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Aged Garlic Supplementation Improves the Anxiety and Depressive-Like Behavioral Manifestations Induced by Maternal Separation in a Rodent Model

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Abstract: Maternal separation (MS) is a commonly used paradigm in rodents that has been widely established as a model to study the long-term effects of early-life stress on brain function and behavior, eventually resulting in heightened anxious- and depressive-like behavior. The present study aimed to assess whether supplementation with aged garlic extract (AGE) could reduce the symptoms of adverse effects in mice. We used a battery of behavior tests such as forced swim test (FST), tail suspension test (TST), elevated plus maze (EPM) and open field test (OFT) to evaluate anxiety- and depressive-like behaviors. Our data demonstrated that aged garlic extract treatment markedly ($p<0.05$) improved the depressive-like behaviors of maternally separated mice. In particular, AGE treatment induced a marked ($p<0.05$) decrease in the immobility time in forced swim test and tail suspension test, which is indicative of a reduction in depressive-like behavior. In addition, AGE-treated mice spent more time in the open arms in the elevated plus maze and in the central area in the open field test, indicating a decrease in anxiety-like behavior. These findings indicate that AGE may represent a potential target for treating early life stress's damage to mental health. The beneficial effects of such exposure likely reflect the anti-inflammatory and anti-oxidant effects of AGE, which could potentially guard against stress-induced neuroinflammation and oxidative harm within the brain. More studies are necessary to elucidate the detailed molecular mechanism of AGE's therapeutic effects and evaluate its translational potential in subjects at high risk of stress-related psychiatric disorders.

Key words: Aged garlic extract, maternal separation, early-life stress, anxiety, depression, rodent model.

Introduction

Adverse experiences in early life are a key risk element for emotion-related psychopathologies such as anxiety and depression that emerge through the lifespan. Adverse childhood experiences (ACEs), such as abuse, neglect or parental separation, also have the potential to impact brain maturation and psychological health in deep and lasting ways (Harms and Pollak, 2020). These early stressors adversely affect developing stress response systems, predisposing individuals to increased vulnerability to mood disorders in later life (Smith and Pollak, 2020). The significant societal burden resulting from early life stress related sequelae underlines the critical need for interventions to prevent and ameliorate the negative consequences (Allen *et al.*, 2017). An understanding that early experiences influence mental health trajectories is of critical value to establish a

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framework of focused interventions and the support of protective factors in people at risk for early adversities (Abrishamcar *et al*., 2024). The literature to date indicates that there are developmental alterations in the prefrontal cortex, hippocampus, hypothalamus and amygdala (and their interactions) early in life that create vulnerabilities to physical and mental health problems across the lifespan (Smith and Pollak, 2020).

Anxiety disorders and depression are two of the biggest contributing factors to the worldwide public health burden, affecting hundreds of millions in the world. It is estimated that 279 million individuals are experiencing depressive disorders, and 301 million individuals are experiencing anxiety disorders (Rajkumar, 2022). These conditions are very common and are associated with a significant burden of disability and impaired quality of life. The economic burden of mental health issues is also staggering (Kimball *et al*., 2018). A substantial amount of evidence that suggests identifying and targeting contributors to the development of these disorders, including early life stress (ELS), is critical. Considering the high rates and burden of depression and anxiety conditions, especially when stress from early life is present, it is essential to establish and deliver effective preventive interventions (Hughes *et al*., 2020). Interventions that can reduce the deleterious effects of early life stress, promote resiliency and prevent the emergence of mood disorders in high-risk individuals are urgently needed (Rapee, 2013). Such interventions can operate at different levels, from individual coping mechanisms to family support and to larger, community-based programs (Bolton *et al*., 2023). By tackling the underlying problems that trigger mental health issues and providing support precisely when and where it's needed, the long-term damage caused by early life stress can be eased, benefiting both people and communities (Harms and Pollak, 2020).

Maternal separation (MS) in rodents is one of the widely employed and well-validated animal model for investigating the long-lasting efficacy of early adversity on brain development and behavior (Shin and Lee, 2023). By keeping rodent pups apart from their mothers for a predetermined period during early development, researchers can simulate certain aspects of early life stress and examine the resulting alterations in behavior, neuroendocrine function, and neurobiology (Cortes and Sullivan, 2014). This model induces increased anxiety- and depression-like behaviors, reflecting disruptions in emotional regulation (Wei *et al*., 2023). These behavioral alterations are accompanied by neurobiological alterations, including impaired functioning of the hypothalamic-pituitary-adrenal axis (Zanta *et al*., 2023), dysregulation of neurotransmitter systems, changes in synaptic plasticity, and neuroinflammation. Interestingly, maternal separation has also been connected to exacerbation of neuropathic pain in mice later in life (Sadler *et al*., 2021). The specific consequences of maternal separation can vary depending on the duration and timing of the separation, as well as the strain and age of the animals.

