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Neuroprotective effects of limonene (+) against Aβ42-induced neurotoxicity in a Drosophila model of Alzheimer’s disease

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Summary

Forest bathing is suggested to have beneficial effects on various aspects of human health. Terpenes, isoprene based-phytochemicals emitted from trees, are largely responsible for these beneficial effects of forest bathing. Although the therapeutic effects of terpenes on various diseases have been revealed, their effects on neuronal health have not yet been studied in detail. Here, we screened 16 terpenes that are the main components of Korean forests using Drosophila Alzheimer’s disease (AD) models to identify which terpenes have neuroprotective effects. Six out of the 16 terpenes, ρ-cymene, limonene (+), limonene (-), linalool, α-pinene (+), and β-pinene (-), partially suppressed the beta amyloid 42 (Aβ42)-induced rough eye phenotype when fed to Aβ42-expressing flies. Among them, limonene (+) restored the decreased survival of flies expressing Aβ42 in neurons during development. Limonene (+) treatment did not affect Aβ42 accumulation and aggregation, but did cause to decrease cell death, reactive oxygen species levels, extracellular signal-regulated kinase phosphorylation, and inflammation in the brains or the eye imaginal discs of Aβ42-expressing flies. This neuroprotective effect of limonene (+) was not associated with autophagic activity. Our results suggest that limonene (+) has a neuroprotective function against the neurotoxicity of Aβ42 and, thus, is a possible therapeutic reagent for AD.

Key words: Alzheimer’s disease, amyloid β42, Drosophila, limonene (+), terpene
INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by progressive impairment of the memory, thinking, and oral capabilities that commonly affects adults above the age of 65, although early onset is possible.\(^1\) The hallmarks of AD are the formation of beta-amyloid (Aβ) plaques and neurofibrillary tangles.\(^2\) Aβ, a cleaved, disease-causing form of the amyloid precursor protein, forms oligomers to produce reactive oxygen species (ROS), induces aggregation of the tau protein, and consequently induces neuronal cell death.\(^3\) It is also known that neuroinflammation is integral to the progression of AD.\(^4\)

There is currently no cure for AD. However, there are various drugs that target the symptoms of AD, mainly acetylcholinesterase inhibitors and a N-methyl-D-aspartate receptor (NMDAR) antagonist.\(^5\) Acetylcholinesterase inhibitors, also known as cholinesterase inhibitors, are a group of drugs that inhibit acetylcholinesterase. When consumed, the drug causes the accumulation of acetylcholine, increasing neurotransmission in the brains of AD patients. Acetylcholinesterase inhibitors approved by the FDA include donepezil, galantamine, and rivastigmine.\(^6,7\) Another drug, memantine, belongs to the NMDAR antagonist family and specifically targets the neurotransmitter glutamate.\(^8\) When the NMDAR channels are opened, memantine can enter and act on the excitotoxic effects of glutamate by reducing the influx of calcium ions and reducing oxidative stress present in postsynaptic neurons.\(^9\) Although these drugs help to slow down deterioration of a patient’s health, some patients may experience side effects, such as nausea, diarrhea, abdominal pain, anorexia, and dizziness.\(^10\) Moreover, the therapeutic effects of the drugs are only moderate and temporary.\(^5\)

Forest bathing is also suggested to have therapeutic effects for neurodegenerative diseases, by decreasing both heart rate and blood pressure and increasing parasympathetic
nerve activity in affected individuals.\textsuperscript{11} Furthermore, exposure to the forest environment significantly reduces interleukin-6 and elevates the level of glutathione peroxidase,\textsuperscript{12} which are involved in the defense against pathogens and the reduction of oxidative stress, respectively.\textsuperscript{13,14} In addition, forest bathing has been proven to decrease malondialdehyde levels, which is a sign of oxidative stress.\textsuperscript{15}

