

Prophylactic effect of the aqueous extract of *Pimpinella anisum* on the behavior of Wistar rats exposed to mercury

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Abstract

The purpose of this study was to evaluate the prophylactic effect of *Pimpinella anisum* (green anis) on neurobehavioral status following mercury chloride intoxication during the developmental period. For this purpose, rats exposed to 100 mg/L of HgCl₂ during the gestation and lactation period. A group of rats was treated with the anis extract for 15 days before becoming intoxicated with mercury. In contrast, one group was orally administered aqueous anis extract for 15 days after intoxication. The forced swimming test, the open field test and the Morris pool respectively recorded an increase in immobility time, a decrease in the number of cross-cells ($p < 0.001$), ($p < 0.05$) and an increase in latency ($p < 0.01$), ($p < 0.001$), ($p < 0.001$) and decreased time spent in the target frame during the probe test ($p < 0.01$) and increased latency in the visible test ($p < 0.01$) in HgCl₂-exposed rats compared to control rats. However, preventive and curative aniseed-based treatment reduced the rate of depression, increased locomotor activity and improved learning performance. In conclusion, the aqueous extract of *Pimpinella anisum* could have a corrective effect on some neurological disorders caused by mercury.

Keywords: immobility time; learning; locomotor activity; mercury; neurobehavior; *Pimpinella anisum*

Introduction

Mercury (Hg) is one of the metallic trace elements with unique physico-chemical properties. This metal is of public health concern because it poses a high risk to the environment and human health (Bjørklund *et al.*, 2017). It can cause significant changes in most tissues and organs. Due to its perpetual existence in the environment, all living organisms are exposed to mercury in one way or another because it is found in various sources such as seafood, food, water, air, sediment, soil, dental amalgam and vaccines (Chehimi *et al.*, 2012; Hazelhoff *et al.*, 2018). It has several toxic effects: on the central nervous system, immune system, kidneys and liver. Mercury exposure has also been associated with gastrointestinal disorders and cardiovascular disease (Hazelhoff *et al.*, 2018).

However, plants represent a source of new bioactive molecules with therapeutic potential. One of the main originalities of medicinal plants lies in their ability to produce a wide variety of natural substances. Indeed,

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they also accumulate so-called secondary metabolites whose physiological function is not always obvious but represents an important source of bioactive molecules usable by humans (Kosalec *et al.*, 2005; Macheix *et al.*, 2005). Anise or *Pimpinella anisum*, is an aromatic plant belonging to the Apiaceae family (Bekara *et al.*, 2016). It has been proven that extracts and essential oils of this plant have various biological activities, such as carminative, antiseptic, antidepressant, antispasmodic, antifungal, antibacterial, antioxidant, insecticide, diuretic, cough and insomnia (Tepe *et al.*, 2015). In medieval Persian medicine, this plant and in particular the essential oil of its fruit was used for the treatment of certain neurological diseases, including convulsions and epilepsy (Gorji *et al.*, 2001).

The aim of the present study was to compare the neuroprotective and neuro-correcting effects of the aqueous extract of green anise in young Wistar rats exposed to mercury chloride during development period.

Materials and Methods

Plant material and preparation of the aqueous extract

The dry and ripe seeds of *Pimpinella anisum* were purchased from local herb market in Saida (Algeria) and were identified and authenticated by an expert taxonomist. A voucher specimen was deposited at the herbarium of the department of Biology at Saida University (Algeria). Subsequently, the seeds were ground using a grinder for the preparation of the aqueous extract by decoction. We put 100 g of the crushed seeds in 1000 ml of distilled water, the whole is boiled with continuous stirring for 15 minutes (Hosseinzadeh *et al.*, 2013). The mixture is then filtered through Whatman Paper No. 1 then the resulting extract is freeze-dried in an alpha 1-2 lyophilizer to obtain an aqueous extract of *Pimpinella anisum* with 17.18% yield.

