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The Efficacy of *Syzygium aromaticum* Essential Oil in Cognitive Disorders against Manganese Chronic Exposure in Rats during Development

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Abstract

The essential oil of *Syzygium aromaticum* has been widely used in traditional medicine to treat a variety of diseases, including some neurological disorders. This study aims at testing, *in vivo*, the possible anxiolytic and antidepressant effects, of the *Syzygium aromaticum* essential oil against chronic manganese chloride (4.79 mg/l) intoxication during the gestation and lactation period, in Wistar rat pups. Wistar rat pups were exposed to manganese via their dams' drinking water from postnatal day (PND) 1 to (PND) 21. After their weaning, the rats exposed to manganese received injections of essential oil of *Syzygium aromaticum* (0.1 ml/kg) for 18 days. The level of anxiety, depression and locomotor activity were studied. Locomotor activity (open field test), anxiety (elevated plus maze tests), and depression (forced swimming test) were evaluated. The results of the present study indicate that Manganese exposure induces, on the one hand, impairments of body (p <0.001) and of brain weight (p<0.05).On the other hand, it increases level of anxiety (p<0.05), depression (p <0.001) and locomotor hyporactivity (p<0.001), when compared to control rats. Administration of essential oil of *Syzygium aromaticum* leads to a reduction in the level of anxiety (p<0.05), of depression (p<0.001) and corrects locomotor hyporactivity (p<0.05) in rats exposed to manganese beforehand. These results suggest that essential oil of S*yzygium aromaticum* can employ as a natural, protective agent against neuro-toxicity induced by manganese chloride during the gestation and lactation periods.

Keywords: anxiety, clove, depression, locomotor activity, manganese chloride

Introduction

Manganese (Mn) is a naturally occurring trace metal commonly found in the environment. It is the twelfth most abundant element in the earth's crust, present in rocks, soil, water and food. It does not occur naturally in a pure state and the most important Mn-containing minerals are oxides, carbonates and silicates (Post, 1999). Mn plays an important role in numerous enzymatic reactions as a cofactor (Keen, 1995). It is also found in a variety of biological tissues and it is necessary for normal functioning of a variety of physiological processes including amino acid, lipid, protein and carbohydrate metabolism (Erikson *et al*., 2005).

Moreover, Mn intoxication is associated with a neurological disorder known as manganism, a brain disorder characterized by psychological and neurological abnormalities that resemble Parkinson's disease (Donaldson, 1987). While it is known that Mn exposure in humans such as hair manganese concentration have been associated with learning disabilities, attention deficits, and hyperactivity (Barlow, 1983; Collipp *et al*., 1983). High Mn exposure produces behavioral changes, such as emotional lability, memory loss, aggressive behavior, impairments in attention, as well as tremor, bradykinesia, postural deficits, and dystonia (Cawte, 1985).

Neonatal rats have the ability to accumulate high levels of Mn in their brains, which will increase the risk of neurotoxicity and develop more pronounced brain pathology than adults do with equivalent or lesser Mn exposures (Chandra and Shukla, 1978; Dorman *et al*., 2000; Seth *et al*., 1977; Shkla *et al*., 1980). The behavioural effects of Mn during early development are not fully characterized. Mn neurotoxicity is characterized by dopaminergic neurodegeneration and it decreases extracellular dopamine levels (Marreilha dos Santos *et al*., 2012). Dopamine is involved in a variety of processes, including cognition, motor activity, motivation and reward, mood, attention, and learning (Jones and Miller, 2008).

The identification of new active plant derived natural compounds (essential oil) could increase the number of available chemotherapeutic agents, thereby reducing the frequency of resistance phenomena and providing alterna tive drugs with greater acceptance (Alawa *et al*., 2003).

