*Biomacromol. J., Vol. 7, No. 1, 35-43, July 2021.*

***Biomacromolecular Journal***
www.bmmj.org

# Myrcene Consumption and Restrain Stress: An *In Vivo* Comparative Study on an Alzheimer's Disease Model and *In Vitro* Investigation on Abeta Fibrils

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*(Received 31 May 2022, Accepted 15 July 2022)*

## ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease characterized by amyloid plaques and neuronal death. Natural therapies have always attracted attention against AD. Herein, Myrcene, as a natural monoterpene, was applied to examine its protective and therapeutic effects on a rat model of AD along with short-term restraint stress. In order to create Alzheimer's rat model, bilateral injection of Amyloid β1-42 was performed into rats' hippocampus. Both therapeutic (post-AD induction) and preventive effects of Myrcene consumption (100 mg/kg) were investigated on the antioxidant and behavioral parameters as well as neurogenesis and brain amyloid plaque formation. Meanwhile, the effects of restraint stress was observed. Moreover, the effect of Myrcene (100 µM) was observed on Aβ1-42 fibrils *in vitro*. Alzheimer's-induced group showed impairment in the memory and antioxidant parameters along with amyloid plaque formation and loss of neuronal cells. Administration of Myrcene, in both treatment and protective modes increased neurogenesis, reduced amyloid plaques, and improved antioxidant parameters as well as memory even during applying restraint stress. Therefore, Myrcene showed capability of improving AD signs *in vivo* as well as direct anti-fibril effect *in vitro* and therefore could be considered as neuroprotective agent.

**Keywords:** Myrcene, Restraint stress, Aβ42, Memory, Amyloid plaques, Neurogenesis

## INTRODUCTION

Alzheimer's disease (AD) has led to a crisis in aging society as the most common progressive neurodegenerative disease associated with learning, memory, and neuronal impairment [1,2]. Both amyloid-β (Aβ) generation and aggregation as well as oxidative stress have shown to play crucial roles in the pathogenesis of AD. Aβ is a potent neurotoxic peptide and a major constituent of the aging plaques [3]. Meanwhile, there is growing evidence that chronic stress may increase the risk of developing AD [4,5]. It has been reported that chronic stress decreases cell survival and neurogenesis, promotes dendritic atrophy, and causes long-term potentiation and cognitive deficits [6,7]. Previous studies have shown that restraint stress can contribute to impaired memory and learning [8,9].

Over decades of studies, natural compounds, such as monoterpenes, have demonstrated strong antioxidant properties as well as neuroprotective effects [10-14]. Myrcene acts as a monoterpene in many plants, including bay, ylang-ylang, wild thyme, parsley, and hops. It also offers many pharmacological properties, including analgesic, anti-inflammatory, and antioxidant effects [15]. Furthermore, Myrcene has shown neuroprotective and anti-inflammatory effects [16] by reducing oxidative damage and improving brain damage [17]. As many studies suggest that inflammatory pathways and oxidative stress play key roles in the pathogenesis of AD [3], herein, the latter effects of Myrcene have been investigated on Alzheimer's rat models. In the present study, both therapeutic and

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preventive effects of different doses of Myrcene, as a natural compound, were investigated on Aβ42 fibrillation using Alzheimer's rat brain as an *in vivo* model system. Moreover, the effect of restraint stress on various Alzheimer's disease indices was investigated with or without Myrcene treatment. In addition, the possible destabilizing mechanism of this compound was examined on Aβ42-pre-formed fibrils as an *in vitro* experiment.

## MATERIALS AND METHODS

### Compounds

Myrcene and Aβ1-42 were purchased from Sigma (St. Louis, MO, USA). The Aβ1-42 (1 mg $ml^{-1}$) was dissolved in double sterile distilled water and placed in an incubator at 37 °C for one week [13]. Myrcene (100 mg/kg) was dissolved in double sterile distilled water and injected peritoneally into rats using an insulin syringe.

