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# Acute and subacute toxicity evaluation of ethanolic extract from fruits of *Schinus molle* in rats

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**Abstract**

Ethanolic and hexanic extracts from fruits and leaves of *Schinus molle* showed ability to control several insect pests. Potential vertebrate toxicity associated with insecticidal plants requires investigation before institutional promotion. The aim of the present study was to evaluate the acute and subacute toxicity of ethanolic extracts from fruits of *Schinus molle* in rats. The plant extract was added to the diet at 2 g/kg body weight/day during 1 day to evaluate acute toxicity and at 1 g/kg body weight/day during 14 days to evaluate subacute toxicity. At the end of the exposure and after 7 days, behavioral and functional parameters in a functional observational battery and motor activity in an open field were assessed. Finally, histopathological examinations were conducted on several organs. In both exposures, an increase in the arousal level was observed in experimental groups. Also, the landing foot splay parameter increased in the experimental group after acute exposure. Only the subacute exposure produced a significant increase in the motor activity in the open field. All these changes disappeared after 7 days. None of the exposures affected the different organs evaluated. Our results suggest that ethanolic extracts from fruits and leaves of *Schinus molle* should be relatively safe to use as insecticide.


*Keywords:* *Schinus molle*; Toxicity; Functional observational battery; Motor activity; Rats

## 1. Introduction

*Schinus molle* – some of whose vulgar name are "aguaribay", "gualeguay" or "molle" – is an American species native of South America (Heywood, 1993) belonging to the Anacardiaceae family (Cabrera et al., 1965). A few genders are native to mild North America and Eurasia (Heywood, 1993).

All parts of the plant have been used in traditional medicine for the treatment of several pathologies: as antibacterial, antiviral, topical antiseptic, astringent, digestive, purgative, diuretic (Duke, 1985), for toothache, wound healer, rheumatism and menstrual disorders, also as stimulant and antidepressant (Yelasco-Negueruela, 1995) and for respiratory and urinary infections (Perez and Anesini, 1994).

Animal studies also showed that extracts from leaves and fruits of *Schinus molle* possess hipotensor effect in rats (Bello et al., 1996). Other experiments demonstrated that extracts from leaves produced analgesic and central depressor effects in rats (Barrachina et al., 1997).

The *Schinus molle* plant also demonstrated insecticidal properties. Essential oils from leaves of *Schinus molle* showed repellent activity on *Musca domestica* Linnaeus (Wimalaratne et al., 1996). In our laboratory, we observed that hexanic extracts from leaves and fruits of *Schinus molle* var *areira* had repellent effects on neonate larvae of *Cydia pomonella* (Chirino et al., 2001), an apple worm considered the key-pest in fruit tree farming of several world regions (Sheldeshova, 1967). Furthermore, the petroleum ether and ethanolic extracts from leaves and fruits of the *Schinus molle* var *areira* had repellent and adulticidal activity on *Tribolium castaneum* and *Blatella germanica* (unpublished data). In recent experiments, we also observed that hexanic extracts from leaves and fruits of *Schinus molle* were highly repellent for first nymphs of *Triatoma infestans*, and fruit

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extracts had ovicidal activity (Ferrero et al., 2006). These results suggest that *Schinus molle* var *areira* must be taken into account to be used in Chagas' vector control.

Considering this evidence, the *Schinus molle* plant could be very useful for some pest control, thus avoiding the use of synthetic pesticides with environmental and human safety hazards. For this reason, in order to continue assessing their potential insecticide use, it is necessary to investigate their safety through toxicity studies. In the present work, we evaluated the acute and subacute toxicity of ethanolic extract from fruits of *Schinus molle* var *areira* in rats by means of a functional observational battery (FOB) and by assessing the motor activity in an open field. The FOB is a series of observational and manipulative tests designed to assess the neurological integrity of the test subject (Moser et al., 1988), whereas motor activity is an apical measure of neurobehavioral function (MacPhail, 1987). Finally, the histopathological examination was realized on several tissues.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats of 12 weeks (acute exposure) and 8 weeks old (subacute exposure) were used. They were maintained under constant temperature conditions (22 ± 1 °C) in a 12-h light:12-h dark cycle (lights on at 07:00 h), provided with food and water ad lib.

