, Sirtuin 6, and Seladin 1 has been reported in the pathogenesis of AD. Current therapies include acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor antagonists, which are only temporarily beneficial. Therefore, it seems that more studies should be conducted to determine the exact mechanisms of drugs to deal with the diseases’ multifactorial features that we face. Methods: In this study, 42 adult rats were randomly divided into 7 groups and received drugs intraperitoneally and orally according to the protocol as follows: scopolamine group, clavulanic acid group, memantine group, scopolamine + memantine group, clavulanic acid pre- and post-treatment, and normal saline group. The Morris water maze method was performed to evaluate the spatial memory of animals, and the terminal deoxynucleotidyl transferase dUTP nick end labeling assay and real-time polymerase chain reaction were performed to study neuronal cell apoptosis and gene expression, respectively. Results: Significant differences were observed in the spatial memory of rats that received clavulanic acid prophylactically compared to the Alzheimer’s model on the day of the test. Moreover, the results obtained during the training showed that both memantine and clavulanic acid improved spatial memory by increasing the time of rats present in the platform position and by reducing the swimming time in the scopolamine-induced Alzheimer’s group. Besides, rats that received clavulanic acid and memantine had a greater percentage of healthy cells in comparison with the scopolamine-induced Alzheimer’s group; however, the results were more significant for clavulanic acid. Furthermore, the expressions of BDNF, Seladin 1, and Sirtuin 6 as neuroprotective target genes were modified after clavulanic acid and memantine administrations; similarly, the results obtained from clavulanic acid were more significant. Conclusion: The results show that the administration of clavulanic acid before and after the use of scopolamine can reduce the percentage of apoptotic cells in the hippocampus and also improve the parameters related to learning and spatial memory; however, its effect in the prophylactic state was stronger. The results obtained from memantine revealed that it has neuroprotective potency against AD; however, clavulanic acid had a greater effect. Also, with increased expression of the neuroprotective genes, clavulanic acid could be considered as an option in the upcoming preclinical and clinical research about Alzheimer’s disease.

1. INTRODUCTION

Alzheimer’s disease (AD) is a type of brain disease that usually begins in the elderly; the risk of catching the disease increases with age. It is a progressive neurodegenerative disorder characterized clinically by a decline in cognitive activity and behavior. AD is the most common form of dementia that begins with many symptoms such as loss of information, especially temporary memory in old age, face recognition difficulties, depression, loss of speech, and finally ends with respiratory distress and death.1,2 The respiratory distress is the result of a disruption of the creation of nerve synapses and the necrosis of brain cells in some areas of the brain and the formation of spherical protein structures called aging plaques along with the neurofibrillary tangles in the neuron’s core.3 Studies have shown that the expression of some genes, such as BDNF, Sirtuin 6, and Seladin 1, are dysregulated in patients with AD.4−7

Received: January 12, 2022
Accepted: March 29, 2022
Published: April 13, 2022
Patients who suffer from Alzheimer’s disease are treated with symptomatic and disease-modifying therapies. All approved drugs for the treatment of AD modulate neurotransmitters such as acetylcholine and glutamate. These medications delay the onset and slow the progression of the disease. Several research efforts have been performed to ameliorate memory and promote the clearance of insoluble tau and Ab in transgenic AD mice models.8–10

Standard drug treatments for Alzheimer’s disease include acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor (NMDAR) antagonists.11,12 Cholinesterase inhibitors regulate information processing in the hippocampus and neocortex that are impaired in AD, which itself arises from the loss of cholinergic nerve fibers in the cerebral cortex. The medications in this group are donepezil, galantamine, and rivastigmine.13 Among the approved NMDAR antagonist drugs, memantine is used nowadays as a therapeutic agent for Alzheimer’s disease. Memantine is a noncompetitive inhibitor of NMDAR that blocks the transmission of glutaminergic messages by antagonizing these receptors.14,15

Clavulanic acid is a potent semisynthetic β-lactamase inhibitor isolated from Streptomyces clavuligerus. This drug is used in combination with amoxicillin in order to decrease the β-lactamase enzyme degradative effects. Its most common form is a combination of potassium salts called potassium clavulanate. Clavulanic acid, as a glutaminergic system inhibitor, has been selected as a target drug to evaluate its efficacy in this research.16

