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THE BENEFICIAL EFFECTS OF GINGER AND NIGELLA SATIVA (ANTIOXIDANTS) IN WISTAR RATS AGAINST THE INORGANIC MERCURY (MEMORY IMPAIRMENT AND NEUROBEHAVIORAL TROUBLES)

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This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The current research explores theimprovementand neuroprotection effects of ginger extract and Nigella sativa oil on working memory and fills the knowledge gap on the neurotoxicity of inorganic mercury in Wistar rats.

Methods: The animals were divided into five equal groups, received by orally to 500 mg/kg/day of ginger extract, 2 ml/kg/day of Nigella Sativa oil for 28 consecutive days (01^{st} – 28^{th} day), and 4 mg/kg/day of mercuric chloride ($HgCl_2$) for 21 consecutive days (08^{th} – 28^{th} day). The rats were exposed each on the 18^{th} day and 28^{th} day respectively in novel object recognition test (NOR) and spontaneous alternation behavior test (Y-maze Test).

Results: The rats were weighed on the 29^{th} day and euthanized,the brains in each group were excisedand weighed.The results obtained show a significant decrease in the relativebrain weight in the rats treated with mercuric chloride alone compared to the control rats. The behavioral tests revealed a decrease in the recognition index and the spontaneous alternation in the group (M) compared to groups (T), (G+M), (N+M), and (G+N+M). The groups pretreated with ginger extract and Nigella Sativa oil reversed these cognitive impairments induced by $HgCl_2$ and improved memorization.

Conclusion: We conclude that ginger extract and Nigella sativa oil exerts a neuroprotective and therapeutic effect against memory disorders induced by mercuric chloride.

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Keywords: Mercuric chloride; ginger extract; nigella sativa oil; memory disorders; wistar rats.

1. INTRODUCTION

Mercury is one of the most toxic heavy metals that can be detected in the environment [1].According to the World Health Organization (WHO) [2] mercury is one of the top 10 chemical agents that have caused public health concerns. "Recent studies have shown that mercury may not have a threshold value, and some adverse effects will not occur if it is below this threshold value". Chemically, mercury exists in several forms, all of which are toxic: elemental mercury (or metallic mercury, Hg^0), inorganic mercury compounds, and organic mercury compounds. The different forms of mercury usually determine the exposure, absorption, distribution, and toxicity to target organs [3]. Although the information on the toxicity of methylmercury is well documented, there is very little research on inorganic mercury [4].

The world's population is exposed to environmental inorganic mercury, which can cause toxic effects [5]. It can enter the air by mining mercury deposits. Coal-fired power plants are the largest industrial source of inorganic mercury emissions. Factories that use mercury also release inorganic mercury into the water. Therefore, people living downstream of the mining area or close to dams or factories may be exposed to inorganic mercury [6]. In addition, the general population's exposure comes from dental amalgam and vaccines. Exposure to mercury in dental amalgam has been a worrying issue for decades [7]. Inorganic mercury can cause clinical symptoms related to nervous system dysfunction. It is worth noting that in the general population, the concentration of brain, blood, and urine is related to the number of amalgam surfaces.

Most human neurotoxicity case studies caused by oral exposure to inorganic mercury salts report that ingestion of therapeutic agents containing mercurous chloride can cause neurotoxic effects [8]. In addition, long-term exposure to low concentrations of inorganic mercury can cause or exacerbate degenerative diseases, such as amyotrophic lateral sclerosis, Alzheimer's disease, multiple sclerosis and Parkinson's disease, which has also caused concern [9,10].

