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Amelioration of prenatal lead exposed induced learning & memory impairments by methanolic extract of *Zataria multiflora* in male rats

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Abstract

Purpose of the study: This study was performed to evaluate the effects of *Zataria multiflora* (ZM) on learning & memory of prenatal lead exposed adult male offspring rats.

Methods: Pregnant rats in case group received tap water containing 0.2 % lead acetate throughout the gestation period. Control rats had free access to tap water. Two male offspring from each mother (2 months old, weighing 180-200g) were randomly selected and were treated with either *Zataria multiflora* (50, 200, 400 & 800mg/kg/ i.p./20 d) or saline. Spatial memory of control, saline and ZM-treated rats was evaluated by a training trial and probe test using Morris water maze (6-8 rat/group).

Results: The data showed memory deficits indicated by increased escape latency and a greater travelled distance as well as decrements in the frequency of crossings into target quadrants in antenatally lead exposed male offspring compared to control. ZM treatment (200mg/kg/i.p) ameliorate the memory deficits in male offspring by increasing the time spent and travelled distance in the trigger zone ($P < 0.01$ vs saline). There was no significant difference in swimming speed between groups.

Conclusion: The results showed memory deficits in antenatally lead exposed male offspring. ZM treatment (especially 200 mg/kg) showed the beneficial effects on cognitive behavior indicated as improvement of lead induced memory deficits in prenatally lead exposed male rats. The exact mechanism(s) is not determined yet, but it could be mediated through the anticholinesterase and antioxidant effects and also alterations in CNS neurotransmission in the central nervous system.

Key words: Lead, Learning, memory, *Zataria multiflora*, Rat

1. Introduction

Lead exposure remains one of the most important problems in terms of relevance of exposure and a major public health risk, particularly in developing countries, ¹⁻². Lead exposure in early life is among the major causes of the persistent decrements in intelligence documented in children, adolescents, and young adults, as well as the development of neurodegenerative disease later in life³.

Prenatal lead exposure is associated with adverse effects on neurodevelopment and the effect is most pronounced during the first trimester of pregnancy which needs the maternal plasma of lead level in pregnant women⁴. Also accumulated exposure to lead is associated with cognitive decline in the elderly³.

It is reported that exposure to lead during pregnancy and lactation induces neurobehavioral effects, hyperactivity, decreased exploratory behavior, and deficits in learning and memory in adult offspring⁵. Lead exposure during childhood period could impair both physical development and increase in bone resorption⁶. Previous studies showed that very low-level of prenatal lead exposure was associated with a significant impairment in cognitive function in boys at 36 months⁷. Also blood lead concentrations lower than 5 µg/dL has been associated with deficits in cognitive and academic skills in children ages 6-16 years⁸.

The disturbances in learning ability, adaptive responses and other aspects of behavior following lead exposure may be related to significant changes in some central nervous system (CNS) neurotransmitters such as glutamate, dopamine and acetylcholine⁹⁻¹⁰. Lead exposure cause significant decrease in activity of the acetylcholinesterase and other neurotransmitters in rat brain¹⁰.

Management of lead-induced biochemical and behavioral changes during prenatal lead exposure focuses on removal of the lead source. Sometimes the removal of maternal lead sources is needed by chelation therapy. Recognition and removal of lead sources during the prenatal period can prevent maternal and neonatal morbidity⁵.

The pathogenesis of lead poisoning may be mediated through decrease in activity of the acetylcholine (Sidhu & Nehru, 2003) and disturbance between the delicate pro-oxidant/antioxidant balance that exists within mammalian cells in brain. Production of reactive oxygen species (ROS) is increased after lead exposure in in vitro studies as well as in vivo studies and alteration of antioxidant defense systems in animals and occupationally exposed workers¹¹. Therefore, exogenous supplementation of antioxidant molecules may be among the strategies for decreasing of lead-induced toxicities (Gurer & Ercal, 2000; Sidhu & Nehru, 2003). Today, herbal plants are important part of traditional medicine in many countries and they have been used to improve memory and cognitive function in traditional medicine (Huang et al., 2013). In various studies have shown that plants with high antioxidant and acetylcholinesterase effects could be effective in treatment of memory disorder. One of these plants can be named *Zataria multiflora* (ZM) (Rabiei et al., 2014 ; Rafieian-Kopaei et al., 2013; Sharififar et al., 2012).