Aged garlic extract (AGE) is generated by a prolonged aging method involving raw garlic, resulting in a product with enhanced stability and bioavailability of its bioactive compounds. This AGE shows promise as a therapeutic agent because of its notable anti-inflammatory and antioxidant properties (Recinella *et al*., 2023). Research indicates that AGE may possess anti-inflammatory effects via lowering the production of nitric oxide and pro-inflammatory cytokines (Kim *et al*., 2014). Given the relevance of inflammation along with oxidative stress in the pathophysiology of anxiety and depression, AGE may offer a natural way to mitigate the neurobiological consequences of early life stress. Additionally, studies suggest that AGE possesses vasorelaxant effects (Recinella *et al*., 2023). Increasing reports from some of previous studies have demonstrated that garlic or its bioactive constituents have anxiolytic and antidepressant properties in several stress models (Rahmani *et al*., 2018). For example, garlic essential oil has been demonstrated to ameliorate acute and chronic mild stress models of depression in rats possibly by

modulating monoaminergic neurotransmission and brain-derived neurotrophic factor levels (Huang *et al*., 2019). Furthermore, in diabetic rats, garlic has been found to improve anxiety and depression-like behaviors and diminish brain oxidative stress (Rahmani *et al*., 2018). Such data reinforce the idea that garlic compounds might represent a natural and efficient intervention in the relief of mood disturbance, possibly through their antioxidant capabilities.

This investigation seeks to investigate the effects of aged garlic extract supplementation on specific anxiety- and depressive-like behavioral outcomes in rodent models subjected to maternal separation. Building upon existing evidence that AGE possesses antioxidant and anti-inflammatory properties, as well as indications that garlic or its bioactive compounds may exhibit anxiolytic and antidepressant effects, this study seeks to determine whether AGE can ameliorate the negative behavioral consequences associated with the early life stress induced by maternal separation

Materials and Methods

Drugs and Reagents: Fluoxetine was obtained from Sigma-Aldrich Corporation, while ethanol was procured from Merck. In this investigation, every other reagent utilized was of analytical quality.

Preparation of aged garlic: The garlic bulbs were collected and the cloves were removed and peeled. After having peeled cloves, theses were finely grinded and homogenized with the aid of the blender. The mixture of clover was deeped into a glass container to which was added 20% ethanol of 1000 ml for aging 10 months inside the glass bottle at room temperature. After that, muslin cloth was used to decant the AGE. The filtered decanted extract was filtered with Whatman No. 1 filter paper and subsequently by vacuum suction filter. The filtered solution was further concentrated by boiling in a rotary evaporator to remove the solvent and to obtain the dry AGE extract. Two doses of AGE (150 mg/kg and 300 mg/kg) were used in the study, along with fluoxetine 10 mg/kg).

Animals: Ten weeks aged, female and male Albino mice were procured from ICDDR'b, Bangladesh. Mice were kept under normal experimental environment (temperature: 22 ± 1◦C; relative humidity: 55 ± 5%) for 2 weeks with unfettered access to food and drinkable water. The animals were mated (one male to two females) after 2 weeks of acclimation. The day of labor was referred as postnatal day 0 (PND 0). The mice used in this study were male offspring. Every procedure was carried out in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Maternal separation procedure: The newly born pups were allocated randomly into two groups: non-maternal separation (NMS) and maternal separation (MS). In the MS group, the pups were taken away from their mothers for four hours every day (10:00 to 14:00 h) from PND 2 to PND 21, and they were then brought back to their mothers following the separation period (Wei *et al*., 2023). On a heating pad, the pups had been kept in sanitized cages with bedding throughout this period. In contrast, the NMS group pups remained with their mothers until the weaning period upon completion of PND 21, the pups were put back in their mother's cages and left alone until PND 25. Male mice underwent weaning on PND 25 and allocated to five experimental groups, each consisting of four mice per cage. Treatment started on PND 44 and continued until experiment day PND 60. We did not touch the control animals and they were left undisturbed and were weaned on PND 25 and grouped (four mice per cage) until PND 60 for experiments. All experimental groups involved eight mice.

Study design: The control and maternally separated mice were allocated into five experimental groups at random as follows (n=8/group). Group 1: a control group that received 14 days of standard saline treatment. Group 2: MS mice treated with normal saline for 14 days. Group 3 and 4: MS mice treated with Aged garlic extract (150 and 300 mg/kg, orally) for a duration of 14 days. Group 5: MS mice received fluoxetine (10 mg/kg). Every behavioral test was

performed in adult mice (PND 60). The course of treatment lasted 14 continuous days, from PND 44 (early adulthood) to PND 60 (adulthood).

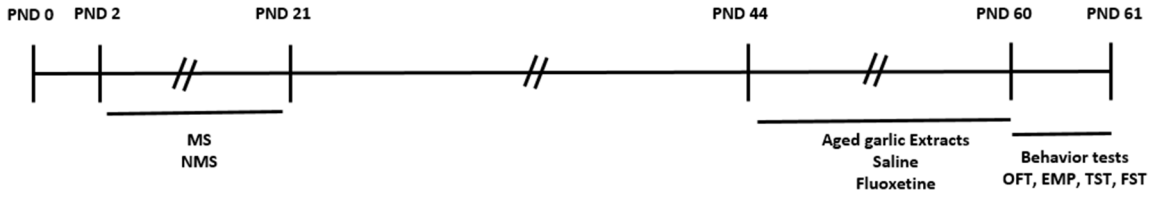

Figure 1. Timeline of the experiment. MS: Maternal separation, NMS: Non-maternal separation, PND: Postnatal day, OFT: Open field test, EPM: Elevated plus maze, TST: Tail suspension test, FST: Forced swim test.

