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ORIGINAL ARTICLE

Effects of the Mint Monoterpene *(R)*-(+)-Pulegone Evaluated by Functional Observational Battery: A Potential Short Method

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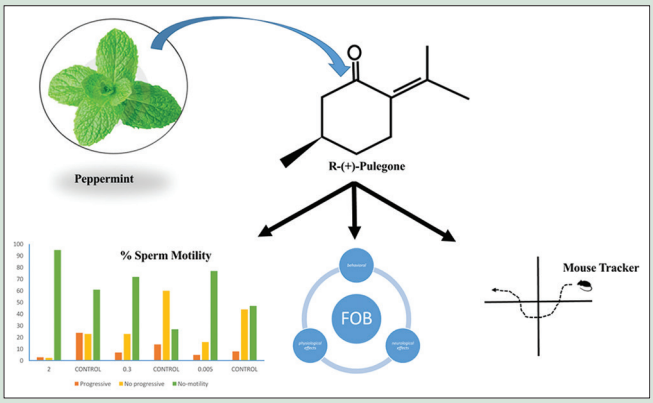
ABSTRACT

Background: Pulegone (PUL) is one of the major constituents of peppermint and pennyroyal. **Objective:** The main purpose of this study was to investigate the early effects of toxicity of PUL. **Materials and Methods:** We assessed single doses of toxicity of PUL (2, 0.3 and 0.05 g/kg body weight), administrated by gavage in C57BL/6 mice, evaluated at 1, 6, and 24 h, their clinical status and behavioral, by functional observational battery (FOB), ambulatory conditions, sperm motility, pathological signs, and organ/body weight (O/Bw). **Results:** No mortality was registered in this *in vivo* study of oral acute toxicity, in which histological changes were found in selected organs, and PUL mainly showed that the highest concentration reduced mice locomotor activity with significant differences when compared with data of the FOB. Sperm motility also diminished, and hepatic as well as renal alterations were found without modifications in clinical status and O/Bw. **Conclusions:** We concluded that PUL could be responsible for these findings and consider that FOB is a useful tool to detect early signs of modifications of physiological and biological parameters in mice.

Key words: Central nervous system, functional observational battery, pulegone, reproduction, terpenes

SUMMARY

- Essential oils are aromatic components (terpenes) obtained from different plant parts such as flower, buds, seed, leaves, and fruits, and they have been employed for a long time in different industries, mainly in perfumes (fragrances and aftershaves), in food (as flavoring and preservatives), and in pharmaceuticals (therapeutic action). They tend to have low mammalian toxicity, less environmental effects, and wide public acceptance. In the present study, the early effects of toxicity of pulegone are evaluated.



Abbreviations Used: PUL: Pulegone, FOB: Functional observational battery, CNS: Central nervous system, OECD: Organization for Economic Cooperation and Development, ANOVA: Analysis of variance, MNI: Mononuclear cell infiltrate, LD_{50}: Median lethal dose

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INTRODUCTION

Natural compounds are more environment-friendly than synthetic products. Therefore, it is necessary to investigate the activity of monoterpenes, like pulegone (PUL). The PUL is a component in a variety of mint species of the Lamiaceae family like *Mentha* spicata *Mentha pulegium*, *Mentha piperita*, *Hedeoma multiflorum*, *Minthostachys mollis*, *Satureja boliviana*, *Satureja odora,* etc.[1] It is also found in Myrtaceae and Verbenaceae families in species such as *Stenocalyx micheli* and *Calamintha nepeta*, respectively.[2]

During the past decade, several plants have received special considerations as a source of potentially useful bioactive components in food (as flavoring and preservatives), pharmaceuticals (due to their therapeutic action) and for medicinal treatments due to their antioxidant and anti-inflammatory properties, as well as in aromatherapy recipes.[3-5] It is also found in marijuana in small amounts.[6] In other species such as *H. multiflorum, M. mollis, S. odora* and *M. pulegium,* PUL is found in higher concentrations (>50%).[7]

To date, several biological properties have been attributed to PUL, including antibacterial action against *Salmonella typhimurium* and *Candida albicans*. Against *C. albicans.* PUL has been shown to be twice as effective than nystatin as antifungal.[8] Its effects as antihistaminic, antipyretic, anticonvulsant,[9] acetylcholinesterase inhibitor,[10] antinociceptive,[11] and insecticide have also been described.[12,13]