These positive effects are partly due to terpenes, which are emitted into the atmosphere by trees and other plants.\textsuperscript{16} Terpenes are the largest class of naturally occurring organic compounds composed of an isoprene unit (C\textsubscript{5}H\textsubscript{8}).\textsuperscript{17} According to the isoprene rule, terpenes are classified based on the number of isoprene units.\textsuperscript{16} Mono-, sesqui-, and di-terpenes represent terpenes that have two, three, and four isoprene units, respectively.\textsuperscript{18} Terpenes have a wide range of biological properties ranging from anti-oxidation, anti-inflammation, and anti-cancer to neuroprotection.\textsuperscript{16,19} Based on the physiological activities of terpenes, the potential therapeutic efficacy of terpenes in a variety of human disease models has been studied. The results of these studies have focused on the beneficial effects of terpenes on immune-related diseases and cancers.\textsuperscript{16} However, there are relatively few studies on the therapeutic effects of terpenes on neurodegenerative diseases, such as AD.

In this study, we used a \textit{Drosophila} AD model to screen 16 terpenes that are commonly produced in Korean forests to investigate the beneficial effects of terpenes on AD. Among them, limonene (+) is shown to have neuroprotective effects against Aβ42-induced cytotoxicity. The neuroprotective activity of limonene (+) against Aβ42 is associated with its antioxidant and anti-inflammatory properties. Our results suggest that limonene (+) can protect neurons against Aβ42-induced cytotoxicity by reducing oxidative stress and neuroinflammation.
MATERIALS AND METHODS

**Fly strains**  The w^1118, glass multimer reporter-GAL4 (GMR-GAL4; eye driver), embryonic lethal abnormal vision-GAL4 (elav-GAL4; pan-neuronal driver), UAS-GFP tagged autophagy-related 8a (UAS-Atg8a::GFP), UAS-Target of rapamycin (TOR) dominant negative (UAS-TOR<sup>TED</sup>), Drosomycin tagged GFP (Drs-GFP) and larval serum protein 2-GAL4 (Lsp2-GAL4; fat body driver) strains were obtained from the Bloomington Drosophila Stock Center. The UAS-\(\alpha\beta 42^{2X}\) strain was a gift from Dr. Pedro Fernandez-Funez (University of Florida, USA). The genotypes of the flies used in this study are elav>\(\alpha\beta 42^{2X}\) (elav-GAL4/++; UAS-\(\alpha\beta 42^{2X}/++\)), GMR>\(\alpha\beta 42^{2X}\) (GMR-GAL4; UAS-\(\alpha\beta 42^{2X}/++\)), and Lsp2>Atg8a::GFP (UAS-Atg8a::GFP; Lsp2-GAL4/++).

**Survival Assay**  To measure the survival of flies during development, more than 200 embryos per genotype were collected on grape juice-agar plates. Fifty embryos were transferred and stored at 25°C in standard upright plastic shell vials containing standard fly media with terpenes (Sigma-Aldrich, USA). The rate of eclosed adult female flies were counted and this experiment was done in duplicate.

**Acridine orange staining**  Acridine orange staining was performed to detect cell death in the sample tissues. The brains or eye imaginal discs of L3 larvae were dissected in phosphate-buffered saline (PBS; Bio Basic Inc., USA) and treated for 5 min in 1.6 × 10<sup>-6</sup> M acridine orange solution (Sigma-Aldrich, USA). The samples were briefly washed with PBS and subsequently examined under an Axiophot2 fluorescence microscope (Carl Zeiss, Germany).
**Immunohistochemistry analysis** The brains of larvae or flies were fixed in 4% paraformaldehyde in PBS for 4 min or 3 h at room temperature. They were then washed in PBST (PBS + 0.1% Triton X-100) and blocked in blocking solution (2% normal goat serum + 2% bovine serum albumin + 0.1% Triton X-100). Anti-Aβ42 (1:200 in blocking solution; Santa Cruz, USA) antibody, anti-reverse polarity antibody [anti-Repo; 1:10 in blocking solution; Developmental Studies Hybridoma Bank (DSHB), USA], and anti-phospho-extracellular signal-regulated kinase (anti-pERK; 1:200 in blocking solution; Cell Signaling Technology, USA) were used as primary antibodies. Alexa-Fluor-488-labeled anti-mouse (1:200 in PBST; Cell Signaling Technology, USA) and Alexa-Fluor-555-labeled anti-rabbit (1:200 in PBST; Cell Signaling Technology, USA) antibodies were used as secondary antibodies.