Zataria multiflora boiss (ZM), commonly known as Avishan Shirazi in Iran, is a member of Lamiaceae family that has grows only in Iran, Pakistan and Afghanistan and has been used in Iranian traditional medicine for its beneficial effects on mental abilities¹². Experimental studies showed that amyloid β -Induced cognitive deficits was reversed by ZM essential¹². The beneficial effects of ZM in the seems to be contribute to its antioxidant, anti-inflammatory and anticholinesterase activities of ZM or its main constituents¹²⁻¹⁴. Thymol, a phenolic compound of oxygenated monoterpenes and carvacrol are the main constituents of the dry plant while the main constituents of the fresh plant include thymol, carvacrol, p-cymene, linalool and gamma-terpinene¹⁵⁻¹⁶. It is reported that ZM extracts in mice showed significant and dose-dependent antinociceptive activity. Also ZM showed remarkable activity against acute inflammation induced by acetic acid in mice and a dose-dependent and significant antinociceptive activity in hot-plate and writhing tests¹⁷⁻¹⁸.

Prenatal lead exposure as well as environmental lead exposure in childhood has been associated with a reduced intellectual development, significant decrease in brain volume, cognition deficits, executive functions, social behaviors, and motor abilities in adults¹⁹⁻²⁰ , so

The prevention of lead-induced biochemical and behavioral changes during prenatal lead exposure is a worldwide strategy and there is an increasing trend to use herbal medicine for either prevention / treatment of cognitive impairments. Since ZM has been used in Iranian traditional medicine for its beneficial effects on mental abilities, so this study was performed to evaluate the effects of ZM on prenatal lead-induced learning & memory deficits in male offspring rats.

2. Materials and Methods

2.1. Animals

All experimental procedures were performed in accordance with the guidelines provided by the experimental animal laboratory and approved by the Ethics Committee of Kerman Neuroscience Research Center (Ethics Code: KNRC-92-57).

Female Wistar rats (3-4 months old, weighing 250-300 g) were used for the current study. Animals were caged in groups of six with ad libitum access to food and water for 2 weeks before mating. They were housed under controlled temperature ($23\pm 1^{\circ}\text{C}$) and 12 -h light-dark cycle (lights on: 7:00-19:00 h). Two females were paired in a cage with a male rat in the late afternoon.

The next morning the female rats were examined for the presence of vaginal plugs. The day in which vaginal plugs was found, has been designated as the gestation day of 0 (GD 0). Then, pregnant rats were randomly divided into 2 groups: Control group which had free access to tap water and Gestation group (G) in which tap water was replaced with a solution containing 0.2 % lead acetate from the first day of gestation and continued until parturition, when tap water was restarted²¹. The number of litters was adjusted to 8 for each dam.

The offspring kept with their mothers and then two male offspring from each mother (2 months old, weighing 180-200g) were randomly selected for the evaluation of ZM effects on their learning & memory behavior (6-8 rat/group).

2.2. Preparation of *Zataria multiflora* extract

Zataria multiflora boiss aerial parts were purchased from a local market in Kerman, Iran, on June, 2014 during spring season. The plant was identified and confirmed by the Pharmacognosy department of School of Pharmacy (Kerman, Iran) as *Z. multiflora* Boiss (Lamiaceae family) and were air-dried and powdered in a grinder. Methanolic extract of *Z. multiflora* was prepared by soaking 250g of the powdered plant in 1000mL of 85% methanol using percolation method for 48 h. The solution thereafter filtered and the filtrate was evaporated in an oven at 40°C. Solvent removal carried out under vacuum afforded a semisolid mass with a yield of 8.6 %. The extract was dissolved in distilled water to prepare a solution containing 1000mg/ml concentration from dry weight of *Z. multiflora*.

2.3. Experimental groups

Male rat offspring were randomly divided into 6 groups:

Ctrl group: male offspring of control groups with no prenatal exposure to lead which received intraperitoneal injection of normal saline for 20 days.

SAL group: male offspring with prenatal exposure to lead which received intraperitoneal injection of saline for 20 days.

Treatment groups: male offspring with prenatal exposure to lead which received intraperitoneal injection of methanolic extract of *Zataria multiflora* (50, 200, 400& 800mg/kg) for 20 days.