Animals and treatment

Experiments were carried out on Wistar rats (obtained from the Department of Biology, Faculty of Sciences, University of Saida) weighing 210 ± 50 g. The animals were housed with free access to water and food in an animal room, with a 12/12-hour light/dark cycle, at 22 ± 2 °C. They were mated one week after their arrival (three females and one male per cage). Prior the intoxication, a group of females were treated with a dose of 500 mg/kg (Al Mofleh *et al.*, 2007) of *Pimpinella anisum* aqueous extract for 15 days by gavage. After one week of cohabitation with males, females were divided into three groups.

Group 1: Pregnant females received distillate water orally.

Group 2: Pregnant females received 100 mg/L of HgCl₂ diluted in distillate water (Chehimi *et al.*, 2012).

Group 3: Pregnant females treated with 500 mg/kg of the extract then received 100 mg/L of HgCl₂ diluted in distillate water.

Experimental design

At birth, the pups of intoxicated females received the solution of HgCl₂ until weaned, while control pups received only distilled water. In order to test the ability of aqueous anise extract (EAA) to attenuate Hg neurotoxicity which induces cognitive deficits, drug therapy was administered, starting 24 hours after withdrawal to determine its curative effect.

At weaning, we got 04 new groups (n = 7) as follow:

Group C: Control rats (issued from control females) received only distilled water.

Group Hg: intoxicated rats with HgCl₂ (issued from intoxicated females) that received intoxicated water orally as vehicle solution.

Group EAA-Hg: treated rats before the intoxication (issued from females treated with a dose of 500 mg/kg body weight of anise extract (EAA) with gavage per day for 15 days (Al Mofleh *et al.*, 2007). After 15

days of treatment with the extract, the females undergo mercury intoxication during gestation and lactation period.)

Group Hg-EAA: intoxicated rats (issued from intoxicated females) that received 500 mg/kg of body weight of anis extract (EAA) with gavage daily for 15 days.

All animals received their dose of extract 30 minutes before the start of behavioural tasks.

The number of animals that suffered was minimised in accordance with the guidelines of the European Council Directive (86/609/EEC).

Neurobehavioral study

Forced swimming test

The forced swimming test is commonly used to assess resignation behaviour in rodents. It is carried out inside a glass cylinder (39 cm high x 20 cm in diameter) containing water at 22 °C. The animal is necessarily forced to swim, and in this situation, it struggles to 'first, then give up before calming down. Stillness therefore reflects the state of resignation that can be associated with "despair". The test duration is 6 min (Kahloula *et al.*, 2013; Elizalde *et al.*, 2008).

Open-field test

General activity was evaluated in the open-field test. The apparatus was constructed of white plywood and measured 72 × 72 cm with 36 cm walls. The lines divided the floor into sixteen 18 × 18 cm squares. A central square (18 cm × 18 cm) was drawn in the middle of the open field. The apparatus was uniformly illuminated with red lights. Each animal was placed individually in the centre of the arena and allowed to explore the apparatus for five minutes. A trial was conducted for three consecutive days and the following variables were recorded: line crossing, centre square entries, rearing (count of times that the animal stood on its hind legs), grooming (time, in seconds, used for the animal to groom), and freezing (time, in seconds that the animal remained immobile, often in a crouching posture, with eyes wide open, and irregular respiration), and defecation (number of faecal produced) (Dauge *et al.*, 1989).

Morris water maze

The Morris water maze task was used to assess cognitive function, in particularly spatial learning and memory abilities. The test was conducted in a round white pool 90 cm in diameter with a 50 cm high wall. The pool was filled to a depth of 30 cm with water made opaque with white, not toxic water-based paint. Water temperature was maintained at 21 ± 1 °C with an aquarium heater. The platform of 10 cm in diameter and 28 cm high (i.e., 2 cm below the water's surface) was used as the hidden goal. The pool was located in a room with numerous extra-maze cues that remained constant throughout the experiment. Animals were tested on days 11-15 after weaning, with four trials per day for five consecutive days. On each trial, the animal was placed in the pool, facing the wall, at one of the four start locations (north (N), south (S), east (E) and west (W)). Start location sequences were randomly assigned each day prior to the start of testing. Rats were allowed to swim to the sub-merged platform placed in the NW quadrant during 60 s. If the platform was not found within the allowed time, the rat was manually placed onto it. Rats were allowed to rest on the platform for 20 s between each trial. A session of four trials was conducted each day from 9:00 to 11:00 A.M. The experimenter was hidden from the view of the animals. The escape latency (time to find the platform) and path length (distance travelled to the hidden platform) were recorded by using a video track system (Viewpoint, France).