The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

### 2.2. Preparation of the plant extract

The plant fruit was collected from Bahía Blanca city, in the south of Argentina, during summer. Botanical identification was performed at the herbarium of Universidad Nacional del Sur (Voucher herbarium specimen number: BBB 10444). Fresh fruits of *Schinus molle* var *areira* (627 g) were macerated in ethanol (3 × 2 l) at room temperature during 72 h. The solvent was evaporated at reduced pressure and the crude extract obtained (119 g) was kept at 4 °C.

### 2.3. Acute and subacute exposure

The plant extract was incorporated into the diet and fed to acute exposure rodents over 1 day at one dose of 2 g/kg body weight/day, and to subacute exposure rodents for 14 days at one dose of 1 g/kg body weight/day. For this, the amount of food consumed and body weight of each rat were daily determined in order to deliver a constant dosing throughout the study. Eight rats were used in each group. Two groups were assigned in both studies. The experimental animals received standard food mixed with plant extract while control rats only received the same standard diet without the plant extract. The elected doses are the maximum doses indicated for both assays of toxicity (OECD, 1995, 2001). The limit test was performed in both assays due to no severe toxic effects we expected for exposure to this plant extract. The parameters measured daily during the exposure period were dietary intake and body weight. At the end of the exposure, behavioral and functional parameters and motor activity were assessed in all animals. Subsequently, the half animals of the all groups were maintained with standard diet without the plant extract for 7 days and again the same parameters were evaluated to determine reversibility, persistence or delayed occurrence of toxic effects. Finally, at the end of both periods, all animals were euthanized by carbon dioxide inhalation, and necropsy observations and histopathological examinations were realized on several tissues: brain, liver, kidney, lung, heart, stomach and intestine. These tissues were weighed immediately after dissection, embedded in paraffin and 5 μm thick sections were stained with hematoxylin and eosin.

### 2.4. Functional observational battery

The FOB included a thorough description of the animals' appearance, behavior and functional integrity (US EPA, 1998). This was assessed through observations in the home cage, while animals were moving freely in an open field, and through manipulative tests. Procedural details and scoring criteria for the FOB protocol have been published (McDaniel and Moser, 1993).

Briefly, measurements were first carried out in the home cage. The observer recorded each animal's posture, activity and palpebral closure. The presence or absence of tremors and convulsions was noted and, if present, described. The presence or absence of spontaneous vocalizations and biting was also noted. The observer then removed the animal, rating the ease of removal and handling. The presence or absence of hindlimb flexor resistance and pressure grade was also noted. Palpebral closure and any lacrimation or salivation were rated. Other abnormal clinical signs were also recorded.

The animal was next placed in an open field arena having a piece of clean absorbent paper on the surface and allowed to freely explore for 3 min. During that time, the observer ranked the rat's arousal, gait score, activity level and rears as well as any abnormal postures, unusual movements and stereotypy. At the end of the 3 min, the number of fecal boluses and urine pools and presence or absence of diarrhea on the absorbent paper were recorded. Next, sensorial responses were ranked according to a variety of stimuli (click stimulus using a metal clicker, approach and touch rump with a blunt object, pinch of the tail using forceps, constriction of the pupil to a penlight stimulus and touch of the corner of the eye and the inside of the ear with a fine object). Also, several motor reflexes were evaluated (flexor and extensor thrust reflexes, forelimb hopping, proprioceptive positioning, forelimb and hindlimb extensions). Degree of surface and aerial righting were rated next. In landing foot splay, the tarsal joint pad of each hindfoot was marked with ink and the animal was then dropped from a height of 30 cm onto a recording sheet. This procedure was repeated three times. The distance from center-to-center of the ink marks was measured (cm) and the average of the three splay values was used for statistical analysis.

### 2.5. Motor activity

An open field of 50 cm × 50 cm × 60 cm whose floor was divided into 12 cm × 12 cm squares by black lines was used. The number of squares entered with all four paws, rearings, groomings and fecal boluses were scored each 5 min for 15 min. After each animal was removed, the open field was carefully cleaned with a damp cloth.