2.4. Morris water maze apparatus and procedures

After the last treatment on day 20, male offspring were subjected to behavioral testing for spatial learning & memory using Morris water maze. In most studies Morris water maze is used for evaluation of neural mechanisms of spatial learning and in animal models²². Morris' water maze (MWM) apparatus is a circular black and transparent pool which was painted with nontoxic materials (160 cm diameter ,80 cm high and 40 cm deep and filled with 22±1°C water).

The maze was divided geographically into four equal quadrants and held release points that were designed at each quadrant as N, E, S, and W. A square hidden black platform (10 cm diameter) was submerged beneath (1.5 cm) the water surface in the middle of the target quadrant in the pool. Visual cues were placed around the pool. The animal motion was recorded and sent to the computer by a camera mounted above the center of the maze. The swimming speed and time latency to reach the platform and also the length of swimming pathway were recorded semi-automatically by a commercial software (Noldus, Netherlands; version:6 XT).

2.5. Spatial learning measurements procedure

Each animal was handled daily for 3 days prior to the initiation of the experiments. Then the rats were habituated to the water maze for 60 sec without a platform. Each rat performed a trial test daily for 4 consecutive days. In each trial the rats were placed randomly at the middle of the circular edge in a randomly selected quadrant in MWM apparatus and released facing the side wall at one of four positions (the boundaries of the four quadrants, labeled N, S, E and W). On each trial, the rat was allowed to swim until it found and remained on the platform for 20 sec. If animal had not found the platform after 60 sec the experimenter would assist the rat to find platform and allowed to stay on the platform for 20 sec. Then the rat was removed from the pool, dried with a towel and located in its holding cage. The next trial was done after 20 to 30 sec of animal rest. Parameters such as latency and the traveled distance to find the platform were recorded in each trial.

2.6. Probe test evaluation

Probe test was performed to evaluate spatial memory retention on the 5th day (24 hr after the last trial). The experimenter conducts a probe trial in which the escape platform is removed from the pool and each rat was placed into the pool from the opposite quadrant and allowed to swim freely for 60 second. Usually, the well-trained rats spent most of the swimming time in the target quadrant of the pool across the former location of the platform. The time swum in target quadrant, swimming speed and the number of times rat crossed the platform area in the probe phase were recorded.

A visible platform test is performed for the evaluation of any possible sensory and motor coordination deficits or motivation abnormality of male offspring. In this test, the ability of animals to escape to a visible platform was evaluated (the platform was raised 2 cm above the water level time and was visible with aluminum foil)²³.

2.7. Plasma lead measurement

In another set of experiments, lead levels was evaluated in separate groups of pregnant rats (7rat/group) that were randomly divided into 2 groups: Control group had free access to tap water and Gestation (G) in which tap water was replaced with a solution containing 0.2 % lead acetate from the first day of gestation and continued until parturition²¹. Blood samples from both control and lead exposed rats were collected from orbital sinus after parturition and serum concentration of lead was measured by atomic absorption spectrophotometry²⁴.

2.8. Statistical Analysis

The data were presented as mean \pm SEM of 7 rats in each group. T-test was used to compare the mean differences between two groups and two-way analysis of variance (ANOVA) test was used to compare the mean differences between groups. Tukey post hoc multiple comparison test was performed to assess differences between experimental groups. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Serum lead concentration in pregnant rats.

Our results showed significant difference in the serum lead concentration in control and lead exposed mother rats (235.76 $\mu\text{g/L}$) by atomic absorption spectrophotometry (Table 1).

Table 1. Serum lead concentration in pregnant rats.

Group	Mean blood lead Conc ($\mu\text{g/L}$)
Ctrl	0
Lead	235.76

Pregnant rats received a solution containing 0.2 % lead acetate from the first day of gestation and continued until parturition. Control group had free access to tap water. Ctrl (Control).

3.2. Zataria multiflora analysis by GC/MS.

We have previously reported that thymol (37.59%), carvacrol(33.65%); para-cymene (7.72%), γ -terpinene (3.88%) and β -caryophyllene (2.06%) were the main components of Zataria multiflora comprising 84.9% of the oil.¹³.

3.3. Spatial learning in prenatally lead exposed male rats

Our results showed training impairment in prenatally lead exposed male rats (SAL group) vs control rats. Analysis of travelled distance at first, second, third and fourth days of training (four trials per day), using repeated measures two-way ANOVA, revealed a significant training process during through the 4 days of experiment [$F(5, 42) = 93.817, p < 0.001$] (Fig 1,2).