A probe test was conducted the day following the end of the training session to further characterize swim task performance. In this test, the platform was removed and the rat was placed next to and facing the S side. Animals were allowed to swim freely for the original training session length of time (60 s) for a single trial. The time spent by animals in the previously correct quadrant (NW) was removed and the rat was placed next to and facing the S side. Animals were allowed to swim freely for the original training session length of time (60 s) for a single trial. The time spent by animals in the previously correct quadrant (NW) was measured.

Visual cue test was performed two hours after the end of the probe test in order to evaluate the visuomotor abilities and the motivation of the animals. It was conducted by extending a large black flag above the water level from the submerged platform. The maximum time allowed was the same as the original training sessions. The escape latency and path length were measured during a single session of four trials (Kahloula *et al.*, 2014).

Determination of blood and brain mercury

The mercury present in the mineralized material is volatilized at high temperature (550 °C). Elemental mercury is formed as vapor by oxygen stream and amalgamated on a trap. After heating the trap to dissociate the amalgam, the mercury vapor is quantified by flameless atomic absorption spectrometry at 253.65 nm.

Statistical analysis

Results were expressed as mean \pm standard error of the mean (SEM). Data were analysed by the two-way analyses of variance (ANOVAs). When a significant difference was found, the Student-Newman-Keuls post-hoc test was conducted. For all analyses, a difference is significant at $p \leq 0.05$.

Results

Forced swimming test

The results of the forced swimming test showed that the Hg intoxicated animals had a significantly higher immobility time than the control animals ($p < 0.001$). However, rats previously treated with anise extract and rats treated after exposition to mercury had a significantly lower immobility time than rats exposed to mercury ($p < 0.001$) (Figure 1).

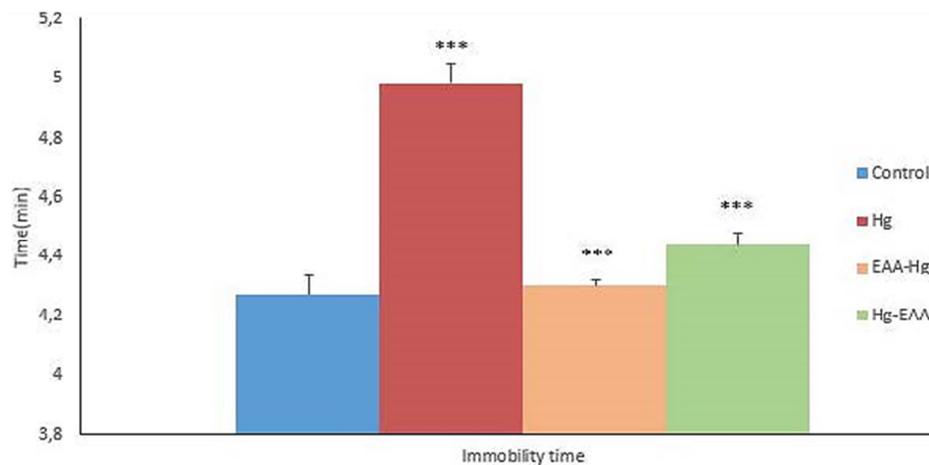


Figure 1. Evaluation of the prophylactic effect of the aqueous extract of *Pimpinella anisum* on depression in Hg intoxicated rats

Values are expressed as mean \pm SEM: *** $P < 0.001$ (Control vs. Hg; Hg vs. EAA-Hg; Hg vs. Hg-EAA)

Open-field test

The locomotor activity test showed that the number of tiles traversed and the number of visits to the centre of mercury-exposed rats were significantly lower than those of control rats ($p < 0.05$).