### 2.6. Statistical analysis

The dietary intake and body weight of rats were tested using an independent Student's *t*-test.

Behavioral test measures in FOB were continuous (providing interval data), ranked (ranked based on a defined scale), descriptive or binary (presence or absence of a sign). Continuous data were tested using an independent Student's *t*-test. The ranked data were analyzed using the Mann–Whitney *U*-test. For descriptive and binary data, each experimental group was compared to the control group using a Chi-square test.

The open field data were submitted to an ANOVA for repeated measures followed by *t*-test for paired samples. Differences between groups in each open field session were analyzed by independent Student's *t*-test. In all cases, resulting probability values <0.05 were considered significant.

## 3. Results

The acute and subacute exposure of ethanolic extract from fruits of *Schinus molle* var *areira* showed that both experimental groups exhibited a significant increase of dietary intake in each day of exposure ($p < 0.05$) compared to control groups. However, when we analyzed the body weight no significant differences were observed between the groups (data not shown).

The data obtained in the FOB are shown in Table 1. Neither acute nor subacute exposure produced alterations in the parameters evaluated in the home cage or during the manipulative tests. Also, no abnormal clinical signs were observed in control and experimental groups. However, in the open field arena both experimental groups exhibited a significant increase in the arousal level ($p < 0.05$) compared to control groups. When we analyzed the landing foot splay, only the acute exposure produced a significant increase ($p < 0.05$) in this parameter. The other parameters evaluated in the open field arena were not altered in the animals exposed. After 7 days, when the same parameters were evaluated again, we observed that the arousal level and landing foot splay altered previously were not modified in relation to control groups (Figs. 1–3). In the other parameters we did not observe any delayed effect as a consequence of exposure.

Motor activity evaluations in the square open field indicated that the acute exposure did not modify the number of squares crossed during a total of 15 min on days 1 and 7 after the exposure (Fig. 4). The ANOVA for repeated measures for comparisons in the number of squares crossed in each 5 min period, only showed significant differences in the number of squares, $F(2,28) = 38.5$, $p < 0.001$. The same was observed when we evaluated the rearings in the square open field (Fig. 5). Also, the ANOVA for repeated measures only showed significant differences in the number of rearings in each 5 min period, $F(2,28) = 45.1$, $p < 0.001$.

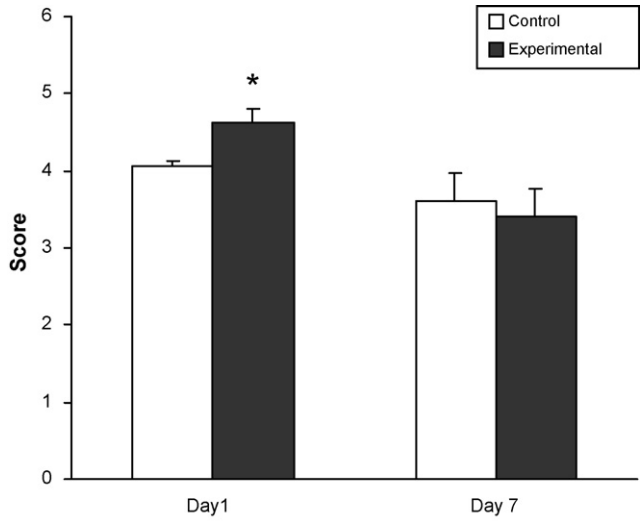


Fig. 1. Arousal level evaluated during the functional observational battery after the acute exposure, at the end of the treatment (day 1) and 7 days later (day 7). Data are expressed as mean ± S.E. of the value scored from the scale used. * $p < 0.05$ compared with control group (Mann–Whitney *U*-test).