**Western blot analysis** For western blot analyses, adult fly heads were homogenized in 2× Laemmli sample buffer, and the lysates were separated using SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes, and the membrane was blocked with 5% non-fat dry milk and probed with indicated primary antibodies. Anti-ERK [1:2,000 in TBST (Tris-buffered saline + 0.1% Tween-20); Cell Signaling Technology, USA], anti-pERK (1:2,000 in TBST; Cell Signaling Technology, USA), anti-phospho-AKT (anti-pAKT; 1:2,000 in TBST; Cell Signaling Technology, USA), Anti-Refractory to sigma P (Ref(2)P; 1:2,000 in TBST; Abcam, USA), and anti-actin (1:2,000 in TBST; DSHB, USA) antibodies were used as primary antibodies. Either a horseradish peroxidase-conjugated ant-mouse IgG or anti-rabbit IgG antibodies were used as a secondary antibody (1:2,000 in TBST; Cell Signaling Technology, USA).
**Measurement of ROS levels**  ROS levels in the larval eye imaginal discs was evaluated with dihydroethidium (DHE; Invitrogen Molecular Probes, USA). The eye imaginal discs of *Drosophila* L3 larvae were dissected in Schneider’s medium at room temperature. The discs were incubated with 30 μM of DHE in Schneider’s medium for 5 min in dark multi-well glass and then washed with Schneider’s medium. The samples were observed under an Axiophot2 fluorescence microscope (Carl Zeiss, Germany).

**Measurement of nitric oxide levels**  Fifteen heads of 3-day-old flies were prepared in homogenization buffer (0.1 M phosphate buffer at pH 7.4, 27 mM KCl) on ice. The samples were homogenized and centrifuged at 10,000 g for 10 min at 4°C. The supernatants were mixed in a 1:1 ratio with Greiss reagent (Sigma-Aldrich, USA) and incubated for 15 min at 25°C. Nitrite levels were measured using spectrophotometry at 550 nm.

**Bacterial strain and infection**  *Escherichia coli* DH5α strains were purchased from Dongin biotech (Republic of Korea). The bacteria were cultivated in Luria Bertani medium at 37°C for 16-20 h and concentrated. Septic injury was performed by prickling the thorax of 3-5-day-old *Drs-GFP* adult males with a thin needle.

**Statistics**  In all experiments, data was quantitatively analyzed for statistical significance using either a Student’s *t*-test (two-tailed) or a one-way ANOVA followed by a Tukey-Kramer multiple comparisons test. Student’s *t*-test was applied for comparisons of two groups. SPSS (ver. 19, Somers, USA) software was used, and *P*<0.05 indicated a significant difference.
RESULTS

Screening the terpenes that alleviate the phenotypes of the AD model flies

In order to evaluate the beneficial effects of terpenes on AD, we tested the neuroprotective effects of 16 terpenes that were reported to be commonly emitted from Korean forests. For our primary screening, we investigated whether the rough eye phenotype caused by Aβ42 can be alleviated when the flies are fed with media containing the respective terpenes. Donepezil was used as the positive control as it was effective in alleviating the rough eye phenotype when fed to Aβ42-expressing flies from the 1st instar larvae, as compared to flies fed with the control (Figs. 1A, B). Among the tested, six terpenes, including ρ-cymene, limonene (+), limonene (-), linalool, α-pinene (+), and β-pinene (-) were shown to relieve the rough eye phenotype when fed to Aβ42-expressing flies (Figs. 1A, B).