### Animals

The present experimental study was carried out on a total of 54 Wistar rats, weighting 200 ± 50 grams, purchased from Pasteur Institute of Iran. Rats were kept in six cages (42 × 26 cm) at a temperature of 20 ± 0.5 °C under 12:12 light-dark cycle. The animals had access to adequate water and food throughout the experiment. The cages were cleaned daily and the temperature, humidity and light cycle were monitored. To avoid stress, the animals were adapted to the conditions and environment one week before the experiment. To induce AD, animals were anesthetized by ketamine and xylasin injection and placed within the stereotactic device. Using stereotaxy and brain atlas [18] to localize hippocampus, 2 µl of beta-amyloid solution was injected with a hamilton syringe in the ventricle of the animal's brain. Injection was slowly carried out in the CA region on both sides of the hippocampus. After one week, amyloid plaques were formed in the animal's brain which were visible by the use of histological methods (detailed below). Experimental procedures were carried out strictly in accordance with the guidelines from the International Association of Veterinary Editors.

### Animals Grouping

Experimental groups were defined as follows (n = 6):

1. Control Group I (Ctr): Animals received routine water and food and underwent no Alzheimer's surgery.
2. Control Group II (S + W): Animals underwent Alzheimer's surgery but distilled water was injected into the hippocampus instead of Aβ.
3. Alzheimer's Group (Aβ): Animals underwent surgery and 2 µl of Aβ was injected bilaterally into the hippocampus.
4. Sham Group (Aβ + W): The animals received Aβ and distilled water intraperitoneally after one week of surgery for 14 days.
5. Restraint Group (R): Animals were subjected to restraint stress for five hours per day in a special tube for 14 days without undergoing surgery.
6. Alzheimer's + restraint Group (Aβ + R): AD animals were subjected to restraint stress.
7. Experimental Group I (Therapeutic) (Aβ + T +100): AD was induced in animals and Myrcene (100 mg/kg) was injected for 14 days intraperitoneally after one week of AD induction.
8. Experimental Group II (Protective) (Aβ + P +100): Animals were first injected with Myrcene (100 mg/kg) for 14 days intraperitoneally before AD induction. Then, AD was induced in the animals who were assessed one week later.
9. Experimental Group III (Aβ + R + 100): AD was induced and animals received Myrcene (100 mg/kg) for 14 days intraperitoneally and were subjected to the restraint stress at the same time.

### Behavioral Test

To perform the shuttle box experiment, a box consisting of two dark and light compartments of equal size (26 × 26 cm) and separated by a sliding lid (8 × 8 cm) was used. The inhibitory avoidance method was employed to investigate the memory status in laboratory rats on two consecutive days: the first day of training was followed by the second day of testing. In this experiment, the animals were placed in the bright compartment for 5 s. After 5 s, the guillotine door was opened and the animals were allowed to enter the dark compartment. It was given 10 s to return to the cage after being exposed to the light and dark environments. After 30 min, the rats were placed in the shuttle box. After entering the dark compartment in this area (50 HZ, 1 Ma for 5 s), the animals underwent electric shock

and were then returned to the cage. Two minutes later, the rats were placed in the shuttle box. At this stage, the rats were placed in the bright compartment and the guillotine door was opened and the latency to enter the dark compartment was recorded. Each test was performed at a maximum of three times for each rat. The testing stage began 24 h after the training phase. No electric shock was performed in the testing session. The maximum latency for entering the dark compartment was 300 s [19,20].

### Histological Test

At the end of the experiment, the rats were anesthetized, and their brains were removed and stored in 10% formalin for 24 h. The brains were then embedded with paraffin. Hematoxin-eosin staining was used to evaluate neurogenesis. Thioflavin S staining method was employed to detect amyloid plaques, and the images were observed by a fluorescence microscope [21]. Image J software was used to count the amyloid plaques.

### Restraint Stress Experiment

To induce restraint stress, the rats were restrained for 5 h, at a certain time of day, in a polypropylene tube (3 × 3 × 10 cm) with multiple punctures to allow ventilation for two weeks [22].

