Acta Alimentaria 55 (2026) 1, 45–55
DOI: 10.1556/066.2025.00227

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AKADÉMIAI KIADÓ

Anethole improves spatial memory and reduces oxidative stress in rats on a calorie-dense diet

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ORIGINAL RESEARCH PAPER

Received: August 7, 2025 • Accepted: December 27, 2025
Published online: February 5, 2026

ABSTRACT

The fragrant organic monoterpene anethole is a component of anise and fennel essential oils. The research intended to evaluate the effects of anethole on spatial memory and oxidative stress in rats on a calorie-dense diet (CDD). Male Wistar rats ($n = 50$) were allocated into 5 groups: Control, CDD, CDD + A62.5, CDD + A125, and CDD + A250. The dietary intervention lasted for 10 weeks, during which anethole at doses 62.5, 125, and 250 mg kg^{-1} was daily orally administered to the last three groups. In the 9th week, the open field test (OFT) for locomotor activity and the spatial object recognition test for spatial memory assessment were carried out. At the end of the experiment, the endogenous antioxidant enzyme superoxide dismutase (SOD) was measured in serum, and the thiobarbituric acid reactive substances (TBARS) were measured in whole-brain homogenate as markers of lipid peroxidation. The OFT showed no significant differences in the locomotor activity across the groups. The consumption of CDD resulted in a decline in spatial memory manifested by the decrease of the recognition index, while anethole treatment prevented this effect in a concentration-dependent manner. In CDD group, the activity of SOD was significantly reduced ($P < 0.05$ vs. Control), while the brain TBARS were significantly elevated ($P < 0.05$ vs. Control). SOD level in CDD + A250 group was comparable to that of the control group. TBARS levels in all anethole-treated groups did not differ significantly from the control value. In conclusion, anethole prevented CDD-induced spatial memory impairment and reduced oxidative stress in rats on a calorie-dense diet.

KEYWORDS

anethole, calorie-dense diet, locomotion, oxidative stress, rats, spatial memory

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Note: E-mail was updated on 12 March 2026.

1. INTRODUCTION

Western society's diet model, which has become very popular nowadays, is a 'bad apple' to our health. It is a critical situation, as it affects not only adults but also children and adolescents. The increased caloric intake through the consumption of high-energy foods containing animal fats and simple carbohydrates, as well as the low physical activity have a negative impact on our health (Poston and Foreyt, 1999; Pi-Sunyer, 2009). The negative health outcomes include cardiovascular diseases, diabetes, cancer, osteoarthritis, chronic liver and kidney disease, brain damage, sleep apnoea, etc. Consuming high-calorie diet results in spatial memory impairment and induces anxiety in experimental animals (Reyzov et al., 2019). Visceral obesity is associated with chronic low-grade inflammation and oxidative stress – key constituents of obesity-related co-morbidities (Patil et al., 2025). According to Shefer et al. (2013), excessive nutrition is linked to brain disease through early inflammation of the hypothalamus, hippocampus, and cortex. The complex interplay between obesity, inflammation, and oxidative stress in the brain on one hand, and cognitive impairment on the other, is reviewed by Naomi et al. (2023). Oxidative stress is the condition, in which there is an imbalance between the generation of free radicals and the protective effects of antioxidants in the organism. The overproduction of free radicals and activated oxygen molecules can be eliminated by biological antioxidants. Superoxide dismutase (SOD) is the endogenous antioxidant enzyme that facilitates the transformation of superoxide radicals into hydrogen peroxide and molecular oxygen (O_2). After that, hydrogen peroxide is converted to water by other enzymes like catalase or glutathione peroxidase, which act downstream of SOD (Weydert and Cullen, 2010).