However, as shown in Fig. 6, the subacute exposure produced a significant increase in the number of squares crossed during a total of 15 min ($p < 0.02$) compared to the control group; this alteration was reverted after 7 days of exposure finished. When we compared the number of squares crossed in each 5 min period, the ANOVA for repeated measures showed significant differences between the number of squares, $F(2,28) = 13.2$, $p < 0.001$, and between the groups, $F(1,14) = 5.5$, $p < 0.05$. Subsequent Student's *t*-test demonstrated a significant increase in

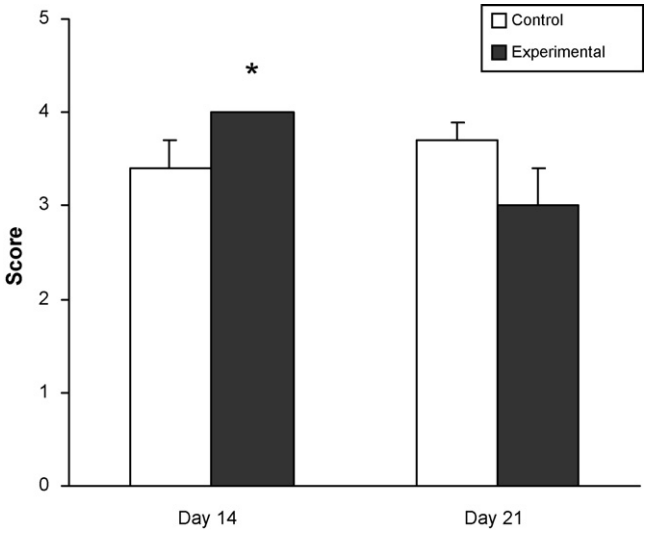


Fig. 2. Arousal level evaluated during the functional observational battery after the subacute exposure, at the end of the treatment (day 14) and 7 days later (day 21). Data are expressed as mean ± S.E. of the value scored from the scale used. * $p < 0.05$ compared with control group (Mann–Whitney *U*-test).

Table 1
Parameters evaluated in the functional observational battery

| Endpoints | Acute exposure | | Subacute exposure | |
|---|---|---|---|---|
| | Control | Experimental | Control | Experimental |
| Home cage observations | | | | |
| Activity (R) | 1.0 | 1.0 | 1.0 | 1.0 |
| Normal body posture (D) (%) | 100 | 100 | 100 | 100 |
| Palpebral closure (R) | 1.0 | 1.0 | 1.0 | 1.0 |
| Tremors (R) | 1.0 | 1.0 | 1.0 | 1.0 |
| Convulsions (D) (%) | 0 | 0 | 0 | 0 |
| Biting (D) (%) | 0 | 0 | 0 | 0 |
| Vocalizations (B) (%) | 0 | 0 | 0 | 0 |
| Hand-held observations | | | | |
| Ease of removal from cage (R) | 1.2 | 1.2 | 1.2 | 1.2 |
| Ease of handling rat in hand (R) | 1.1 | 1.0 | 1.1 | 1.0 |
| Lacrimation (R) | 1.0 | 1.0 | 1.0 | 1.0 |
| Salivation (R) | 1.0 | 1.0 | 1.0 | 1.0 |
| Normal fur appearance (D) (%) | 100 | 100 | 100 | 100 |
| Hindlimb flexor resistance (B) (%) | 100 | 100 | 100 | 100 |
| Limb pressure grade (B) (%) | 100 | 100 | 100 | 100 |
| Open field observations | | | | |
| Activity level (R) | 3.0 | 3.2 | 2.6 | 3.0 |
| Rearing (R) | 3.0 | 3.0 | 2.0 | 2.6 |
| Arousal (R) | 4.1 | 4.6* | 3.4 | 4.0* |
| Gait description (R) | 1.0 | 1.0 | 1.0 | 1.0 |
| Normal body posture (D) (%) | 100 | 100 | 100 | 100 |
| Unusual movements (D) (%) | 0 | 0 | 0 | 0 |
| Stereotypy (D) (%) | 0 | 12.5 | 0 | 25 |
| Fecal boluses (C) | 1.9 | 1.4 | 0.4 | 0.9 |
| Urine pools (C) | 0.2 | 0.7 | 1.0 | 1.0 |
| Diarrhea (B) (%) | 12.5 | 0 | 0 | 0 |
| Click response (R) | 1.9 | 1.9 | 2.0 | 1.9 |
| Approach response (R) | 1.6 | 2.0 | 2.0 | 2.0 |
| Touch response (R) | 1.1 | 1.4 | 1.4 | 1.5 |
| Tail pinch response (R) | 2.1 | 2.2 | 2.2 | 2.0 |
| Pupillary reflex (B) (%) | 100 | 100 | 100 | 87.5 |
| Palpebral reflex (B) (%) | 100 | 100 | 100 | 100 |
| Pinna reflex (B) (%) | 100 | 100 | 100 | 100 |
| Flexor reflex (B) (%) | 100 | 100 | 100 | 100 |
| Extensor thrust reflex (B) (%) | 100 | 100 | 100 | 100 |
| Forelimb hopping (B) (%) | 100 | 100 | 100 | 100 |
| Propioceptive reaction (B) (%) | 100 | 100 | 100 | 100 |
| Forelimb extension (B) (%) | 100 | 100 | 100 | 100 |
| Hindlimb extension (B) (%) | 100 | 100 | 100 | 100 |
| Surface righting reaction (D) (%) | 100 | 100 | 100 | 100 |
| Aerial righting reaction (D) (%) | 100 | 100 | 100 | 100 |
| Hindlimb landing foot splay (C) | 5.3 | 6.5* | 6.1 | 6.7 |