### Biochemical Assessments

At the end of the experiment, when the rats were knocked out, blood samples were collected. Each sample was first coagulated at room temperature for 30 min and then centrifuged at 300 rpm at 37 °C for 10 min. After preparing the blood serum, superoxide dismutase (SOD) activity and malondialdehyde (MDA) level were measured using German laboratory kits (ZELLBIO). SOD activity measurement was made based on the enzyme ability to inhibit the auto-oxidation of pyrogallol, which was checked at 420 nm [23], while MDA reacts with thiobarbituric acid (TBA) in serum to produce a state of MDA-TBA, which was then measured by colorimetric (OD = 532) method [24].

### *In Vitro* Experiment

Aβ42 peptide was first dissolved in deionized water (DW) to a final concentration of 1 mg $ml^{-1}$. In order to make mature fibrils, tubes containing monomers were incubated at 37 °C for 4 days. In order to check the destabilization potential of Myrcene, aliquots of 1 mg $ml^{-1}$ of four-day-old pre-formed Aβ fibrils were further incubated with the 100 µM of Myrcene at 37 °C for 1 and 3 weeks. In all experiments, the water bath containing the tubes of samples was being gently stirred by a Teflon magnetic bar [25].

### Transmission Electron Microscopy

About 5 µl of 1 mg $ml^{-1}$ samples were adsorbed onto copper 400 mesh F-C grids. After 2 min, the excess liquid was removed using a paper filter, and then 5 ml of 1% uranyl acetate was added to the grids. Excess dye was removed after 2 min and the dried samples were then observed by Hitachi HU-12A electron microscope (Hitachi, Japan) at 75 Kv.

### Statistical Analysis

Data analysis was carried out using ANOVA and Tukey's test in SPSS V.21. Results were expressed as Mean ± SEM and $p < 0.001$ was considered as the significant level.

## RESULTS

### Effects of Myrcene on Biochemical Parameters and Behavioral Index

Results demonstrated that the activity of SOD was lowered in the disease-induced (Aβ) group compared with other groups. Meanwhile, there was a significant increase of SOD activity in the group receiving Myrecene 100 mg/kg in the therapeutic mode (Aβ + T + 100) compared with the AD-induced and the control groups ($p < 0.001$). Also, SOD activity was increased in the group receiving Myrecene 100 mg/kg in the protective mode (Aβ + P + 100) compared with the control and Aβ groups ($p < 0.001$). Also, results showed a significant increase of SOD activity in the Aβ + R + 100 group compared with the R, Aβ, and Aβ + R groups ($p < 0.001$) (Fig. 1).

On the other hand, serum levels of MDA in the groups receiving Myrecene in both treatment and protective modes were significantly decreased compared to Ctr, S + W, R, Aβ, Aβ + W, and Aβ + R groups ($p < 0.001$). Moreover, results showed a significant decrease in the Aβ + R + 100