The natural antioxidant defense system plays an important role in eliminating many toxic effects in the body. Medicinal plants are known for their powerful antioxidant properties and continue to be considered as effective sources of supplementary medicines [11]. Ginger is one of the commonly used spices in the world. It has been cultivated for thousands of years and can be used safely in cooking, as well as in folk and home remedies for medicinal purposes. It is widely used in traditional medicine. It has been reported that ginger or it's extract has certain pharmacological activities, including analgesic, antioxidant, anti-inflammatory, anticonvulsant and neuroprotective effects [12]. Nigella sativa, commonly known as nigella, is widely used to treat different diseases. Nigella oil is a high-value medicinal solvent traditionally used to treat many diseases. Evidence from animal and human studies shows that nigella oil has a wide range of pharmacological properties, including antioxidant, anti-inflammatory, neuroprotective, immune regulation and anti-tumor, curative effect on neurodegenerative diseases and memory enhancement [13].

The aim of the present study is to assess the effects of ginger extract and Nigella sativa oil on inorganic mercury-induced working memory disorders in Wistar rats using novel object recognition test (NOR) and the spontaneous alternation behavior test (Y-Maze Test).

2. MATERIALS AND METHODS

2.1 Chemicals

Mercuricchloride ($HgCl_2$),**(**ACS reagent ≥99.5% CAS CAS 7487-94-7) and Ginger extract (Natural, FG. CAS 84696-15-1) used in this experiment were purchased from (Sigma-Aldrich, St Louis, MO, USA), The Nigella Sativa oil cold pressed product by El Capitan (CAP Pharm., Egypt).

2.2 Animalcare

The experiments were carried out on male Wistarratsweighing 265-295grams, taken from the Pasteur Institute of Algiers, rats were bred in the animal house of University of Annaba throughout the period of the experiment. Standard rat's food diet and water were available. The animals were kept at constant room temperature (21-25 °C), a 13h/11h dark/light cycle and a hygrometry of 45-55%.

2.3 Procedure of Experiment

Twenty-five adult healthy ratsrandomly divided into five groups(each n = 5): group (T) or untreatedcontrol group, group (M) receives distilled water (1ml/kg/day) for 7 days (D01-D07) and mercuric chloride (4mg/kg/day) for 21 days (D08-D28), group (G+M) receives ginger extract (500mg/kg/day) for 28

days (D01-D28) and mercuric chloride (4mg/kg/day) for 21 days (D08-D28), group (N+M) received Nigella Sativa oil (2ml/kg/day) for 28 days (D01-D28) and mercuric chloride (4mg/kg/day) for 21 days (D08-D28) group (G+N+M) received a ginger extract (500mg/kg/day) and Nigella Sativa oil (2ml/kg/day) during the whole experiment (D01-D28) and mercuric chloride (4mg/kg/day) for 21 days (D08-D28).All these treatments were administered by orally.

Rats were subjected to behavioral tests in the following order: novel object recognition test (NOR), spontaneous alternation behavior (Y-maze test) on day 18 and day 28 of experimentation respectively.

In the morning of the 29th day, the animals were weighed and euthanized, all brains in each group were excised and weighed. The brain weight/body weight ratio×100 was calculated and expressed as relative brain weight.

2.4 Novel Object Recognition Test (NOR)

The novel object recognition test (NOR),also known as the object recognition test (ORT),was originally described by Ennaceur and Delacour in 1988 and used primarily in rats [14], however, since then, it has been successfully adapted for use in mice [15]. This behavioral test assesses non-spatial working memory [16].

Before starting the test, each animal is accustomed for 5 min during one day to the open field device consisting of a base surrounded by Plexiglas parapets whose measures are respectively 100cm×100cm×40cm. Two trials of 5 minutes each are performed per test session. The first trial corresponds to the familiarization phase and consists in presenting the animal with two identical objects arranged symmetrically in the two ends of the box. After this test, the rat is put back in its cage for one hour [16]. In the second trial corresponding to the test phase, one of the two objects presented is replaced by a new object whose size, color, shape and material used is different [17]. The box and objects are cleaned with hydroalcoholic gel or 70% ethanol between each trial in an effort to avoid olfactory cues. Time spent interacting with the object (e.g., touching, climbing, and sniffing the object at a distance ≤ 2 cm) is manually counted by the experimenter [18,19].