The essential oil extracted from the dried flower buds of clove, *Syzygium aromaticum L*. Merr. and Perry (Myrtaceae), is

used as a topical application to relieve pain and to promote healing (Chaieb *et al*., 2007). Previous research has shown that clove has several therapeutic properties, such as anti-inflammatory, neuroprotective action, antihyperglycemic, nervous stimulant, tonic antimutagenic, aphrodisiac, bactericidal, nematicidal, fungicide, anti-toothache, anti-oxidant, analgesic, anesthetic and hypotensive properties (Mani *et al*., 2012; Dashti-R et Morshedi, 2009). Several works have demonstrated that the major component of the essential oil of *Syzygium aromaticum* is eugenol and lesser amounts of beta-caryophyllene and eugenol acetate (Chaieb *et al*., 2007; Prashar *et al*., 1999). Some studies have shown that acute administrations of clove oil enhances learning and memory recall and can reverse short-term and long-term memory deficits (Dashti-R et Morshedi, 2009; Halder *et al*., 2011).

The purpose of the present study was to investigate the efficacy of *Syzygium aromaticum* (clove) essential oil in attenuating the neurotoxic effects of Mn in rats.

Materials and methods

Plant Material and Preparation of Aqueous Suspension

Dried clove buds *Syzygium aromaticum* were purchased from local herb market in Saida (Algeria) and were identified and authenticated by an expert taxonomist. We used 50 g of dried clove buds *Syzygium aromaticum* that were processed by steam distillation, over a period of four hours, in an all-glass apparatus, to obtain essential oil with 5.5% yield.

Animals and Treatment

Experiments were carried out on Wistar rats (obtained from Charles River) weighing 200±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). On pregnancy day 0, the dams were divided into three groups: one group received 4.79 mg/l Manganese chloride tetrahydrate ($MnCl_2$ $4H_2O$) in drinking distilled water during gestation and lactation (Mn), the second group received $MnCl_2$ and clove essential oil (MEO), whereas, rats in the control group received distilled water without $MnCl_2$ (C), as described previously. At birth, the Mn pups continued to receive $MnCl_2$ during lactation until postnatal day (PND) 21 and the rats were weaned on this day. Immediately after behavioral evaluation, the rats were decapitated and blood samples were taken for glucose blood determination. The number of animals that suffered was minimised in accordance with the guidelines of the European Council Directive (86/609/EEC).

Experimental Design

Chronic Administration Prior to Assessment

Animals were exposed to manganese during gestation and lactation (Mn) and the control animals received distilled water. In order to test the ability of clove essential oil (CEO) to attenuate Mn neurotoxicity induced cognitive deficits, drug therapy was administered, beginning 24 hours after weaning. Randomly chosen animals in each group were injected (i.p.) with 0.1 ml/kg of body weight CEO (n = 7; n = 7, respectively) or distilled water (vehicle) (n = 7). Every animal was injected once a day, on days 1-18 after weaning. During PND 32-39 all subjects were given their injection 30 minutes prior to the beginning the behaviour tasks.

Elevated Plus Maze

The anxiety was evaluated in the elevated plus maze test. The elevated plus maze apparatus consisted of two open arms, 50 × 10 cm (length × width) and two closed arms, 50 × 10 × 50 cm (length × width × height) with an open roof arranged in such a way that the two arm types were opposite to each other. The maze was elevated from the floor. For the test, each animal was placed in the centre of the maze, facing one of the closed arms, the number of entries into the two arms, the time spent in the open and the closed arms were registered for five minutes (Pellow *et al*., 1985).

Forced Swimming Test

Swimming sessions were conducted by placing the rats in individual glass cylinders (39 cm height × 20 cm diameter) containing water at 22 °C and 30 cm deep, so that rats could not support themselves by touching the bottom with their paws or tail. Two swimming sessions were conducted: An initial 15 minute pre-test followed 24 hours later by a six minute test. Following each swimming session, the rats were removed from the cylinders, dried with paper towels and placed into heated cages for 30 minutes, and then returned to their home cages. Test sessions were run between 12:00 and 15:00 hours, and videotaped, for later scoring. We recorded the immobility and floating time (Porsolt *et al*., 1977).

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 center 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, Center 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 fecal boli produced) (Dauge *et al*., 1989).

Biochemical Assay

The dosage of glucose is made on the serum after separation of the total blood. The blood glucose is estimated by using the organic kit (kit Bio systems).

Statistical Analysis

Results were expressed as mean ± 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 was considered significant at $p \leq 0.05$.