During the acquisition phase, as shown in Fig. 1, the data showed that there was a significant difference in travelled distance in SAL animals vs Ctrl on fourth day; [$F(5, 42) = 2.497, p < 0.05$] (Fig. 1A). Also, the SAL animals (prenatally lead exposed male rats) needed more time to find hidden platform than the Ctrl rats on (first day; [$F(5, 42) = 2.261, p < 0.05$], second day; [$F(5, 42) = 3.304, p < 0.01$]) (Fig 1B).

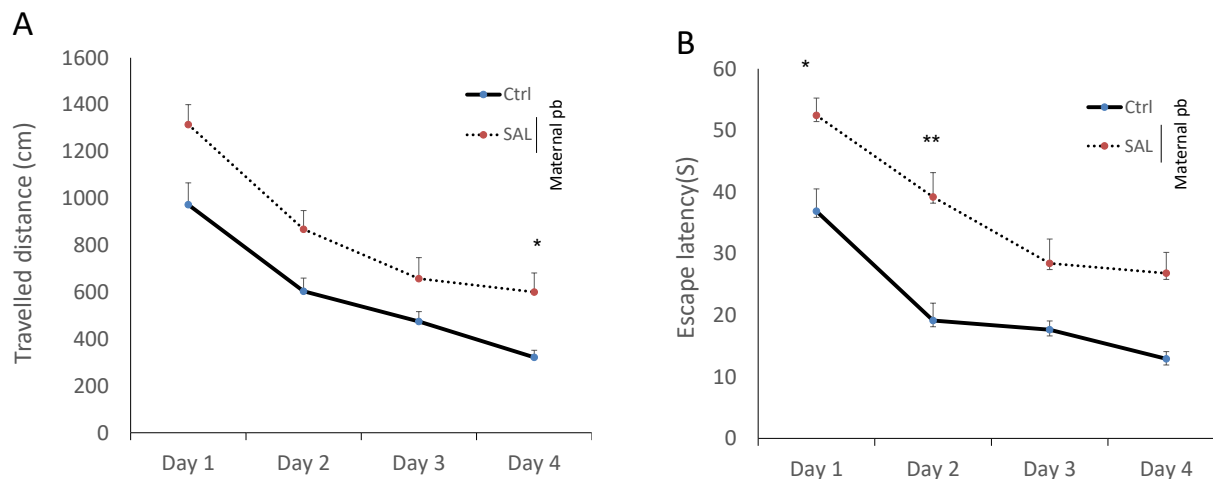


Figure 1. The effects of lead on travelled distance (A), the average escape latency (B) during spatial learning to find the hidden platform in the Morris water maze in antenatally lead exposed adult male offspring. Adult male offspring rats Ctrl and SAL groups received saline. Data are expressed as the Mean \pm SEM (7 rats/group). Ctrl (Control), SAL (Saline), Maternal pb (lead exposure in prenatal). * $p < 0.05$, ** $p < 0.01$ vs Ctrl.

3.4. The effects of ZM (50, 200, 400, 800 mg/kg) on the average escape latency to find the hidden platform and travelled distance in antenatally lead exposed male offspring (spatial learning).

As shown in Fig. 2, during the acquisition phase, there was no significant difference in travelled distance and escape latencies between ZM treated rats with Ctrl and SAL.