Behavioral studies

Forced swimming test: The forced swim test (FST) is one of the most widely employed tests for evaluating the influence of antidepressants on depressive-like behaviors (Hwang *et al.*, 2020). The FST consists of measuring the natural response of the mice to swim, when they are submerged in an inescapable cylinder. Prior to the test, mice were acclimatized for 15 min in a clear, water-filled cylinder (30 cm depth). Similarly the procedure was performed on the test day, the mice were monitored for 5 minutes and their activity was video taped and scored later. The duration in seconds in which the mice spent being immobile during the 5 min test was measured. Immobility is their total lack of movement, except for the small movements of the hindlimbs required to keep the mouse's head above the water.

Tail suspension test: The tail suspension test (TST) is a popular experimental model utilized to evaluate the antidepressant activity (Aslam, 2016). TST is performed in the dark to reduce disturbances. Mice are taped by the tail and immobilized so that they cannot free themselves or reach surrounding objects. The test is 6 min long, and the time spent immobile, which is indicative of escape-related behaviors, is recorded. Mice are now housed singly in plastic boxes to prevent interaction. Body movements are video recorded and only movements that engage the hind legs are counted as locomotion. Between sessions the suspension rack is washed with a sterilizing solution.

Open field test: The locomotor activity of mice was evaluated using open field testing (OFT) (Guo *et al*., 2020). The apparatus was a Plexiglas square enclosure (50 cm length, 50 cm width, 38 cm height) with an open top. The base of the arena was subdivided into 25 smaller square units by lines. Mice were introduced to the device for 5 minutes the day before testing to acclimate. On the test day, each mouse explored the center of the arena for five minutes. Thigmotaxis, or time spent in the periphery, is a reliable anxiety indicator in mice. Increased thigmotaxis (more time in outer zones) indicates increased anxiety. To prevent odor cues, the device was cleaned with 70% ethanol in water and dried between trials.

Elevated plus maze: Anxiety levels in the mice were measured using the Elevated Plus-Maze (EPM) test – a test conducted on mice that capitalizes on their natural drive to explore open spaces, while also avoiding open spaces. The EPM equipment was previously described (Carobrez *et al*, 2014). Each mouse's activity from the time when it was placed at the center of the apparatus was recorded over 5 min. Duration of stay in open and closed arm was analyzed by researchers. The central time spent by the mice was not considered in the analysis. To eliminate olfactory cues, the maze was cleaned with 70% ethanol after each trial and then was air-dried.

Statistical analysis: Data analysis was done with GraphPad Prism version 8.0 and IBM SPSS Statistics 25. The means ± standard deviation (SD) are used for presenting quantitative data. To evaluate group differences Kruskal-Wallis H test and one-way analysis of variance were used, with treatment as the independent variable, followed by Tukey's post-hoc

multiple comparison test. Significance was set at P < 0.05 (5% significance level) and P < 0.10 (10% significance level).

Results

Aged Garlic Extract treatment improved depression-like behaviors in male offspring induced by maternal separation.

AGE treatment attenuated maternal separation-induced depression-like behaviors in mice. The forced-swim and tail suspension tests were used to discover depression-like behaviors. In the forced swim test, control group (non-mother separation) showed lower the mean level of immobility time (μ_{saline}= 63.87) than maternal separation saline group's mean immobility time ($\mu_{MS\ saline}$=111.00). Following the application of FLX, AGE 150, and AGE 300, a notable decrease in mean immobility time μ_{FLX}=69.50, $\mu_{AGE\ 150}$=91.50, $\mu_{AGE\ 300}$=79.625 respectively was observed across the three remaining maternal separation groups were shown in figure 2.

There was significant ($p<0.05$) difference in mean duration of immobility among the five experimental groups as shown in table 1. The results of the ANOVA F test were also supported by the Kruskal-Wallis H test. The mother-separated saline group showed a significant ($p<0.05$) elevation of

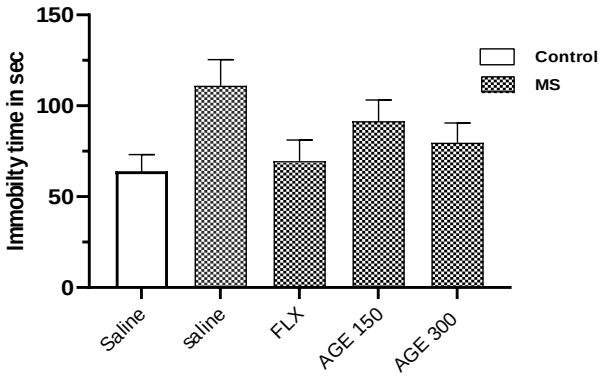

Figure 2. Average immobility time among the five groups of mice for the forced swim test. The data were reported as mean ± SD. MS: maternal separation, FLX: Fluoxetine, AGE: Aged garlic extract.

