



Sub-acute Cinnamaldehyde rectal administration induces non-toxic effects in neurological and physiological responses in C57BL/6 mice

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Abstract: Trans-Cinnamaldehyde, the main component of cinnamon essential oil, is widely used in food and traditional herbal medicine. The objective of this study was to investigate the subacute toxic effects of trans-Cinnamaldehyde (Cinn; 2000 mg/kg/day) administered intrarectally for 15 consecutive days in male C57BL/6 mice, a route of administration not previously evaluated in this model. Mice were monitored for survival, preclinical signs, and behavior. Neurotoxic potential was assessed using a functional observational battery (FOB), ambulatory activity, and spontaneous locomotion. Physiological parameters included sperm motility, liver function, relative liver weight, and macroscopic and microscopic liver morphology. Food and water intake were recorded and considered in the analysis. Subacute intrarectal administration of Cinn did not produce neurotoxic, hepatotoxic, or reproductive adverse effects. The treatment was well tolerated and associated with reduced body weight gain without changes in food or water intake. In conclusion, these results support the safety of Cinn and suggest potential applications in metabolic research, particularly in the context of obesity.

Keywords: Trans-Cinnamaldehyde; intra-rectal; functional observational battery; toxicity; mice.

1. Introduction

The genus *Cinnamomum* comprises about 250 species. Cinnamon, obtained mainly from *Cinnamomum zeylanicum* (native to Sri Lanka and southern India) and *Cinnamomum cassia* (Chinese cinnamon), both belonging to the Lauraceae family, is widely used in the food industry due to its unique organoleptic properties. Historical records describe its medicinal use in Chinese texts dating back more than 4000 years.

The essential oil from cinnamon bark is rich in trans-Cinnamaldehyde (Cinn) a phenylpropanoid that represents its major component (Yap et al., 2015; Liu et al., 2023). Cinn has been granted Generally Recognized as Safe (GRAS) status by the FDA and exhibits multiple pharmacological properties. Reported activities include antimicrobial (even against resistant strains), antitumor, antidiabetic, antioxidant, and anti-inflammatory effects, along with actions on the central nervous, cardiovascular, respiratory, immune, and digestive systems (Utcharyaki et al., 2016; Muhammad & Dewettinck, 2017; Lu et al., 2018; Mahmoudi et al., 2022; Mo et al., 2023; Vaz et al., 2023; Gandhi et al., 2023; Figueiredo et al., 2023).

The rectal route is considered appropriate for drugs that are unstable in the stomach or affected by pH and gastric enzymes. It is commonly used for suppositories containing laxatives, antipyretics, anticonvulsants, and treatments for hemorrhoids. Although rectal absorption can be inconsistent, it remains a useful alternative when other routes are impractical. Compared to oral gavage, rectal administration may also reduce handling stress in experimental animals. However, this route is rarely employed in preclinical models, and there are no previous reports evaluating rectal administration of Cinn (Karunajeewa et al., 2007; Dundar et al., 2008). Several reports have described neurological effects of Cinn, including preclinical studies on memory (SoukhakLari et al., 2019), investigations in mammalian neurodegenerative disease models (Jiao et al., 2024), and studies on anxiety and stress (Etaee et al., 2019), although these findings were obtained using others different routes of administration.

Therefore, the present study aimed to investigate the subacute in vivo effects of rectally administered Cinn (2000 mg/kg/day for 15 days) in male C57BL/6 mice. Clinical status (morbidity and mortality), behavioral and neurological outcomes (Functional

Observational Battery, FOB), and spontaneous locomotor activity (SLA) were assessed. In addition, sperm motility, pathological parameters, organ weight index, and liver morphology were evaluated.

Abbreviation

Cinn: trans-cinnamaldehyde; FOB: functional observational battery; CNS: central nervous system

2. Materials and Methods

2.1. Animal preparation

Experiments were conducted in adult male C57BL/6 mice (30 ± 2.5 g; 8–10 weeks old). Animals were randomly housed in pairs in standard cages ($30 \times 20 \times 15$ cm) under controlled conditions (24 ± 2 °C; 12:12 reverse light/dark cycle), with free access to standard chow and water. Female mice were excluded to avoid hormonal variability. All experiments were performed between 16:00 and 17:00 h during the dark phase, under dim red light (25 W), which is not detected by mouse vision (McLennan & Taylor-Jeffs, 2004). An enriched environment was provided (Brenes et al., 2008).