Previously, we reported that ectopic expression of Aβ42 in neurons decreased the survival rate of flies during development. Therefore, we conducted a secondary screening to observe if feeding the selected terpenes would increase the survival rate of the flies expressing Aβ42 in neurons during the developmental stage. The survival rate of the Drosophila was measured in duplicate after being fed with low concentrations (50 μg/mL) and high concentrations (200 μg/mL) of the six terpenes selected during the first screening. As a result, we found that limonene (+) most effectively increased the survival rate of Aβ42-expressing flies (Fig. 1C).

The concentration of limonene (+) that we used for screening is closer to the concentrations obtained from food than to the concentrations that would be inhaled in the forest. In general, lower concentrations of limonene (+) are known to be emitted from the forest compared to the concentration of limonene (+) present in fruit. Therefore, we investigated the effects of limonene (+) at various concentrations, including low...
concentrations similar to those emitted from forests. Unlike that at high concentrations, at low concentrations, limonene (+) did not protect flies from the effects of Aβ42 (Fig. 1D).

Suppression of Aβ42-induced cell death in Drosophila AD models by limonene (+) feeding

Previously, we reported that both the rough eye phenotype and decrease in viability in Aβ42-expressing flies have been associated with cell death. Therefore, we investigated if limonene (+) is able to inhibit cell death by observing its ability to alleviate both the rough eye phenotype and decrease in viability. Drosophila expressing Aβ42 in neurons or the developing eyes were raised in a medium containing limonene (+) (200 μg/mL), and then subjected to AO staining of their brains or the imaginal discs. As shown in Fig. 2, both limonene (+) and donepezil significantly reduced cell death in the brain and the eye imaginal discs as compared to DMSO, a vehicle.

As the aggregation of Aβ42 causes neurofibrillary tangles and is important for neuronal loss, we examined the effect of limonene (+) on the accumulation and aggregation of Aβ42 in the brain of AD model flies. Immunohistochemistry with anti-Aβ42 antibody and thioflavin S staining showed that the amount and the aggregation of Aβ42 in the brain of limonene (+)-fed flies did not differ from the DMSO-fed control group (Figs. 2E, F). Taken together, these results suggest that limonene (+) alleviates both the rough eye phenotype and decrease in viability by reducing cell death without affecting Aβ42 accumulation and aggregation.

Reduced amount of ROS in a Drosophila AD model by limonene (+) intake

As Aβ42-induced ROS generation is essential for neuronal death in AD and limonene (+) is
known to have an antioxidant effect, we investigated whether the inhibitory effect of limonene (+) on cell death is due to its antioxidant effect. As shown in Fig. 3, where ROS levels were measured through DHE staining, limonene (+) feeding reduced the amount of ROS in the eye imaginal discs of AD model flies compared to the control.

This result suggests that the antioxidant properties of limonene (+) are important for the neuroprotective action of limonene (+) in the AD model flies.

The effect of limonene (+) on Aβ42-induced ERK activation

Given that the hyperactivation of the ERK signaling pathway is one of the pathological changes in both human AD patients and Drosophila AD models, the effects of limonene (+) treatment on the Aβ42-induced ERK activation in the brain of Drosophila AD models were investigated. As previously reported, the ERK phosphorylation, which was restricted to neurons (Elav-positive), and not glial cells (Repo-positive) (Fig. 4A), is increased in the brains of Aβ42-expressing flies (elav> Aβ422X) compared to that of control flies (elav-GAL4) (Fig. 4). As well as, limonene (+) feeding clearly reduced the elevated ERK phosphorylation level to that of the control flies. However, limonene (+) feeding does not affect ERK phosphorylation in the heads of the control flies (elav-GAL4) (Figs. 4B, C), which implies that limonene (+) does not act directly as an ERK inhibitor. Given that markedly increased oxidative stress in the brains of individuals with AD causes phosphorylation of ERK and that limonene (+) has antioxidant activity, it is expected that limonene (+) would reduce ERK phosphorylation in the brains of Aβ42-expressing flies by ROS scavenging. To test this hypothesis, we performed experiments to examine whether limonene (+) could inhibit ERK phosphorylation induced by H2O2 in the fly brain. As shown in Fig. 4D, we found that ERK phosphorylation levels were increased in the heads of flies fed H2O2 and that the increased ERK phosphorylation by H2O2 was inhibited by limonene (+) intake. This indicates that
ingested limonene (+) can inhibit ERK phosphorylation through its antioxidant activity. Interestingly, the antioxidant effects of limonene (+) were more pronounced when consumed for a long time than when consumed for a short time. The inhibitory effects of limonene (+) on ERK phosphorylation were not obvious upon ingestion for 3 days, whereas ERK phosphorylation was almost completely inhibited upon ingestion for 7 days (Fig. 4D).