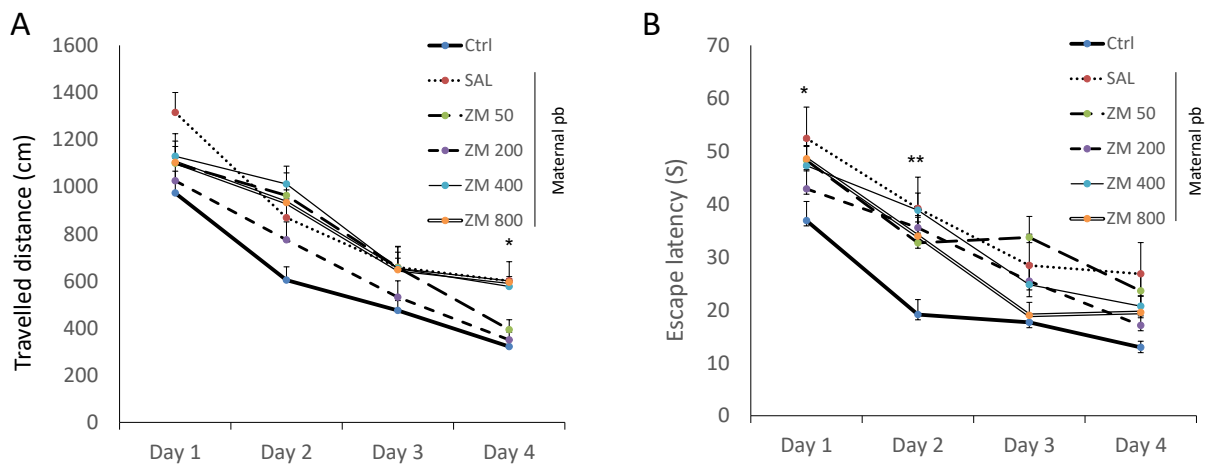


Figure 2. The effects of *Zataria multiflora* on travelled distance (A) and the average escape latency (B) during spatial learning to find the hidden platform in the Morris water maze in antenatally lead exposed adult male offspring. Adult male offspring rats received *Zataria multiflora* (50, 200, 400, 800 mg/kg/ i.p./ 20 d). Ctrl and SAL groups received saline. Data are expressed as the Mean \pm SEM (7 rats/group). Ctrl (Control), SAL (Saline), ZM (*Zataria multiflora*), Maternal pb (lead exposure in prenatal). * $p < 0.05$, ** $p < 0.01$ vs Ctrl.

3.5. The effects of ZM on travelled distance, the time spent and crossings to target quadrants in antenatally lead exposed male offspring during the probe test (memory retention).

Data showed that the travelled distance in trigger zone in SAL and ZM 50 was significantly lower than Ctrl ($p < 0.001$, $p < 0.05$, respectively) (fig 3A). ZM 200 and ZM 400 significantly increased the travelled distance in trigger zone as vs SAL ($p < 0.01$, $p < 0.05$, respectively) (fig 3A). The time spent in trigger zone in SAL, ZM 50, ZM 800 was significantly lower than Ctrl rats ($P < 0.001$, $P < 0.05$, $P < 0.05$ in SAL; ZM 50 and ZM 800 vs Ctrl, respectively) (fig 3B). ZM 200 and ZM 400 treatment result in a significant increase in the time spent in trigger zone compared to SAL rats ($p < 0.01$, $p < 0.05$ respectively) (fig 3B).

There was a significant difference in crossings to target quadrants among SAL ($p < 0.001$) and ZM 50 ($p < 0.05$) vs Ctrl rats (fig 3C).

The swimming speed in target quadrant showed no significant difference among Ctrl, SAL & ZM treated offspring.