Substances with antioxidant and anti-inflammatory effects can ameliorate obesity-induced negative health consequences. A potential candidate in this context could be anethole. It is a natural monoterpene found in some essential oils such as anise and fennel. Anethole is a clear, colourless to amber liquid. Anethole is widely used in food industry with its pronounced sweetish taste and specific flavour. Emerging evidence indicates its anti-inflammatory, antibacterial, anti-neurodegenerative, and analgesic effects and low toxicity (Marinov and Valcheva-Kuzmanova, 2015). Anethole has been demonstrated to scavenge and neutralise free radicals, reduce lipid peroxidation, chelate metal ions - thereby preventing metal-accelerated oxidation processes - and protect mitochondria against oxygen deprivation (Khodadadian and Balali-Dehkordi, 2024). It has been shown that anethole through modulation of neurotransmissions by monoamines, gamma-aminobutyric acid, and glutamate, as well as through its anti-inflammatory and antioxidant actions, affects the central nervous system and manifests memory-enhancing, anxiolytic, antidepressant, anticonvulsant, and antinociceptive effects (Khodadadian and Balali-Dehkordi, 2024).

The current investigation was conducted with the purpose to determine the effect of anethole on the spatial memory, locomotor activity, and oxidative stress in rats fed a calorie-dense diet.

2. MATERIALS AND METHODS

2.1. Experimental animals

The study was carried out on 50 male Wistar rats kept in the animal housing facility of the Medical University of Varna under standard laboratory conditions, in ventilated rooms at a

light-dark cycle of 12 h and temperature ranging from 20 to 25 °C, with unrestricted availability of food and water, housed in standard plastic cages. The tests were carried out in a manner that was compliant with national and international laws and policies based on the European Directive for the protection of animals in scientific research (2010/63/EU) and permission from the Bulgarian Food Safety Agency (Document No 177/07.07.2017).

2.2. Experimental procedure

Animals initially weighing 250–310 g were allocated into 5 groups ($n = 10$): Control, CDD, CDD + A62.5, CDD + A125, and CDD + A250. Over the duration of 10 weeks, animals from the control group obtained a standard laboratory rat diet and drinking water, and the rest of the groups were fed a CDD with increased content of saturated fats and simple carbohydrates. The CDD was prepared by enriching standard pellets with 17% lard and 17% fructose. Furthermore, instead of drinking water, the rodents which were provided CDD were given a 10% fructose solution (Gancheva et al., 2015). The calorie content was 279 kcal per 100 g for the standard diet and 405 kcal per 100 g for the CDD with additional 40 kcal per 100 mL fructose solution consumed. The CDD + A62.5, CDD + A125, and CDD + A250 groups received an oral treatment every morning through a soft probe with anethole as follows: 62.5 mg kg^{-1}, 125 mg kg^{-1}, and 250 mg kg^{-1}, respectively, by dissolving the substance in sunflower oil at a volume of 0.3 mL/100 g body weight. The groups Control and CDD obtained the solvent administered with identical method at the same volume. During the 9th week, the open field test (OFT) for assessment of locomotor activity and the spatial object recognition test (SORT) for evaluating spatial memory were performed.

2.3. Open field test (OFT)

OFT has been widely used to study the general locomotor activity in rodents (Crusio, 2001). The open field consists of an enclosed wooden arena with dimensions 100 × 100 × 40 cm divided into 20 × 20 cm squares. After placing the rat in the centre of the field, it was allowed to explore for 5 min. After each animal testing, the arena was cleansed with 70% ethanol. Results were recorded manually. The number of horizontal movements (the squares the animal crossed with all four paws) and vertical movements (the number of rearings, including propping up against the field walls with the front paws) were counted (Belovicova et al., 2017).