Binary (B) and descriptive (D) data expressed as percentage of incidence (Chi-square test); ranked (R) data expressed as the mean score of the scale used (Mann–Whitney *U*-test); continuous (C) data expressed as mean value (Student's *t*-test).
* $p < 0.05$ compared with control group.

the number of squares in the subacute exposed animals in second ($p < 0.02$) and third ($p < 0.05$) 5 min period compared to the control group. The rearing evaluations did not show any differences in the total 15 min after the exposure or 7 days after it (Fig. 7). The ANOVA for repeated measures only showed significant differences in the number of rearings in each 5 min period, $F(2,28) = 48.2$, $p < 0.001$.

When we analyzed the emotionality parameters as the number of groomings and fecal boluses, no measures demonstrated any significant differences between control and experimental groups in either acute or subacute exposure (data not shown).

The histopathological examinations of brain, liver, kidney, lung, heart, stomach and intestine in all animals, did not show any changes at the end of any of the periods.

## 4. Discussion

Rats exposed to ethanolic extract from fruits of *Schinus molle* var *areira* in acute and subcute exposure showed an increase in food intake. Fruits from *Schinus molle* are used as adulterant of piper grains and also due to their agreeable flavor they are used in syrups and beverage preparations (Taylor,

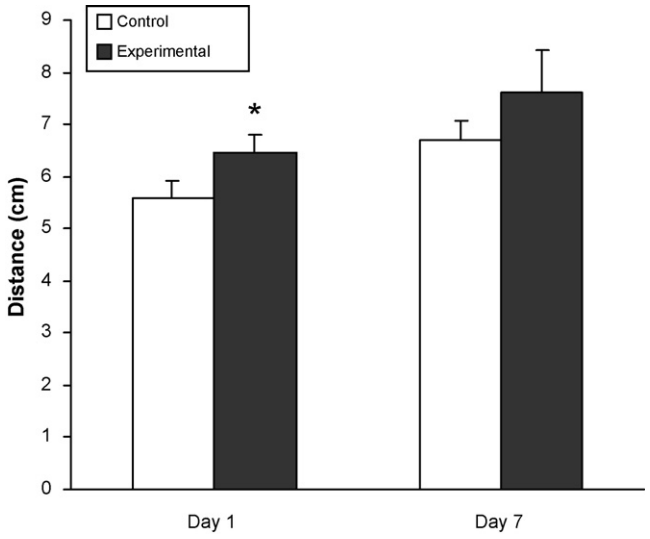


Fig. 3. Landing foot splay evaluated during the functional observational battery after the acute exposure, at the end of the treatment (day 1) and 7 days later (day 7). Data are expressed as mean ± S.E. of the distance in cm measured between the feet of the rat upon landing. $^{*}p < 0.05$ compared with control group (Student's *t*-test).