Combining input information to the brain and phenomenon-based learning creates spatial memory. This information can be stored in many parts of the brain, including the neocortex and hippocampus, so these parts have a fundamental role in creating spatial memory. Spatial memory helps animals remember and recall the information that they have acquired.17,18 Many evidence-based studies have shown that spatial memory is being gradually decreased with age and other reasons, most of which are deficiencies that impair learning and spatial memory due to altering the hippocampal morphology and function.19,20 It has been proven that patients who suffer from AD often show spatial memory loss, including that they do not know where they are and forget where they left their belongings. They often show signs of confusion, which is also a feature of spatial memory impairment.21

To create models of Alzheimer’s disease, a tropane alkaloid called scopolamine with antagonistic properties on muscarinic receptors is used in the laboratory animal models. After an intraperitoneal (i.p) injection of scopolamine, the cholinergic neurotransmitter system is blocked, causing cholinergic deficiency and memory impairment in the rats. Recently, it has been reported that memory loss by scopolamine causes oxidative stress in the rat’s brain. Thus, scopolamine-treated rats are used as animal models for the study of antidementia...
Scopolamine can also cause apoptosis in rat hippocampal cells.\textsuperscript{25,26} Because of the increasing prevalence of AD and reduced spatial memory performance in these patients, especially in industrialized societies, and the report of the World Health Organization (WHO) on an increasing number of early-onset Alzheimer’s disease, we aimed to investigate the possible prophylactic and therapeutic effects of clavulanic acid on AD.

## 2. RESULTS

### 2.1. Clavulanic Acid and Memantine’s Effect on the Spatial Memory of Rats

In the control groups, rats received normal saline with a volume of 30 international units i.p for 10 d. From the seventh day, the training in the Morris water maze was begun. The training was 3 d for each rat, and each one was trained only once a day. The duration of each experiment was 60 s, and if the rat could find the position, the experiment was completed; otherwise, the experiment was extended until the end of the test (60 s), so every rat had the opportunity to stay in the position for 20 s to learn the spatial position of the platform. Finally, the average percentage of time the rats spent in the target position was reported relative to the total time of the experiment. The results obtained from the control group showed that the rats spent more time in the platform position in the following days of the experiment by finding the spatial position of the platform in the maze, and if they could find the platform, they tended to stay on that position until the experiment was ended. This indicated that the learning pathway and spatial memory were normal in this group of rats (Figure 1).

In the group of rats that received memantine and clavulanic acid, control rats received 10 mg/kg of both drugs for 10 consecutive days. During the training, the average percentage of time the rats spent in the target position was calculated relative to the total time of the experiment. The results of the study in these groups showed that these rats had an improved learning process and that the time that animals spent on the target platform on the third day of the training was significantly increased compared to the first day of the training and control group. Also, the results of studying the learning process in this group of rats indicated that swimming time during the training had a decreasing trend in comparison with the first day of the training and control group (Figure 2).

### 2.2. Scopolamine Induced an Alzheimer’s Disease-like Condition in Rats

The results of the training in rats receiving scopolamine showed that the average percentage of
time the rats spent in the target position did not change during the training, indicating that rats were unable to learn the spatial position of the platform. Also, the duration of swimming on the third day of the training did not change in comparison with the first day, indicating that scopolamine disrupted the learning mechanism in these animals. In fact, the scopolamine disrupts the learning ability in rats, causing them to lose the ability to maintain and recall the position in the Morris water maze (Figure 3).

2.3. Clavulanic Acid and Memantine’s Effect on Spatial Memory in Scopolamine-Induced Alzheimeric Rats. To evaluate the effects of memantine and clavulanic acid on scopolamine-induced Alzheimeric rats, rats that had received scopolamine were treated with memantine and clavulanic acid for 10 consecutive days. The results showed that both memantine and clavulanic acid improved the spatial memory performance of rats in scopolamine-injected groups to remind the rats of the spatial position of the platform, which was deduced by increasing the time that rats spent in the target position during the training. It was also indicated that the ability of rats in this group to learn the spatial position of the platform was improved after the consumption of memantine and the injection of clavulanic acid, obtained from the reduced swimming time of rats during the training. However, the results were more significant for rats treated with clavulanic acid compared to memantine-treated groups (Figure 4).