Evaluation of rat's recognition memory was expressed as percentage of recognition index (RI), according to the following formula: RI = [TN/ (TN+TF)] x 100. Were TN: the time spent to exploring the novel object, and TF: the time spent to exploring the familiar object.

2.5 Spontaneous Alternation Behavior (Y-Maze Test)

This test is used to measure spatial working memory that depends on proper hippocampal functions, as confirmed by the severe memory impairment after excitotoxic lesion of this brain region [20]. The spontaneous alternation test made by black Plexiglas (5 cm width, 35 cm length,10 cm height) consisted of identical three arms with 120°. Animals were placed on the same arm facing the wall of the arm and this arm was designated as a start arm for 6 minutes under dim lightening condition and its behavior was recorded with a camcorder. The inside of Y-maze was cleaned with 70% ethanol between trials and allowed to dry. The entry of the animal is counted as soon as its four legs are in the arm [21]. Spontaneous alternation consists of a sequential entry in the three arms. The higher the score, the better the working memory capacity of the animal [22].

Percent alternation was calculated by dividing the number of alternations by the number of possible alternations [number of alternation/ (number of total arms entries-2)] x100**.**

Rats making less than 10 visits are excluded from the test.

2.6 Statistical Analysis

All data collected during the behavioral testing of the animals were expressed as mean ± SEM. Results comparisons were performed using One-way ANOVA, followed byapost-hoc test (Dunnett's test). GraphPad Prism 9.0.0 was the statistical package used for data analysis.

Significance was defined as a *p*-value less than 0.05 (*p<0.05; **p< 0.01; ***p< 0.001)

3. RESULTS

3.1 Variation of Relative Brain Weight

Statistical analysis of these results revealed a significant (p< 0.05) decrease in the relative brain weight of the mercuric chloridetreated group (M) compared to the control group (T) (0.627±0.045 vs 0.506±0.019). However, we found insignificant increases (p> 0.05) in the relative brain weight of groups pre-traited with the ginger extract and Nigella Sativa oil (G+M; N+M; G+N+M) compared to the mercuric chloridetreated group alone (M) (0.530±0.011; 0.548±0.023; 0.566±0.028 vs 0.506±0.019) (Fig. 1).

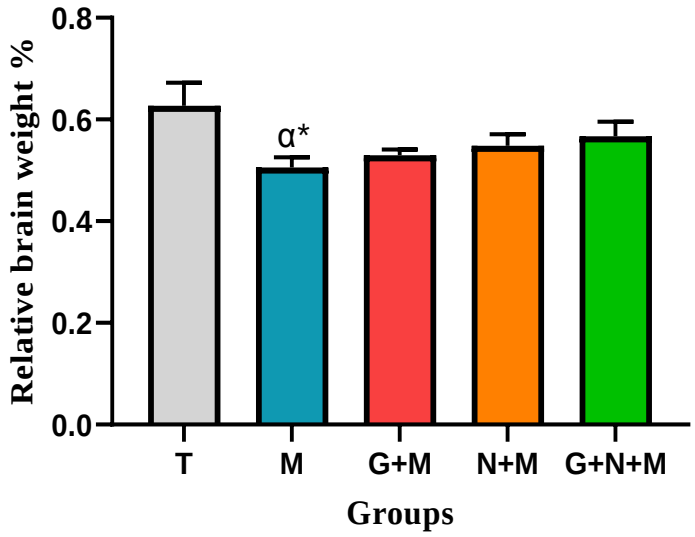

Fig. 1. Ratio of brain weight to body weight in control and treated rats (n=05; mean ± SEM)
α: Comparison with control group (T); β: Comparison with the group treated with mercuric chloride alone (M)

3.2 Performance in the Novel Object Recognition Test (NOR Test)

After 18 days of treatment with ginger extract and Nigella Sativa oil (D01-D18), and 11 days of treatment with mercuric chloride (D08-D18), the behavior of rats showed no significant difference (p> 0.05) between the treated groups (M, G+M, N+M, G+N+M) and the control group (T) in the novel object recognition test(45.28±3.99; 57.30±4.55; 55.12±3.66; 59.62±4. 52 vs. 53.34±2.98), whereas our results reveal that the control group (T) and groups pretreated with antioxidants (G+M, N+M, G+N+M) spend more time exploring the novel object compared to the group treated with $HgCl_2$ (M) (53.34±2.98; 57.30±4.55; 55.12±3.66; 59.62±4.52 vs. 45.28±3.99). (Fig. 2).