Results

Administration of $MnCl_2$ showed a significant decrease ($p<0.001$) in the body weight of exposed rats compared to the Mn-CEO and control groups, respectively. The brain weight also decreased significantly ($p<0.05$) in the Mn-treated rats compared to the Mn-MEO and control groups, respectively (Tab. 1).

Furthermore, the test of plus maze revealed that Mn administration reduced significantly the time in the open arms ($p<0.05$) and the number of entries into the open arms ($p<0.05$) (Fig. 1).

In addition, we registered a significant increase of immobility time in Mn-treated animals, in the forced swimming test (FST), compared to the controls ($p < 0.001$) (Fig. 2). Likewise, we noted a significant reduction ($p<0.001$) of immobility time in the Mn-CEO group compared to the Mn group (Fig. 2).

The results showed that females exposed to 4.79 mg of $MnCl_2$ during pregnancy and lactation allowed to rec ord a significant increase ($p<0.05$) of the glycaemia rate, with a mean of 1.58 ± 0.07 compared to control rats, 0.96 ± 0.01 (Fig. 3). On the other hand, no significant difference was observed in terms of blood glucose concentration in Mn-poisoned rats that were treated with CEO, compared to the control rats ($p>0.05$) (Fig. 3).

The Student's t test, in an open-field test, demonstrated that exposure to Mn during pregnancy and lactation inc reased the latency time ($p<0.01$), On the other hand, there was significant decrease in line crossing ($p<0.05$), center square entries ($p<0.05$) and rearing ($p<0.05$), but there was no significant difference in grooming ($p>0.05$) and defecation ($p>0.05$) compared to the control group (fig.4). However, after the CEO treatment the results relative to this test indicated a significant reduction of all parameters (measuring latency time ($p<0.001$), line crossing ($p<0.05$), rearing ($p<0.05$) excepting the center square entries ($p>0.05$), grooming ($p>0.05$) and defecation ($p>0.05$) compared to the Mn-vehicle group (Fig. 3).

Tab. 1. Body weight (g) and brain weight (g) of control (c) and Manganese - exposed rats and Mn - treated rats by clove essential oil (Mn - CEO) during gestation and lactation

Groups (g)	Control	Mn	Mn-CEO
Body weight	111,98±3,66	94,18±8,03***	103,42±2,36***
Brain weight	1,73	1,61*	1,78***

Data are mean±S.E.M. *** p <0.001, * p<0.05, (Mn vs. Control) p<0.001, (Mn vs.Mn-CEO) p<0.001, (Mn vs. Control) p<0.05, (Mn vs. Mn-CEO) p<0.05

Discussion

This study was carried out to provide, on the one hand, a comprehensive assessment of the effects of Mn exposure during pregnancy and lactation on the behaviour of weaned pups that were submited to a range of tasks. On the other hand, to attenuate the Mn neurotoxicity - this is translated into anxious and depressive states - by using clove essential oil treatment on rats. Several studies have revealed that Mn is an essential element due to its participation in numerous enzymatic reactions (Aschner *et al*., 2006). Nevertheless exposures to exceedingly high levels of this metal have proven to be neurotoxic. Prenatal Mn exposure is extremely dangerous and its can result in behavioral and neurological changes in the developing central nervous system.

Clove (*Syzygium aromaticum*) oil is mainly constituted of eugenol, beta-caryophyllene, eugenol acetate, as well as alpha-humulen (Ozturk *et al*., 2006). All these active agents may be responsible for the effects observed in this study.

The present findings show that Mn exposure during developmental period causes a loss of both body and brain weight. This shows that Mn induces reduction food intake, which is due to the action of Mn on certain neuro-centers responsible for the control of hunger in the brain. Even the hyperglycemia reduces the hunger sensation. It has been indicated that the endocrine and biochemical mechanisms underlying the growth suppression, produced mainly by gestational and lactational Mn exposure, are related to the decreases in growth hormones and associated factors (Bagga et Patel, 2012 ; Kern, 2009 ; Marreilha *et al*., 2012 ; Molina *et al*., 2011 ; Oszlánczi *et al*., 2010).

