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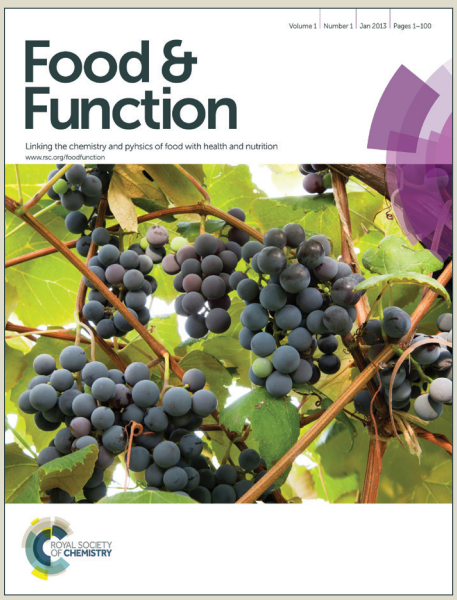

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Anxiolytic and antidepressant like effects of natural food flavour (E) - methyl isoeugenol

Abstract

(E) - methyl isoeugenol (MIE) is a natural food flavour that constitutes 93.7 % of its essential oil *Pimenta pseudocaryophyllus* leaf. The leaf extracts of this species are used as a calming agent. As a ubiquitous food additive, application of MIE in treating mood disorders seems to be globally attractive. Hence, we sought to evaluate general pharmacological activities, anticonvulsant, anxiolytic and antidepressant like effects and possible mechanisms of MIE actions. Administration of MIE was carried out prior to the exposure of male Swiss mice to general behavioural tests, barbiturate sleep, PTZ - induced convulsion, light dark box - LDB, elevated plus maze - EPM, wire hanging, open field - OF and forced swimming test - FST. Involvement of monoamine system was studied through mice pretreatment with WAY100635 (antagonist of 5-HT1A), α-methyl-p-tyrosine (AMPT; depletor of catecholamine) or p - chlorophenylalanine (PCPA; serotonin depletor storage). There was no record of neurotoxic effect or animal's death in the course of general pharmacological tests. MIE at 250 and 500 mg/kg potentiated hypnotic effect of sodium pentobarbital. However, MIE did not protect against PTZ - induced convulsion. Except for MIE at 500 mg/kg, parameters evaluated in the LDB, EPM and OF demonstrated anxiolytic like property of MIE. This effect was blocked by WAY100635 pretreatment. MIE at 500 mg/kg elicited a reduction in locomotor activity of the mice in the OF. Anti - immobility effect of MIE 250 mg/kg in the FST suggested its antidepressive like property. Unlike AMPT, pretreatment with PCPA reversed antidepressant like effect of MIE. Our findings demonstrated anxiolytic and antidepressant like properties of (E)-methyl isoeugenol and suggested the participation of serotonergic pathways.

Keywords: food flavour, (E) - methyl isoeugenol, serotonergic pathways, anxiety, depression

1. Introduction

Mood disorders belong to the most common psychiatric diseases with lifetime prevalence of up to 20% worldwide.[1] Considering the low remission rates with current treatments (about 30%) and high rate of non-response to the currently available first-line medication, the development of new therapeutic agents becomes a necessity.[2,3] The cases of non-adherence to prolong treatment of these diseases[4] could be overcome through the consumption of a functional food. This food could provide basic nourishment and health benefit.[5,6] Since time immemorial, Plants of medicine and food. Plant resources in traditional societies, especially wild greens, serve dual purposes as food and medicine.[7] Studies on the potential health benefit aspects of traditional foods show that such plants have specific pharmacological effects.[7] The gathering or cultivation, preparation, and consumption of these species are rooted in the emic perceptions of the natural environments coupled with available resources, local cuisine and medical practices, taste appreciation, and cultural heritage.[8-15] The links between food and medicine among different cultures were evident in the superb work of Etkin and Ross[16] on the medicinal plant uses among the Hausa ethnic group in Nigeria, where out of 235 noncultivated medicinal plants, 63 taxa were also used as food. Studies have demonstrated how the overlap of food and medicine are related to the ingestion of phytochemicals and explain diverse cultural food behaviours and health outcomes[9,17-20.]

Over several decades, essential oils (also known as volatile oils) from plants have been used in the form of aromatherapy to balance the mind, body and spirit as well as to prevent or cure diseases.[21] Popular use of aromatic plants for healing cut across many cultures, including ancient China, India, and Egypt.Essential oils and their isolated

compounds have been reported to possess psychotropic effects.[22] The essential oil mixture of the Chinese herbal prescription SuHeXiang Wan (SHXW) and the *Aconus gramineus* rhizome protect against epilepsy.[23,24] Barocelli and collaborators[25] demonstrated an analgesic activity of the essential oil of Lavender. Reinaldo has documented anticonvulsant activity of essential Oils and their Constituents in his work.[30] This documentation showed that common essential oil constituents such as eugenol, methyleugenol, isoeugenol[26] possess anticonvulsant property in experimental models. α-Asarone, a phenylpropanoid, also presented effective anticonvulsant activity.[27] Sell and Carlini demonstrated anesthetic action of methyleugenol and other eugenol derivatives found in the volatile oil fraction of *Myristica pagans* in mice.[28]

Paula and collaborators reported the presence of a phenylpropanoid derivative (E) - methyl isoeugenol (MIE) and its predominance (93.9%) in the essential oils of *Pimenta pseudocaryophyllus*.[29] The characteristic fragrances of this species have been attributed to the presence of MIE.[31] Previous ethnopharmacological and neuropharmacological studies have reported nerve tonic and calming properties as well as *anxiolytic* and antidepressant like activities of an organic extract and essential oils of *P. pseudocaryophyllus*.[32-35] Hence, in the present study we sought to evaluate the effect of MIE on the CNS (depressive or stimulatory) and investigate anticonvulsive, antianxiety and antidepressive like properties of MIE. The neural mechanisms of MIE were studied by using appropriate pharmacological tools.

2. Material and Methods

2.1 Drugs and Treatment

(E) - methyl isoeugenol (MIE; Sigma-Aldrich, St. Louis, MO, USA), diazepam (DZP; Cristália, Itapira, SP, Brazil), buspirone (BUS; Cristália, Itapira, SP, Brazil), pentylenetetrazole (PTZ; Sigma-Aldrich, St. Louis, MO, USA), imipramine (IMI;

Cristália, Itapira, SP, Brazil), p-chlorophenylalanine (PCPA; Sigma-Aldrich, St. Louis, MO, USA), α-methyl-p-tyrosine (AMPT; Sigma-Aldrich, St. Louis, MO, USA), Polyoxyethylenesorbitan monooleate (Tween 80; Sigma-Aldrich, St. Louis, MO, USA), N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclohexanecarbox-amide (WAY100635 or WAY; Sigma-Aldrich, St. Louis, MO, USA), were used in the present study. Drugs were prepared freshly and dissolved in a vehicle [a mixture of 0.9% NaCl and 2% (v/v) Tween-80 (polyoxyethylene sorbitan monooleate)]. Mice received 0.1 mL per 10 g b.wt. (10 mL/kg) orally. All control animals received vehicle on the same regimen as the treated groups.