High doses of pennyroyal oil have been associated with effects on the central nervous system (CNS) such as toxicity and coma, renal effects, actions on the inhibitory system of cytochrome P-450 and lysosomal enzymes, increase of spontaneous activity and gastritis.[14-16] PUL is metabolized by hepatic microsomal monooxygenases to reactive metabolites responsible for hepatotoxicity.[17]

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The search for new methods to control food spoilage is a promising area of research. This study provided evidence that neurobehavioral tests could be used for rapid screening of different PUL concentration, administered by gavage on clinical status (morbidity or mortality) using a functional observational battery (FOB) in a mice mode. In addition, its effects on locomotor activity, sperm motility, pathological parameters, macroscopic morphology, and organ/body weight index were assessed.

MATERIALS AND METHODS

Animal preparation

Experiments were performed in adult male C57BL/6 mice (average body weight 30 ± 2.5 g, aged 8–10 weeks), randomly distributed and placed in standard polycarbonate cages (30 cm × 20 cm × 15 cm). The animals were housed at 21°C with cycles of 12 h light/dark and 55%–75% humidity as well as continuous access to standard food and water *ad libitum*.

The animals were handled gently to attain all possible abbreviation of distress. All efforts were made to avoid unnecessary suffering and the experimental procedures were carried out in strict compliance with the U. S. National Institutes of Health guidelines for the experimental use of animals. All studies were approved by a local committee for animal use at the University of Cordoba. The mice were weighed just before being placed in the partition cage and controls were weighed on the same day.

Study protocol and *in vivo* chemistry studies

Chemical characteristics of pulegone, preparation, and assayed product

Assessment of doses was performed in accordance with an adaptation of the guidelines published by the Organization for Economic Cooperation and Development (OECD) 423, 2001.[18,19] (*R*)-(+)-Pulegone (purity 99%) [Synonyms: (*R*)-2-Isopropylidene-5-methylcyclohexanone; (*R*)-*p*-Menth-4(8)-en-3-one; *p*-Menth-4(8)-en-3-one], MW 152.23, was stored refrigerated and protected from the light (Sigma-Aldrich, St. Louis MO). The single doses were as follows: 2, 0.3, and 0.05 g/kg of PUL, and were administrated by gavage. These dosages were adapted from another study conducted in mice.[18,19]

The tested substance was diluted in soybean oil and prepared daily because the animals were treated on different days but similarly as described later, protected from light, and sealed with parafilm.

All mice groups started at 12:00 h AM, and the experiment commenced with the administration of oral PUL or vehicle, and FOB evaluation was performed 1, 6, and 24 h after exposure to the samples, by gavage to achieve more stable tissue levels than could have been achieved with injections.

Clinical observations and survival

Since PUL is a highly volatile element (vapor pressure 138 mm Hg at 25°C) and to avoid the smell of PUL with the minty smell, the cages were placed in the other room during treatments. Weight changes of individuals were calculated and compared with control animals as stated in paragraph 26 of OECD guidelines 423.[19] All animals were evaluated individually at least once, at the time of PUL administration and 1, 6, and 24 h by means of clinical examination and detection of mortality/morbidity. The measurements were made starting from 1 h, due to the properties the terpenes to diffuse rapidly through the body of the animal.[20] The observations included, but were not limited to, changes in the skin, fur, eyes and mucous membranes, respiratory, circulatory, autonomic and CNS functions, somatomotor activity, and behavioral patterns. Detailed physical examination including observation of any variation in behavior, gait, neurological effects such as posture or clonic/tonic movements, stereotypes, bizarre behavior, and permanent or semi-permanent signs, was conducted before dose administration, along with the experiments, and before histological analysis and necropsy.

Macroscopic and histological analyses

Body weight, sign of abnormality, and mortality were observed after the administration in the first, 6th h and once daily for 24 h. Once the mice were sacrificed by CO_2 inhalation, the organs of the liver, kidneys, spleen, and stomach were removed and cleaned with saline, weighed and preserved in 10% formalin for histopathology analyses, fixed onto glass slides and stained with hematoxylin and eosin for histological examination.[21]

Measurement of locomotor activity

Previously adapted mice to the cage environment, locomotor activity was measured at 1, 6, and 24 h after PUL administration with a video-camera of 0.1s resolution positioned inside the standard polycarbonate cages (30 cm × 20 cm × 15 cm). After administration of PUL or vehicle, mice were immediately placed into the locomotors activity chambers and data were automatically recorded for 5 min.