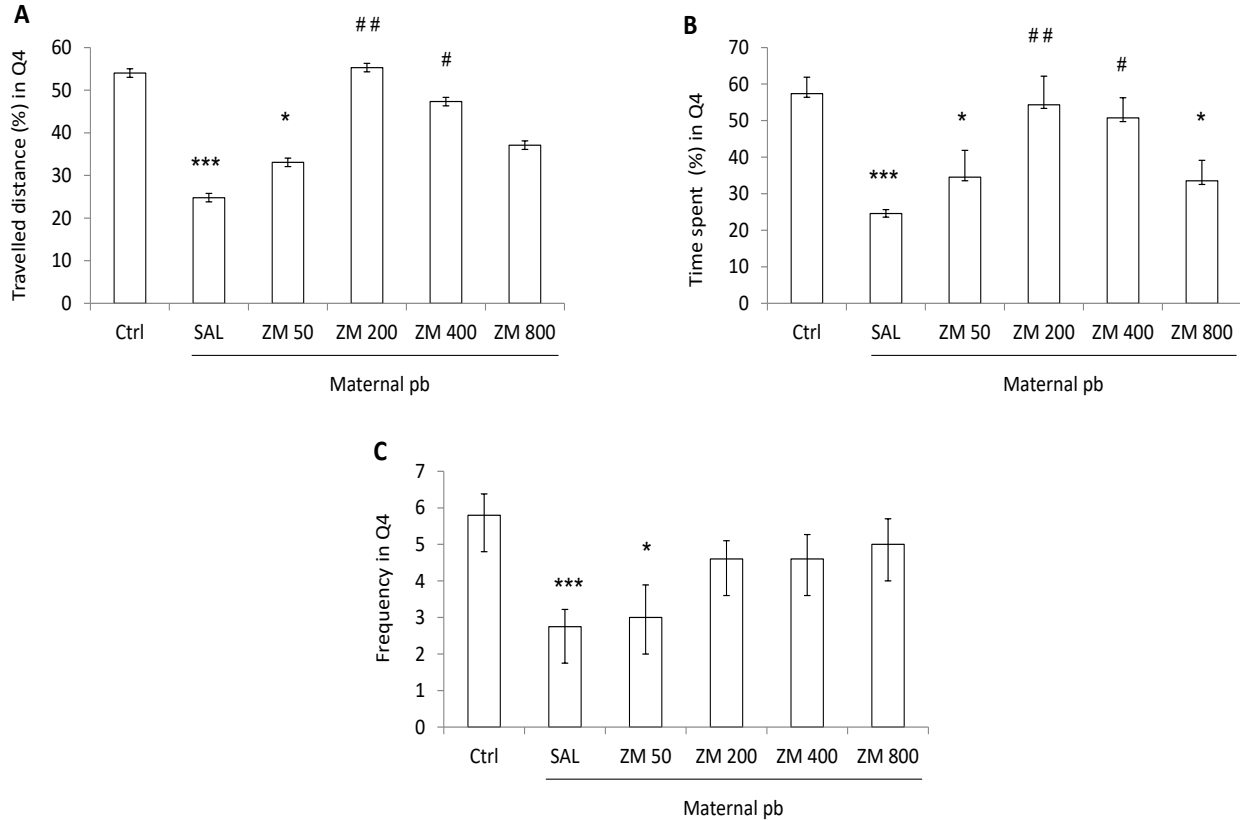


Figure 3. The effects of *Zataria multiflora* on the travelled distance (A), the time spent (B) and the crossings (C) in trigger zone in antenatally lead exposed adult male offspring during the probe test (memory retention). Adult male offspring rats received *Zataria multiflora* (50, 200, 400, 800 mg/kg/ i.p./ 20 d). Ctrl and SAL groups received saline. Data are expressed as the Mean \pm SEM (7 rats/group). Ctrl (Control, SAL (Saline), Maternal pb (lead exposure in prenatal). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Ctrl group. # $p < 0.05$, ## $p < 0.01$, vs SAL group.

3.6. Escape latency to find visible platform

Our results showed no significant difference in the time spent to find the visible platform in Ctrl, SAL and ZM treated male offspring (Table 2).

Table 2: The time spent to find the visible platform in antenatally lead exposed adult male offspring rats

Group	Escape Latency (s)
Ctrl	31.1 ± 4.16
SAL	37 ± 6.3
ZM 50	43.7 ± 4.03
ZM 200	52.1 ± 4.9
ZM 400	43.4 ± 7.1
ZM 800	33.6 ± 4.3

Comparisons of escape latency to escape on to the visible platform in Morris water maze in antenatally lead exposed adult male offspring rats, using one-way analysis of variance (ANOVA) (the differences were not significant). Adult male offspring rats received *Zataria multiflora* (50, 200, 400 & 800mg/kg/ i.p./ 20 d). Ctrl and SAL groups received saline. Data are expressed as the Mean ± SEM of 8 rats/group. Ctrl (Control), SAL (Saline), ZM (*Zataria multiflora*), Maternal pb (lead exposure in prenatal).

4. Discussion:

Lead exposure is one of the most important public health risk during the first trimester of pregnancy in most countries which is associated with a significant impairment in cognitive function in both childhood and adolescence period. So, prevention of lead-induced behavioral changes during prenatal lead exposure is the best possible strategy. In this study, the effects of methanolic extract of ZM on lead-induced deficits in cognitive behavior of prenatally lead exposed male offspring rats were evaluated by MWM. Data revealed elevated serum lead concentration during the gestation period (235.76 µg/L). Our results indicate that lead exposure during gestation period results in long lasting deficits in spatial reference learning in adult male offspring. Escape latency to find the hidden platform, as an indication of learning index, was significantly increased in prenatally lead exposed male offspring. Also the results of the probe test showed a significant increase in escape latency, travelled distance, as well as decrements in

crossings frequencies to target quadrants in the prenatally lead exposed male offspring. These deficits in cognitive behavior can be attributed to spatial performance. However, there was no significant difference in swimming speeds and ability of rats to find the visible platform, which shows that prenatal lead exposure did not affect the locomotion or sensorimotor coordination.