2.4. Spatial object recognition test (SORT)

SORT is a modified version to the novel object recognition task aiming to assess the spatial memory (Vogel-Ciernia and Wood, 2014). SORT facilitates the evaluation of memory by utilising the inherent tendency of animals to explore novel spatial configurations. The experiment was conducted in the day after the OFT, using a rectangular part of the open field arena, 65 × 45 cm, surrounded by 40-cm walls. As the rats were familiar with the arena, there was no need of habituation. Two sessions were performed: training and testing, each lasting for 3 min. The training session was conducted using two identical objects (parallelepipeds attached to the floor without the possibility of being moved) placed symmetrically. Exploration was defined as the time spent in pointing the animal's nose towards the item from a distance of 1 cm or less, actively approaching and sniffing the item, and climbing on the item without sitting on it.

After each rat, the arena and the objects were carefully cleansed using a 70% ethanol solution. The test phase was conducted 30 min following the training one. During the test session, one of the items was relocated to a position that differed from its location during the training session. The investigation duration of the item with the novel location (B) and the object with the familiar location (A) were recorded. An indicator of spatial memory was the recognition index, which was determined by dividing time B by the entire time of exploration (A + B): B/(A + B).

2.5. Tissue preparation

Animals were given diethyl ether anaesthesia at the end of the 10th week of the experiment (Aguwa et al., 2020). Blood was collected from the sublingual veins, centrifuged at a speed of 2,000 rounds per minute for 10 min in order to obtain serum, which was stored at a temperature of −20 °C until the biochemical analysis. After cervical dislocation and a cranial dissection, the brain was blotted to remove excess fluid and frozen. At the time of biochemical analysis, the whole-brain sample was minced in a beaker and homogenised with ice cold Tris/HCl buffer, 50 mM, pH 7.4 (ratio of 1 g tissue per 5 mL buffer).

2.6. Serum superoxide dismutase (SOD) activity

The measurement of the activity of SOD was conducted using commercial kits (Boster Biological Technology Co., Ltd.) for enzyme-linked immunosorbent assay (ELISA) and performed according to the manufacturer's instructions using BioTek 800 TS microplate reader. The principle of the method is that tetrazolium salt is used to identify superoxide anions generated by hypoxanthine and xanthine oxidase. The amount of SOD needed to produce a 50% dismutation of the superoxide ion is defined as a single unit. The results are presented in U mL^{-1}.

2.7. Thiobarbituric acid reactive substances (TBARS)

The levels of TBARS, which serve as an indicator of lipid peroxidation, were quantified in the whole-brain tissue homogenate, which was centrifuged at a speed of 2,000 r.p.m. for ten min at a temperature of 4 °C. TBARS concentration was determined in the supernatants colorimetrically (spectrophotometer CE2021, Cecil Instruments Ltd, UK) at a wavelength of 532 nm, following the procedure described by Ohkawa et al. (1979). This approach quantifies the absorbance of the colour produced from the interaction between thiobarbituric acid and lipid peroxides. The amounts of TBARS were quantified in units of nmol per gram of tissue. Malondialdehyde serves as the reference standard due to being the primary compound produced from the peroxidation of membrane fatty acids.

2.8. Statistical analysis

The findings from the experiments are presented as a mean ± standard error of the mean (SEM) and were analysed by Student's *t*-test, one-factor analysis of variance (One-way ANOVA), and Dunnett's post-test. The assessment of the dose-dependent effect of anethole was performed using a post-test for linear trend. A statistical significance threshold of $P < 0.05$ was accepted. The statistical software GraphPad Prism 5.00 was used (Graph Pad Software, Inc., CA, USA).

3. RESULTS AND DISCUSSION

3.1. Open field test

Figure 1 presents the results from the OFT. The horizontal (Fig. 1A) and vertical (Fig. 1B) locomotor activity were affected neither by the CDD, nor by the anethole treatment, as shown by one-way ANOVA ($F = 1.342$, $P = 0.2701$ for horizontal activity and $F = 0.5457$, $P = 0.7030$ for vertical activity).