However, the number of tiles crossed and the number of centre visits of treated pups with aqueous extract of aniseed and intoxicated with mercury (EAA-Hg) were not significantly elevated compared to rats exposed to mercury ($p > 0.05$). In addition, the number of tiles crossed and the number of centre visits by

animals exposed to Hg and treated with aniseed (Hg-EAA) were elevated to those of Hg exposed animals (($p < 0.05$), ($p > 0.05$)) respectively.

The number of righting and the number of grooming of the poisoned animal group are insignificantly lower than those of the control animal group ($p > 0.05$).

The number of righting and the number of grooming of young rats treated with aniseed prior to mercury poisoning were high compared to those of young rats exposed to Hg, but in statistical terms there was no significant difference ($p > 0.05$).

For the group of animals intoxicated and subsequently treated with aniseed extract showed a significant increase in the number of recovery and grooming (($p < 0.05$), ($p < 0.01$)) respectively.

The number of defecations of the intoxicated lot was significantly higher than that of the control lot ($p < 0.001$). In addition, the number of defecation of animals treated batches: EAA-Hg and Hg-EAA was significantly lower than the batch of animals exposed to mercury (($p < 0.01$); ($p < 0.001$)) respectively.

Finally, the latency time of the Hg group was non-significantly elevated compared to the control group ($p > 0.05$). Rats of the EAA-Hg group had a high latency time to that of the Hg group ($p > 0.05$). The latency time of the Hg-EAA rats was lower than that of the Hg rats. Statistically, no significant difference was noted for the latency time ($p > 0.05$).

Table1. Parameters of open field test

Groups	Number of tiles crossed	Number of visits to the center	Adjustment	Grooming	Defecation	Latency time
control	154± 11.35	4.33±2.15	8.16±2.85	3.66±0.80	1.5±0.67	0
Hg	88± 19.35*	0.33±0.21*	3.83±0.74	3.5±0.34	5.66±0.76***	1.06±0.61
EAA-Hg	105± 11.05	1.5±0.56	4.33±1.28	2.66±1.11	4±0.36*	6±3.79
Hg-EAA	106.16± 20.08*	2±0.36	8.5±0.76*	5.33±0.21**	1.5±0.42***	1±0.63

Values are expressed as mean ± SEM: ***P < 0.001, **P < 0.01, *P < 0.05

Morris water maze

The results recorded during the four days of spatial learning showed that the latency time of the intoxicated rats is significantly higher than that of the control rats during the 2nd, 3rd and 4th day of learning ($p < 0.01$), ($p < 0.001$), ($p < 0.001$) respectively.

The latency time of animals treated with aniseed aqueous extract before intoxication was insignificantly elevated compared to that of intoxicated animals on the first day of acquisition ($p > 0.05$). We recorded a decrease in the latency time of EAA-Hg rats compared to Hg rats during the last three days of training with a significant decrease on the 3rd day of acquisition ($p < 0.01$).

In addition, the Hg-EAA batch presented a latency time lower than that of the Hg batch. In statistical terms we recorded a significant difference during the 3rd and 4th day of spatial learning ($p < 0.01$), ($p < 0.05$) respectively (Figure 2).

The probe tests

During the probe test phase, we recorded a latency time of the animals exposed to mercury in the NO target frame significantly lower than that of the control animals ($p < 0.01$). Furthermore, the latency time of rats treated with EAA including EAA-Hg and Hg-EAA have a significantly higher latency time than that of Hg rats in the target frame ($p < 0.05$), ($p < 0.01$) respectively (Figure 3).