Mice were gently handled to minimize distress and acclimated for two weeks before dosing. During the pre-test period, food and water intake and functional observational battery (FOB) parameters were recorded (Zaio et al., 2019). All procedures were approved by the Institutional Animal Care and Use Committee (CICUAL), Faculty of Medicine, National University of Córdoba, and followed NIH guidelines.

2.2. Study protocol & in vivo chemistry studies

Administration: Animals were randomised into groups based on body weight one week before the beginning of the experiment. Vehicle and test substance formulations were administered as a single dose by intra-rectal intubation using small disposable pipette tips (100 µl).

Day 0: the beginning of dose administration. Individual doses were based on body weight on day 0. Animals received a daily dose of 2000 mg kg⁻¹ Cinn dissolved in edible sunflower oil (90–110 µl) for 15 days (370,000 ppm). Toxicological studies on Cinnamon have predominantly been conducted following recognized international regulatory guidelines and, according to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS (Rev.4; 2011)), corresponding to category 4 (LD50 is between 300–2000 mg/kg), which provides a safety factor of approximately more than 100 times the expected human exposure to Cinnamon ("OECD Annual Report 2008", 2008; Zhang et al., 2020) (Fig. 1).

Chemical: Standard Cinn was purchased from Sigma Aldrich (USA) trans-Cinnamaldehyde (3-Phenylprop-2-enal; C₉H₈O; ≥ 96 %); W228613; 132.16, was stored refrigerated and protected from the light.

Drug preparation: Cinn was diluted in sunflower oil and prepared daily, protected from light.

2.3. Parameters evaluated

2.3.1. Preclinical observations and survival

Animals were examined daily for 15 days (except on FOB test days 1, 9, and 15). Preclinical signs included general appearance, fur, eyes, oral and nasal cavities, secretions/excretions, autonomic activity (lacrimation, piloerection, pupil size, respiratory pattern), and detection of palpable masses. Mortality and morbidity were recorded.

2.3.2. Body and organ weight

Body weight was recorded on day 0 and before necropsy. After

euthanasia (CO₂ inhalation), the liver was excised, blotted dry, and weighed. Relative liver weight (RLW) was calculated as:

$$RLW (\%) = \frac{\text{Liver weight (g)}}{\text{Body weight at sacrifice (g)}} \times 100$$

RLW=liver weight (g) body weight at sacrifice (g)

2.3.3. Food and water intake

Measured every 3 days, starting from the pre-test period (Zaio et al., 2019). Feed efficiency (body weight gained per food consumed) was also calculated.

2.3.4. Macroscopic and histological analysis

A full necropsy was performed. Liver samples were fixed in 10% buffered formalin, processed through ethanol and xylene, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Sections were examined under light microscopy (160×).

2.3.5. Serum biochemical assays

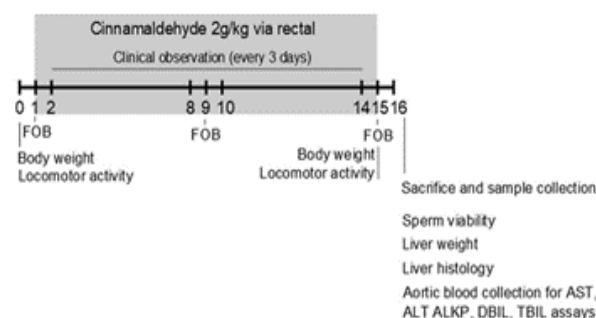
Blood was collected by aortic puncture in EDTA tubes. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBil), and direct bilirubin (DBil) were quantified using commercial kits (Abbott Lab, Buenos Aires, Argentina).