3.3 Performance in the Spontaneous Alternation Behavior (Y-maze Test)

In the spontaneous alternation behavior test, the percentage of alternation is highly significantly increased(p< 0.01)in rats in the (G+M and G+N+M) groups and significantly increased(p< 0.05) in rats in the (N+M) group compared with rats in the (M) group after 28 days of pretreatment with ginger extract and Nigella Sativa oil (D01-D28) and 21 days of treatment with mercuric chloride (D08-D28) (58.02±3.66; 62.50±4.24 vs 40.84±3.82) and (56.72±1.41 vs 40.84±3.82). There was a slight decrease in the percentage of alternation of the (M) group compared to the control group (T) (48.34±3.58 vs 40.84±3.82), in contrast the (G+N+M) group noted a significant increase(p< 0.05) in the percentage of alternation compared to the control group (T) (48.34±3.58 vs 62.50±4.24). (Fig. 3).

4. DISCUSSION

Mercury is a major neurotoxin, which mainly affects brain tissue, causes brain damage, and ultimately affects the functions of cerebral cortex neurons, cerebellum, and visual cortex, and may also lead to behavioral and cognitive changes [23.24]. Our results reported a significant decrease in the relative brain weight in $HgCl_2$-treated rats when compared to that control rats. Reduction in relative or absolute organ weight changes after exposure to an agent is an indication of the toxic effect of that agent [25]. The mercuric chloride $HgCl_2$ has strongly affected the cells of the brain tissue in animals leads to neurotoxicity [26]. Despite the low lipid solubility, it can cross the blood-brain barrier and enter the central nervous system (CNS) and cause different neurologic disorders [7]. The precise mechanism of inorganic mercury accumulation in the brain following chronic exposure is not well known. Szumafiska et al 1993. suggested that the impaired activity of Na/K ATPase in the micro vessels of the cerebral cortex can be a possible pathway for inorganic mercury absorbance [27]. Another hypothesis is that amino acid transporters, especially cysteine transporters, the same as methyl mercury used to cross the blood-brain barrier [9]. The report of Xu et al. [24] pointed out that the brain areas most commonly affected by mercury poisoning are the cerebral cortex and

cerebellum. Functionally, the cerebral hemisphere and the cerebellum are responsible for regulating primary sensory function and motor coordination, balance and postural stability, respectively. However, it is worth studying that the hippocampus is also related to memory and learning, because any damage to these parts of the brain may lead to neurological dysfunction [28]. In the present study, results show that exposure to$HgCl_2$(4 mg/kg/day) for 11 days (08^{th} - 18^{th}day) causes the rats to develop object recognition and memory deficits. When tested in NOR, these animals are expected to spend more time exploring new objects in the arena. However, compared with control rats, rats exposed to mercury for 11 days spent less time exploring new objects, suggesting that the recognition memory is destroyed. In addition to the NOR test, in the Y-maze test, we verified that control rats had a higher percentage of spontaneous alternation than rats treated with mercuric chloride for 21 days (8^{th} - 28^{th}days) suggesting that mercury, on the other hand, also affects spatial working memory. Similar results on the neurotoxicity of $HgCl_2$ have been described by Aragão et al., [29] such that chronic low-levels exposure to inorganic mercury during the adulthood in rats promoted cognitive impairment related to hippocampal cell density reduction, oxidative stress, cytotoxicity, and induced cell apoptosis [29]. The suggested mechanisms for the toxicity of inorganic mercury include the combination of mercury ions with sulfhydryl groups leading to a decrease in glutathione levels and thiol consumption leading to an increase in reactive oxygen species (ROS), which ultimately leads to an increase in oxidative stress and neurotoxicity [30,31]. In fact, oxidative stress is closely related to the neurobehavioral effects induced by mercury chloride [32].