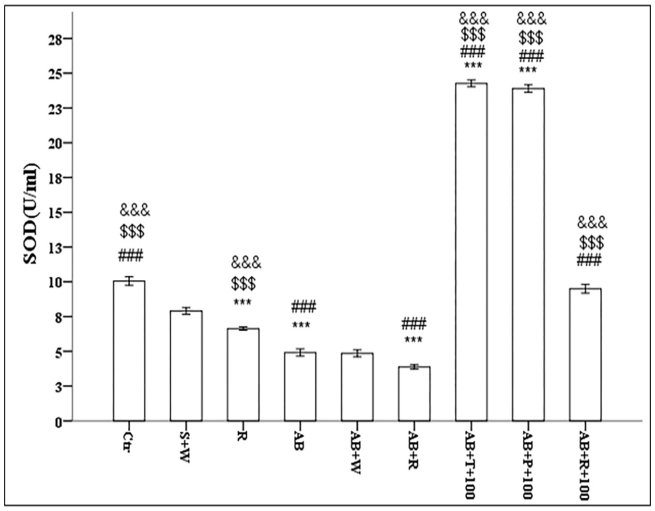


**Fig. 1.** Overall serum activity of SOD in different groups. Ctr: control group; S + W: rats underwent surgery with water being injected into brain; R: Animals were subjected to restraint stress; AB: Aβ1-42 was injected into brain to induce Alzheimer's disease; AB + W: Alzheimer's-induced animals receiving water as Myrcene solvent; AB + R: Alzheimer's-induced animals were subjected to restraint stress; AB + T + 100: Alzheimer's-induced animals treated with 100 mg/kg Myrcene; AB + P + 100: Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); AB + R + 100: Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress. ***: $p < 0.001$ : Comparison of control group (Ctr) and the other groups; ###: $p < 0.001$ Comparison of restraint group (R) and the other groups; $$$: $p < 0.001$ Comparison of Alzheimer's group (Aβ) and the other groups; &&&: $p < 0.001$ Comparison of Alzheimer's group receiving restraint (Aβ + R) and other groups.

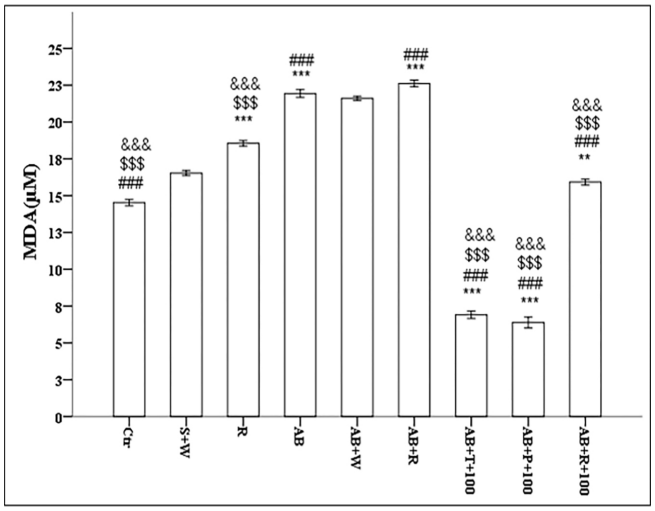


**Fig. 2.** Overall serum level of MDA in different groups. Ctr: control group; S + W: rats underwent surgery with water being injected into brain; R: Animals were subjected to restraint stress; AB: Aβ1-42 was injected into brain to induce Alzheimer's disease; AB + W: Alzheimer's-induced animals receiving water as Myrcene solvent; AB + R: Alzheimer's-induced animals were subjected to restraint stress; AB + T + 100: Alzheimer's-induced animals treated with 100 mg/kg Myrcene; AB+P+100: Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); AB + R + 100: Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress. ***: $p < 0.001$ : Comparison of control group (Ctr) and the other groups; ###: $p < 0.001$ Comparison of restraint group (R) and the other groups; $$$: $p < 0.001$ Comparison of Alzheimer's group (Aβ) and the other groups; &&&: $p < 0.001$ Comparison of Alzheimer's group receiving restraint (Aβ + R) and other groups.

group compared with R, Aβ, and Aβ + R groups ($p < 0.001$) (Fig. 2).

Results of shuttle box behavioral test showed a significant improvement of memory in the groups receiving Myrecene in both treatment and protective modes compared to other groups ($p < 0.001$) (Fig. 3).

## Effects of Myrcene on Neurogenesis

Histological examination of the CA1 region of the hippocampus showed that Ctr, S + W and R groups contained a large number of normal pyramidal cells (Figs. 4 and 5b, c, d). In contrast, there was an evident decline in the number of pyramidal cells in the Aβ, Aβ + W and Aβ + R groups as shown in the Figs. 5e, f, g. Results of microscopic examination showed that the number of pyramidal cells was increased in the Aβ + T + 100, Aβ + P + 100, and Aβ + R + 100 groups ($p < 0.001$) (Figs. 4 and 5h, i). Moreover, there was an evident increase in the number of pyramidal cells in the Aβ + R + 100 compared to the Alzheimer's group (Figs. 4 and 5j).