2.3.6. Sperm motility

Cauda epididymides were dissected, minced in isotonic saline at 37.5 °C, and incubated in Tyrode's solution. (in mM: CaCl₂: 1.8; MgCl₂: 0.929; KCl: 2.68; NaHCO₃: 11.903; NaCl: 136.86; NaH₂PO₄: 0.287; glucose: 5.56; penicillin 0.5 mg/ml and streptomycin 0.075 mg/ml. PH: 7.3–7.5; 270–290 mOsm) (all reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA). Sperm motility was evaluated at 23 ± 2 °C using a Makler chamber under an inverted microscope (200×). Results were expressed as the percentage of motile (progressive + non-progressive) versus non-motile spermatozoa (Carrascosa et al., 2001).

2.3.7. Spontaneous locomotor activity (SLA)

Mice preadapted to the open field were recorded for 5 min on days 1 (before treatment) and 15 (1 h post-treatment) using a video camera (0.1 s resolution). Movement was analyzed with Tracker software. The results were presented as variation in speed and variation in distance traveled (day 15 minus day 1 locomotor activity).



[FOB: functional observational battery; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALKP: alkaline phosphatase; DBIL: direct bilirubin; TBIL: total bilirubin.]

Fig. 1. Experimental protocol.

2.4. Functional Observational Battery (FOB)

Observations of the FOB were made and documented during treatment 15 days after rectal administration of Cinn. The FOB was developed based on a procedure commonly used by the Environmental Protection Agency to evaluate potential toxicants. It provides an overall behavioural profile that allows the assessment of a wide range of compound effects (Zaio et al., 2019). Observations were made on days 1, 9 and 15 of the experiment to detect any toxic effects, i.e. general changes in the eye and mucous membranes, hair and skin colour, food and water consumption, respiratory and circulatory systems, tremors, convulsions, bizarre behaviour and permanent or semi-permanent signs, salivation, diarrhoea, lethargy, abnormal behaviour, motor activity, neurological effects such as posture or clonic/tonic movements, sleep and coma. We used the FOB to assess a range of factors including behavioural, physiological and neurological characteristics in an *in vivo* rodent model.

The following parameters were observed in all animals:

-Behavioural effects: home cage observation [posture, convulsions/tremors, biting, and palpebral (eyelid) closure] (categorical); transfer behaviour (categorical).

-Neurological effects: ear reflection (categorical), bite (ordinal), tail position (ordinal).

-Physiological effects: piloerection (ordinal), breathing (categorical).

Efforts were made to ensure minimal variation in sound levels, temperature, humidity, lighting, odours, time of day and environmental distractions. Mice from different groups were handled in the same way and under the same conditions. The procedure used was a modification of previously published procedures but essentially followed the methods described by (Irwin, 1968).

A well-trained person blinded to the group being tested was responsible for conducting the behavioural tests. In the afternoon before the experiment, the mice were separated and placed in cages, identified and kept on a shelf away from other mice, with water *ad libitum* and food. On the day of the experiment, the mice were brought into the experimental room. The undisturbed behaviour of each animal was observed in a standard box for 5 min.

2.5. Statistical analysis

In the FOB, categorical variables were classified as normal or abnormal. These data were organized into contingency tables and analyzed using the Chi-squared test of homogeneity.

Continuous variables were first tested for normality with the Shapiro-Wilk test. Differences between treatments were analyzed by factorial analysis of variance (ANOVA), followed by Duncan's multiple range test for post hoc comparisons. A *p*-value < 0.05 was considered statistically significant.

All statistical analyses were performed using InfoStat software (version 2018; Córdoba, Argentina).

3. Results

3.1. Animal survival, preclinical observation, relative organ weight, macroscopic evaluation, biochemicals & histopathological parameters

Daily administration was performed according to our previous study to investigate the effects caused by acute administration of Cinn (Zaio et al., 2018). No mortalities were observed in mice during the 15-day treatment period with Cinn at a dose of 2,000 mg/kg bw by the intrarectal method. None of the mice showed any

apparent morbidity, adverse effects, or preclinical signs of toxicity such as changes in eyes, respiratory rate, autonomic (salivation, sweating, and piloerection), and stereotypic activities after administration of Cinn throughout the 15-day experimental period. No preclinical signs of toxicity were observed in the normal control group. Animals in the treatment group showed eczematous lesions in the perianal region accompanied by scratching excoriations and perilesional alopecia without anus involvement, which were absent in the other two groups. This could be explained by the irritant properties of Cinn at the cutaneous and mucosal level and not by an effect of the method used, as lesions were absent in the other groups (Bickers et al., 2005). In all cases, the feces of the experimental animals were dark-colored. Any minor changes or activities observed in the mice during the study period can be considered normal findings for C57BL/6 mice. All animals survived until scheduled euthanasia.