Table 1. One-way ANOVA, Kruskal Wallis -H and pairwise post hoc comparisons among the five groups of mice of the forced swim test.

Tests		Multiple comparison	p value
One-way ANOVA	Kruskal Wallis-H		
$F(4,35) = 20.59$ (p=0.000)*	$H = 26.68$ (p=0.000)*	$H_0 : \mu_{saline} \geq \mu_{MS\ saline}$ $H_1 : \mu_{saline} < \mu_{MS\ saline}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{FLX}$ $H_1 : \mu_{MS\ saline} > \mu_{FLX}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 150}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 150}$	0.001**
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 300}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 300}$	0.000*

*, and ** Indicates significant at 5% and 10% level of significance respectively. H_0 and H_1 are null and alternative hypothesis.

mean immobility time in multiple comparisons, reflecting the stress induced by maternal separation. In addition, a noticeable reduction ($p<0.05$) was found in the average duration of immobility of the mice in the FLX, AGE 150, and AGE 300 groups in relation to the mother-separated saline group, suggesting an antistress action of FLX, AGE 150, and AGE 300 on mice.

In the tail suspension test, the non-mother separation group exhibited an average immobility time of μ_{saline}=66.62. The mother-separated saline group displayed a significantly higher mean immobility time ($\mu_{MS\ saline}$=98.75) compared to the non-separated group (μ_{saline}=66.62). Furthermore, a reduction in average immobility time was noted for the FLX (μ_{FLX}=67.50), AGE 150 ($\mu_{AGE\ 150}$=82.50) and AGE 300 ($\mu_{AGE\ 300}$=75.25) groups when compared to the $\mu_{MS\ saline}$=98.75 were shown in figure 3.

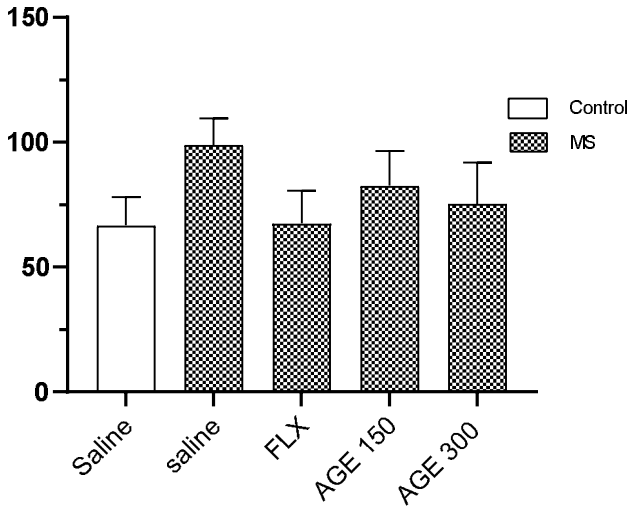

Figure 3. Average immobility time among the five groups of mice for the tail suspension test. The data were reported as mean ± SD. MS: maternal separation, FLX: Fluoxetine, AGE: Aged garlic extract.

Table 2. One-way ANOVA, Kruskal Wallis -H and pairwise post hoc comparisons among the five groups of mice of the tail suspension test.

Tests		Multiple comparison	p value
One-way ANOVA	Kruskal Wallis-H		
$F(4,35) = 7.80$ (p=0.000)*	$H = 17.34$ (p=0.002)*	$H_0 : \mu_{saline} \geq \mu_{MS\ saline}$ $H_1 : \mu_{saline} < \mu_{MS\ saline}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{FLX}$ $H_1 : \mu_{MS\ saline} > \mu_{FLX}$	0.001*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 150}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 150}$	0.065**
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 300}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 300}$	0.005*

*, and ** Indicates significant at 5% and 10% level of significance respectively. H_0 and H_1 are null and alternative hypothesis.

The findings of the one-way ANOVA ($F_{(4,35)}$ = 7.80) and Kruskal-Wallis H test (H = 17.34) indicated a statistically significant ($p<0.05$) difference among the five mean immobility times observed across the five distinct groups of mice were shown in table 2. Subsequent pairwise multiple comparisons (Post Hoc Tukey's) demonstrated statistically significant increases in the mean immobility time within the mother-separated saline group ($\mu_{MS\ saline}$=98.75) in comparison to the control group mice (non-mother separated, μ_{saline}=66.62). This observed significant reduction in the average immobility time suggested the development of stress resulting from maternal separation. Statistically significant ($p<0.05$) decreases in the mean immobility time were also evident for both FLX (μ_{FLX}=67.50) and AGE 300 ($\mu_{AGE\ 300}$=75.25) relative to the $\mu_{MS\ saline}$=98.75, thereby indicating the efficacy of FLX and AGE 300 in mitigating stress among the mice. A statistically significant decrease in the mean immobility time was also observed for AGE 150 ($\mu_{AGE\ 150}$=82.50); however, this finding reached statistical significance ($p<0.10$) only at the 10% significance level.

Aged Garlic Extract treatment improved anxiety-like behaviors in male offspring induced by maternal separation.