Each cage was recorded from the center of the cage top, and dim red light (power indicator light) was positioned above each cage. The movements of each mouse in the cage were measured with Software Mouse Tracker. Locomotor activity was recorded before completion of the FOB.

Evaluation of pulegone by means of a functional observational battery

Observations of the FOB were carried out and documented during treatment at 1, 6, and 24 h after oral administration of PUL. The FOB was prepared based on a procedure commonly used by the Environmental Protection Agency to evaluate potential toxins. It provides an overall behavioral profile that allows the assessment of a wide range of compound effects.

We used the FOB to evaluate many factors, addressing behavioral and neurological characteristics in an *in vivo* rodent model. The scoring scale for FOB is shown in two different tables. The FOB test measurements were categorized to determine a profile of behavioral and neurological parameters.

Home-cage observations were numbered by ordinal or categorical measurements. The following parameters were observed in all animals: behavioral effects: home-cage observation (posture, convulsions/tremors, biting, and palpebral [eyelid] closure) (categorical), transfer abilities (categorical), difficulties in locomotor activity (categorical), startle reaction (ordinal) (touch response, irritability, aggression, and freezing).

Behavioral and neurological effects: posture (categorical), ear reflection (categorical), bite (ordinal), tail position (ordinal), pupillary reflex (categorical), posture, reaction rate, piloerection (ordinal), respiratory rhythm (categorical), close eyelid (categorical), lacrimation (categorical), and other stereotyped compulsive movements (any repetitive movement that does not fall under other categories of stereotyped behavior).

Efforts were made to ensure minimal variations in sound level, temperature, humidity, lighting, odors, time of the day, and environmental distractions. Mice of different groups were handled in the same way and under the same conditions. The procedure applied was a modification of previously published procedures but essentially in line with methods described by Irwin.

The person responsible for the performance of behavioral tests was qualified and well trained in observation and rating of rodent behavior and was blind to the studied groups.

Blind assessment of each animal began with the observation of undisturbed behavior in a transparent cylindrical viewing jar

(11 cm in diameter). All data were recorded on standardized data sheets and subsequently entered into a computer system for analysis.[22]

Sperm collection and analysis of motility

The epididymis was carefully separated from the testis and cauda severed. Cauda was finely minced with anatomical scissors in 1 ml of isotonic saline at 37.5°C in a center well at 37.5°C, and then it was completely squashed with tweezers for 3 min to expel the sperms. Sperm concentration and motility were assessed at 23°C ± 2°C in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) under an inverted microscope (Olympus, Japan) at ×200, as previously described in our laboratory.[23,24] The results are expressed as the percentage of motile cells (progressive plus nonprogressive spermatozoa). No fewer than 200 gametes were examined.

Statistical analysis

In the FOB, categorical variables were set as normal versus abnormal. Ordinal measures were scored using an ordinal scale with 1 = normal/no doses effect and increasing numbers until 3. None of the three doses of PUL produced any of the following responses: salivation, convulsions, writhing, circling, stereotypic behaviors, bizarre behaviors, defecation, and urination. For this reason, these measures were omitted from further analysis. A vehicle control group was tested for each compound.

Data from the control groups were combined into a single vehicle group, in which all compound doses were compared separately. The normal distribution of the data was confirmed with the Kolmogorov–Smirnov test. The statistical significance of the differences between treatment and vehicle was determined by factorial analysis of variance followed by Duncan's multiple-range test. Differences were considered statistically significant when $P < 0.05$. The categorical values in FOB results were formulated as contingency tables and judged by the Chi-squared test of homogeneity. The differences were considered statistically significant when $P < 0.05$. Calculations were performed with Info-Stat software (Córdoba, Argentina, 2018).