2.2 Animals

Experimental animals were male Swiss mice (27 - 35 g) provided by central animal house, Federal University of Goiás. Animals were kept for acclimatization under 23 ± 2°C (12 hr light-dark cycles) with access to standard diet and water *ad libitum*. Experiments were carefully conducted by a trained researcher to minimize animal´s pain or distress in compliance with the experimental protocol (number 104/08) as approved by the Ethical Committee of the Federal University of Goiás and in agreement with the relevant national and international laws.[36]

2.3 Pharmacological approaches

2.3.0 General pharmacological test

This test was conducted by using a modified method that was adopted by Malone.[37] This preliminary test permits us to observe general behavioural change, estimate effective doses in our subsequent tests and report any sign of MIE - induced toxicity. Animals were treated through subcutaneous - s.c, intraperitoneal - i.p, or oral - p.o route with MIE (4, 20, 100 or 500 mg/kg) or vehicle and observed periodically for 7 days.

2.3.1 Sodium pentobarbital sleep induction

Mice (n = 10) were treated orally with vehicle 10 mL/kg, MIE (125, 250 or 500 mg/kg) or diazepam (1 mg/kg) 1 hour prior to the intraperitoneal administration of sodium pentobarbital (50 mg/kg). Sleep latency and duration (time to the loss of righting reflex and voluntary recovery of the righting reflex, respectively) were recorded as parameters to assess the depression or stimulation of CNS**.**

2.3.2 Pentylenetetrazol-induced seizure test

The anticonvulsant activity of MIE was evaluated by using the model of pentylenetetrazol-induced seizure. Mice were randomly divided into five groups (n = 10) and subjected to oral administration of vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 3 mg/kg). After 1 hr of drug administrations, pentylenetetrazol (PTZ 70 mg/kg i.p.) was administered to each animal. Behavioural changes in the animals were videotaped for 30 minutes and analyzed later. Parameters like latency or threshold to the first myoclonic, duration of the seizure were recorded. The survival (%) is calculated by using the formula; [(N - nd)/N] X 100 where N indicate total number of animal; nd, the number of death recorded. The severity of the seizure was taking as a measure of collective changes in mice behaviour (myoclonic jerks, vocalization, straub, akinesia, tremor, leap, paralysis, clonic seizure, rigidity and tonic extension of the hind limbs with death). Each of this behavioural parameter was scored by a trained researcher.

2.3.3 Light dark box test (LDB)

Mice were treated orally with vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 1 mg/kg). The animals were placed at the centre of the light area facing the opening of the dark area after 1 hr of oral treatment. The number of transitions between the two compartments and the time spent in the light area were recorded for 5 min.[38]

2.3.4 Elevated plus maze test (EPM)

Groups of mice (n=10) were treated orally with vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 1 mg/kg). The animals were later placed individually at the centre of the plus maze (after 1hr of oral administration) and observed for 5 min.[39] The time spent and the numbers of entries into the open arms were recorded for statistical analysis.

2.3.5 Wire Hanging Test

The wire hanging test is an in vivo preclinical model to evaluate pharmacological effect of drugs on motor function (motor impairment or coordination) of experimental animal. Mice were randomly divided into five groups (n = 10) and subjected to the oral administration of vehicle (10 mL/kg), MIE (125, 250 or 500 mg/kg) or diazepam (DZP 3 or 5 mg/kg). The test begins with the animal hanging from an elevated wire by their forepaws at a height of ~20 cm above the floor to prevent the animal from climbing down. The animal is placed at the centre of the wire; the time that elapsed until the animal fell was recorded three times and the cutoff time was set at 60 s. The latency to the falls was recorded and analyzed.

2.3.6 Open field exploratory activity

After oral administration of MIE (125, 250 or 500 mg/kg), diazepam (DZP 1 mg/kg) or vehicle, mice were exposed to a circular open field (a 50 cm high wooden wall with the division of the base area of 62.80 cm^2 into 8 equal sectors). The apparatus was clean up with 10 % alcohol at the end of each experiment. Parameters like total crossing, immobility time, number of grooming, rearing activity, crossing at the centre and time spent at the centre were scored in the course of 5 min and later analyzed statistically.

2.3.7 Forced Swimming Test

The detail of the FST in the present study has been described in our previous study.[35] All animals were subjected to swimming for 6 min, and the duration of immobility was recorded during the final 4-min interval of the test. The immobility period was considered to be the time spent by the mouse floating in the water and making only those movements necessary to keep its head afloat. The test sessions were recorded by a video camera while the parameter (immobility time) was later scored and analyzed.

2.3.8 Mechanism of anxiolytic like effect of MIE

After 30 min of N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclohexanecarboxamide - WAY100635 0.3 mL/kg, i.p or NaCl 0.9% - SAL, i.p pretreatments, mice were treated orally with vehicle 10 mL/kg or MIE 250 mg/kg prior to their exposure to EPM.

2.3.9 Mechanism of antidepressive like effect of MIE

In experiment 1, mice were pretreated intraperitoneally with NaCl 0.9% - SAL or AMPT 100 mg/kg and treated orally with vehicle or MIE (250 mg/kg) after 4 hours interval. In experiment 2, mice were pretreated intraperitoneally with NaCl 0.9% - SAL or PCPA 100 mg/kg for four consecutive days prior to vehicle or MIE treatments. Following 1 hour of oral treatment in both experiments 1& 2, animal were subjected to forced-swimming test.

2.4 Statistics analysis

Parametric data are expressed as means ± S.E.M following appropriate statistical analysis (Unpaired Student's *t*-test, one way ANOVA followed by the Dunnett´s test as post hoc test or two way ANOVA followed by the Bonferroni as post hoc test. Analysis of convulsions severity scores was realized by using Kruskal-Wallis test followed by Dunn's

multiple comparison tests. Quantitative data are expressed as the median and interquartile range (Q1–Q3). Significance difference between or among groups were set at $p < 0.05$.[40]

3. Results

3.1.0 General pharmacological test of MIE effect on mice

In the general pharmacological test, the effects elicited (abdominal contortion, environmental alienation, ataxia, sedation, analgesia, loss of paw grip, an increase and a reduction in exploratory activity by MIE 100 or 500 mg/kg were time and route of administration (s.c, i.p or p.o, table 1) dependent. At MIE 500 mg/kg, we observed sedation, analgesia and loss of paw grip via s.c. In addition, at this dose, sedation was observed via i.p or p.o route (table1). However, all these behavioural manifestations disappeared after 4 hours. These pharmacological effects of MIE did not lead to animal death in the course of 7 day - observation irrespective of the administration route and dose (table1).

3.1.1 Effect of MIE on sleep induced by sodium pentobarbital

The administration of diazepam 1 mg/kg or MIE 500 mg/kg elicited a decrease in the sleep latency ($F (4, 42) = 7.5$, $p < 0.001$, one way ANOVA, fig. 1A). Also fig. 1B demonstrated a dose dependent increase in sleep duration after oral treatment of MIE [$F (4, 42) = 9.6$, $p < 0.001$, one way ANOVA]. The Dunnett´s post-hoc test revealed a significant increase in sleep duration by MIE 250 mg/kg ($p < 0.05$) and MIE 500 mg/kg ($p < 0.001$).