Using elevated maze and open-field tests, we evaluated the degree of anxiety-like behaviors in each mouse group. Figure 4 presented the mean time spent in the center during the open field test for the five mouse groups. The average time in the center for the saline group was μ_{saline}=104.88, whereas the mother-separated saline group showed a lower value of $\mu_{MS\ saline}$=59.12. Furthermore, the mean times for the FLX, AGE 150, and AGE 300 groups were μ_{FLX}=94.75, $\mu_{AGE\ 150}$=80.87, and $\mu_{AGE\ 300}$=87.62, respectively, all exceeding the $\mu_{MS\ saline}$=59.12 value. The subsequent table illustrates the outcomes of both the one-way ANOVA and the non-parametric Kruskal-Wallis H tests, utilized to ascertain whether the mean differences between the five mice groups were statistically significant.

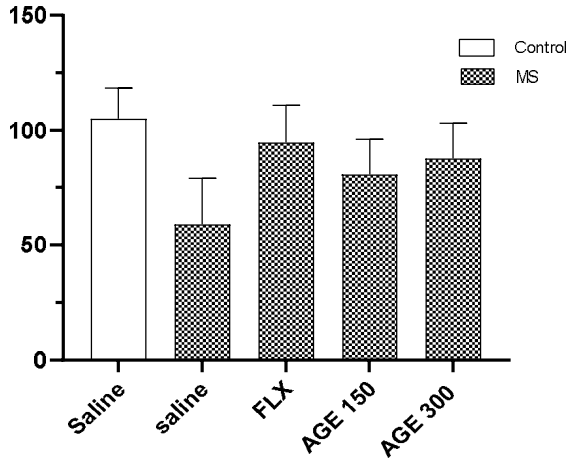

Figure 4. Average time spent in the center of five groups of mice for the open field test. The data were reported as mean ± SD. MS: maternal separation, FLX: Fluoxetine, AGE: Aged garlic extract.

The ANOVA F test ($F_{(4,35)}$ = 9.02) revealed a significant ($P<0.05$) difference in the means among the five mouse groups were shown in table 3. This finding was corroborated by the Kruskal-Wallis test (H = 17.44). Pairwise multiple comparisons indicated that the mean time spent in the center was significantly ($P<0.05$) reduced in the mother-separated saline group ($\mu_{MS\ saline}$=59.12) compared to the non-mother-separated group (μsaline=104.88), suggesting stress-induced by maternal separation.

Conversely, a significant (P<0.05) increase in the mean time spent in the center was observed in the FLX (μ_{FLX}=94.75), AGE 150 ($\mu_{AGE\ 150}$=80.87), and AGE 300 ($\mu_{AGE\ 300}$=87.62) groups compared to the saline group $\mu_{MS\ saline}$=59.12, indicating a reduction in stress due to maternal separation and highlighting the effectiveness of FLX, AGE 150, and AGE 300.

In the non-mother separated control group shown in figure 5, the average time spent in the periphery was μsaline=135.12. Compared to this, the mother-separated saline group showed an increase in the mean time spent in the periphery (μMS saline=180.87). Conversely, the FLX (μ_{FLX}=145.25), AGE 150 ($\mu_{AGE\ 150}$=159.25), and AGE 300 ($\mu_{AGE\ 300}$=152.37) groups demonstrated a decrease in mean peripheral time relative to μMS saline=180.87. The subsequent table 4 presents the results of both the one-way ANOVA and the non-parametric Kruskal-Wallis H tests, which were utilized to ascertain whether the mean differences between the five mice groups were statistically significant.

Table 3. One-way ANOVA, Kruskal Wallis -H and pairwise post hoc comparisons among the five groups of mice of the open field test.

Tests		Multiple comparison	p value
One-way ANOVA	Kruskal Wallis-H		
$F(4,35) = 9.02$ (p=0.000)*	$H = 17.44$ (p=0.002)*	$H_0 : \mu_{saline} \geq \mu_{MS\ saline}$ $H_1 : \mu_{saline} < \mu_{MS\ saline}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{FLX}$ $H_1 : \mu_{MS\ saline} > \mu_{FLX}$	0.001*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 150}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 150}$	0.038*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 300}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 300}$	0.005*

*, and ** Indicates significant at 5% and 10% level of significance respectively. H_0 and H_1 are null and alternative hypothesis.

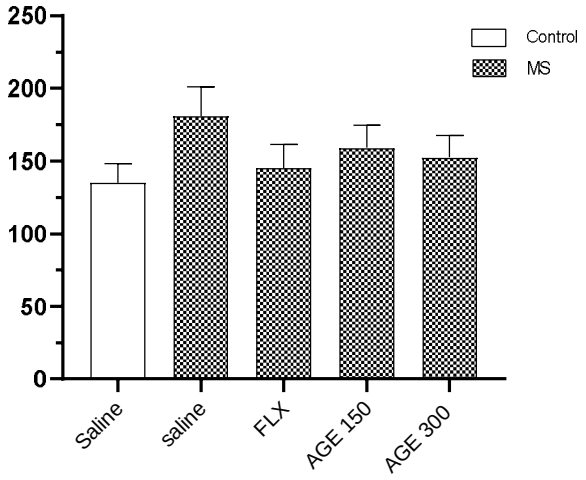

Figure 5. Average time spent in the peripheri of five groups of mice for the open field test. The data were reported as mean ± SD. MS: maternal separation, FLX: Fluoxetine, AGE: Aged garlic extract.