RESULTS

Animal survival, clinical observation, relative organ weight, macroscopic evaluation, and histopathological parameters

Neither treatment-related morbidity/mortality and lethal effects of PUL were observed under clinical examination of the mice. Behavioral abnormalities such as catalepsy and scratching were not observed in any animal during the experiment period. During observation times, at 1, 6, and 24 h after dose administration, the animals that had been administered 0.3 and 0.05 g/kg of PUL were more active and behaved normally than mice that received a higher dose; all the mice consumed standard food and water amounts (data not shown). Normal weight gain occurred in treated and control groups (data not shown). No statistical significance was found in terms of absolute (g) and relative weight (%) of almost all isolated organs when comparing treated and control mice (data not shown).

Moreover, macroscopic examination of vital organs did not reveal any abnormality. A slight alteration in the intestine was detected in subjects that received 0.05 and 0.3 g/kg PUL, showing a diffuse mononuclear cell infiltrate (MNI). In the kidneys of mice that received 2 and 0.3 g/kg, cortical congestion, showing hemorrhages and interstitial cell proliferation [Figure 1]. When analyzing the stomach, a slight MNI was found in mice that received 0.3 g/kg and gastric atrophy in those that were administered 0.05 g/kg. Finally, PUL caused diffuse congestion, vascular dilation and MNI in the liver, only in subjects that received the highest dose [Figure 2].

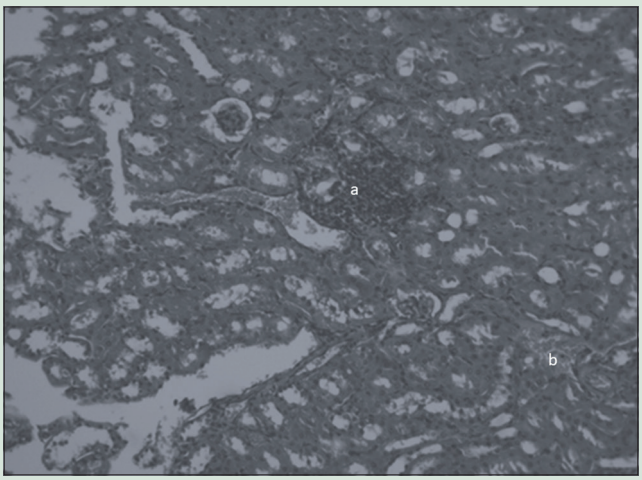

Figure 1: Kidney section of mice treated with oral pulegone (2 g/kg) showing (a) inflammatory infiltrate cells and (b) hemorrhages (H and E, ×100)

Effects of different doses of pulegone on locomotor activity

The spontaneous motor activity of the animals was assessed; ambulatory activity constitutes a type of locomotor activity in mice, as shown in Table 1.

Motor activity decreased with the highest dose (2 g/kg) of PUL. PUL had a clear influence on overall motor activity over the entire period (1, 6, and 24 h) compared to the control group, as shown in Table 1.

Effects of pulegone on behavior, as recorded in the functional observational battery

Table 2 shows specific data of the FOB that showed significant results. Information collected during mice observation after gavage was analyzed comparing the control group with treated animals always, including the dose at which the effects occurred. When appropriate (i.e. ordinal or categorical data), the direction of the effect is also indicated (more details provided in Supplementary Material).

Measures that remained unaffected by any of the compounds are not shown: Convulsions or spasms circling, bizarre behaviors, stereotyped behaviors, lacrimation, salivation, urination, excretion, and writhing. The evolution of these parameters over time was also evaluated for each group.

PUL clearly induced the most relevant effects with the highest dose; it produced a decrease of autonomic effects (breathing) CNS activity (ambulation) and sensorimotor reactivity (startle reaction) with increased not retracted ear reflection. PUL impaired the muscular tone/equilibrium domain. Effects on CNS excitability occurred at the lower PUL doses, with increased tail elevation. PUL produced significant effects over CNS activity on other parameters like home-cage observation and transfer behavior.

Effects of different doses of pulegone on sperm motility

In terms of sperm motility, treatment with PUL significantly reduced the number of free-swimming sperm in C57BL/6 mice. As shown in Table 3, a decrease in sperm motility was noted in around 89% of the subjects after the highest dose (2 g/kg). In addition, the percentage of nonprogressive sperm decreased with the three doses tested when compared to control.