Changes in body weight after 15 days of Cinn treatment are shown in [Figure 2](#). The body weight of the Cinn group was significantly lower than the vehicle and control groups (*p*<0.002). The relative weight of the liver in the Cinn group was slightly higher than the control and vehicle groups ([Figure 2](#)). However, the macroscopic examination of the liver did not reveal any abnormality, while the histopathological examination revealed a slight alteration, a non-significant difference was observed in the evaluation of sinusoidal congestion and percentage of binucleated hepatocytes. Similarly, no changes in biochemical parameters, inflammatory infiltrate, cholestasis, hemorrhages, or architecture were observed (data not shown).

Cinn induced no significant effects in liver biochemical parameters levels analyzed in serum, such as aspartate aminotransferase (AST/GOT), alanine aminotransferase (ALT/GPT), etc. ([Table 1](#)).

3.2. Effects of Cinn on food and water intake

Concerning food and water intake, Cinn increased consumption of food (15.3 %) and water (16.7 %) when compared to the Vehicle group (*p*<0.05) ([Table 2](#)).

3.3. Effects of Cinn on spontaneous locomotor activity (SLA)

Cinn induced non-significant effects on spontaneous locomotor activity, evaluated as speed and distance variations between measurements (day 15 minus day 1 locomotor activity) ([Table 3](#)). Although the negative values indicate that the animals exhibited reduced locomotor activity on day 15 post-treatment, the mean velocity values were close to zero, and the variation in distance traveled showed negative values ranging between 879 and 1179 cm. If there are no significant differences between groups, it is likely that the treatment has not had a specific impact on locomotion, and the observed reduction is a general phenomenon, unrelated to the intervention.

3.4. Effects of Cinn on sperm motility

In terms of sperm motility, Cinn induced non-significant differences in the parameters evaluated. All groups exhibited sperm motility percentages within the following ranges: immotile sperm (56–77%), non-progressive motility (20–39%), and progressive motility (3–7%). No statistically significant differences were observed among the groups ([Figure 3](#)).

3.5. Effects of Cinn on FOB parameters

Treated animals showed no evidence of behavioral, neurological, and physiological effects, during the FOB procedures. As shown in [Table 4](#), intrarectal administration of Cinn (2 g/kg) for 15 consecutive days did not produce significant alterations in most parameters of the Functional Observational Battery (FOB) compared to the control and vehicle groups. However, a

significant increase ($p < 0.05$) in piloerection was observed in the Cinn-treated mice on day 9. Although no statistical differences were detected for other behavioral parameters, slight changes were noted in home cage observations, transfer behavior, and tail position.