**Reduction of inflammatory response by limonene (+) intake in AD model flies**

There is growing evidence that abnormally increased inflammation in the brain of AD patients plays an important role in the progression of the disease. It is also well known that limonene (+) exerts anti-inflammatory properties in various mammalian cells and mouse disease models. Therefore, we investigated whether limonene (+) intake affects the inflammatory response in the AD model flies. First, we examined the effect of limonene (+) on the increase in the number of glial cells by \(\text{A}\beta\text{42}\) expression. As shown in Figs. 5A and B, the number of glial cells increased in the brain of AD flies compared to that in the control flies, and limonene (+) intake almost completely returned the number of glial cells to control levels. Secondly, we examined the effect of limonene (+) intake on the nitric oxide (NO) level. Consistent with the result from the glial cell number, \(\text{A}\beta\text{42}\) expression increased NO levels in the fly head, and the elevated NO was reduced to the control level via limonene (+) intake (Fig. 5C).

Additionally, we investigated whether limonene (+) could reduce inflammation induced by microbe infection by measuring the expression levels of Drosomycin tagged GFP (\(\text{Drs-GFP}\)). Limonene (+) intake significantly decreased \(\text{Drs-GFP}\) expression induced by DH5\(\alpha\) infection in the *Drosophila* adult thorax (Figs. 5D, E), suggesting that limonene (+) exerts anti-inflammatory property in vivo.

Taken together, these observations might be explained by the inhibitory effect of
limonene (+) on increased neuroinflammation induced by Aβ42.

**Limonene (+) intake does not activate autophagy in Drosophila**

It has been previously reported that limonene (+) induces autophagy in SH-SY5Y neuroblastoma cells,\(^{39}\) and that autophagy is beneficial for the cytotoxicity of Aβ42.\(^{40}\)

Therefore, we investigated whether limonene (+) also causes autophagy in *Drosophila*, using the autophagy reporter, *Atg8a::GFP*, which is expressed in the fat body. Unlike in human cells, limonene (+) did not induce autophagy in *Drosophila*, although the autophagy was strongly activated by expressing the TOR dominant negative form in the fat body (*Lsp2>UAS-TOR\(^{TED}\); Fig. 6A). Moreover, chloroquine, an autophagy inhibitor, treatment only partially suppressed the increased survival rate of Aβ42-expressing flies via limonene (+) intake (Fig. 6B). Additionally, limonene (+) intake did not affect the activity of autophagy-related factors in the fly brain (Fig. 6C). Taken together, these results indicated that limonene (+) intake does not activate autophagy in *Drosophila*. 
DISCUSSION

A number of previous studies have shown that forest bathing is beneficial for human health and suggest that terpenes, which are generally emitted from forests, have potential to act as an important mediator for the beneficial effects of forest bathing.\textsuperscript{16,41} In fact, studies using various disease models have shown that terpenes are expected to have mitigative and therapeutic effects on various diseases.\textsuperscript{16} However, there have only been a few comparative studies on screening various terpenes. Moreover, the effects of terpenes on neurodegenerative diseases, such as AD, have not been well-studied. Thus, in this study, we investigated the neuroprotective effects of 16 terpenes, which are known to be common in Korean forests, on Aβ42 neurotoxicity, through a screening with a \textit{Drosophila} AD model. As a result, six terpenes expected to have neuroprotective activity could be identified through primary screening. Among them, we investigated the mitigative effects of limonene (+), the most potent terpene, on neurological phenotypes of AD model flies. Here, the identification of Aβ42 cytotoxicity-inhibiting substances could be successfully conducted by studying various mechanisms of action, starting from phenotype, suggesting that \textit{Drosophila} is a useful model system for screening various materials that have beneficial effects on neurodegenerative diseases. It is also expected that the methods of our study will be applied in future to analyze the efficacy of terpenes for various diseases.