Despite the results obtained during the training, the results obtained from the probe trial, the day that platform was removed from the maze, showed that the spatial memory in clavulanic acid-treated Alzheimeric rats had no significant difference with the scopolamine-induced Alzheimeric group, while the results were significant for the memantine-treated ones (Figure 5).

Figure 5. Effects of memantine, clavulanic acid, scopolamine, and post- and pretreatments performed by clavulanic acid, and memantine post-treatment on rats’ remaining time in Q2 on the day of the experiment. * p < 0.05, *** p < 0.001.

2.4. Clavulanic Acid Pretreatment’s Effect on the Reduction of Spatial Memory Performance on Scopolamine-Induced Rats. The results of this group showed that injection of clavulanic acid before scopolamine modulates the destructive effect of scopolamine on spatial memory in rats. These results were obtained by increasing the time that rats spent on the target platform during the training and also by decreasing the average swimming time of the animals during the training (Figure 6). Similarly, the results showed that the group receiving a clavulanic acid pretreatment could delay the Alzheimer’s disease onset by inhibiting the effects of scopolamine (Figure 5).

2.5. Memantine and Clavulanic Acid’s Effect on Neuroprotective Target Genes downregulated in Rats with AD. On the one hand, the results obtained from real-time polymerase chain reaction (PCR) showed that memantine and clavulanic acid, except for clavulanic acid on Sirtuin, had no significant effect on neuroprotective target genes including BDNF, Sirtuin 6, and Seladin 1 in normal rats who received these drugs compared with the control group. On the other hand, the results for these drugs were significant when injected post-treatment and revealed that they could increase all target genes’ expression; however, the result of clavulanic acid was more significant for all three genes than that of memantine when compared to scopolamine-induced positive groups. The results for pretreatment of clavulanic acid showed that its pretreatment administration could increase the expression of all three genes in comparison with rats that only received scopolamine (positive group) (Figure 7).

3. MATERIALS AND METHODS

3.1. Drugs Treatment and In Vitro Modeling of Alzheimer’s Disease in Rats. The experimental study was performed on adult male Sprague-Dawley (SD) rats that weighed 150–200 g. Animals were obtained from the pharmacology department of the Zanjan University of Medical Sciences. Rats were kept in special clear plastic cages at 23 °C. Adequate water and food were supplied except during the training. Each animal was used only once and was excluded from further experimentation under the ethical principles of working with animals (Ethics Code No. IR.ZUMS.REC.1396.227). Scopolamine has a role in learning/memory impairments as well as in the induction of amyloid-beta deposition, synaptic dysfunction, and oxidative stress, all of which are the most common forms of dementia affecting people with AD, so it is used to create a laboratory animal model for memory disorders including AD. Scopolamine hydrobromide was purchased from Sigma Inc. and diluted to a concentration of 0.01 mg/mL. Then, memantine under the brand name Namenda and clavulanic acid under the brand name potassium clavulanate were prepared daily to minimize the possibility of any contamination.

3.2. Rats Grouping. Rats were randomly divided into seven groups to implement memory assessment models, and experiments were done. Each group consisted of six adult male SD rats weighing 150–200 g. The groups were as follows:

1. Healthy rats receiving normal saline.
2. Memantine-receiving rats.
3. Clavulanic acid-receiving rats.
4. Scopolamine-injected rats (Alzheimer’s model).

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Figure 6. Protective effects of clavulanic acid pretreatment against scopolamine by evaluating the swimming time and the time that rats stayed in Q2 during the first 3 d of the experiment. (A) The mean percentage of the time that rats spent in the Q2. (B) Mean swimming time. (10 mg/kg of clavulanic acid was injected, and half an hour later, 2 mg/kg of scopolamine was injected i.p) * p < 0.05, ** p < 0.01.