Table 1: Effects of pulegone at evaluation points (hours), on locomotor activity of C57BL/6 mice (cm/seg)

Dose (g/kg)	Treatments			Control		
	1 h	6 h	24 h	1 h	6 h	24 h
0.05	3.68±0.71 (6)	5.03±1.18 (5)	3.64±0.34 (4)	5.27±0.61 (6)	6.68±0.28 (7)	4.68±0.28 (4)
0.03	7.19±0.44 (8)	6.31±0.28 (7)	6.83±0.44 (8)	7.31±0.69 (5)	6.75±0.52 (7)	6.87±0.65 (8)
2	2.39±0.75* (6)	2.21±0.63* (5)	4.41±0.39* (4)	6.32±0.37 (8)	6.15±0.33 (3)	6.71±0.68 (3)

Data expressed as mean±SEM. *Significant differences with control at the 5% level by Duncan's multiple-range test within the same row. In parentheses, number of animals tested. SEM: Standard error of the mean

Table 2: Effects of pulegone in a functional observational battery procedure on C57BL/6 mice†

Domain	1 h	6 h	24 h
Breathing	*↓	*↓	**↓
Piloerection	#↑	-	-
Eyelid closure	-	*↑	-
Lacrimation	-	*↑	-
Not retracted ear reflection	*↑	*↑	*↑
Ambulation	*↓	*↓	*↓
Startle reaction	*↓	*↓	*↓
Posture†	*	*	*
Tail position	#	#	#
Home cage observation	#	@	#
Transfer behavior	-	*	@
Reaction- rate	-	*	*

*,@ and # denote significance at $P \leq 0.05$ level compared to control values; *Affected only at highest dose; #Affected only at lower dose; @Affected only at middle dose; **Dose-dependent, affected at ≥2 doses; ##Affected at 2 lower doses; ↓↑Arrows denote direction of change in measure compared to vehicle; -: No effect seen at any level. Doses: 2, 0.3 and 0.05 g/kg. *n*=6 mice/dose. †More detail in supplemental material online

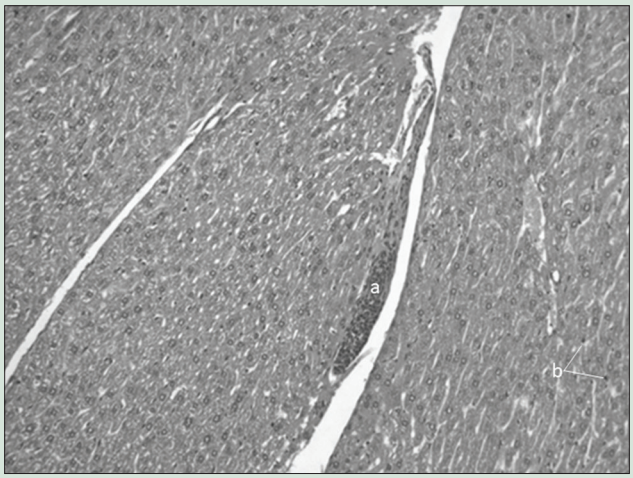

Figure 2: Liver segments from mice treated with oral pulegone (2 g/kg) showing (a) inflammatory infiltrate cells and (b) proliferation of Kupffer cells (H and E, ×100)

DISCUSSION

The oral assay showed that PUL administration did not result in any treatment-related mortality or abnormal clinical signs. Our findings provide the rationale for experimentation that PUL can induce behavioral effects measured by FOB on mice; all of this was evidenced by a decrease of locomotor activity, alterations in sperm motility and physiological functions after PUL administration.

There are some limitations of our study when intending to explain the differences among the results obtained after different doses of PUL. First, it was a single-dose study, and it is possible that multiple doses of PUL are necessary to produce more effects. We used high concentration (2 g/kg) of PUL than the median lethal dose (LD_{50}) because the purpose of this analysis was to describe any change of FOB and others parameters studies. However, some early physiological changes occurred in FOB in low concentration, as shown in Tables 2 and 3.

Other restrictions of our study were the length of the evaluation period and route of PUL administration. These parameters may be important to detect adverse side effects.