Table 4. One-way ANOVA, Kruskal Wallis -H and pairwise post hoc comparisons among the five groups of mice of the open field test.

Tests		Multiple comparison	p value
One-way ANOVA	Kruskal Wallis-H		
F (4,35) = 9.02 (p = 0.000)*	H = 17.44 (p = 0.002)*	$H_0 : \mu_{saline} \geq \mu_{MS\ saline}$ $H_1 : \mu_{saline} < \mu_{MS\ saline}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{FLX}$ $H_1 : \mu_{MS\ saline} > \mu_{FLX}$	0.001*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 150}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 150}$	0.039*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 300}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 300}$	0.005*

*, and ** Indicates significant at 5% and 10% level of significance respectively. H_0 and H_1 are null and alternative hypothesis.

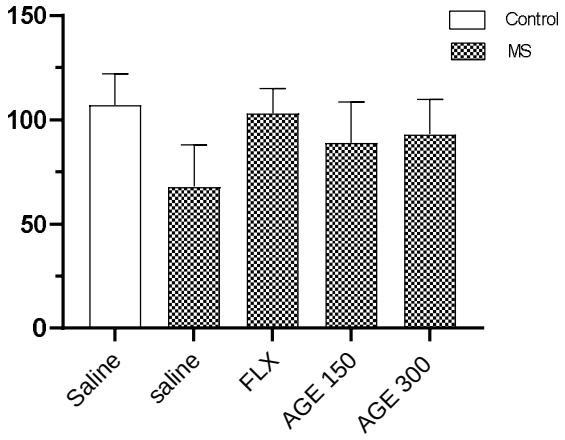

Figure 6. Average time spent on the open arm of mice of five groups for the elevated plus maze test. The data were reported as mean ± SD. MS: Maternal separation, FLX: Fluoxetine, AGE: Aged garlic extract.

The mean time spent on an open arm in the elevated maze test for the five mouse groups is presented in figure 6. The control group's mean time spent on an open arm was μ_{saline}=107.00. In the mother-separated groups, a reduction in the mean time spent was noticed in the saline group $\mu_{MS\ saline}$=67.88, suggesting stress. Conversely, the FLX (μ_{FLX}=103.13), AGE 150 ($\mu_{AGE\ 150}$=88.17), and AGE 300 ($\mu_{AGE\ 300}$=39.00) groups showed an increased average time spent compared to the $\mu_{MS\ saline}$=67.88 group.

To determine whether the group means varied significantly, a one-way ANOVA was conducted, supplemented by the non-parametric Kruskal-Wallis H test. Subsequently, post-hoc Tukey's tests were employed for pairwise multiple comparisons. The outcomes are presented in the table 5 below.

Statistical analyses, including one-way ANOVA and kruskal-wallis H tests, revealed significant ($p<0.05$) differences in the meantime among the five experimental groups of mice. Specifically, pairwise comparisons indicated a significant decrease ($p<0.05$) in average time for the mother-separated saline group ($\mu_{MS\ saline}$=67.88) relative to the non-mother-separated group (μsaline=107.00), suggesting the presence of stress induced by MS. Moreover, the administration of FLX and AGE 300 led to a substantial increase in the median time, indicating a reduction in the stress response resulting from MS. A significant rise in

average time was also noted in the AGE 150 group ($p<0.10$).

According to figure 7, the control group of mice spent an average of $\mu_{saline}=133.00$ seconds in the closed arm. Following maternal separation, mice given saline indicated an increase in the meantime ($\mu_{MS\ saline}=172.12$) spent in the closed arm. The FLX, AGE 150 and AGE 300 treatment groups exhibited mean times in the closed arm of $\mu_{FLX}=136.88$, $\mu_{AGE\ 150}=151.13$, and $\mu_{AGE\ 300}=147.00$ seconds, respectively, which were less than $\mu_{MS\ saline}=172.12$ but approached $\mu_{saline}=133.00$.

Table 5. One-way ANOVA, kruskal wallis -H and pairwise post hoc comparisons among the mice of five groups of the elevated plus maze test.

Tests		Multiple comparison	p value
One-way ANOVA	Kruskal wallis-H		
F (4,35) = 6.51 ($p = 0.001$)*	$H = 14.17$ ($p = 0.005$)*	$H_0 : \mu_{saline} \geq \mu_{MS\ saline}$ $H_1 : \mu_{saline} < \mu_{MS\ saline}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{FLX}$ $H_1 : \mu_{MS\ saline} > \mu_{FLX}$	0.001*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 150}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 150}$	0.061**
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 300}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 300}$	0.021*

*, and ** Indicates significant at 5% and 10% level of significance respectively. H_0 and H_1 are null and alternative hypothesis.