We have shown here that limonene (+) intake can alleviate the \textit{Drosophila} eye degeneration and reduced survival during development both induced by Aβ42. Limonene (+) also inhibited Aβ42-induced cell death in the larval brain and eye imaginal discs, suggesting that the phenotypic mitigative effects of limonene (+) in AD model flies are due to inhibition of Aβ42-induced cell death. Limonene (+) is known to have anticancer activity, antinociceptive activity, and antidiabetic activity, and is beneficial for metabolic syndrome.
and diseases of the gastrointestinal and respiratory tracts. However, in our knowledge, this study is the first report on the neuroprotective effects of limonene (+) against the toxicity of Aβ42. Although there are few to no detailed studies suggesting the inhibitory effect of limonene (+) on Aβ42 toxicity, a previous study has reported that extracts of natural herbs containing a large amount of limonene (+) inhibit the cytotoxicity of Aβ42 and protect nerve cells. It has also been reported that limonene (+) causes neuronal differentiation, inhibits inflammation associated with AD, and has strong antioxidant properties. Based on these findings, it is not surprising that limonene (+) has a neuroprotective effect on Aβ42 toxicity. However, our finding that limonene (+) does not only affect the cell level, but also affects neuroprotection in in vivo models suggests that the ingested limonene (+) can be delivered to the brain in an effective amount without degrading in the digestive tract. This emphasizes the usefulness of limonene (+) in both the prevention of AD and the development of therapeutic agents. Thus, there is a need for further studies on the feasibility of using limonene (+) in AD treatment with mammalian models.

Among the terpenes we have used, linalool is well-known for its therapeutic efficacy in AD models. Recent studies have shown that linalool could alleviate neuropathological and behavioral impairment in AD model mice. In our study, the beneficial effects of linalool were also partially confirmed in the Drosophila AD model. However, confined to our results in the Drosophila model, the effects of linalool on the cytotoxicity of Aβ42 were not as high as the effects of limonene (+). Additionally, the amount of linalool produced in forests is generally much smaller than that of limonene (+). Therefore, further research into the effects of linalool has not been conducted here. Nevertheless, we cannot deny that linalool is still a valid candidate for AD therapy and that further research is needed.

In this study, we found a neuroprotective effect of limonene (+) on Aβ42 cytotoxicity, but did not elucidate the detailed mechanism of this neuroprotective action. However, our
results suggest that antioxidant and anti-inflammatory properties of limonene (+) may play an important role in neuroprotection. As with many other phenolic phytochemicals, limonene (+) acts as an antioxidant, its activity being the potential main mechanism contributing to the beneficial effect of limonene (+) on human health. Indeed, an in vitro study showed protective effects of limonene (+) on H$_2$O$_2$-induced chromosome breakage and loss along with DNA damage on human lymphocytes and V79 cell lines. Furthermore, in a number of other studies, limonene (+) has been shown to be beneficial to health by inhibiting oxidative stress. Our results also confirm that ROS is significantly reduced in AD flies fed with limonene (+), which means that limonene (+) has antioxidant activity not only in vitro, but also in vivo. On the other hand, it has also been reported that limonene (+) oxidation products, such as 4-oxopentanal, 3-isopropenyl-6-oxo-heptanal, and 4-acetyl-1-methylcyclohexene, induce oxidative stress and inflammation in human bronchial and alveolar cell lines. However, limonene (+) is generally considered as a safe flavoring agent and has fairly low toxicity. Consistently, in our study, no toxicity of limonene (+) was observed at the concentrations we used for Drosophila studies. Moreover, the ingestion of limonene (+) reduced the amount of ROS produced. These results suggest that the effect of limonene (+) on oxidative stress may vary depending on the concentration.