3.1.2 Pentylenetetrazol-induced seizure test

One way analysis of variance showed significant increase in the latency to the myoclonic convulsion [$F (4, 45) = 11.04$, $p < 0.001$, fig. 2A]. Post-hoc test (Dunnett´s test) did not show significant alteration in the latency to the myoclonic convulsion by MIE treatment 125, 250 or 500 mg/kg ($p > 0.05$), unlike diazepam - DZP 3 mg/kg ($p < 0.001$) as

compared to the vehicle treated group (fig 2A). One way analysis of variance showed a change in seizure duration [$F(4, 45) = 13.93$, $p < 0.001$ fig. 2B]. Except for DZP 3 mg/kg ($p < 0.001$), seizure duration was not altered significantly by MIE treatments ($p > 0.05$, Dunnett´s post hoc test, fig. 2B). The severity of convulsion [fig. 2C, represented by median (25^{th} percentile – 75^{th} percentile)] induced by PTZ was not influenced significantly by MIE administration [MIE at 125 mg/kg, 15.5 (11-17); 250 mg/kg, 18 (14 – 25.5); 500 mg/kg, 20 (15.5 – 23.0); however, DZP 3 mg/kg, 6.5 (3.2 – 9.7) shows a significant decrease in severity as compared to vehicle treated group 15.5 (10.7 – 21.2). Also, % of animal protected by the administration of MIE (125, 250 or 500 mg/kg) dwindled (60, 40 and 30 %, respectively) or DZP 3 mg/kg – 100 % as compared to the vehicle treated group 60 % (fig. 2D). The scoring of seizure severity and protection against its occurrence is displayed on table 2.

3.1.3 MIE effects on mice behaviour in the light dark box - LDB

The treatment with MIE (in different doses) increased the number of transition with $F(4, 35) = 6.67$, $p < 0.001$, fig. 3A and time spent in the light area of the light-dark box with $F(4, 35) = 6.19$, $p < 0.001$, fig. 3B (one way ANOVA). The Dunnett post-hoc test showed a significant increase in transition by MIE at 125 mg/kg ($p < 0.05$) and 250 mg/kg ($p < 0.01$). The reference drug diazepam 1 mg/kg increased ($p < 0.01$) both of these parameters (fig. 3A & B).

3.1.4 Behavioural alterations elicited by MIE in the elevated plus maze - EPM

In the elevated plus maze, MIE administration at the dose of 500 mg/kg reduced total arm entries ($p < 0.05$) with $F(4, 45)$ value of 3.86 (one way ANOVA fig. 4A). The number of open arms entries was altered significantly [$F(4, 45) = 3.48$, $p < 0.05$, one way ANOVA, fig. 4B] by MIE 250 mg/kg and diazepam 1 mg/kg treatment ($p < 0.05$); Also, the time spent on the open arms was increased [$F(4, 45) = 4.99$, $p < 0.001$, one way

ANOVA, figure 5 C] by MIE 125 mg/kg ($p < 0.05$), 250 mg/kg ($p < 0.01$) and diazepam 1 mg/kg ($p < 0.001$).

3.1.5 Effect of MIE on mice performance in the wire hanging test

MIE administration did not elicit significant changes in the values of latency of fall as represented by median (25^{th} percentile – 75^{th} percentile) on figure 5 [MIE 125 mg/kg, 25.0 (11.5 – 35.5); 250 mg/kg, 18 (10 – 54.7); 500 mg/kg, 7.5 (6.7 – 17.0)] or DZP 3 mg/kg 18.0 (14 – 53.4). However, DZP 5 mg/kg [8.0 (6.7 – 11.2)] reduced this parameter significantly as compared to the vehicle treated group 37.5 (9.0 – 52.5).

3.1.6 Effect of MIE on mice behaviour in the open field

The parameters evaluated in the open field were altered significantly by MIE or diazepam treatments; total crossing in the open field [$F (4, 45) = 8.07$, $p < 0.001$, fig 6A], freezing time [$F (4, 45) = 5.14$, $p < 0.01$, fig. 6 B], grooming activity [$F (4, 45) = 3.17$, $p < 0.05$, fig. 6 C], number of rearing [$F (4, 45) = 4.37$, $p < 0.05$, fig. 6 D], time spent at the centre of open field [$F (4, 45) = 4.18$, $p < 0.01$, fig. 6 E], and crossing at the centre of open field [$F (4, 45) = 4.81$, $p < 0.01$, fig. 6F] by using one way ANOVA. MIE 500 mg/kg reduced total crossing ($p < 0.05$) and number of rearing ($p < 0.01$) while the freezing time was increased ($p < 0.01$); Both MIE 250 mg/kg and diazepam 1 mg/kg reduced the number of grooming ($p < 0.05$ and $p < 0.01$, respectively). MIE 125, 250 mg/kg and diazepam 1 mg/kg increased the number of crossing at the centre of the open field ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively). The time spent at the centre of the open field was increased by MIE 250 mg/kg and diazepam 1 mg/kg ($p < 0.05$ and $p < 0.01$, respectively).

3.1.7 MIE effect on mice performance in the Forced Swimming Test

MIE or IMI administration elicited significant alteration in the immobility time in the FST [F (4, 45) = 5.27, $p < 0.01$, fig. 7]. Dunnett post hoc test showed significant reduction in immobility time by MIE 250 mg/kg ($p < 0.05$) and IMI 30 mg/kg ($p < 0.01$).

3.1.8 Mechanism of anxiolytic like property

The effect of pretreatment (SAL and WAY100635 - independent variables) and treatment (Vehicle, MIE 250 mg/kg and BUS 10 mg/kg – independent variable) on time spent in the open arms (dependent variables, fig. 8A) and the percentage of open arms entries (dependent variables, fig 8B) of the EPM were demonstrated. The data obtained on time spent in the open arms of the EPM revealed interaction between the independent variables [F (2, 54) = 6.39, $p < 0.01$, two-way ANOVA]. In fig 8A, Bonferroni post hoc test showed an increase in the time spent in the light compartment by MIE [i.e SAL + MIE vs SAL + Vehicle, $p < 0.05$] and buspirone – BUS treatments [i.e SAL + BUS vs SAL + Vehicle, $p < 0.05$]. However, the effect of both MIE and BUS on this parameter was blocked completely by WAY100635 pretreatment [i.e SAL + MIE vs WAY100635 + MIE and SAL + BUS vs WAY100635 + BUS, $p < 0.05$]. The data obtained on the percentage of open arms entries revealed interaction between the independent variables [F (2, 54) = 25.44, $p < 0.01$, two-way ANOVA]. In fig 8B, Bonferroni post hoc test indicated an increase in the percentage of open arms entries in the groups SAL + MIE ($p < 0.05$) and SAL + BUS ($p < 0.05$) as compared to the group that received SAL + Vehicle (control group). The effect of BUS and MIE on the percentage of open arms entries were attenuated by WAY100635 pretreatment [i.e SAL + BUS vs WAY + BUS and SAL + MIE vs WAY + MIE, $p < 0.05$, respectively].