Figure 7. Memantine and clavulanic acid regulate neuroprotective-related target genes in rats with AD. (A) Relative BDNF gene expression. (B) Relative Seldrin 1 gene expression. (C) Relative Sirtuin 6 gene expression. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Figure 8. TUNEL assay results for clavulanic acid pre- and post-treatment and memantine post-treatment. (A) Light microscope images of the stained cells. (B) Bar plot of TUNEL positive cells against the treated groups. Images were taken by an optical microscope with a magnification of 40X. * p < 0.05, **** p < 0.0001.
5. Scopolamine-injected rats receiving memantine.
6. Pretreatment of clavulanic acid in scopolamine-injected rats
7. Post-treatment of clavulanic acid in scopolamine-injected rats.

Briefly, in rats that did not receive scopolamine, 10 mg/kg clavulanic acid and 10 mg/kg memantine by i.p injection were given for 10 consecutive days, and the experiments were performed from the seventh to the ninth day of the injection according to the protocol. In scopolamine-induced Alzhimeric rats, first, they were injected with 2 mg/kg of scopolamine intraperitoneally for 10 consecutive days, then they received clavulanic acid and memantine as described above, and the experiments were performed. In rats who received clavulanic acid prior to the injection of scopolamine, 10 mg/kg of clavulanic acid was injected intraperitoneally, and half an hour later, 2 mg/kg of scopolamine was injected intraperitoneally, and the experiments were performed.

3.3. Behavioral Study. After the rats were grouped according to which of the different drugs were given, the Morris water maze test was performed for the behavioral study. For this aim, a water-filled tank was divided into four quadrants (Q1–Q4), and a platform was positioned in the Q2 quadrant. Each rat was placed in a random quadrant that was traced by the software. From days 7–9, the rats were trained. If the rat could find the maze in the target quadrant, it was allowed to stay on the platform for 3 s to assess the position of the platform and then was exited from the maze. Otherwise, if the rat was not able to find the platform during this time, it was manually guided to the platform to stay on the platform for 30 s to remember the position of the platform. On day 10, the platform was removed to determine if the rats could recall where they were and how long they would spend in the Q2 quadrant trying to find the platform. Finally, the spatial memory improvement of the rats was evaluated.

3.4. TUNEL Assay. The TUNEL assay procedure was based on studies evaluating the effect of drugs on hippocampal cell death.27,28 Briefly, after conducting behavioral studies, the brains of all rats were extracted and maintained in 10% formalin to perform the TUNEL assay. Genomic DNA breaks during the apoptotic process, forming two molecular strands of mononucleosome and oligonucleosome as well as terminal strands that are labeled enzymatically at the 3′-OH end. The TUNEL test was performed using the Apo-Direct kit (BD Biosciences). First, the incisions obtained from the microtome were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). The fixed cells were incubated with the blocking solution (3% H2O2 in methanol) for 10 min at room temperature and then washed with PBS. The cells were then incubated in ice permeable solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min. After that, the samples were resuspended in 50 μL of terminal deoxynucleotidyl transferase (TdT) and nucleotide (TUNEL reaction mixture) and incubated in a dark area for 1 h. Then, 50 μL of horseradish peroxidase (HRP)-labeled Converter POD (POD = peroxidase) solution was added, and the cells were kept at 37 °C in a bain-marie bath for 30 min. Henceforth, after washing, 100 μL of a 3,3′-diaminobenzidine (DAB) solution was added and incubated in a dark place for 10 min. Finally, the stained cells were analyzed under a light microscope.

3.5. RNA Extraction, cDNA Synthesis, and Real-Time PCR. The procedure was conducted based on conventional methods of RNA extraction and the real-time PCR method.28 In brief, target gene primers including BDNF, Sirtuin 6, and Seladin 1 were purchased from Cinnagene Inc. The sequences are summarized in Table 1.

Table 1. Primer Sequences

| name           | sequences                                      |
|----------------|-----------------------------------------------|
| Rpl13          | F: 5′-AAGAAGGGAGACAGTTCTGCTG-3′               |
| BDNF           | F: 5′-ATCCAGTGGAAAGCCGGA-3′                   |
| Seladin 1      | F:5′-CGAGTCTATGTCCTCCCCACAGT-3′              |
| Sirtuin 6      | F:5′-CTTTATGTGTCGCGGCCG-3′                   |
|                | R: 5′-GGGTCGAAATATCTGCGGAG-3′                |

After the rats were grouped according to the manufacturer’s protocol. Then, a conventional PCR was performed to reverse transcribe the RNAs to cDNAs using a cDNA synthesis kit (Exiqon). Finally, the real-time PCR reaction was conducted by using the SYBR Premix Ex in a light cycle 96 (Roche) instrument. The extracted brain samples of rats that had received normal saline were considered as our control. The mRNA level of β-actin was considered as an internal control.