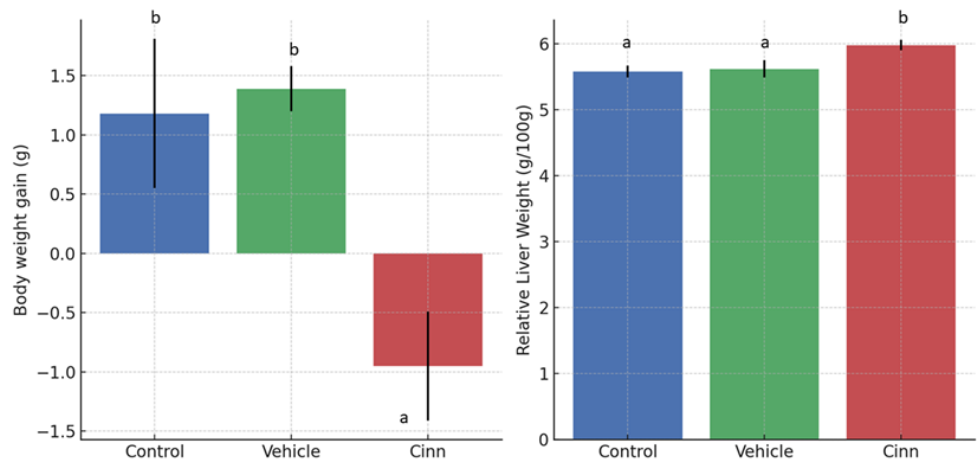


Fig. 2. Effect of trans-Cinnamaldehyde (2000 mg kg⁻¹ body weight/day) on body and liver weight parameters from C57BL/6 mice (n=8). [The initial weight was recorded before starting the different treatments (**day 1**). At the end of the treatment (**day 15**) the final weight was recorded in the following groups of mice: Control, mice uptake water; Cinn: animals administered with trans-Cinnamaldehyde and, Vehicle, mice administered with sunflower oil. Relative liver weight was determined after 15 days of treatment for all groups. Data are expressed as Mean $\pm$ SEM. Different letters indicate significant differences according to Duncan's test.]

Table 1. Effect of trans-Cinnamaldehyde (2000 mg kg⁻¹ body weight/day) on liver biochemical parameters from C57BL/6 mice (n= 8).

Parameters	Control	Vehicle	Cinn	<i>p</i> -value
ALT/GPT (U/L)	16.00 $\pm$ 2.23	17.88 $\pm$ 1.17	15.88 $\pm$ 0.85	0.4669
AST/GOT. (U/L)	55.38 $\pm$ 12.22	51.13 $\pm$ 5.75	61.63 $\pm$ 9.74	0.6658
ALKP(U/L)	<5	<5	<5	
Direct bilirubin (mg/dl)	<0.1	<0.1	<0.1	
Indirect bilirubin (mg/dl)	<0.1	<0.1	<0.1	

[The animals were divided into the following groups of mice: Control, mice uptake water; Cinn: animals administered with trans-Cinnamaldehyde and, Vehicle, mice administered with sunflower oil. The data are expressed in percentages as Mean $\pm$ SEM. According to Duncan's post hoc test, there were no significant differences $p > 0.05$. The number of animals evaluated is indicated in parentheses. Aspartate aminotransferase (AST/GOT); alkaline phosphatase (ALKP); and alanine aminotransferase (ALT/GPT).]

Table 2. Effect of trans-Cinnamaldehyde (2000 mg kg⁻¹ body weight/day) on uptake of food and water in C57BL/6 mice (n=8).

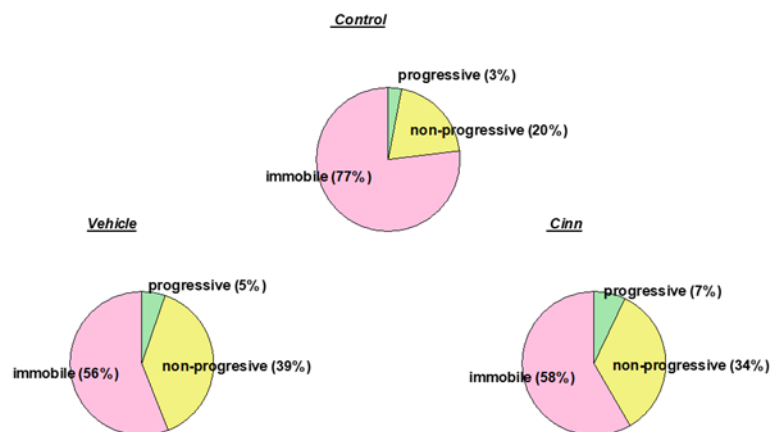
Intake (g)	Control	Vehicle	Cinn	<i>p</i>
Food	49.88 $\pm$ 1.68 a, b	45.22 $\pm$ 1.68 a	52.14 $\pm$ 1.68 b	0.025
Water	82.55 $\pm$ 2.16 b	70.26 $\pm$ 2.16 a	82.01 $\pm$ 2.16 b	0.0008

[The animals were divided into the following groups of mice: Control, mice uptake water; Cinn: animals administered with trans-Cinnamaldehyde and, Vehicle, mice administered with sunflower oil. The data are expressed in percentages as Mean $\pm$ SEM. The number of animals evaluated is indicated in parentheses. Different letters indicate significant differences according to Duncan's test.]