Schrankel investigated the administration of 470 mg/kg PUL in rats and 300 mg/kg in mice; the PUL was administered by oral gavage, and all animals died at day 5.[25] These deaths were attributed to liver toxicity. In other studies, the mean body weight of the tested groups was similar to those of vehicle controls.[26] On the other hand, in our investigation, no mortality was found, and this difference it could be due to the short period of evaluation carried out in this study. Our results showed the absence of mortality or relevant clinical findings in all groups within the entire period (24 h after PUL oral administration).

Moreover, Lasrado *et al.* did not observe adverse effects after oral administration of the highest dose of dry spearmint extract tested, in Sprague–Dawley rats.[27]

We also tried to confirm our results and that PUL reached significant plasma concentration after 1 h of administration using the histopathological evaluation specifically provided an understanding of how PUL is hepatotoxic, with quantitative evaluation (data not show). Our results indicated that PUL at maximal doses could be potentially nephrotoxic as well.[1] It is well known that the death of hepatic cells leads to rupture or damage of cell membranes and subsequent release of enzymes into the bloodstream, thereby increasing the levels of marker enzymes in serum.[28]

PUL has been reported to induce oxidative stress and liver injury in mice and rats; the most important metabolites are menthofuran, *p*-cresol-and other compounds, which have been suggested to be responsible for most of their side effects. High doses cause damage to lungs, kidneys, liver, and CNS; furthermore, oxidative stress might also precipitate underlying diseases and other behavior alterations.[26,29,30]

In the kidneys, tubular and glomerular hemorrhages were noticed, with a spontaneous and slight MNI. The possible effects could be attributable; however, the drift in the genetic constitution of the animals might have influenced this result.[31]

The FOB scores are based on variations in appearance or behavior. Without more invasive assay one can only speculate about the mechanisms of these detected effects. Moreover, when interpreting the results of the FOB examination, it is important to consider that these considerations should not be evaluated as single parameters but rather as a complex system since the FOB is influenced by several unspecific parameters such as age, gender, use of different mice strains, and circadian rhythm. These aspects certainly require careful consideration when designing FOB studies. Moreover, the extremely high doses that were used in this study (up to 2 g/kg body weight) could be related to the volatility of PUL. Although the chemical was administered by gavage,

Table 3: Effects of pulegone on sperm motility of C57BL/6 mice

Dose (g/kg)	Treatments			Control		
	Percentage sperm motility		Percentage no-motility	Percentage sperm motility		Percentage no-motility
	Progressive	Nonprogressive		Progressive	Nonprogressive	
0.05	5.3±2.9 (10)	15.6±5.9* (10)	76.7±8.7* (10)	7.8±5.8 (10)	43.6±5.4 (10)	47.0±5.1 (10)
0.3	6.6±3.7 (9)	22.4±8.5* (9)	71.1±9.2* (9)	13.4±3.0 (10)	59.7±6.7 (10)	27.1±6.7 (10)
2	3±3* (5)	2.3±1.45* (5)	94.7* (5)	23.2±6.3 (7)	22.9±6.5 (7)	60.8±11.6 (7)

*The number of motile sperm was analyzed by Mackler chamber showing significant differences between treated and controls in the same row: $P \leq 0.05$ by ANAVA and Duncan's multiple-range test. Medias±SEM and in parentheses, the number of experiments. ANOVA: Analysis of variance; SEM: Standard error of the mean

the response to this stimulus could well explain all the neurobehavioral effects that were detected even though there was no evidence of an effect on body weight or food or water consumption.

To obtain reproducible results, these unspecific parameters were reduced to a minimum in our study. The selection of physiological parameters, as well as the time of evaluations, may be critical to detect specific effects.

Our experiments showed changes in FOB as early as 1 h after PUL administration; FOB tests may be sensitive using short dosage schedules. When mice were evaluated for behavioral, and neurological changes after oral administration of PUL, abnormal behavioral responses like home cage observations were noted after the lower dose, especially in CNS activity and excitability (tail position and home-cage observation), also in autonomic effects (piloerection). These include motor activity, tremor, and sedation and muscle relaxation.

Mice that received the highest dose of PUL displayed a significantly decreased startle response when compared to controls ($P < 0.05$). Similar results were obtained on analysis of mice with more pronounced ambulation impairment, which also displayed a significant decrease in breathing. In our study, these behaviors were used as indices to assess the effects of PUL over CNS and as a parameter of anxiety and fear.