3.1.9 Mechanism of antidepressive like property

Figure 9A showed the effect of pretreatment (SAL or AMPT - independent variable) and treatment (Vehicle or MIE 250 mg/kg – independent variable) on the immobility time (dependent variables) in the forced swimming test - FST. The data obtained did not demonstrate interaction between the independent variables [$F(1, 36) = 6.02$, $p > 0.05$, using a two-way ANOVA] on the immobility time. Bonferroni post hoc test showed a decrease in immobility time in the groups SAL + MIE as compared to control group (i.e SAL + Vehicle, $p < 0.05$). AMPT did not reverse the anti-immobility effect of MIE (i.e SAL + MIE versus AMPT + MIE showed a p value > 0.05). Figure 9 B showed the effect of pretreatment (SAL or PCPA) and treatment (vehicle or MIE 250 mg/kg) on the immobility time in FST. The data obtained on immobility time did not show interaction between the independent variables [$F(1, 36) = 2.14$, two-way ANOVA, $p > 0.05$]. Bonferroni post hoc test showed a decrease in immobility time in the group SAL + MIE ($p < 0.05$) but not in SAL + PCPA ($p > 0.05$) as compared to the control group (i.e SAL + Vehicle). PCPA pretreatment blocked the anti-immobility effect of MIE (i.e SAL + MIE versus PCPA + MIE, $p < 0.05$).

4. Discussion

The growing interest in natural product could be traced to their perceived safety.[41] Up to date, aromatic plants are largely explored as functional ingredients in the pharmaceutical, cosmetic, food and feed industries.[42] Despite the presence of (E)-methyl isoeugenol's (MIE) in the essential oil and crude extract that has been acclaimed to possess calming effect and anxiolytic like property,[32-35] there has been no pharmacological data on the biological activities of MIE to the best of our knowledge. Being a naturally occurring food flavour, therapeutic application of MIE for the treatment of neural disorders seems to be more acceptable to the use of available pharmacotherapies. Since food safety issues is crucial to human health,[43] the present study also revealed behavioural alterations that are elicited by MIE at different doses.

The report on the behavioural alteration in the general pharmacological tests seems to be dependent on dose and the route of administration. The oral treatment of MIE 100 mg/kg increased exploratory activity while at 500 mg/kg, MIE elicited sedative effect and a decrease in exploratory activity. The effects of intraperitoneal and subcutaneous administration of MIE were characterized by sedation. The oral route of administration and the dose ranges of MIE in our subsequent experiments were chosen based on the popular application of the leaf extract and previous work on the essential oil of *Pimenta pseudocaryophyllus*.[31]

The sodium pentobarbital sleep induction test showed an increase (in a dose dependent manner) in the sleep duration by oral administration of MIE. Potentiation of the hypnotic effect of barbiturate sleep is an indication of central nervous system depressive activity.[34] This data reinforces the assumption of MIE involvement in the activity of the organic leaf extract.[35] Being a CNS depressive compound like diazepam, we hypothesized anti-seizure property of MIE. Our hypothesis is further supported by the antiseizure property of aromatic compounds like methyleugenol, eugenol and 1-nitro-2-phenylethane[30,44] that share similar chemical structure with MIE. We assumed that the presence of phenylpropanoid structure could be associated with their anti-convulsant property. Hence, we conducted pentylenetetrazole (PTZ) induced convulsion test; a predictive animal model that is widely used in the search for new antiepileptic drugs.[45,46] However, contrary to our expectation, oral administration of MIE did not protect against the PTZ induced convulsion. It is intriguing to observe that at the highest dose there was a decline in the percentage of animal protected, an increase in the severity and duration of convulsion. Since the dose of diazepam tested in this study did not induce sedative or myorelaxant effect, we hypothesized that the sedative tendency of MIE at the highest dose could be responsible for the potentiation of PTZ effects. Based on the mechanism of action of PTZ, the effect of MIE on CNS perhaps did not involve GABA A receptor.

The study of antianxiety like property of unknown compound could be achieved through environmental manipulation that elicits aversive behaviour and the resultant conflict with the innate desire of the animal to explore. The light dark box is an established animal model for the detection of compound with potential anxiolytic like property.[38] Parameters like number of transition between the light and dark compartments and time spent in the light area of the box are used to make antianxiety inference.[47] In this study, except for the highest dose, MIE induced an increase in the number of transition and time

spent in the light area of LDB. The elevated plus maze (EPM) was further used to detect possible anxiolytic and anxiogenic like effects.[48] Mice treated with MIE (125 or 250 mg/kg) increases time spent on the open arms. MIE at 500 mg/kg reduced total arm entry in the EPM. Based on these data there are indication that MIE interferes with motor coordination of the animals at the dose of 500 mg/kg.

In the wire hanging test, the effect of oral administration of MIE on motor activity of mice was evaluated. In this test, the data obtained on the latency of falls, after MIE administration, did not demonstrate significant alteration. These results further suggest that the anxiolytic-like effect of MIE at lower doses (125 and 250 mg/kg) did not elicit myorelaxant or sedative effect. However, the effect of MIE at 500 mg/kg on motor activity seems inconclusive based on the EPM data. In order to further unravel possible sedative effect of MIE at 500 mg/kg or stimulatory at 125 and 250 mg/kg, animals were exposed to the open field test. This animal model could be used to ascertain if the increase in number of transition in the LDB and an increase in the open arm entries are mere stimulatory response. This model is of importance to our subsequent study of MIE in the forced swimming model. An agent with stimulatory effect could reduce immobility time in the forced swimming test while sedative agent or dose could interfere with the animal performance in this model. Our data showed a reduction in rearing activity, total crossing and an increase in freezing time at MIE 500 mg/kg. These results suggest sedative or myorelaxant effect at this dose. On the other hand, MIE 125 or 250 mg/kg increased crossing and time spent at the centre significantly (effects that suggest a reduction in aversiveness and anxiety in this animal model) without significant increase in total crossing (sum of the crossing at the centre and periphery of open field). The observations in the open field further confirm anxiolytic like property of MIE at lower doses (125 or

250 mg/kg) and sedative property at the highest dose of 500 mg/kg. These results seem to be consistent with the data under general pharmacological tests.

In the present study, a U-shaped dose - response and dose dependent response were reported with MIE administrations. The pattern of behavioural responses to the doses administered could be dependent on animal model or associated with physiological alterations. MIE at 500 mg/kg potentiated sleep induced and elicited a sedative like effect in the open field and EPM but could not alter behavioural response in the LDB. In contrast, at 250 mg/kg, the anxiolytic like effect of MIE on animal were consistent in the LDB, EPM and open field tests. On the basis of animal model, MIE effects were dose dependent in sodium pentobarbital sleep induction test while a U-shaped dose – response was reported in the LDB. On the whole, we hypothesized that different doses of drug (MIE) administered could produce physiological changes which can be observed through an alteration (specific or non-specific) in animal behaviour. However, the observation and quantification of behavioural responses are susceptible to the influence of external factors/ sensitive of animal models.