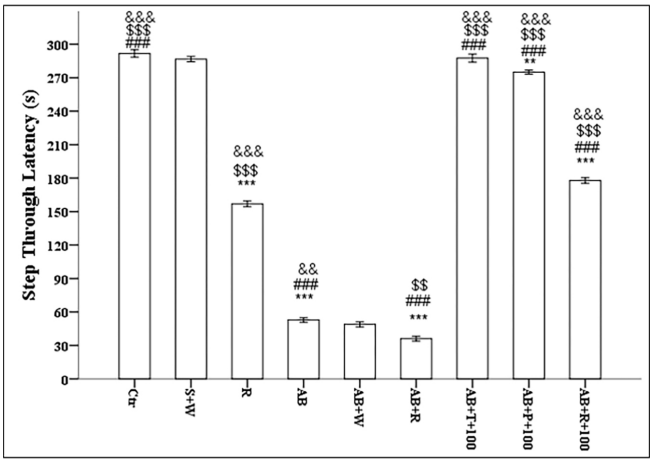


**Fig. 3.** The mean latency to enter the dark chamber on the test day. Ctr: control group; S + W: rats underwent surgery with water being injected into brain; R: Animals were subjected to restraint stress; AB: Aβ1-42 was injected into brain to induce Alzheimer's disease; AB + W: Alzheimer's-induced animals receiving water as Myrcene solvent; AB + R: Alzheimer's-induced animals were subjected to restraint stress; AB + T + 100: Alzheimer's-induced animals treated with 100 mg/kg Myrcene; AB + P + 100: Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); AB + R + 100: Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress. ***: $p < 0.001$ : Comparison of control group (Ctr) and the other groups; ###: $p < 0.001$ Comparison of restraint group (R) and the other groups; $$$: $p < 0.001$ Comparison of Alzheimer's group (Aβ) and the other groups; &&&: $p < 0.001$ Comparison of Alzheimer's group receiving restraint (Aβ + R) and other groups.

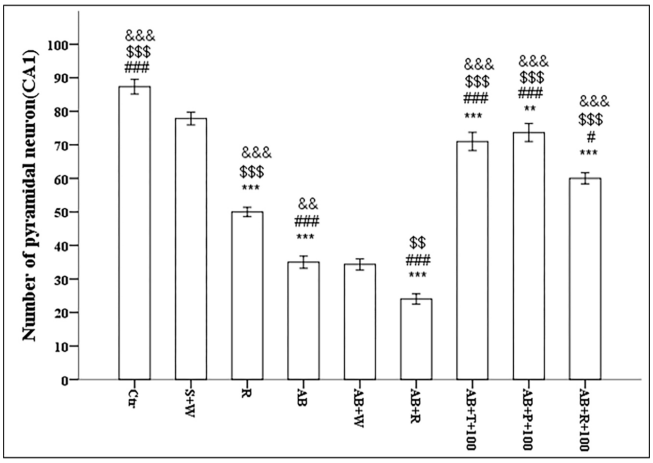


**Fig. 4.** Number of neurons in different groups. Ctr: control group; S + W: rats underwent surgery with water being injected into brain; R: Animals were subjected to restraint stress; AB: Aβ1-42 was injected into brain to induce Alzheimer's disease; AB + W: Alzheimer's-induced animals receiving water as Myrcene solvent; AB + R: Alzheimer's-induced animals were subjected to restraint stress; AB + T + 100: Alzheimer's-induced animals treated with 100 mg/kg Myrcene; AB + P + 100: Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); AB + R + 100: Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress. ***: $p < 0.001$ : Comparison of control group (Ctr) and the other groups; ###: $p < 0.001$ Comparison of restraint group (R) and the other groups; $$$: $p < 0.001$ Comparison of Alzheimer's group (Aβ) and the other groups; &&&: $p < 0.001$ Comparison of Alzheimer's group receiving restraint (Aβ + R) and other groups.