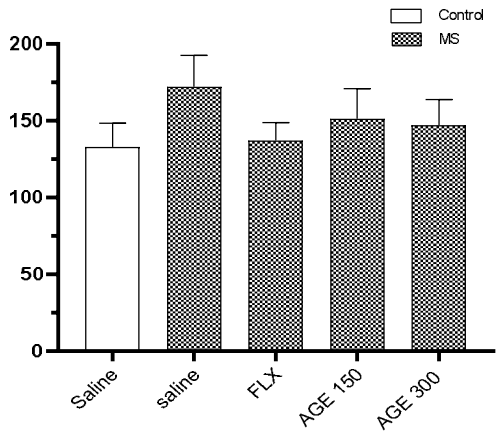

Figure 7. Average time spent on the closed arm of five groups of mice for the elevated plus maze test. The data were reported as mean ± SD. MS: Maternal separation, FLX: Fluoxetine, AGE: Aged garlic extract.

To determine the significance of mean differences, an analysis of variance and the Kruskal-Wallis H test were employed. Furthermore, Post hoc Tukey's tests were conducted to assess the effects of FLX, AGE 150, and AGE 300. The test outcomes are presented in the subsequent table 6.

The results demonstrated that both ANOVA and the Kruskal-Wallis H test significantly differ at 5% level among the 5 groups of mice, suggesting significant differences among the mean values of the five groups of mice. Post hoc analysis by Tukey's test for non-mother vs mother-separated groups revealed a significant rise in the mean time in the closed arm for the mother-separated group. This indicates stress development after maternal separation. Furthermore, the mean time invested in the closed arm for the AGE

300, FLX group was substantially less than that of the remaining groups, which suggests FLX + AGE 300 exerted an anxiolytic effect. There was also a decrease in the mean time for holdings of the AGE 150 group, although the disparities are only significant at the 10% significance level.

Table 6. One-way ANOVA, kruskal wallis -H and pairwise post hoc comparisons among the five groups of mice of the elevated plus maze test.

Tests		Multiple comparison	p value
One-way ANOVA	Kruskal wallis-H		
$F(4,35) = 6.51$ (p=0.001)*	$H = 14.17$ (p=0.005)*	$H_0 : \mu_{saline} \geq \mu_{MS\ saline}$ $H_1 : \mu_{saline} < \mu_{MS\ saline}$	0.000*
		$H_0 : \mu_{MS\ saline} \leq \mu_{FLX}$ $H_1 : \mu_{MS\ saline} > \mu_{FLX}$	0.001*
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 150}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 150}$	0.061**
		$H_0 : \mu_{MS\ saline} \leq \mu_{AGE\ 300}$ $H_1 : \mu_{MS\ saline} > \mu_{AGE\ 300}$	0.021*

*, and ** Indicates significant at 5% and 10% level of significance respectively. H_0 and H_1 are null and alternative hypothesis.

Discussion

In the present study, aged garlic supplementation has been able to rescue anxiety- and depressive-like behaviours in maternal separation rodents. The findings imply that aged garlic extract might have a therapeutic potential in ameliorating the negative consequences of early life stress on mental health. Our findings reveal that AGE reduced depressive-like behaviors, such as immobility duration in the forced swim and tail suspension tests, and time spent in the open arm in the elevated plus maze test, as well as in the open field test, in mice following repeated AGE treatment. Maternal separation in rodents provides a valuable model for investigating the long-term impacts of early-life stress on brain function and behavior. This model induces increased anxiety- and depression-like behaviors, reflecting disruptions in emotional regulation (Wei *et al*., 2023). One possible intervention that has demonstrated the potential to ameliorate the detrimental consequences of MS and depression is aged garlic extract. Research has shown that AGE has strong anti-inflammatory and antioxidant capabilities which work to counteract the effects of MS and depression on the brain.

The forced swim test is widely used behavioral tests to determine the antidepressive effects of drugs in rodents and the test has been actively utilized in the study of depression. Additionally, we discovered in the FST that the mice in which MS was performed, spent a higher amount of time being immobile in the water tank than the control mice. This elevated immobility time is regarded as a surrogate marker of "behavioral despair" and the interpretation is a depression-like behavior in these animals. In contrast, MS mice co-treated with fluoxetine (10 mg/kg) or AGE (150 and 300 mg/kg) did not demonstrate depressive-like behaviors as they had more time for mobility in water. This indicates that the pharmacological treatments were capable of reversing the aversive behavior resulting from MS, probably via their antidepressant-like mechanisms.

The tail suspension test is a reliable method for evaluating the antidepressant potential of experimental drugs. Rodents subjected to MS in this study exhibited a significantly increased duration of immobile behavior compared to the control group. Conversely, MS mice given fluoxetine (10 mg/kg) or AGE (150 and 300 mg/kg) did not display depressive-like behaviors, showing a longer mobility time. The results indicate that the effects of AGE on depressive-like behaviors in mice were comparable to those of fluoxetine, suggesting that AGE may possess similar antidepressant-like properties.

The open field test is a widely known behavioral model for measuring locomotor functions, exploratory behavior, and anxious behaviors in rats or mice. In the present study, MS animals showed a reduced locomotor activity as compared to controls. Furthermore, the results show that treatment with Flx 10 mg/kg or AGE restored the performance in locomotor deficits of MS mice. MS mice receiving Flx or AGE spent more time and moved more extensively in the central area of the open field arena, indicating that the drugs treatment recovered movement impairments and anxiety-like behavior that were exerted by MS.