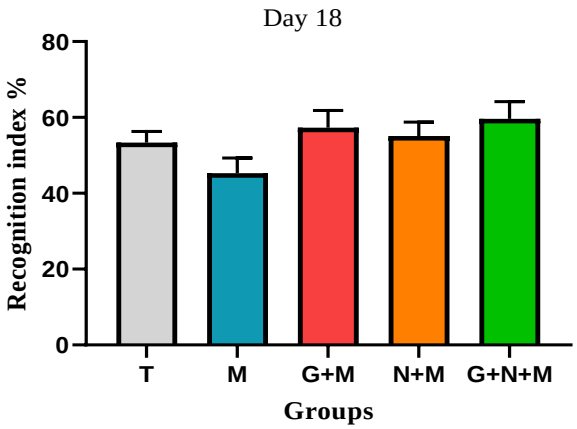

Fig. 2. Recognition index (RI) in control and treated rats in the NOR test on the 18^{th} day of experimentation (n=05; mean ± SEM)

α: Comparison with control group (T); β: Comparison with the group treated with mercuric chloride alone (M)

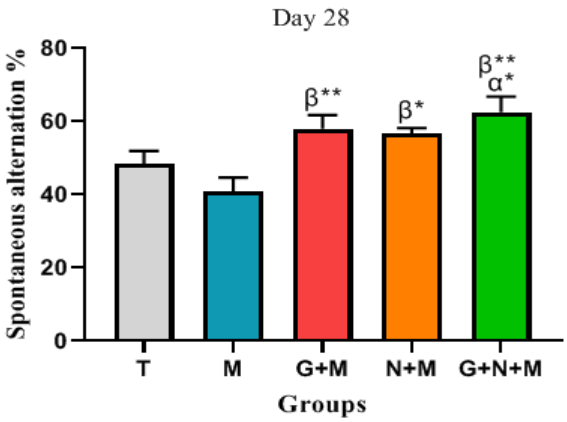

Fig. 3. Percentage of spontaneous alternation in control and treated rats in the Y-maze test on the 28^{th} day of experimentation (n=05; mean ± SEM)

α: Comparison with control group (T); β: Comparison with the group treated with mercuric chloride alone (M).

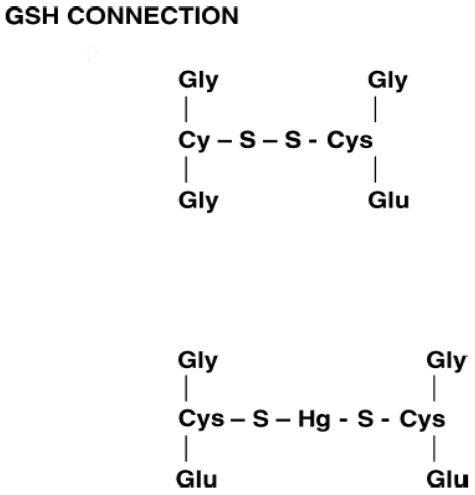

Fig. 4. Inorganic mercury (Hg^{2+}) binds to the thiol ligands of two molecules of reduced glutathione to form a complex similar to that of oxidized glutathione [7]

Glutathione (GSH) is a tripeptide formed by the condensation of glutamate, cysteine and glycine. It's presence in all cells and its rapid intracellular renewal prove its importance [33].Mercury has toxic effects on the central nervous system, leading to changes in neurotransmission [34]. Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) of mammals and is related to cognition, memory [35,36]. A previous study showed that exposure to mercuric chloride in adult rats induced a significant increase in glutamate transport in the hippocampus and motor cortex [37]. Excessive extracellular glutamate levels can induce brain damage and excessive activation of ionotropic glutamate receptors through a pathway called excitotoxicity, thereby inducing neuronal death [36-38]. Also, High levels of extracellular glutamate can induce oxidative stress, which can lead to neurodegenerative diseases by stimulating the production of reactive oxygen species (ROS), mitochondrial hyperpolarization, and lipid peroxidation in neuronal cells [36-39]. Indeed, changes in glutamate transport are one of the main phenomena related to mercury toxicity in the central nervous system [33-37].