## Effects of Myrcene on Amyloid Plaque Formation

Thioflavin Staining S indicated the absence of amyloid plaques in healthy groups (Figs. 6 and 7a, b, c). In contrast, as shown in Figs. 7d, e, f, amyloid plaques were increased in the Aβ, Aβ + W, and Aβ + R groups. Results of the studies revealed a significant reduction in the number of plaques ($p < 0.001$) in the Aβ + T + 100, Aβ + P + 100, and Aβ + R + 100 groups (Figs. 6 and 7g, h, i).

## Destabilizing Effects of Myrcene on Pre-formed Aβ42 Fibrils *In Vitro*

The second part of this study involved monitoring destabilizing effect of Myrcene 100 µM on four-day-old pre-formed Aβ42 fibrils for 1 and 3 weeks of incubation *in vitro*. It was first demonstrated that Aβ42 fibrillation proceeded from monomeric species (Fig. 8a) to longer and well-matured fibrils after 4 days (Fig. 8b). Based on TEM images, there was a notable decrease in Aβ fibrils in the presence of Myrcene after one (Fig. 8c) and three weeks (Fig. 8d). TEM images demonstrated that shorter and thinner fibrillary structures were formed in the presence of Myrcene after 3 weeks of incubation. As a result, it seemed that longer incubation time was required to create instability of fibril formation in the presence of Myrcene 100 µM.

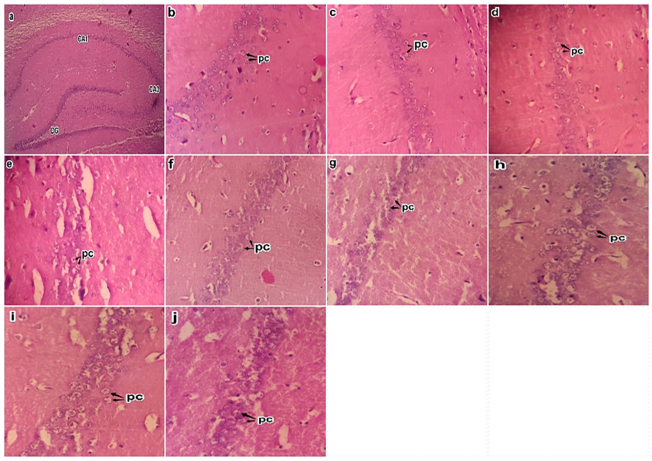


**Fig. 5.** Brain tissue section used to determine neuron numbers in CA1 region. Staining has been done with Hematoxilline Eosine. Pc is indicative of pyramidall cells. a: control group (x40); b: control group (x400); c: (S + W) rats underwent surgery with water being injected into brain; d: (R) Animals were subjected to restraint stress; e: (AB) Aβ1-42 was injected into brain to induce Alzheimer's disease; f: (AB + W) Alzheimer's-induced animals receiving water as Myrcene solvent; g: (AB + R) Alzheimer's-induced animals were subjected to restraint stress; h: (AB + T + 100) Alzheimer's-induced animals treated with 100 mg/kg Myrcene; i: (AB + P + 100) Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); j: (AB + R + 100) Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress.