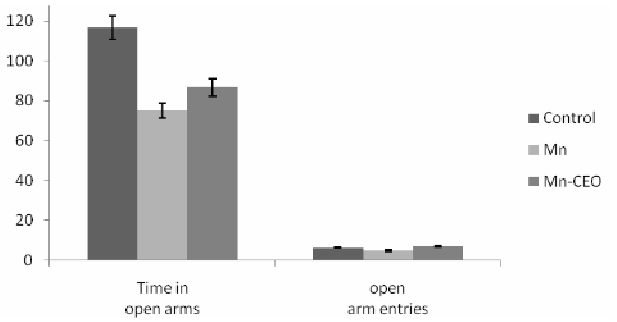

Fig.1. Effect of Mn exposure and clove essential oil during pregnancy and lactation period on anxiety behaviour (plus maze test). Data are mean ± S.E.M. * p<0.05; (Mn vs. Control) p<0.05; (Mn vs Mn-CEO) p<0.05

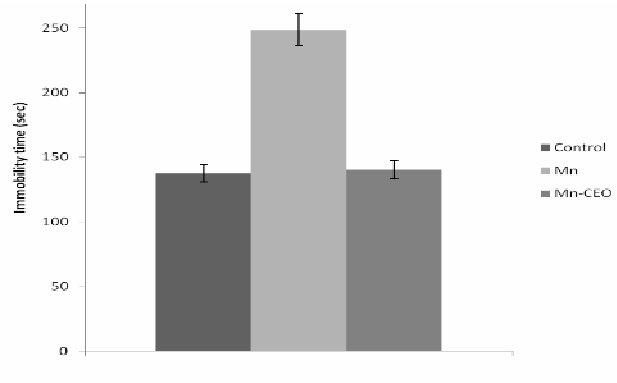

Fig.2. Effect of Mn exposure and clove essential oil during pregnancy and lactation period on depression. Data are mean ± S.E.M. *** p<0.001. (Mn vs Control) p<0.001; (Mn vs Mn-CEO) p<0.001

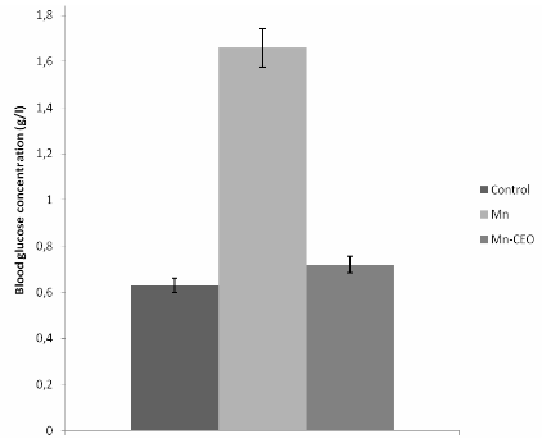

Fig. 3. Blood glucose level (g/l) of control (C) and manganese exposed during gestation lactation (Mn) and treated rats by clove essential oil (Mn-CEO).Data are mean ± S.E.M. * $p<0.05$. (Mn vs. Control) $p<0.05$

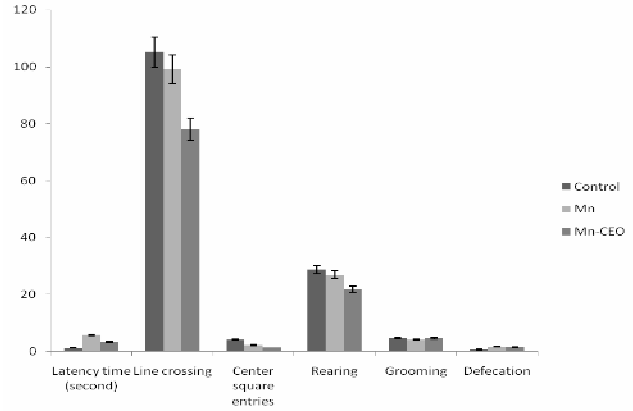

Fig. 4.Effect of the exposure to Mn and clove essential oil treatment during pregnancy and lactation on Locomotor activity. Data are mean±S.E.M. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (Mn vs. Control) $p<0.01$, $p<0.05$ (Mn vs. Mn-CEO) $p<0.001$, Mn-CEO – Mn-treated rats by clove essential oil

Anorexia caused by this metal could be justified by the disruptive effects of Mn homeostasis in thyroid hormones, specifically the reduction of growth hormone and rise in rate of thyroid hormone (T_3, T_4) and TSH may be influenced by the tubero in fundibular pathway which is one of the four major dopamine pathways in the brain. (Molina *et al*., 2011; Kern, 2009).