3.2. Spatial object recognition test

The Student's *t*-test revealed a substantial decline ($P < 0.001$) in the recognition index in CDD group (0.39 ± 0.033) compared to control group (0.57 ± 0.028). A one-way analysis of variance showed that the groups receiving anethole treatment had significantly better performance in the spatial memory task compared to the control group ($F = 3.802$, $P = 0.0182$). Dunnett's post-test revealed a statistically significant increase in the recognition index for groups CDD + A125 (0.59 ± 0.04) and CDD + A250 (0.61 ± 0.06) compared to the CDD group (0.39 ± 0.03), with a *P*-value less than 0.05. The post-test analysis for linear trend showed a dose-response relationship between the concentration of anethole and its effect. The statistical significance supporting this finding was $P = 0.0028$ (Fig. 2). The recognition index of all anethole-treated groups did not differ significantly compared to the control group (ANOVA: $F = 0.5806$, $P = 0.6318$).

3.3. Serum superoxide dismutase (SOD) activity

As shown in Fig. 3A, serum SOD activity in the CDD group (0.23 ± 0.02 U mL^{-1}) was significantly reduced ($P < 0.05$) compared with the control value (0.31 ± 0.01 U mL^{-1}). The levels of the antioxidant enzyme remained consistently low in groups CDD + A62.5 (0.23 ± 0.02 U mL^{-1}) and CDD + A125 (0.22 ± 0.01 U mL^{-1}). The SOD level in the CDD + A250 group (0.29 ± 0.02 U mL^{-1}) was not significantly different from that of the control group.

3.4. Brain thiobarbituric acid reactive substances (TBARS) levels

Brain TBARS levels are presented in Fig. 3B. Consumption of CDD caused a significant increase in brain TBARS (13.8 ± 1.1 nmol g^{-1}) compared to the control level of 9.9 ± 0.9 nmol g^{-1} ($P < 0.05$, Student's *t*-test). The administration of anethole resulted in TBARS levels in the brain homogenate of 11.5 ± 1.5 nmol g^{-1} for CDD + A62.5 group, 11.6 ± 1.1 nmol g^{-1} for CDD + A125 group, and 11.3 ± 1.3 nmol g^{-1} for CDD + A250 group. These values did not differ significantly from the control one (ANOVA: $F = 0.4218$, $P = 0.7386$).

3.5. Discussion

To our knowledge, the present study is the first one focusing on the effects of anethole on locomotor activity and spatial memory in rats receiving a calorie-dense diet. The present research found that anethole did not affect locomotor activity. Changes in locomotor activity are important behavioural indices to consider when interpreting the results from other behavioural tests where locomotion is involved. In the current investigation, the locomotor activity of experimental groups did not differ significantly from one another. Therefore, we could conclude that the differences in the animal performance in SORT were not related to changes in