3.6. Statistics. SPSS software ver. 16 was used to analyze the obtained data. A homogeneity of variance test was performed to evaluate the homogeneity of the data. Then, a one-way Anova test was used to analyze the results, and a post-hoc test was used to compare the groups. In all experiments, P < 0.05 was considered significant.

4. DISCUSSION

Alzheimer’s disease is a type of brain dysfunction that gradually degrades the patient’s mental abilities. The most obvious type of dementia is memory impairment, which is seen in patients with AD. Memory impairment is initially limited to recent events and learning, but old memories are also damaged gradually. AD is a multifaceted disease that results from a combination of factors such as age, genetics, and environmental factors. These factors either cause Alzheimer’s disease onset or its progression.29 Approximately 15 million people worldwide currently suffer from AD, and by 2040 the number is anticipated to reach 80 million.30,31

The mechanisms by which scopolamine induces Alzheimer’s disease include elevated acetylcholinesterase and decreased acetylcholine in the synaptic space, increased cytotoxicity signals, Tau levels, free radicals, oxidative stress, and amyloid-beta, all of which indicates that scopolamine acts as a potent contributor to Alzheimer’s disease by increasing toxicity and cell death.26,32 In this study, an i.p injection of scopolamine was performed to disturb memory and reduce learning ability, and based on previous studies and in line with our study, it leads to a significant reduction in memory in rats. According to the results of our study, the injection of scopolamine not only impaired spatial memory in rats but also impaired learning ability during the training, which was in line with previous findings that showed the injection of scopolamine disrupts immediate and working memory as in AD.33,34

Memantine is one of the candidate drugs in the treatment of AD. Memantine prevents neuronal cell death and Alzheimer...
disease’s progression by preventing elevated glutamate levels. Since we aimed to evaluate the effect of drugs on hippocampal cell apoptosis, memantine was a suitable drug for this study.\textsuperscript{15} Studies have shown that memantine can improve cognitive (mental) dysfunction. It has also been revealed that memantine increases the ability to manage daily tasks and reduces the necessity for patients with AD to have care.\textsuperscript{35–37} In the study performed by Block et al., they injected 20 mg/kg memantine into rats and then induced brain ischemia on them. The results obtained from the study showed that the swimming time and distance in the water maze in rats receiving memantine were increased compared to rats in which only ischemia had been induced.\textsuperscript{38} Also, the results of another study revealed that the nerve damage of the hippocampus in rats with AD was significantly reduced by memantine.\textsuperscript{39} It has also been shown that treatment with a neuroprotective agent such as memantine could reduce the function and morphological effects of brain ischemia.\textsuperscript{40} Ali et al. investigated the effect of memantine on scopolamine-induced memory disruption. For this purpose, a group of mice received scopolamine, after which the memantine was injected. In line with our study, it was revealed that memantine attenuates scopolamine-induced memory dysfunction.\textsuperscript{41}

Clavulanic acid is a safe neuroprotective drug with strong blood-brain-barrier permeability and considerable antioxidant and antitoxic effects, which announce it as a potential therapeutic drug for neurodegenerative disorders and diseases. However, because of its hepatotoxicity the consumed doses should be potentially low. Besides, the effects of clavulanic acid on neurotoxin-induced animal models of Parkinson’s disease have revealed that this drug protects hippocampal nerve cells’ death against neurotoxic drugs.\textsuperscript{42–44} It has also been proven that clavulanic acid improved behavioral motor function in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic drug.\textsuperscript{45} The main possible mechanism for its efficiency in AD can be suggested as its NMDAR antagonism, which can be compared with that of memantine. Although few studies have been performed on the effect of clavulanic acid on Alzheimer’s disease, Huh et al. showed that clavulanic acid improved behavioral defects in all models of human neurological disease.\textsuperscript{43} It was also determined that clavulanic acid protects nerve cells’ death. The results of studies revealed that clavulanic acid as a compound that modulates central nervous system (CNS) activity could be a neuroprotective agent in improving the performance of neurotoxin-induced animal models.\textsuperscript{44} However, the underlying neuroprotective mechanisms of clavulanic acid are not yet clarified, and further research is needed to elucidate the potential of clavulanic acid as a treatment for Alzheimer’s. In line with conducted studies, the results of our study for the first time revealed clavulanic acid improves the cognitive functions of rats with AD. Moreover, we showed that this drug protects the hippocampus cell death against scopolamine.