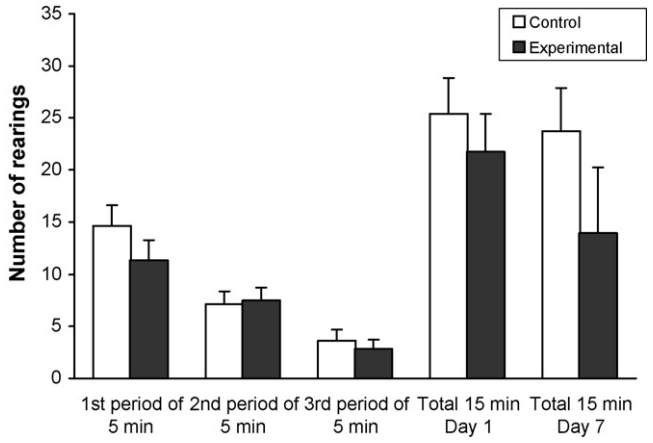


Fig. 5. Motor activity evaluated in the open field after the acute exposure. Data are expressed as mean ± S.E. of the number of rearings recorded during each period of 5 min (data analysed using ANOVA for repeated measures followed by *t*-test for paired samples and by independent Student's *t*-test) and in the complete period of 15 min at the end of the treatment (day 1) and during the 15 min of the evaluation made 7 days later (day 7) (data analysed using independent Student's *t*-test).

2005). The latter may be the reason for the increase in food intake.

However, when the body weight was analysed no differences were observed between control and experimental groups. A similar situation was shown by Belmain et al. (2001) when dusts from roots of *Securidaca longipedunculala* (Polygonaceae) and leaves from *Chamaecrista nifgricans* (Leguminoseae), useful as insecticide against stored grain pests, were offered to rats.

The increase in the arousal level observed in experimental groups in both exposures and also in the number of squares crossed in the open field in the animals after subacute exposure, indicated that the ethanolic extract from fruits produced a stimulant effect in rats. This stimulant effect observed in the exposed animals could explain the absence of differences in body weight between control and experimental groups in spite of increase in food intake observed in rats exposed to ethanolic extract from fruits.

It is known that the activation of mesolimbic dopamine pathways stimulates the locomotor activity (Vezina and Kim, 1999). Jamaluddin and Poddar (2001) showed that aldrin exposition produced an increase in this activity due to an activation of dopamine pathway. The same authors also obtained evidence that gabaergic and cholinergic systems were implicated in the hiperlocomotion.

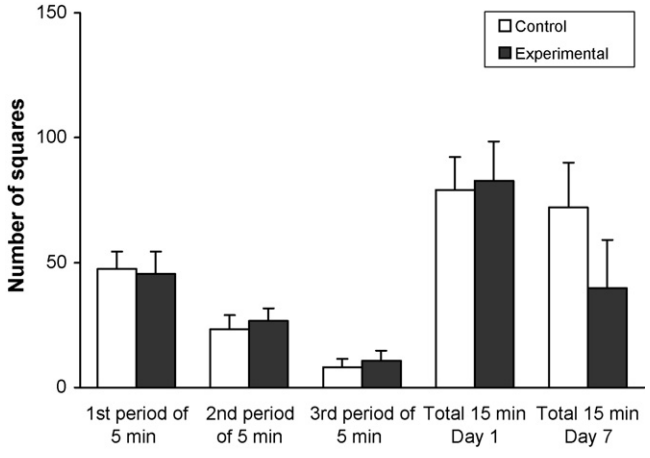


Fig. 4. Motor activity evaluated in the open field after the acute exposure. Data are expressed as mean ± S.E. of the number of squares entered by the rat, recorded during each period of 5 min (data analysed using ANOVA for repeated measures followed by *t*-test for paired samples and by independent Student's *t*-test) and in the complete period of 15 min at the end of the treatment (day 1) and during the 15 min of the evaluation made 7 days later (day 7) (data analysed using independent Student's *t*-test).