In addition, some works suggest that several neurotransmitters are involved in the regulation of food intake, such as serotonin, dopamine, norepinephrine and glutamate could play the primary role in the control of satiety (Diané *et al*., 2005; Harris *et al*., 1998; Torres and Nowson, 2007). In the same series of experiments, we observed a significant reduction in brain weight in intoxicated rats ($MnCl_2$) compared to controls. These results could be due to delayed development of the internal organs, brain included (Bisson, 2012). Similar observations have been reported by different authors (Zhang *et al*., 2003). Even the hyperglycemia reduces the hunger sensation. It has been indicated that the endocrine and biochemical mechanisms underlying the growth suppression, produced mainly by gestational and lactational manganese exposure, are related to the decreases in growth hormones and associated factors. Neonatal and growing brains are more susceptible to Mn toxicity. Absorption of Mn from the gut is generally high in the first week of life (Cawte, 1985). Moreover, excretion of Mn is minimal during the first 18 days of life (Cawte, 1985). These factors contribute to the considerably greater risk of neurotoxicity for neonates when exposed to excess Mn.

The administration of the essential oil of clove in rats previously exposed to ($MnCl_2$) allowed to observe a normal recovery in weight and brain weight, which could highlight the beneficial effect of the plant. It is probably due to the effect of eugenol on food intake that stimulates the brain development.

Furthermore, on the test of elevated plus-maze, which is a test to assess the relative degree of anxiety, we noted that the offspring exposed to $MnCl_2$ during pregnancy and lactation had a preference for confined and dark spaces and were fleeing open and bright spaces. This was reflecting a significantly high level of anxiety compared to control animals.

Firstly, some physiological and psychological responses to stress are mediated by the hypothalamic-pituitary-adrenal (HPA) axis and brain monoaminergic systems. This anxiety behaviour could be explained on the one hand, by iron deficiency that is induced by chronic exposure to Mn (Molina *et al*., 2011) and, on the other hand, by, reduced the amount of iron in the brain during this period of development which causes an altered a monomaninergic fonction particulary dopaminergic (Beard *et al*., 2002). In addition, a decrease in the expression of GABA due to exposure to Mn and an increase in extracellular GABA can cause marked changes in behaviour related to anxiety (Anderson *et al*., 2008).

Some study that was designed to evaluate the anti-stress effect of eugenol, shows that Eugenol seven-day pre-treatment in anxious rats decreased their stress indicating a preferential effect on the HPA axis specially monoaminergic systems (Garabadu *et al*., 2011). Moreover, during the forced swimming test we observed that the time of immobility in the Mn exposed group was signifcantly higher than the one in the control group. This could be due to the fact that Mn acts like a depressive element on the monoaminergic systems particulary serotoninergic system in different brain areas, mainly the striatum, hippocampus, and hypothalamo-hypophysaire axis. This result was in good agreement with those who had observed that Mn treated rats during both pregnancy and lactation had a depressive-like behaviour that was detected in the forced swimming test. Some authors explained this as being the effect of Mn on serotonin (5-HT) which is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) and was reported to be decreased in the globus pallidus at the Basal Ganglia (Kimura *et al*., 1978; Moreno *et al*., 2009). Most of antidepressant drugs in clinical use promote an increase in 5-HT availability directly affecting serotonin turnover in the brain, inhibiting serotonin reuptake and also interacting with 5-HT_1A and 5-HT_2 receptors (Elhwuegi *et al*., 2004; Rauhuz *et al*., 2008).