Since MIE administration did not protect the animals against PTZ induced convulsion, we assume non-involvement of GABA A receptor in its mechanism of actions. Hence, we decided to investigate the participation of serotonergic system by using pharmacological antagonist of 5-HT1A receptor. Evaluation of this particular receptor becomes interesting based on its involvement in the anxiolytic like property of the organic extract of *Pimenta pseudocaryophyllus*.[34] The neural mechanism of clinically prescribed anxiolytic drug like buspirone has been associated with 5-HT1A receptor.[49] The WAY100635, a selective antagonist of 5-HT1A, pretreatment blocked anxiolytic like effect of MIE in the EPM. Interestingly, 5-HT1A receptor had been implicated in the antidepressant property of azapirones.[50,51] Previous study has demonstrated

antidepressant like property of in our laboratory on the organic extract of *Pimenta pseudocaryophyllus*.[35] Hence, we proposed to study the effect of MIE administration on animal behavior in the forced swimming test (FST).

In the FST, the lowest dose of 60 mg/kg was introduced to substitute 500 mg/kg dose whose effect was characterized by sedation in the previous experiments. MIE at 250 mg/kg reduced the immobility time, thereby demonstrating antidepressive-like activity. In order to elucidate the involvement of monoamine in the effect of MIE, monoamine depletion approach were employed. The anti-immobility effect of MIE remained unaltered by α-methyl-p-tyrosine (depletor of catecholamine storage) pretreatment.[52,53] In contrast, pretreatment of mice with parachlorophenylalanine (serotonin depletor), attenuated anti-immobility effect of MIE.

Conclusion:

Our findings demonstrated anxiolytic and antidepressive like properties of (E)-methyl isoeugenol and suggested the participation of serotonergic pathways.

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Table 1. General pharmacological tests

Observation time after acute administration	Dose (mg/kg)	Administration routes/Observations		
		s.c	i.p	p.o
15min	**4, 20 or 100**	N	N	N
	500	Reduced exploration	N	N

30 min	**4 or 20**	N	N	N
	100	Ataxia, contortion	Environmental alienation	Increased exploration
	500	Sedation, analgesia, loss of paw grip,	Sedation	Reduced exploration
1hr	**4 or 20**	Effects after 30 min of administrations persists		N
	100			Increased exploration
	500			Sedation
4 hr – 7 days	Total recovery from the effects of MIE administration without sign of toxicity in the course of the 7 day observation			

N - No observable behavioural alteration as compared to vehicle treated group

Table 2. Parameters for the scoring of PTZ induced behavioural alterations

	Parameter	**Score**
1	Absence of convulsive behaviour	0
2	Myoclonic jerks	1
3	Vocalization	2
4	Straub	3
5	Akinesia	4
6	Tremor and leap	5
7	Paralysis of hindlimbs	6
8	Clonic seizures with loss of righting reflex	7
9	Rigidity and tonic extension of the hind limbs with death	8
Other parameters		
10	Latency to first myoclonic jerk	seconds
11	Duration of crisis	seconds
12	Survival or percentage of animals protected	[(N - nd)/N] X 100

N - total number of animal; nd - number of death recorded.

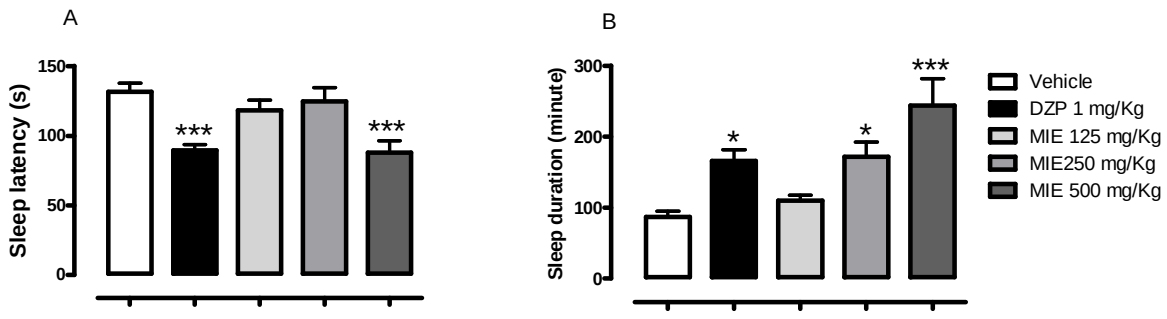


Figure 1. Effect of vehicle, diazepam – DZP 1 mg/kg or (E) - methyl isoeugenol (MIE) 125, 250 or 500 mg/kg on latency (A) and duration (B) of sodium pentobarbital (50

mg/kg) induced hypnosis. Results are expressed as mean ± SEM; n = 8-10 in each group. * and *** indicate $p < 0.05$ and $p < 0.001$ respectively as compared with vehicle treated group (One way ANOVA followed by Dunnett´s post hoc test).