The results of this study show that the pretreatment with ginger extract (500 mg/kg/day) and Nigella Sativa oil (2 ml/kg/day) prevented brain damage and improved short-term memory in rats against neurotoxicity of mercuric chloride ($HgCl_2$).In previous study conducted by Wattanathorn et al [40] found that ginger extract increases hippocampal neuron density and improves cerebral blood flow, resulting in improved spatial memory in the right common carotid artery occlusion stroke model [40]. Similarly, Ghayur and Gilani reported that ginger causes vasodilation. Therefore, the improvement of spatial memory in our study may be due to the ability of ginger to promote cerebral blood flow and the high content of strong antioxidant polyphenols including in particular gingerols and shoagaols [41]. Another interpretation, the mechanisms of ginger-mediated cognitive enhancement are through nerve growth factor (NGF)-induced signaling pathways. Ginger administration caused an increase in the level of NGF in the hippocampus of mice, and induced the phosphorylation of extracellular signal-regulated kinase (ERK) and cyclic AMP response element binding protein (CREB); NGF-specific antibodies inhibited the ERK and ERK induced by ginger in the hippocampus CREB activated. Therefore, it can be concluded that ginger has a synaptic effect through NGF-induced ERK/CREB activation, thereby enhancing memory [42]. The cerebral cortex and hippocampus play an important role in working memory [43]. The effects of ginger on cognition may be associated with changes in the monoaminergic and cholinergic systems in various areas of the brain, including the prefrontal cortex and hippocampus [43].Ginger increases the levels of norepinephrine, epinephrine, dopamine, and serotonin in the cerebral cortex and hippocampus [44], where dopamine and norepinephrine play a key role in digital working memory. Acetylcholine and serotonin in the hippocampus were simultaneously activated during spatial working memory tasks [43]. The active component of ginger, 6-gingerol, inhibits cholinesterase activity; this effect increases acetylcholine (ACh), which plays an important role in learning and memory [45], thus, it can be concluded that the antioxidant effects of ginger can lead to cognitive enhancement effects [40].