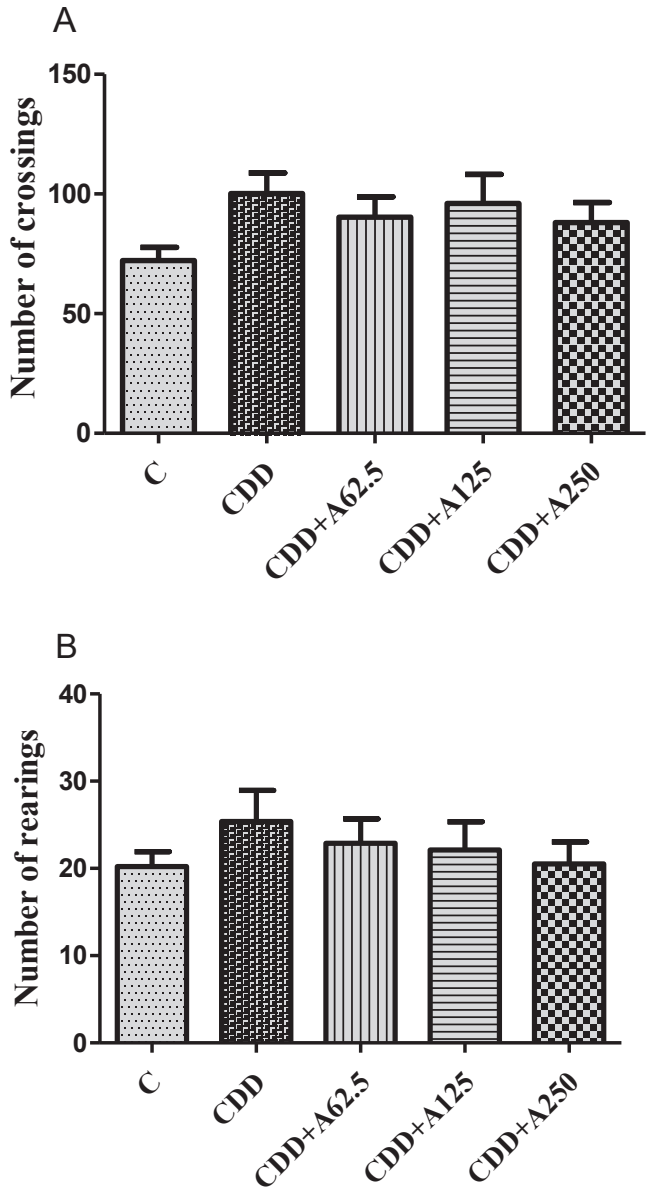

Fig. 1. Effect of anethole (A) administered to rats on a calorie-dense diet (CDD) at doses of 62.5 mg kg^{-1} (CDD + A62.5), 125 mg kg^{-1} (CDD + A125), and 250 mg kg^{-1} (CDD + A250) on the number of crossings (panel A) and rearings (panel B) in the OFT

locomotion. This result is consistent with the findings of Ritter et al. (2014), who also reported no changes in locomotor activity in rats treated with anethole. Hassanzadeh et al. (2022) reported similar results in NMRI (Naval Medical Research Institute) mice injected

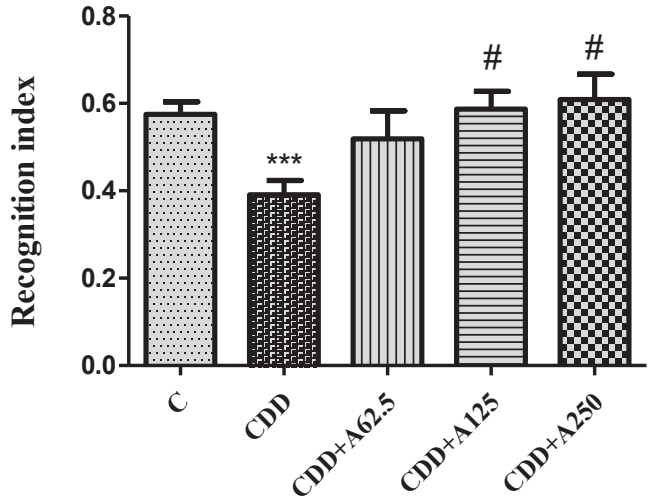

Fig. 2. Effect of anethole (A) administered to rats on a calorie-dense diet (CDD) at doses of 62.5 mg kg^{-1} (CDD + A62.5), 125 mg kg^{-1} (CDD + A125), and 250 mg kg^{-1} (CDD + A250) on the recognition index in the SORT; $^{***}P < 0.001$ *vs.* Control; $\#P < 0.05$ *vs.* CDD

intraperitoneally with trans-anethole at doses of 25 and 50 mg kg^{-1}. The locomotor activity of both unstressed and stressed mice was unaffected by trans-anethole (Dhingra and Sudha, 2019). A similar result was obtained when fennel essential oil (82.08% anethole content) was administered intraperitoneally to albino mice at doses of 100–400 mg kg^{-1} (Abbasi-Maleki and Maleki, 2021).