The elevated plus maze is a standard test to measure anxiety-like behavior in rodents. It has been reported that the spending time by the mouse in the open arms of the plus maze is the index of anxiety, and the mice showing reduced time in the open arms have higher level of anxiety. When compared to the control (NMS) group, mice that received MS exhibited more anxiety-like behavior because they spent a longer period in the elevated plus maze's closed arms as opposed to its open arms. However, the anxiety-like behavior was ameliorated in the MS group when co-treated with fluoxetine or with AGE (150 or 300 mg/kg).

The present study provides evidence that aged garlic supplementation can improve anxiety- and depressive-like behaviors in maternally separated rodents, adding to an expanding literary collection demonstrating the garlic's potential as a beneficial dietary intervention to control stress-related conditions. These findings are consistent with previous research showing that AGE helps to lessen acute stress (Tsai, 2019). For example, Shih-Jen Tsai's study demonstrated the efficacy of AGE in mitigating acute stress, and Rahmani et al. (Rahmani *et al*., 2018) found that garlic improved anxiety- and depressive-related behaviors in diabetic rats. This suggests a broader application of garlic in managing mood disorders, especially those exacerbated by physiological stressors.

Huang *et al.* demonstrated that garlic essential oil may have a potential intervening effect on stress-induced depression through modulating neurotransmitters, thereby providing a possible mechanism for the behavioral improvements observed on the behavioural tests (Huang *et al.*, 2019). This monoaminergic neurotransmission and brain-derived neurotrophic factor alteration (Huang *et al*., 2019) would be a direct effect on brain function relevant to mood modulation. In addition to these advantages, the anti-inflammatory and vasorelaxant activities of AGE could be taken into account (Recinella *et al*., 2023). Maternal separation and depression symptoms are frequently related to high inflammatory and vascular dysfunction, and the action of AGE may participate in the stress overall reducing effects as observed in our study (Recinella *et al.*, 2023).

Several potential mechanisms could underlie the observed effects of aged garlic supplementation. One avenue to explore is the impact of aged garlic on the microbiota in the digestive system and whether this affects anxiety (Rincel and Darnaudéry, 2019). It is becoming more well acknowledged that the gut-brain axis plays a crucial role in the emergence and expression of anxiety and depression (Rincel and Darnaudéry, 2019). Furthermore, garlic's antioxidant and anti-inflammatory effects (Recinella *et al*., 2023) may provide safety to the brain from oxidative stress, as demonstrated in diabetic rats (Rahmani *et al.*, 2018). In the brain, garlic therapy increased SOD and GPx activity while decreasing MDA levels that is related to lower anxiety and depressive-like behaviors. The role of hydrogen sulfide (Tain *et al.*, 2022), a gaseous signaling molecule produced by garlic-derived compounds, could also be considered. One potential preventive measure for hypertension could be an early H2S-targeted intervention. Future studies should focus on quantifying the principal cytokines (e.g., IL-6, TNF-α) and oxidative stress markers (e.g., MDA, SOD) and also explore these potential pathways to elucidate a comprehensive understanding of how aged garlic exerts its beneficial effects. Further research should also investigate the comparative efficacy of AGE and fluoxetine, administered both individually and in combination.

The present study provides important knowledge, but some limitations must be recognized. First, the dose and duration of aged garlic supplemented in the current study should not be translated to estimate the best human dosages. Second, the maternal separation model is the most commonly used model for ELS studies, as it is an animal model it is unable to fully replicate the complications of early-life stress experienced in human participants. Lastly, more future investigations are required to explain the detailed mechanisms of aged garlic supplementation potency and the long-term consequences on anxiety and depressive-like behaviors. AGE encompasses a diverse array of bioactive elements, and the identification of these key components may open the path for more precisely targeted and efficacious therapeutic applications. Garlic bulbs and aged garlic extract powder incorporate prebiotics, potentially serving as sustenance for advantageous gut bacteria. Comprehending the mechanisms through which aged garlic modulates the gut microbiota, and whether this modulation contributes to its effects on anxiety and depression, constitutes a promising trajectory for forthcoming research endeavors. In conclusion, clinical trials that involve human subjects are essential to validate these observations and to determine the most effective dosage and length of aged garlic supplementation for the treatment of anxiety and depression in human populations.

Conclusion

In conclusion, these results offer strong evidence that aged garlic extract supplementation can ease such anxiety and depressive-like behaviors in MS rodents, a valuable model of early-life adversity. These are consistent with behavioral tests forced swim test, tail suspension test, elevated plus maze, and open field test findings, from which it can be concluded that AGE might provide potentially therapeutic intervention for relieving the harmful influences of early life stress on mental health. The results align with the already known anti-inflammatory and antioxidant properties of AGE, which likely contribute to its neuroprotective effects. Additional experiments are necessary to investigate these underlying mode of action of AGE and to assess AGE as a preventive or treatment agent for stress-induced mental health disorders.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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