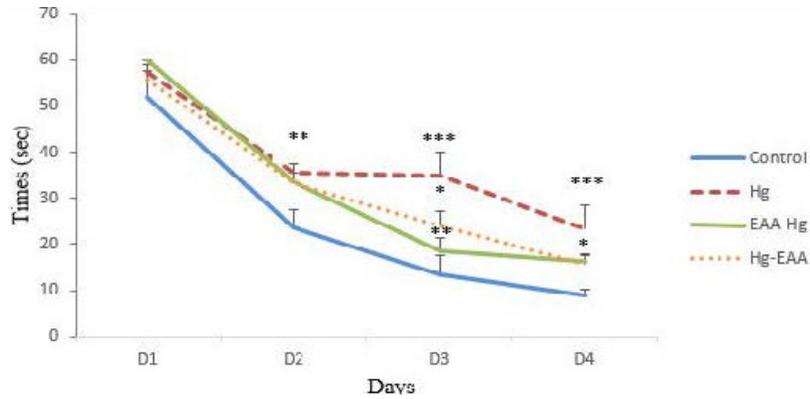


Figure 2. Evaluation of the curative effect of the aqueous extract of *Pimpinella anisum* on spatial learning in Hg intoxicated rats

Values are expressed as a mean \pm SEM: Day 2: **P < 0.01 (control vs. Hg). Day3: ***P < 0.001 (control vs. Hg); **P < 0.01 (Hg vs. Hg-EAA). *P < 0.05 (Hg vs. EAA-Hg). Day 4 ***P < 0.001 (control vs. Hg); *P < 0.05 (Hg vs. Hg-EAA).

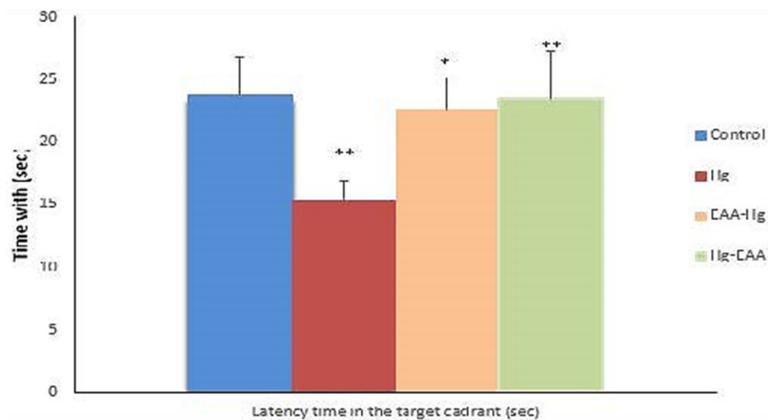


Figure 3. Time spent in the (NO) frame during the probe test by control, poisoned and poisoned treatment rats

Values are expressed as mean \pm SEM: **P < 0.01 (control vs. Hg); *P < 0.05 (Hg vs. EAA-Hg); **P < 0.01 (Hg vs. Hg-EAA).

The visible test phase

During the visible platform test, young rats exposed to Hg during the gestation and lactation period take longer time to reach the platform than the control rats despite the latter being marked by a visual clue ($p < 0.01$). However, animals in the EAA-Hg and Hg-EAA groups have a significantly lower latency time to reach the platform than animals in the Hg group ($p < 0.01$) (Figure 4).

Blood and brain mercury assay results

The atomic absorption spectrophotometry technique revealed variations in blood and brain mercury levels in the mercury exposed group compared to the control group and compared to the treated intoxicated and intoxicated treated groups. These results show that the mercury concentration in poisoned rats is significantly elevated to those of control rats. However, treatment with aniseed extract reduced this concentration in the blood and brain. The recorded results are in Table 2.