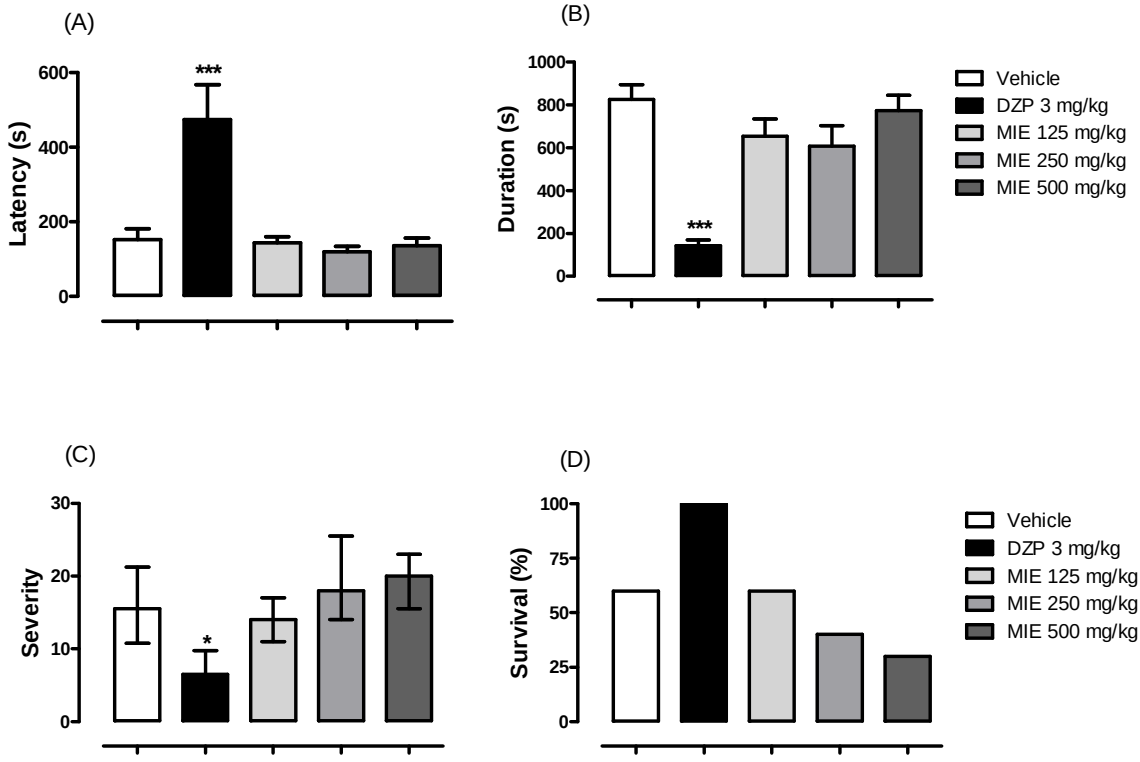


Figure 2. Data on the latency to the first myoclonic convulsion (A), and the duration of convulsion (B) were analyzed by one-way ANOVA followed by Dunnett as post hoc test. Data are represented as mean ± SEM, n=10; Non parametric data on the severity (C) were analyzed using Kruskal-Wallis test followed by Dunns as post hoc test (data are represented as median (25^{th} percentile – 75^{th} percentile), n=10. Bar graph (D) showed % of the animals that were protected against pentylenetetrazol (PTZ). * and *** indicate $p < 0.05$ and $p < 0.001$ respectively as compared with vehicle treated group

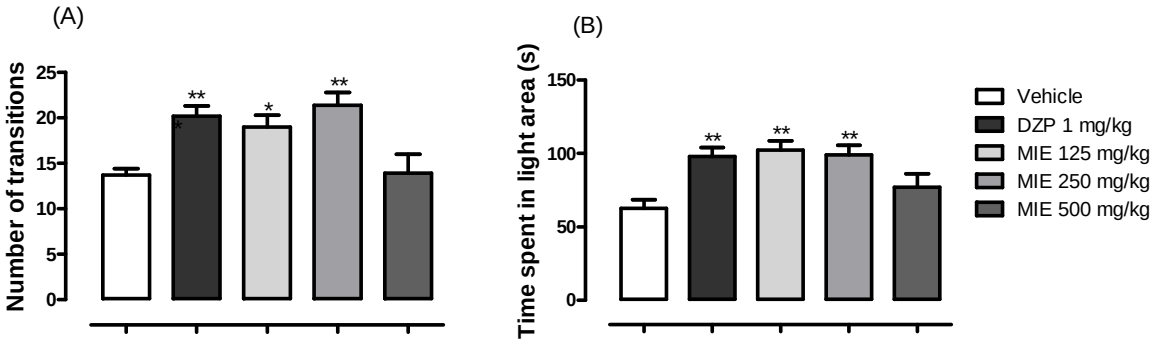


Figure 3. Effect of oral administration of vehicle, (E) - methyl isoeugenol (MIE) or diazepam (DZP) on the number of transition (A), and time spent in the light area (B) of the light dark box. Results are expressed as mean ± SEM; n = 8; *$p < 0.05$ and **$p < 0.01$versus vehicle treated group using one way ANOVA followed by Dunnett´s post hoc tests.

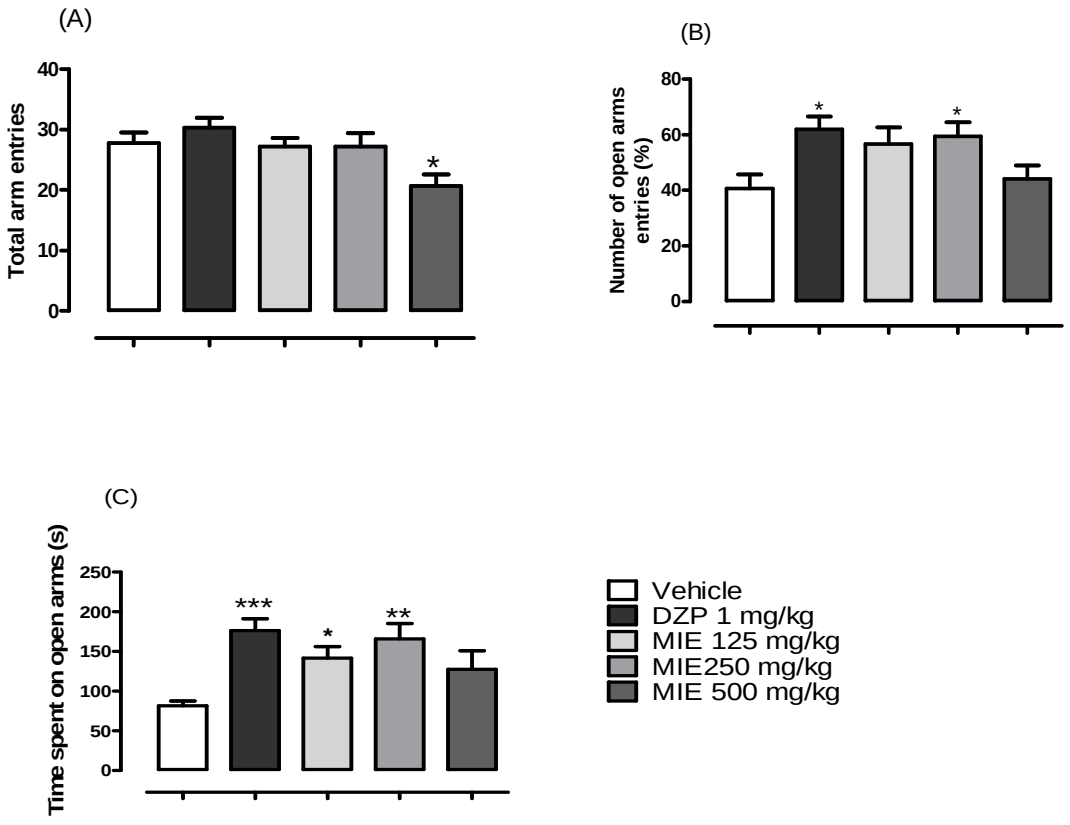


Figure 4. Effect of the oral administration of vehicle, diazepam (DZP), or (E) - methyl isoeugenol (MIE) on the mice behaviour in the elevated plus maze. Parameters like total arm entries (A), number of open arms entries (B), and time spent on the open arms (C) were evaluated. Results are expressed as mean ± SEM; n = 10; *p < 0.05, **p < 0.01 and ***p < 0.001 versus vehicle using one way ANOVA followed by Dunnett´s post hoc tests.