PUL is used as a flavoring compound, in perfumery and aromatherapy, and was designated as a psychoactive compound with the profile of an analgesic drug.[32] Anxiety and depression are considered the most prevalent psychiatric disorders worldwide. These are clinic illnesses related to the CNS. The lack of locomotor activity is typical of drugs that reduce CNS activity such as anxiolytics, neuroleptics, hypnotics, and sedatives;[33] since ambulatory activity is a type of locomotor activity in mice, its use as the behavioral index has been well established. Our investigation revealed that oral administration of PUL caused a significant decrease in ambulatory activity, as shown in Table 1.

Previous studies have suggested that PUL has ambulation-promoting actions and CNS effects on ambulation response, which might invalidate and appear to be contradictory with our results, in terms of locomotor activity.[34,35]

In addition, da Silveira *et al.* have reported that PUL increases mice locomotor activity and immobilization time, whereas in our study, high doses caused a significant decrease in ambulation after 1 h of PUL administration; this reduction persisted during 24 h.[35] However, other authors have reported that PUL induces significant muscle relaxation in the intestine, sedative, and antipyretic effects, and increases the latency of convulsions.[35-38] In addition, from this perspective, it may be natural to consider that the decrease of locomotor activity after high concentrations of PUL is an adverse event that produces an apparent effect on behavior.

It is important to note that an alteration of physiological parameters can be reflected on ambulation status only in the absence of systemic abnormality. Since behavior is influenced by the functioning of other organ systems (e.g., hepatic, renal, and endocrine systems), toxin-induced alterations in these organs, like those produced by menthofuran, might also be reflected in changes of general behavior.[39]

Furthermore, the results obtained from the observation of locomotor activity can be correlated with the description of behavior. Toda and Morimoto described that immediately after exposure to the essential oils, the group exposed to peppermint aroma presented a significantly lower perception of stress.[40]

In agreement with these authors, one of the most remarkable aspects of our results is that PUL appears to act by some physiological mechanisms and stress-related behaviors, with reduction of breathing frequency, eyelid closure, elevated pelvis, behavior transfer, ambulation, startle reaction, and response to escape. Again, PUL could exhibit some toxic properties in experimental mice and might have the same properties as depressant drugs on mice when administering doses above 2 g/kg.

Moreover, one of the most relevant results of this study is that PUL has effects on the reproductive physiology of males. A marked reduction of motility in spermatozoa from the cauda epididymis was noted after all doses tested [Table 2]. Sperm progressive motility in one of the main factors influencing *in vitro* fertilization rates.[23]

Fraser and Ahuja could attribute this pattern of deterioration to changes in the metabolic activity that occurred during the training process, probably due to modifications in cellular metabolic parameters, as suggested.[41] It is also possible that the reduction in motility registered in our study could be due to the properties of PUL to modify hormones, enzymes or serum iron used to obtain energy for sperm motility.[41] This process depends on the coordinated propagated flagella wave and Ca^2+, whose function is to provide propelling force for sperm to penetrate the pellucid area and produce the cumulus phenomenon known as hyperactivated motility. An adequate number of spermatozoa with normal functions are necessary for successful fertilization, and any alteration may lead to infertility.[41]

CONCLUSIONS

The main benefit from our research, and evidence of this observation obtained through FOB, is the rapid analysis time of the results, the possibility on the future of the reduction the animals and time to use for experiments in preparation of new substance, and the subsequent implications for compounds development from natural source obtained from PUL.

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Conflicts of interest

There are no conflicts of interest.

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Table 1. Effects of PUL on mice, recorded in a functional observational battery (% values).

		Post-treatment (hours)		
Doses (gr/Kg)		**1**	**6**	**24**
Control	**Breathing**			
	Irregular	0	0	0
	Normal	100	100	100
0.005	Irregular	10	0	20 *
	Normal	90	100	80
0.3	Irregular	0	0	0
	Normal	100	100	100
2	Irregular	50 *	40 *	37.5 *
	Normal	50	60	62.5
Control	**Piloerection**			
	Yes	0	0	0
	No	100	100	100
0.005	Yes	60 *	30	20
	No	40	70	80
0.3	Yes	41.7 *	0	0
	No	58.3	100	100
2	Yes	0	20	12.5
	No	100	80	87.5