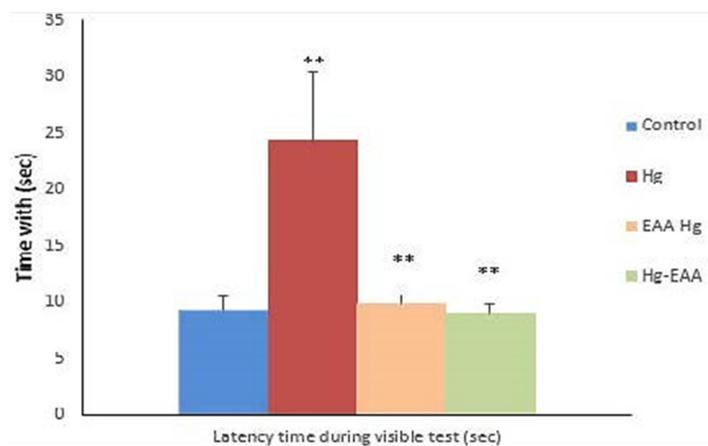


Figure 4. Latency time during the visible platform in control, intoxicated and intoxicated treated rats. Values are expressed as mean \pm SEM: **P < 0.01 (control vs. Hg); **P < 0.01 (Hg vs. EAA-Hg); **P < 0.01 (Hg vs. Hg-EAA)

Table 2. Evaluation of blood mercury and cerebral mercury

	Mercuremia	Mercury cerebral
Control	0.20 $\mu\text{g.L}^{-1}$	3.10 $\mu\text{g.L}^{-1}$
Hg	8.11 $\mu\text{g.L}^{-1}$	20.09 $\mu\text{g.L}^{-1}$
EAA-Hg	4.63 $\mu\text{g.L}^{-1}$	12.91 $\mu\text{g.L}^{-1}$
Hg-EAA	2.89 $\mu\text{g.L}^{-1}$	8.64 $\mu\text{g.L}^{-1}$

Discussion

Mercury is an environmental pollutant linked to a high health risk. Studies on this metal have shown that it can harm several systems such as: cardiovascular, respiratory, renal, nervous, reproductive and immune (Azevedo *et al.*, 2016).

On the other hand, aromatic and medicinal plants are known for their interesting biological properties and are used in various fields such as medicine, pharmacy, cosmetology and agriculture (Fyad *et al.*, 2013).

The result of the forced swimming showed that the Hg exposure induced an increase in the time of immobility, which is the key parameter to estimate the degree of resignation. This increase was significant compared to the time of immobility recorded in the control group. This results in a state of hopelessness caused by the mercury. Mercury vapor readily enters the brain and causes tremors and depression (Langford *et al.*, 1996). In contrast, subjects with chronic occupational exposure to Hg are more depressed than subjects occasionally exposed or non-existent (Soleo *et al.*, 1990).

However, the immobility time of rats from Hg-exposed females treated (EAA-Hg, Hg-EAA) with aqueous extract of *Pimpinella anisum* was significantly less than that of Hg poisoned rats, with more efficacy for the preventive treatment. Oral administration of the aqueous extract of green anis seeds significantly increased the mobility time. Different doses of aqueous and ethanolic extract of the fruit *Pimpinella anisum* have proven their activity as an antidepressant (Shahamat *et al.*, 2016). In addition, the essential oil of this plant may have an antidepressant effect (Kahloula *et al.*, 2013).

The results obtained from the open field test show that early exposure to mercury caused hypoactivity in intoxicated rats compared to control rats. This was shown by the decrease in the number of tiles crossed and the number of visits to the centre. This locomotor hypoactivity was accompanied by an increase in the number of defecations and latency time compared to the control batch. Several studies have shown that exposure to

MeHg can produce behavioural deficits related to locomotor activity and motor performance in adult mice (dos Santos *et al.* 2016).

However, the animals exposed to Hg and treated with EAA, to know the preventive and curative treatment, had a higher number of cells crossed than the exposed animals as well as an increase in the number of visits to the centre, the number of adjustments and the number of grooming. According to the work of Kahloula *et al.* (2013), the essential oil of anise could have a correcting effect of the locomotion deficit of rats poisoned by lead. Our results are in agreement with those of (Kahloula *et al.*, 2013; dos Santos *et al.*, 2016).

The results of the Morris pool test show that the learning time of the intoxicated group was significantly increased during the last 3 days of the test compared to that of the control group. This indicates that intoxicated animals have a deficit of spatial learning and memorization.