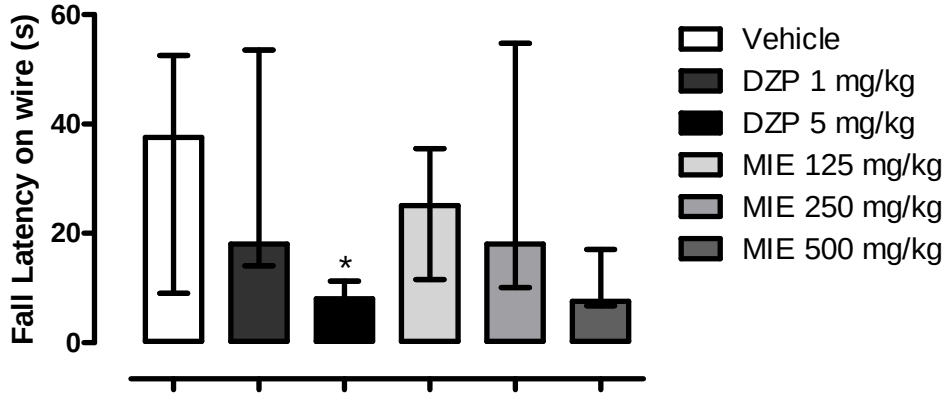
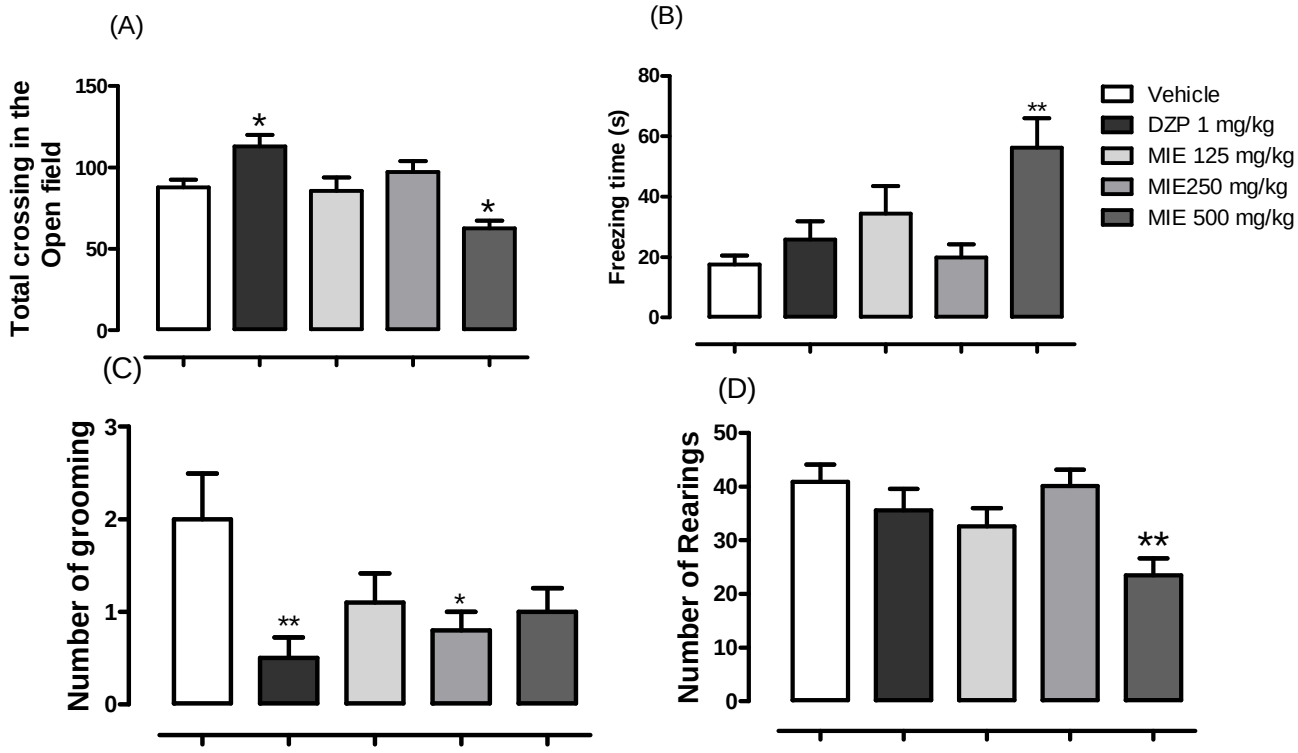


Figure 5. Effects of the oral treatment with vehicle, (E) - methyl isoeugenol (MIE) or diazepam (DZP) on motor activity of mice exposed to wire hanging test. Data are analyzed by Kruskal-Wallis test followed by Dunns as post hoc test and expressed as median (25^{th} percentile – 75^{th} percentile), n=10.



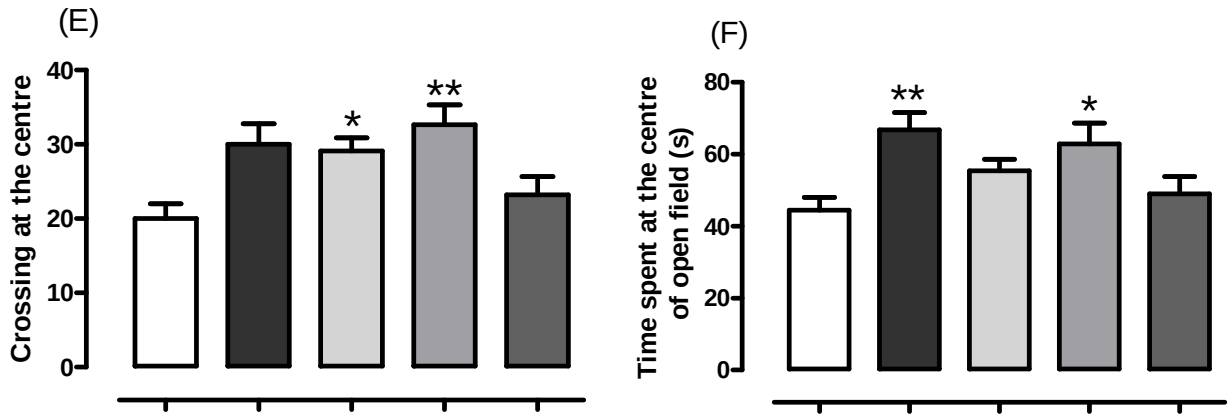


Figure 6. Effects of oral treatments of vehicle, diazepam (DZP) or (E) - methyl isoeugenol (MIE) on the total crossing (A), freezing time (B), number of grooming (C), number of rearing (D), crossing at the centre (E) and time spent at the centre (F) of the open-field. Each column represents mean ± SEM of 10 mice. *p < 0.05, **p < 0.01 as compared to the vehicle treated group (one-way ANOVA followed by Dunnett´ s post hoc test).