	Eyelid closure			
Control	Open	100	100	100
	Close	0	0	0
0.005	Open	90	90	100
	Close	10	10	0
0.3	Open	100	100	100
	Close	0	0	0
2	Open	70	60 *	100
	Close	30	40	0
	Lacrimation			
Control	Yes	0	0	0
	No	100	100	100
0.005	Yes	0	0	0
	No	100	100	100
0.3	Yes	0	0	0
	No	100	100	100
2	Yes	20	30*	0
	No	80	70	100
	Ear reflection			
Control	Retracted	100	100	100
	Not retracted	0	0	0
0.005	Retracted	100	100	100

	Not retracted	0	0	0
0.3	Retracted	100	100	100
	Not retracted	0	0	0
2	Retracted	50	50	62.5
	Not retracted	50*	50*	37.5*
	Bite			
Control	Yes	7.69	0	0
	No	92.31	100	100
0.005	Yes	0	0	0
	No	100	100	100
0.3	Yes	25	0	0
	No	75	100	100
2	Yes	0	0	0
	No	100	100	100
	Tail position			
Control	Crushed	66.7	100	83.3
	Horizontal	33.3	0	16.7
	Elevated	0	0	0
0.005	Crushed	30	40	50
	Horizontal	0	0	0
	Elevated	70 *	60 *	50 *
0.3	Crushed	25	100	91.7
	Horizontal	75	0	8.3

	Elevated	0	0	0
2	Crushed	100	80	87.5
	Horizontal	0	0	0
	Elevated	0	20	12.5
Home cage observations				
Control	Normal	100	66.67	75
	Jump	0	33.33	25
	Without activity	0	0	0
0.005	Normal	80 *	100	100 *
	Jump	0	0	0
	Without activity	20	0	0
0.3	Normal	66.67	83.33	50
	Jump	33.3	16.67*	50
	Without activity	0	0	0
2	Normal	60	60	50
	Jump	0	0	0
	Without activity	40	40	0
	Posture			
Control	Flat pelvis	0	0	0
	Normal	100	100	100
	Back raised up	0	0	0
0.005	Flat pelvis	0	0	0
	Normal	100	100	100

	Back raised up	0	0	0
0.3	Flat pelvis	0	0	0
	Normal	100	100	100
	Back raised up	0	0	0
2	Flat pelvis	30 *	30 *	37.5 *
	Normal	70	70	62.5
	Back raised up	0	0	0
Control	**Transfer behavior**			
	Without movement	0	0	0
	Little movement	0	0	0
	Normal	91.7	100	83.33
	Excited	8.3	0	16.7
0.005	Without movement	10	0	0
	Little movement	20	20	20
	Normal	70	80	80
	Excited	0	0	0
0.3	Without movement	0	0	0
	Little movement	0	0	0
	Normal	100	100	41.67
	Excited	0	0	58.33 *
2	Without movement	20	20	12.5

	Little movement	30	30	25
	Normal	50	50 *	62.5
	Excited	0	0	0
	Ambulation			
0	No	0	0	0
	Yes	100	100	100
0.005	No	0	0	0
	Yes	100	100	100
0.3	No	0	0	0
	Yes	100	100	100
2	No	40 *	50 *	37.5 *
	Yes	60	50	62.5
	Startle reaction			
Control	Yes	100	100	100
	No	0	0	0
0.005	Yes	70	100	100
	No	30	0	0
0.3	Yes	100	91.7	100
	No	0	8.3	0
2	Yes	30 *	10 *	37.5 *
	No	70	90	62.5

	Reaction-rate			
Control	No reaction	0	0	0
	Tail suspension	25	8.3	8.3
	Hind legs	75	91.7	91.7
	Tail and legs	0	0	0
0.005	No reaction	18.2	0	0
	Tail suspension	18.2	18.2	0
	Hind legs	54.5	81.8	100
	Tail and legs	9.1	0	0
0.3	No reaction	0	0	0
	Tail suspension	58.3	0	16.7
	Hind legs	41.7	100	83.3
	Tail and legs	0	0	0

2	No reaction	27.8	40	33.3
	Tail suspension	36.4	10	11.1
	Hind legs	36.7	50 *	55.6 *
	Tail and legs	0	0	0

Statistical significance: *p<0.05 (compared to control values)