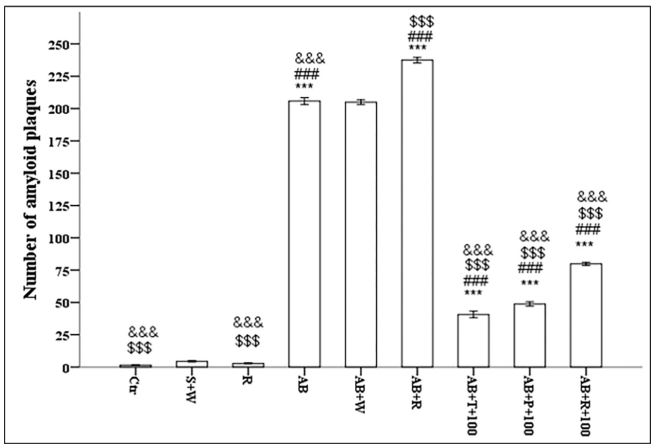


**Fig. 6.** Number of amyloid plaques in the hippocampus CA1 region in different groups. Ctr: control group; S + W: rats underwent surgery with water being injected into brain; R: Animals were subjected to restraint stress; AB: Aβ1-42 was injected into brain to induce Alzheimer's disease; AB + W: Alzheimer's-induced animals receiving water as Myrcene solvent; AB + R: Alzheimer's-induced animals were subjected to restraint stress; AB + T + 100: Alzheimer's-induced animals treated with 100 mg/kg Myrcene; AB + P + 100: Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); AB + R + 100: Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress. ***: $p < 0.001$ : Comparison of control group (Ctr) and the other groups; ###: $p < 0.001$ Comparison of restraint group (R) and the other groups; $$$: $p < 0.001$ Comparison of Alzheimer's group (Aβ) and the other groups; &&&: $p < 0.001$ Comparison of Alzheimer's group receiving restraint (Aβ + R) and other groups.

## DISCUSSION

Administration of Myrcene was effective in counteracting the deleterious effects of AD on memory, neurogenesis, amyloid plaque formation, and biochemical parameters in the Alzheimer's animal model. It seemed that Myrcene had the potential for destabilizing fibrils prior to their formation based on *in vitro* experiments. It was also found that restraint stress could accelerate the AD-related consequences which were improved by Myrcene.

Injection of Aβ fibrils onto animals' brains is now an established method of generating AD model [26]. Aβ42 is a potent neurotoxic peptide and the main structure of plaque leading to neurological dysfunction, memory impairment, inflammation, and oxidative stress in AD patients [27,28]. These signs could be counteracted by potential anti-amyloid natural compounds [25,26]. Monoterpenes have emerged as a class of compounds that could prevent neurotoxicity of progressive neurological diseases, such as AD and Parkinson's disease [29,30]. Moreover, monoterpenes are important chemical compounds in the essential oils of plants and possess various therapeutic properties, including antioxidant and anti-inflammatory effects [31,32]. *Bene hullec*, rich in Myrcene, has shown great antioxidant activity and free-radical-scavenging capacity [33,34]. Earlier

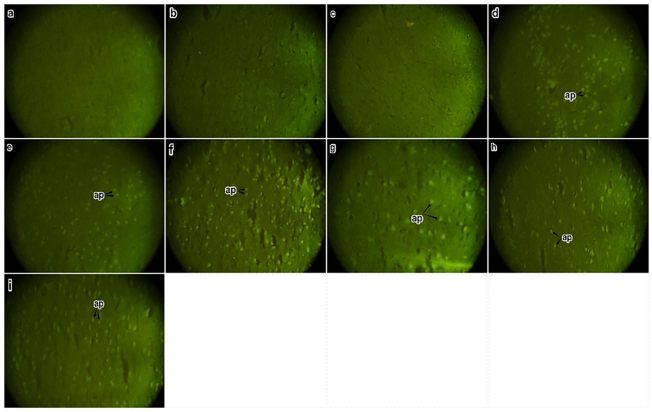

**Fig. 7.** Thioflavin S staining of amyloid plaques in the hippocampus CA1 region. ap is indicative of amyloid plaques. a: control group; b: (S+W) rats underwent surgery with water being injected into brain; c: (R) Animals were subjected to restraint stress; d: (AB) Aβ1-42 was injected into brain to induce Alzheimer's disease; e: (AB + W) Alzheimer's-induced animals receiving water as Myrcene solvent; f: (AB + R) Alzheimer's-induced animals were subjected to restraint stress; g: (AB + T + 100) Alzheimer's-induced animals treated with 100 mg/kg Myrcene; h: (AB + P + 100) Alzheimer's-induced animals receiving protective dose of Myrcene (100 mg/kg); i: (AB + R + 100) Alzheimer's-induced animals receiving both Myrcene (100 mg/kg) and restraint stress.