In addition, in the phase of the probe test, the intoxicated group takes little time in the target frame that contains the platform compared to the control group. In the visible test phase, the young rats exposed to mercury take much longer than the young control rats to reach the platform despite the latter being marked by a visual clue. This results in the presence of visual disturbances in the group exposed to Hg.

Developmental and intelligence performance are reduced in children of women exposed to high levels of mercury during pregnancy (Kjellström *et al.*, 1989; Grandjean *et al.*, 2008).

Also, a neuropsychological study carried out on 7-year-old children of mothers exposed to methylmercury in fish during pregnancy showed decreased attention, language, motor speed and visuospatial function (Grandjean *et al.*, 1997; Grandjean *et al.*, 2008).

As well, it causes degeneration of sensory nerve fibers, demyelination and increased collagen resulting from incomplete and irregular repair of these fibers (Harada *et al.*, 1995). The mercury is redistributed throughout the infant's body. This potent neurotoxicant accumulates in different compartments of the developing brain, including the hippocampus, cerebral cortex, cerebellum and retina (Grosman *et al.*, 2010). Inorganic mercury disrupts glutamate transport and glutamine synthetase (GS) activity (Grosman *et al.*, 2009).

However, the Hg-EAA batch has a significantly lower learning time during the 3rd and 4th days compared to the Hg batch, with a non-significant decrease during the first two days of learning.

Regarding the preventive treatment (EAA-Hg) we recorded a significant decrease during the 3rd day of acquisition.

For the probe test and the test of the visible platform, there is a significant difference between the treated groups (EAA-Hg, Hg-EAA) and the Hg group during the probe test phase, the animals of the EAA-Hg group and Hg-EAA spend more time than the animals of the Hg group in the NO target frame (which contains the platform), this is due to the memorization of the location of the platform.

During the visible testing phase, the EAA-Hg and Hg-EAA lots take little time compared to the Hg lot to reach the platform which is marked by a visual clue. This indicates that aniseed has a beneficial effect on memorization and impaired vision.

Many medicinal virtues are attributed to anise, an infusion of its seeds or a few drops of its essential oil can be prescribed to treat many disorders and can have an effect on the nervous system (Samojlik *et al.*, 2012).

The results of the mercury assay showed high levels of mercury in the poisoned rats compared to the control lot.

Hg ions are transported equivalently in plasma, complexed to albumin, and in red blood cells after binding with haemoglobin and glutathione (Vesterberg *et al.*, 1993). The concentration of cerebral mercury in the intoxicated group was very high in comparison with the control group and even in comparison with mercuriaemia this can be interpreted by the accumulation of this metal in the brain.

The CNS is considered to be the target organ in humans when inhaling mercury vapor. The severity and reversibility of damage depend on the intensity and duration of exposure (Bensefa *et al.*, 2011). Tissue damage is also correlated with increased accumulation of the toxicant in brain regions indicating the development of a neurodegenerative disorder caused by mercury intoxication. These effects were confirmed by an electron microscopic study of the brain which showed a discontinuous myelin sheath around the axon, including

swollen mitochondria, a swollen nucleus, and nuclear membrane changes with treatment for mercuric common bile duct (Rao *et al.*, 2011).

However, preventive and curative treatments with an aniseed extract have reduced mercuremia and brain mercury concentration. According to El-Sayed *et al.* (2015), green anise has a strong capacity to chelate multivalent metal ions, this chelating effect against minerals would have been the cause of a corrective effect.

Conclusions

Exposure of Wistar rats to mercury chloride during the gestation and lactation period revealed neurotoxic effects manifested by a change in neurobehavioral state, as: depression, hypoactivity and cognitive impairment. In addition, the curative treatment with the aqueous extract of *Pimpinella anisum* was more beneficial than the preventive treatment. The treatment was able to correct the depressive effect, improve locomotor capacities and spatial learning.

Authors' Contributions

AW and KK designed and performed the experiments and also wrote the manuscript. ADEH performed the statistical analysis. MB and AW and TN, carried out the behavioural tests. SM and KK reviewed the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

The number of suffering animals were minimized in accordance with the guidelines of the European Council Directive (86/609/ EEC).

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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