Seladin-1 has recently been known to be downregulated in Alzheimer’s disease. This gene has been shown to protect cells against apoptotic cell death. Livonen et al. showed that seladin-1 was downregulated in patients with AD, in which this reduction was correlated with hyperphosphorylation of Tau protein.\textsuperscript{46,47} It has been revealed that seladin 1 has downregulated in brain regions selectively degenerated in AD, so its restoration could be beneficial in patients and models of AD.\textsuperscript{48} The results obtained from our study demonstrate that either memantine and clavulanic acid increase the expression of seladin 1, which was downregulated in scopolamine-induced rats. It was also clarified that both drugs improved memory performance and reduced hippocampal cell death; however, these results were more prominent for clavulanic acid.

The role of sirtuin in AD has come to light. Sirtuin regulates the neuroinflammation and neuronal degradation that are seen in AD. Its correlation with DNA repair, telomere maintenance, and genome integrity has also been seen, which are associated with longer longevity. Recent studies have proposed sirtuin 6 as a potential therapeutic target in AD. Downregulated sirtuin levels have recently been reported to have correlated with increased Aβ production and deposition in patients with AD.\textsuperscript{49} It has recently been reported that sirtuin 6 has reduced expression in AD models, where its upregulation improves memory function.\textsuperscript{28} In line with studies, we showed that sirtuin 6 has been downregulated in scopolamine-induced rats while memantine increases its expression and improves memory function; however, the clavulanic acid had a more significant effect in comparison with memantine.

BDNF strengthens memory formation by long-term induction of the hippocampus.\textsuperscript{7} Weinstein et al. revealed that elevated levels of BDNF protect the elderly against AD.\textsuperscript{49} Besides, its upregulation has been shown to participate with a reduced risk of AD or dementia.\textsuperscript{50} BDNF takes part in the synaptic growth of the CNS, leading to enhanced memory functions and hippocampal learning. Its reduction has been proven to be involved in the progression of AD, in which the restoration of BDNF led to an improved neurological impairment in patients with AD.\textsuperscript{51} In line with our study, the results of a recently published article showed that BDNF was downregulated in mice who received scopolamine, while induction of memantine could significantly increase its expression alongside an improved memory performance.\textsuperscript{28} Although we showed that memantine has a similar role, the superior role of clavulanic acid has come into view for the first time in our study.

5. CONCLUSION

The results of the present study showed for the first time that clavulanic acid, as an effective drug in scopolamine-induced rats before and after scopolamine injection, can improve spatial memory in these rats and also reduce cell apoptosis. It also reduces hippocampal cell death, which can ultimately prevent the acceleration of Alzheimer’s disease. Also, the results of our study showed that the expression of neuroprotective genes, which is reduced in Alzheimer’s disease, increases after treatment with clavulanic acid, which can be used as an option in the upcoming preclinical and clinical research for treatment of Alzheimer’s disease. In conclusion, although the results of memantine administration were significant, the results of clavulanic acid injection were more considerable, which due to its higher blood-brain barrier permeability and the widespread suppressor role in neuroinflammation may be a promising therapeutic approach for further research in the areas related to Alzheimer’s disease.

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ACS Omega 2022, 7, 13861–13869

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**Notes**

The authors declare no competing financial interest.

Ethics Statement. All ethical principles set by the NIH for this study, and all practical steps of the experiment were performed after the approval of the project with Ethics code ZUMS.REC.1396.227 by the University Ethics Committee.

**ACKNOWLEDGMENTS**

This study was performed and supported at the Zanjan school of pharmacy, Zanjan University of Medical Science, Zanjan, Iran.

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