In the current study, CDD induced a spatial memory impairment. Accumulated evidence suggests that obesity is linked with memory impairment and oxidative stress. Heyward et al. (2016) found that diet-induced obesity led to hippocampus-dependent spatial memory decline but object recognition memory was not affected. Insulin resistance is considered to impair hippocampal physiological function and synaptic plasticity (Stranahan et al., 2008). This finding provides the link between spatial memory and obesity. Prior research has substantiated that the high-fat diet is associated with decreased serum amount of enzymes that fight free radicals, including glutathione S-transferase, SOD, and catalase (Emami et al., 2016). Maciejczyk et al. (2018) presented evidence that prolonged administration of a high-fat diet resulted in an elevation of malondialdehyde (MDA) concentration in the hypothalamus and cerebral cortex of rats. These previous observations are in accordance with the results from this study demonstrating that the CDD reduced serum SOD activity and increased brain lipid peroxidation measured by TBARS. Another possible underlying mechanism of cognitive decline might be the reduction of hippocampal brain-derived neurotrophic factor (Stranahan et al., 2008).

In this investigation, anethole treatment ameliorated the spatial memory impairment in a dose-dependent manner. The duration of treatment (10 weeks) was long enough for the aromatic compound to exert its spatial memory-improving effect. Memory-enhancing and neuroprotective properties of anethole have been demonstrated in previous studies (Marinov and Valcheva-Kuzmanova, 2015). Anethole is a key compound of *Shanzhuyu* (a Chinese

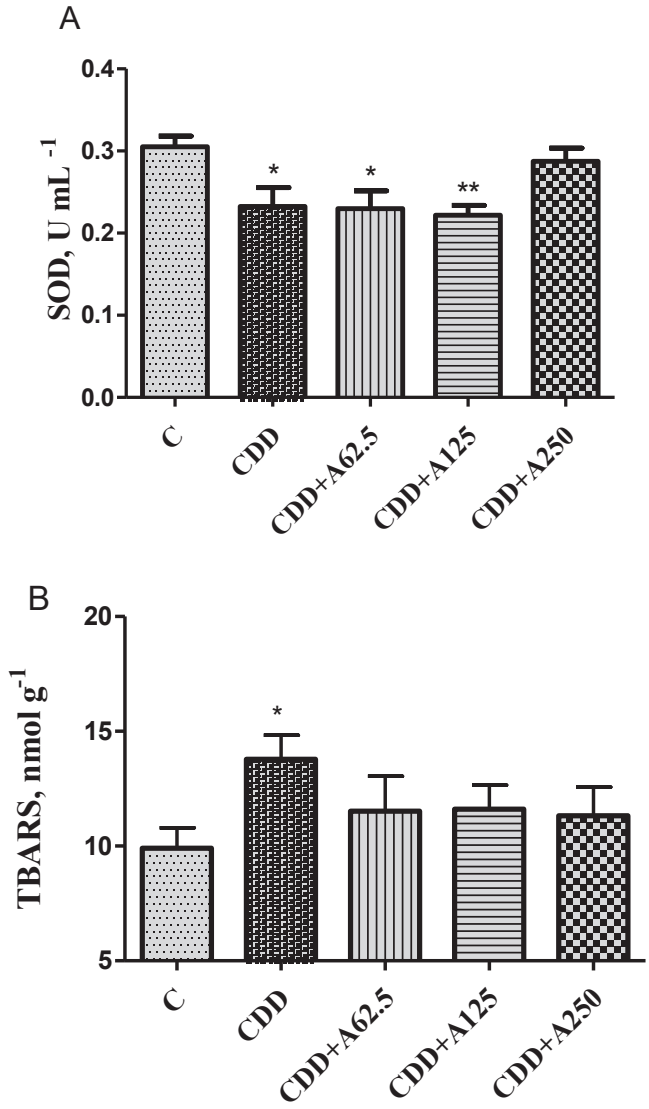

Fig. 3. Effect of anethole (A) administered to rats on a calorie-dense diet (CDD) at doses of 62.5 mg kg^{-1} (CDD + A62.5), 125 mg kg^{-1} (CDD + A125), and 250 mg kg^{-1} (CDD + A250) on serum superoxide dismutase (SOD) (A) and brain thiobarbituric acid reactive substances (TBARS) (B) levels; $^{*}P < 0.05$, $^{**}P < 0.01$, both *vs.* Control