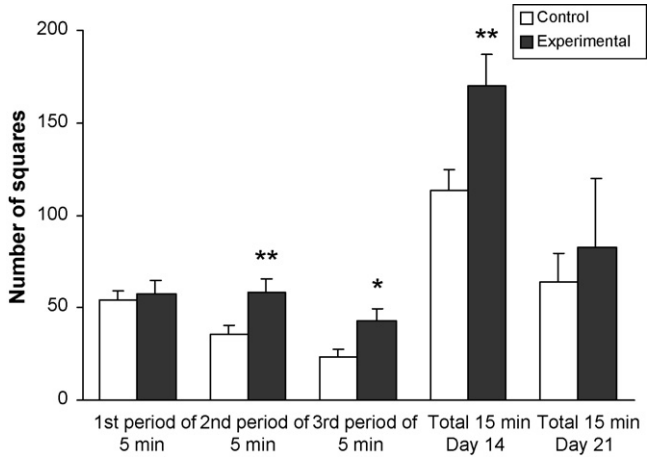


Fig. 6. Motor activity evaluated in the open field after the subacute exposure. Data are expressed as mean ± S.E. of the number of squares entered by the rat, recorded during each period of 5 min (data analysed using ANOVA for repeated measures followed by *t*-test for paired samples and by independent Student's *t*-test) and in the complete period of 15 min at the end of the treatment (day 14) and during the 15 min of the evaluation made 7 days later (day 21) (data analysed using independent Student's *t*-test). $^{*}p < 0.05$ and $^{**}p < 0.02$ compared with control group.

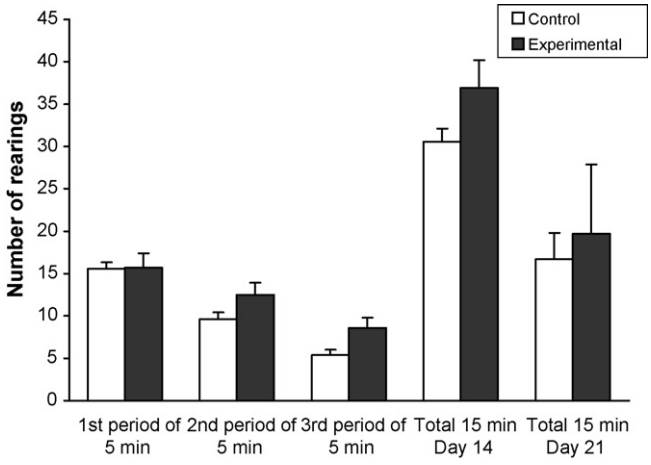


Fig. 7. Motor activity evaluated in the open field after the subacute exposure. Data are expressed as mean ± S.E. of the number of rearings recorded during each period of 5 min (data analysed using ANOVA for repeated measures followed by *t*-test for paired samples and by independent Student's *t*-test) and in the complete period of 15 min at the end of the treatment (day 14) and during the 15 min of the evaluation made 7 days later (day 21) (data analysed using independent Student's *t*-test).

Other pathways, such as serotonergic and glutaminergic, are involved in locomotion regulation (Carey et al., 2004). It is possible that the ethanolic extract from fruits of *Schinus molle* may produce some alteration in the dopaminergic neurotransmition or in other systems, and these changes could be responsible for the hyperactivity observed in the experimental groups.

With regard to the possible activation of some dopaminergic pathways by the ethanolic extract from fruits of *Schinus molle*, while the systemic treatment with dopamine D1 or D2 receptor agonist decreases food intake and the dopaminergic stimulation in the lateral hypothalamus is also associated with inhibition of food intake (Ramos et al., 2005), the D1 or D2 receptor blockade in the nucleus accumbens did not alter the total amount of food consumed although significantly decreased ambulatory activity (Baldo et al., 2002). These evidences suggest that the possible activation of mesolimbic dopaminergic system could explain the increase in the motor activity but not the increase in food intake that we observed.

The acute exposure also produced an increase in the landing foot splay. Successful performance of this parameter depends on stimulation of the visual system, vestibular motor detector and the appropriate muscle tension to support body weight upon landing (Moser and Ross, 1996). The acute exposure could affect any of these functions.

Although in our study we observed alterations in motor activity and in the landing foot splay parameter in the animals exposed, they were transitory because after 7 days of both exposures the experimental groups did not show changes in relation with control groups. Furthermore, the absence of abnormalities in histopathological examinations in all tissues studied, revealed that the ethanolic extract from fruits of *Schinus molle* could be useful as insecticide and safe for human beings. However, future research like potential chronic toxicity associated with this extract will need to be evaluated through long-term bioassays.

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