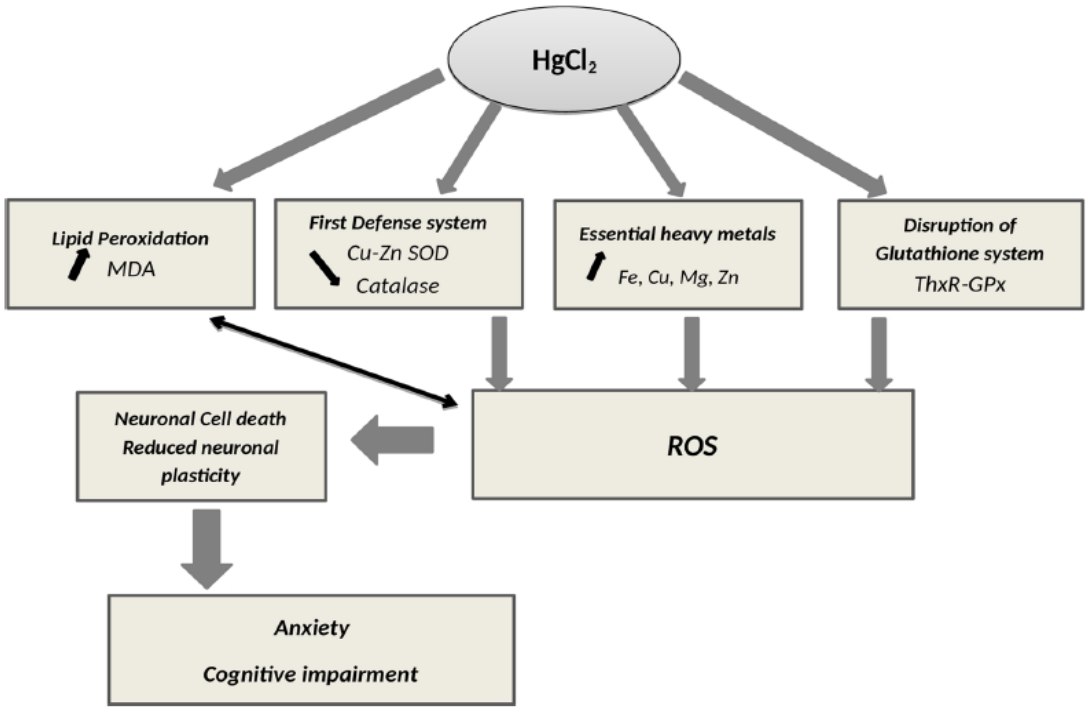

Fig. 5. Summary proposed model of mercuric chloride ($HgCl_2$) neurotoxicity [10]

Nigella Sativa's beneficial effects on memory are probably due to one or more of its components that can protect against cell damage caused by oxidative stress through its free radical scavenging properties [46]. It is reported that by acting as a natural free radical scavenger, by improving the parameters of antioxidant status, and by inhibiting cell membrane damage related to lipid peroxidation that leads to cell death, it has important antioxidant properties for the generation of free radicals can oxidize cells responsible for damage [47]. Nigella Sativa has a strong antioxidant effect and can protect the brain from oxidative stress after lipid peroxidation [48]. Terpinene is also a good inhibitor of lipid peroxidation [49]. Thymoquinone acts as an antioxidant; it inhibits oxidative stress in the hippocampus by reducing lipid peroxidation and improves spatial memory [50]. The active ingredients of Nigella Sativa restore antioxidant enzymes such as glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase in the cognitive impairment induced by streptozotocin [51]. In addition, thymoquinoline and thymoquinone usually exist in the form of glycosidic-bonded aglycones, which easily cross the blood-brain barrier, so they may be related to their neuroprotective effects [52]. Thymoquine appears to be the main neuroprotective component present in Nigella Sativa oil. Other biologically active compounds, namely thymol and carvacrol, also alleviate scopolamine-induced cognitive impairment in rats [53]. It was discovered that nutraceuticals containing thymol and p-cymene have obtained patents for cognitive enhancement properties [54]. Studies have shown that treatment with Nigella Sativa including thymoquinone can improve the morphology of hippocampal neurodegeneration after chronic toluene exposure in rats [55].

Long-term potentiation (LTP) is a form of synaptic plasticity that is now widely accepted as a cellular correlate of memory processing [56]. Long-term potentiation is most easily demonstrated in the hippocampus; it is well known that it is an area of the brain that is fundamentally important in memory acquisition [57]. Our results are consistent with those of the study by Aburawi et al. [58] indicating that the combined treatment of ginger and black seed is most effective in increasing the observed LTP [58]. We suggest that ginger extract and nigella oil improve short-term memory and may be a potential treatment for Alzheimer's disease.

5. CONCLUSION

The current study provides valuable data for a broader understanding of the dangerous effects of $HgCl_2$ on memory, however, the treatment with Ginger extract and Nigella Sativa oil effectively enhanced the working memory of rats investigated in the NOR and Y-maze tests. The amelioration of working memory in rats is undoubtedly accompanied by the improvement

of hippocampal synaptic transmission. Until now, knowledge about the neurotoxicity of mercuric chloride is still not well elucidated. In addition, the molecular mechanism of ginger and Nigella in memory enhancement requires further molecular study.

ETHICAL APPROVAL

All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23), revised 1996.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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