In addition to antioxidative activity, limonene (+) has been shown to have anti-inflammatory effects and our study also showed that there was a reduced inflammatory response in the AD model head after ingestion of limonene (+). However, it is still unclear how limonene (+) inhibits the inflammatory response. A recent study has suggested that systemic treatments of limonene (+) can modulate the inflammatory response by regulating the activation of the TRPA1 channel in astrocytes. In addition, it was reported that the activity of TRPA1 mediates the toxicity of Aβ42. Therefore, the activity of TRPA1 might be inhibited in the glial cells of Drosophila AD models in which limonene (+) is taken, and
as a result, neuroinflammation is alleviated and nerve cell death is likely to be mitigated. In other words, the inhibition of TRPA1 activity by limonene (+) may at least partly explain the alleviation of toxicity of Aβ42 in limonene (+)-fed fly heads. If this hypothesis is verified using a TRPA1-deficient fly, it is anticipated that the exact mechanism of the anti-inflammatory effect of limonene (+) can be found.

In the present study, our results revealed that limonene (+) was effective at reducing Aβ42 neurotoxicity at levels similar to those obtained in the diet but not at low levels, such as those emitted from the forest. This indicates that inhaling limonene (+) from forests is unlikely to be an effective treatment for AD. Rather, high levels of limonene in food or medication are expected to help treat or prevent AD. At the present time, however, there is a lack of complete understanding of the differences between the effects of limonene (+) in fruit flies and humans; moreover, the long-term effects of limonene (+) inhalation as well as the effects of terpene mixture are unknown. Therefore, more extensive follow-up studies on the effects of limonene (+) at low concentration should be conducted.

In conclusion, our study suggests that limonene (+) has a beneficial effect on AD due to its antioxidant and anti-inflammatory properties and may be used for treating or preventing AD.
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Conflict of Interest

The authors declare no conflict of interest.
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Fig. 1. Limonene (+) suppresses the Aβ42-induced reduction of eye size and the decreased survival in Drosophila AD models

(A) Drosophila adult eyes of control (GMR-GAL4) and Aβ42-expressing flies (GMR> Aβ42<sup>2X</sup>) fed with the indicated reagents. Intake of each six terpenes suppresses the Aβ42-induced reduction of the eye. (B) Graph showing the comparative eye size of Aβ42-expressing flies fed with the indicated terpenes (50 μg/mL, n=10) or vehicle (DMSO). (C) The effect of terpene intake (50 μg/mL or 200 μg/mL) on survival rate of Aβ42-expressing flies during development (n≥200). (D) Survival rate of Aβ42-expressing flies fed with limonene (+) at various concentrations. All data in the graphs are expressed as means ± standard error (S.E.) [***p<0.001, **p<0.01, *p<0.05 vs DMSO-fed β42<sup>2X</sup> (B) or DMSO-fed elav>Aβ42<sup>2X</sup> (A); Student’s t-test]. NS, not significant.
Fig. 2. Suppression of Aβ42-induced cell death without affecting Aβ42 aggregation by limonene (+)

(A-D) The effect of limonene (+) intake on Aβ42-induced cell death. (A and B) Representative images of acridine orange (AO)-stained larval brain and imaginal disc of respective groups. (C and D) Graphs showing the relative number of AO-positive signals in the larval brain and imaginal discs of indicated groups (n≥20). (E and F) Comparison of Aβ42 levels (E) and Aβ42 aggregation (F) between limonene (+)-fed and DMSO-fed Aβ42-expressing (elav>Aβ42^{2X}) fly brains. Adult brains of the respective groups were stained with anti-Aβ42 antibody (E) and thioflavin S (F). All data in the graphs are expressed as means ± S.E. [*p<0.05, ***p<0.001 vs DMSO-fed elav>Aβ42^{2X} (C) or DMSO-fed GMR>Aβ42^{2X} (D), Student’s t-test]. Done, 0.1 μg/mL donepezil; Limo, 200 μg/mL limonene (+).
Fig. 3. Limonene (+) intake reduces ROS levels in Aβ42-expressing flies