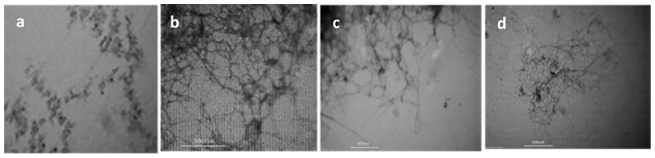

**Fig. 8.** Electron microscope analysis of Aβ42 fibrillation with and without Myrcene. TEM images of Aβ42 fibrillation process after immediate incubation (a) and 4 days (b) in the absence of Myrcene. The four-day-old Aβ42 fibrils incubated with Myrcene 100 μM for 1 week (c) and 3 weeks (d).

studies have reported that oxidative stress is a major factor in the development of AD [35]. In another report, it was found that plants rich in Myrcene have antioxidant and anti-acetylcholinesterase activities as well as anti-amyloid and memory-boosting effects [36].

In the present study, the level of SOD was decreased in AD-induced rats. SOD is considered to be one of the most active enzymes whose activity is sufficient for inactivation of superoxide anions produced during oxidative stress in cells. Abramov *et al.* reported that beta amyloid enters the nerve cells and produces oxygen free radicals ultimately leading to oxidation of proteins and lipids [37]. In aging brain, the memory impairment is also thought to be due to an increased oxidative stress [38] causing the development and progression of Alzheimer's disease [39]. Both aged humans and rodents have demonstrated cognitive impairment being correlated to the accumulation of oxidative damage to lipids, proteins, and nucleic acids [40]. Herein, Myrcene demonstrated the ability of increasing SOD level and therefore could offer protection against Aβ-induced oxidative stress in the hippocampus. Linalool, a monoterpene, has also shown to reverse cognitive deficits [41]. Previous studies have shown that phenolic monoterpenes, such as Thymol, Myrtenol, and Nerol could increase the antioxidant potential [42,43]. Terpenese, such as Carvacrol, γ-Terpinene, and p-Cymene have also shown antioxidant effects *in vitro* [44].

One way to directly measure the free radicals *in vivo* is to quantify the cellular components reacting with the free radicals, such as lipids [45]. An increase in free radicals causes overproduction of MDA whose level is commonly known as a marker of oxidative stress [46]. In the present study, decreased activity of SOD was consistent with an increase in the level of MDA in the Alzheimer's-induced rats. Herein, applying Myrcene in both treated and protective modes exhibited a considerable increase in SOD activity leading to a notable decrease in MDA level. In consistent with our study, previous studies have also shown that chronic stress increases lipid peroxidation in plasma, hippocampus and intestinal mucosa [47]. Reports indicate higher plasma SOD levels and lower MDA levels in improving nerve damage by applying *Pistacia atlantica* rich in Myrcene [48]. Moreover, it has been demonstrated that Myrcene prevents lipid peroxidation and had anti-radical activity [49]. Myrcene has also shown the capability of improving memory disorders [50]. Another study also found that Myrcene protects brain tissue and prevents neurodegeneration and can reduce brain lesions as well as augmented neurogenesis [51,52]. In addition to its

antioxidant and anti-inflammatory properties, the results showed that Myrcene could be effective on the Alzheimer's model through its anti-fibrillation effect *in vitro*. The anti-fibrillation effects of aromatic compounds, especially larger polycyclic chemicals, have been reported on a variety of compounds and on different types of fibrils [25]. Meanwhile, reports indicate that stress has destructive effects on memory and learning [53]. Herein, Myrcene showed capability of improving such stress-related symptoms in AD. Therefore, Myrcene could play an important role in the creation of new biologically-active compounds, including drugs, for improving Alzheimer's disease symptoms.

## CONCLUSSIONS

Myrcene has been demonstrated to counteract deleterious effects of AD in a rodent model when used either protectively or in a therapeutic mode. Meanwhile, application of Myrcene to the restraint stressed-AD rats also improved the conditions. Finally, Myrcene has been shown to have a disaggregating effect on pre-formed amyloid fibrils, which could be of use in other forms of amyloidosis.

## ACKNOWLEDGEMENTS

This study has been performed in the Laboratory Complex of the Science and Research Branch of Azad University. Authors declare no conflict of interest.

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