herb), which has been shown to regulate amyloid deposition, oxidative stress, and apoptosis *via* regulating signalling targets involved in the pathogenesis of Alzheimer's disease (Qu et al., 2020). Anethole, as one of the main components of *Uncaria rhynchophylla,* is considered as a potential candidate for a future anti-amnesic drug, as it has been shown to enhance memory in tests such as Morris water-maze and passive avoidance (Shin and Lee, 2013). In harmony with the

previous statement, administration of anethole (250 mg kg^{-1}) ameliorated rotenone-induced cognitive dysfunction in both novel object recognition test and passive avoidance task (Vastegani et al., 2023). Trans-anethole administration of 50 mg kg^{-1} for 14 days improved memory retention potential in rats with a model of Huntington's disease (Rajan et al., 2020). Trimethyltin chloride-induced long-term potentiation (LTP) impairment could be alleviated by trans-anethole through an action on N-methyl-D-aspartate (NMDA) receptors (Chang et al., 2022).

In this experiment, anethole caused a decrease in brain lipid peroxidation, as shown by the decreased TBARS levels, together with the restoration of serum SOD activity. Thus, the amelioration of oxidative stress probably accounted for the observed enhancement in spatial memory. Such biochemical improvements might preserve hippocampal neuronal integrity and synaptic plasticity, thereby facilitating enhanced cognitive performance. The effects of anethole to reduce oxidative stress have been reported in other studies. The administration of an anethole-rich fraction resulted in a considerable rise in the levels of SOD and reduced glutathione in diabetic nephropathic rats fed a junk food diet (Vellapandian et al., 2022). Following a 3-week period, administration of *Foeniculum vulgare* fruit, containing the primary component trans-anethole, to rats at a dosage of 200 mg kg^{-1} per day, led to a substantial increase in the activities of SOD and CAT (Choi and Hwang, 2004). Additionally, there was a significant decrease in lipid peroxidation, as determined by MDA (Choi and Hwang, 2004). Stressed rats experienced a decrease in brain MDA levels, which was shown to be significant after three weeks of trans-anethole treatment (Dhingra and Sudha, 2019). Ryu et al. (2014) explained the neuroprotective effects of anethole by mitochondrial protection and antioxidant activities. Another study indicated that anethole decreased the nitrite amount and MDA content in the brain in an animal model of epilepsy (Salimian et al., 2022). Raman et al. (2020) also demonstrated memory improvement in anethole-treated socially isolated rats suggesting that an antioxidant activity, inhibition of cholinesterase enzyme and amplification of LTP in the hippocampus might stay behind this effect.

4. CONCLUSIONS

The current investigation revealed that administration of anethole dose-dependently prevented the calorie-dense diet-induced spatial memory impairment in rats. This result could be explained by the capacity of anethole to counteract the calorie-dense diet-induced brain oxidative stress as demostrated by its ability to antagonise the diet-induced elevation of lipid peroxidation products in the brain. The alleviation of oxidative stress by anethole might be attributed to its ability to enhance the superoxide dismutase production demonstrated in this experiment, as well as to its intrinsic antioxidant capacity well documented in other studies.

ACKNOWLEDGEMENT AND FUNDING SOURCES

The study was supported by fund "Science" of Medical University of Varna, project №16011.

The publication was supported by the Fund for Supporting the Publication of Articles and Scientific Works in Impact Factor (IF) Journals by Researchers from the Structure of the Medical University "Prof. Dr. Paraskev Stoyanov"—Varna [No. P-100-415, 2025].

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