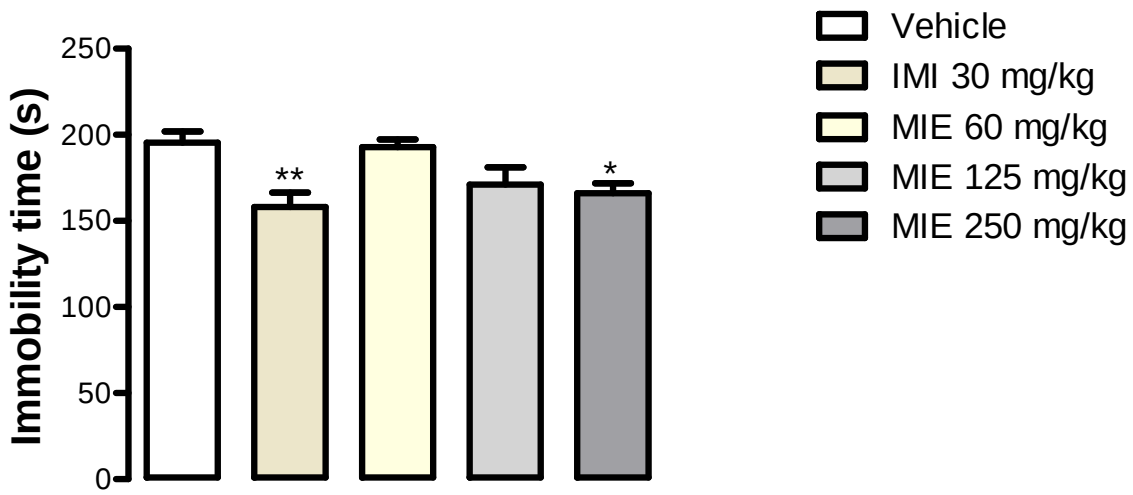


Figure 7. The effect of acute oral administration of vehicle, imipramine (IMI) or (E) - methyl isoeugenol (MIE) on the immobility time in the forced swimming test. Data are analyzed using one way ANOVA followed by Dunnett´s test as post hoc test (A). Each

column represents the mean ± SEM of 10 mice. *p < 0.05, **p < 0.01 as compared to the vehicle treated group (one-way ANOVA followed by Dunnett´ s post hoc test).

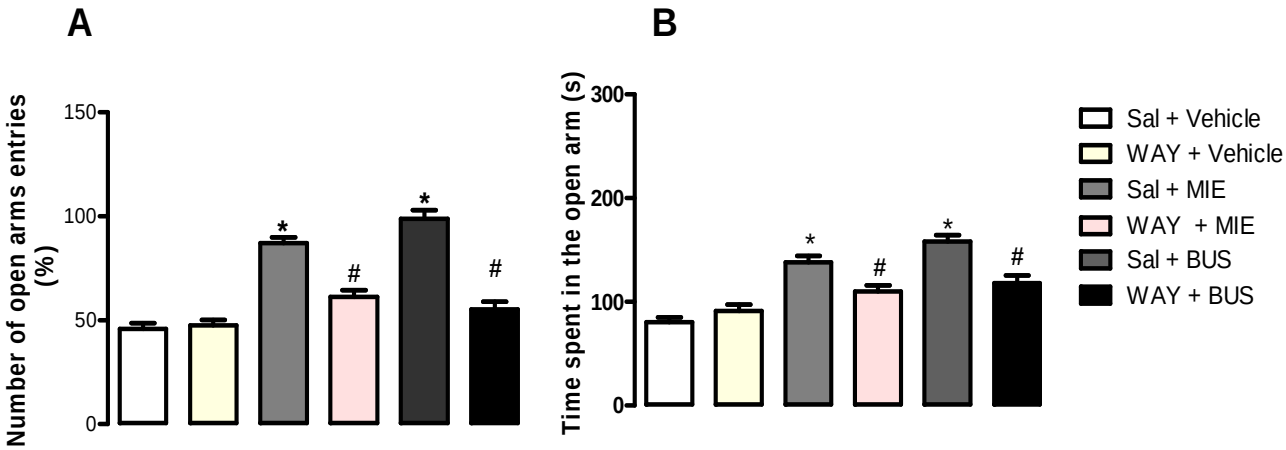


Figure 8. Effects of NaCl 0.9% (SAL) or WAY100635 0.3 mL/kg (WAY) pretreatment on the number of open arms entries (A) and time spent in the open arms (B) of EPM prior to oral treatments with vehicle, (E) - methyl isoeugenol (MIE) 250 mg/kg or buspirone (BUS) 10 mg/kg. Data were analyzed using two way ANOVA followed by Bonferroni post hoc test and expressed as mean ± SEM, n = 10. * $p < 0.05$ versus vehicle treated group while # $p < 0.05$ indicate significant reversal of MIE or BUS effect by WAY pretreatment.

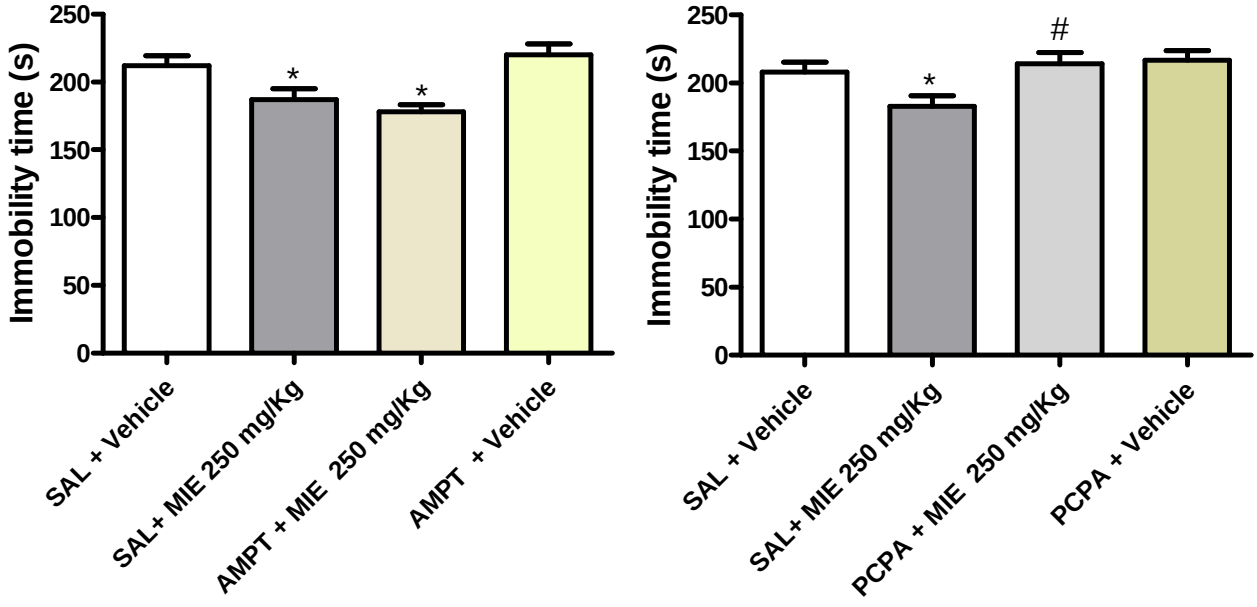


Figure 9. Effects of pretreatment with (A) NaCl 0.9% (SAL) or α-methyl-p-tyrosine 100 mg/kg (AMPT), (B) NaCl 0.9% (SAL) or p-chlorophenylalanine 100 mg/kg (PCPA) prior to oral administration of (E) - methyl isoeugenol – MIE 250 mg/kg or vehicle on the immobility time in forced swimming test. Data are presented as mean ± SEM (n= 10). * $p < 0.05$ versus vehicle treated group while # $p < 0.05$ indicate significant reversal of anti-immobility effect of MIE.