In the present study, we showed that CEO (0.1 ml/kg) has significantly decreased the immobility duration of Mn

exposed rats in the FST and those treated with CEO presented less important depression state than poisoned Mn animals. This was due the effects of clove oil, suggesting an antidepressant-like effect. Some authors report that anti-immobility effect of *Syzygium aromaticum,* specially bis-eugenol (10 and 50 mg/kg, i.p) was prevented by pre-treatment of mice with PCPA. They suggested that the serotonergic system was probably implicated in this effect induced by *Syzygium aromaticum* (bis-eugenol) in the FST. Also, this result indicates that the mechanism underlying the effect bis-eugenol is dependent on the increase in 5-HT levels in the synaptic cleft (Do Amaral *et al*., 2012).

For further, investigation on the effect on stress of Mn, we measured glucose levels in the rat's blood. Our results indicate hyperglycemia in Mn intoxicated rats compared to control rats. It could be argued that Mn chronic administration during the period of development causes a malfunction of energy metabolism that manifested by increased levels of glucose in the blood and is probably due to the liver and muscle glycogenolysis to cover brain energy needs. Our results show that stress effect resulting in hyperglycemia is explained by the action of stress hormones, namely the corticotropin releasing factor , corticotropin and cortisone in the hypothalamus , pituitary and adrenal gland , respectively, activating enzymes of glucose metabolism (Kasdallah *et al*., 2005). The administration of clove essential oil to Mn exposed rats resulted in a decrease of blood glucose level, it showed that this essential oil makes a hypoglycemic activity, among other things (Prasad *et al*., 2005). It represses the expression of genes of phosphor-enol pyruvate-carboxy-kinase (PEPCK) and glucose 6-phosphatase (G6Pase) which encode enzymes control the hepatic gluconeogenesis.

The open field test allowed initially observed that $MnCl_2$ rats have a stressful state into a new and special environment that results in a significant increase in the time latency compared to control rats. Some studies showed the same results (Moreno *et a*l., 2009). In addition, the Mn exposure animals showed significant decrease in crossing line and vertical activity who indicate locomotor hypoactivity, compared to control rats. These results are consistent with different authors (Marreilha *et al*., 2012; Molina *et al*., 2011; Oszlánczia *et al*., 2010).

Therefore, the $MnCl_2$ interacts with GABAergic, glutamatergic and catecholaminergic transmission systems. Indeed, this increased dopaminergic degeneration causes a dysfunction in the striatum, so it was suggested that Mn is transported into dopaminergic neurons via the dopamine transporter (DAT) (Fitsanakis *et al*., 2006). These results in a loss of striatal dopamine which leads D_1 receptors become less active and D_2 receptor become more active (Bagga and Patel, 2012). The D_4 receptors are implied in the vertical activity behaviour (Saldívar - González *et al*., 2009). The imbalance favouring the activation of the indirect pathway leads to over - activation of neurons in the internal Globus Pallidus (GPi), and to excessive inhibition of the thalamus and brainstem regions (Bagga and Patel, 2012).

On the other hand, vertical activity can also assess the emotional state of the animal. Indeed, it has been established in a study that the activity of Mn to target a certain number of cholinergic synaptic mechanisms, also manganese, affects significantly the synapses transport of astrocytic and choline - binding proteins of the astrocytic acetylcholine not only in the central nervous system in cortical regions of the brain such as the hippocampus, the frontal and parietal cortex , but also in the peripheral nervous system (nerves and neuromuscular junctions engines) (Finkelstein *et al*., 2007; Marreilha *et al*., 2012). The administration of the clove essential oil, which contains eugenol as the active ingredient, to rats previously intoxicated by $MnCl_2$ causes a significant decrease in horizontal activity and vertical activity compared to control rats. The reason behind this hypoactivity may be caused by the sedative effect that has eugenol (Avila-Peña *et al*., 2007; Sharma *et al*., 2012). In fact, this sedative effect will cause sedation and motor disorders that result in relaxation and a staggering gait which could reduce the horizontal and vertical locomotor activity.

Conclusions

Our results demonstrate that developmental Mn exposure induces significant perturbation of emotional reactivity that can be improved by treatment with the essential oil of *Syzygium aromaticum*. Further evaluation of the use of clove oil in the treatment neurological disorders is suggested.

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