Our data are in complete agreements with previous reports and lead –induced cognitive behavior impairments has been reported previously in both human and animal studies^{2-4, 25}. The cellular, intracellular and molecular mechanisms of lead neurotoxicity are numerous and the possible mechanisms by which lead exposure causes impaired learning and memorizing abilities may be mediated through different pathways. Lead exposure during pregnancy results in high lead blood level in umbilical cord blood and fetus blood level which cause neurobehavioral impairments in infants and children by affecting the anticholinesterase activity^{14, 26-27}. Lead exposure result in a significant decrease in the intensity of anticholinesterase staining in the dentate gyrus, CA2 and CA3 areas of hippocampus, as well as in the different cell layers of cortex and cerebellum²⁷. Alteration in the CNS neurotransmitters such as dopamine, glutamate, serotonin and norepinephrine are also involved in lead –induced behavioral changes^{9-10, 27}. Also lead - induced toxicity may be mediated through the production of reactive oxygen species (ROS) which is confirmed in both in vitro and in vivo studies in animals and occupationally exposed workers¹¹. Nitric oxide synthase (NOS) activity in the hippocampus, the cerebral cortex and the cerebellum of rats were inhibited by low-level lead exposure (drinking water containing 0.025%, 0.05% and 0.075% lead acetate/ 28 d)²⁵. Lead toxicity could result in oxidative stress, DNA damage and apoptosis²⁸.

The lead-induced cognitive deficits were reversed by administration of ZM essential oil. The beneficial effects of ZM (200mg/kg) on spatial memory was characterized by the increased travelled distance and time spent in quadrant zone as well as an increase in crossings to target quadrants in the prenatal lead-exposed rats. The mechanism(s) by which ZM ameliorate the lead –induced cognitive impairments in antenatally lead exposed rats is not precisely determined, however, our GC/MS analysis of methanolic extract of ZM essential oil showed that thymol (37.59%) and carvacrol (33.65%) were the main constituents of the dry plant of ZM

essential oil, so the beneficial effects of ZM on cognitive behavior of rats could be mainly mediated by these compounds^{14, 26}. Others also reported the carvacrol and thymol as the main constituents of ZM essential oil²⁹⁻³⁰ reported that thymol and carvacrol alleviate the cognitive deficits induced by amyloid β (A β) or scopolamine in the rat models of dementia. The beneficial effects of *Zataria multiflora* on rat models of dementia may be mediated through its anticholinesterase, antioxidant, and anti-inflammatory activities²⁹. Although both carvacrol and thymol possess anticholinesterase activity, however, anticholinesterase inhibitory effect exerted by carvacrol is reported to be 10 times stronger than that exerted by its isomer thymol³¹. Amelioration of cognitive deficits by ZM has been reported by other investigators¹²⁻¹³. Gelatin films were prepared from gelatin solutions (10% w/v) containing ZM essential oil exhibited excellent antioxidant and antimicrobial properties³².

Kavoosi et al (2012) reported that ZM essential oil, thymol and carvacrol significantly reduced nitric oxide (NO) and hydrogen peroxide (H₂O₂) production in lipopolysaccharide (LPS)-stimulated macrophages and thus potential to be used in the therapy of lead-induced oxidative damage mechanism of cognitive deficits³³⁻³⁴. Carvacrol, the main constituent of ZM, antioxidant and nitric oxide scavenging and malondialdehyde scavenging activities may be involved in the reversing of lead-induced cognitive impairments in rats³⁵. Zotti et al (2013) showed that carvacrol, increased dopamine and serotonin levels in the prefrontal cortex and hippocampus in rats and thus can clearly influence behavioral outcome through modulation of neurotransmitters³⁶.

In summary, this study showed that lead exposure during pregnancy caused impaired memory of male offspring rats in Morris water maze test. Administration of *Zataria multiflora* (200 mg / kg) improved lead-induced memory deficits in prenatally exposed male offspring. The exact mechanism(s) underlying the beneficial effects of ZM on lead-induced memory impairment is not determined yet, but it could be mediated through the anticholinesterase activity, antioxidant effects, nitric oxide scavenging and malondialdehyde scavenging activities and alterations in CNS neurotransmission such as dopamine, glutamate, serotonin and norepinephrine in the central nervous system by carvacrol/thymol, the main constituents of ZM. Further research is needed to elucidate the underlying mechanism(s).

Conflict of interest

The Authors have no conflict of interest.

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