(A) In vivo detection of ROS with dihydroethidium (DHE) in the eye imaginal discs of DMSO, limonene (+), or donepezil-fed Drosophila larvae (stage L3). (B) Graph showing the relative number of DHE-positive signals per eye imaginal disc of the Aβ42-expressing larvae (GMR>Aβ42^{2X}) fed with the indicated medium (n≥20). All data in the graph are expressed as means ± S.E. (**p<0.01, *p<0.05 vs DMSO-fed GMR>Aβ42^{2X}; Student’s t-test). Done, 0.1 μg/mL donepezil; Limo, 200 μg/mL limonene (+).
Fig. 4. Limonene (+) intake reduces the ERK phosphorylation that is induced by Aβ42

(A and B) Confocal microscopic images of the brain showing phosphorylation of ERK. (A) Phosphorylated ERK signals are merged with Elav-positive neurons, but not with Repo-positive glial cells. (B) Phosphorylation of ERK is increased in Aβ42-expressing fly heads (DMSO-fed elav>Aβ42^{2X}) compared to control (DMSO-fed elav-GAL4). However, limonene (+) intake reduces the increased ERK phosphorylation levels in Aβ42-expressing fly heads (limonene (+)-fed elav>Aβ42^{2X}). The bottom panels are magnified image of the central region of each adult brain (dotted rectangle). (C) Western blot analysis showing the relative levels of ERK phosphorylation in Aβ42-expressing fly heads (elav>Aβ42^{2X}) that were fed with limonene (+) or DMSO. (D) Western blot analyses showing the relative levels of ERK phosphorylation in the heads of w^{1118} flies fed with limonene (+) or DMSO for 3 or 7 days and then treated with H₂O₂ for 3 days. All data in the graph are expressed as means ± S.E. (**p<0.01 vs DMSO-fed elav-GAL4, ##p<0.01 vs DMSO-fed elav>Aβ42^{2X}; Tukey–Kramer test; n≥20). Limo, 200 μg/mL limonene (+).
Fig 5. Limonene (+) intake reduces glial cell number in Aβ42-expressing larval brains

(A) Representative confocal microscopic images of larval brain stained with the anti-Repo antibody. (B) Graph showing the relative number of Repo-positive cells (n≥20). (C) Comparison of nitric oxide (NO) levels in the adult fly heads of indicated samples (n≥20). (B, C) All data in the graphs are expressed as means ± S.E. (**p<0.01 vs DMSO-fed elav-GAL4, , ##p<0.01 vs DMSO-fed elav>Aβ422X; Tukey–Kramer test). (D) Representative fluorescence microscopic images of flies expressing Drosomycin tagged GFP (Drs-GFP) (E) Graph showing relative expression levels of GFP in the thorax of bacteria-infected flies (n≥9). All data in the graph are expressed as means ± S.E. (***p<0.001 vs sucrose-injected Drs-GFP, #p<0.05 vs DH5α-injected Drs-GFP; Tukey–Kramer test). Limo, 200 μg/mL limonene (+).
Fig 6. Limonene (+) intake does not affect autophagy

(A) Representative images of autophagy activation in larval fat body. The bottom panels are magnified images of the central region of each larval fat body (dotted rectangle). (B) The effect of chloroquine (CQ) treatment on the survival rate of Aβ42-expressing (elav>Aβ42^{2X}) flies fed with limonene (+) (n≥200). (C) Western blot analysis showing the relative levels of phospho-AKT (pAKT) and p62 (Ref(2)P) in heads of Aβ42-expressing flies that were fed with limonene (+). All data in the graph are expressed as means ± S.E. (***p<0.001 vs Limo (-) CQ (-) elav-GAL4, ###p<0.001 vs Limo (-) CQ (-) elav>Aβ42^{2X}; $$$p<0.001 vs Limo (+) CQ (-) elav>Aβ42^{2X}; Tukey–Kramer test). CQ, 1 mg/mL chloroquine; Limo, 200 μg/mL limonene (+).