Table 3: Effect of trans-Cinnamaldehyde (2000 mg kg⁻¹ body weight/day) on spontaneous locomotor activity in C57BL/6 mice (n=4).

Treatments (n:4)	Speed variation (cm/s)	Variation of the distance (cm)
Cinn	-0,19 ± 0,71	-938,39 ± 407,41
Vehicle	-1,7 ± 0,71	- 1178, 89 ± 407,41
Control	-1,15 ± 0,71	-879,27 ± 407,41

[The animals were divided into the following groups of mice: Control, mice uptake water; Cinn: animals administered with trans-Cinnamaldehyde and, Vehicle, mice administered with sunflower oil. The data are expressed in percentages as Mean ± SEM. According to Duncan's post hoc test, there were no significant differences between the data ($p>0.05$). The number of animals evaluated is indicated in parentheses.]

**Fig. 3.** Effect of trans-Cinnamaldehyde (2000 mg kg⁻¹ body weight/day) on sperm motility (%) in C57BL/6 mice (n=8).

[The animals were divided into the following groups of mice: **Control**, mice uptake water; **Cinn**: animals administered with trans-Cinnamaldehyde and, **Vehicle**, mice administered with sunflower oil. The data are expressed in percentages as Mean ± SEM. According to Duncan's post hoc test, there were no significant differences between the data (preogressive: $p=0.9055$; non-progressive: $p=0.1264$; immobile: $p=0.0663$). The number of animals evaluated is indicated in parentheses.]

Table 4. Effects of trans-Cinnamaldehyde (2000 mg kg⁻¹ body weight/day) on record in a functional observational battery (FOB) C57BL/6 mouse (n=8).

Parameters	Day 1			Day 9			Day 15		
	<u>Cinn</u>	<u>Veh</u>	Con	<u>Cinn</u>	<u>Veh</u>	Con	<u>Cinn</u>	<u>Veh</u>	Con
Home cage observations	--	--	--	↑	--	--	↑	--	--
Transfer behavior	--	--	--	--	↑	--	↑	--	--
Piloerection	--	--	↑	* ↑	--	--	↑	↑	↑
Tail position	--	--	--	--	↑	↑	↓	↑	--
Breathing	--	--	--	--	--	--	--	--	--
Ear reflection	--	--	--	--	--	--	--	--	--
Bite	--	--	--	--	--	--	--	--	--

The animals were divided into the following groups of mice: **Control** (Con) mice uptake water; **Cinn**: animals administered with trans-Cinnamaldehyde and, **Vehicle** (Veh), mice administered with sunflower oil. * denote significance at $P\leq 0.05$ level compared to control values. ↓↑Arrows denote the direction of change in measure compared to the vehicle; --: No effect seen.

4. Discussion

Cinnamon has been used for centuries as a spice and as a traditional herbal medicine. In the last decade, it has received special attention as a source of potentially useful bioactive components in foods (as flavouring and preservative), pharmaceuticals (for their therapeutic action), for medicinal treatments due to its pharmacological properties, described as anticancer, anti-hypertriglyceridemic, anti-inflammatory, antitumour, anti-diabetic, as well as in aromatherapy recipes (Dorri et al., 2018).

In this study, subacute intra-rectal administration of Cinn, even at a dose of 2000 mg/kg/day, did not induce significant changes in mice's physiological, histopathological, and biochemical parameters. To our knowledge, this is the first report on the physiological and behavioural effects of 15 days of intra-rectal administration of Cinn, specifically addressed by FOB.

The primary objective of this study was to evaluate the analytical sensitivity of the FOB assay in a mouse model by subacute intra-rectal administration of Cinn. The International Conference on Harmonisation and Tripartite Guideline on Safety Pharmacology Studies for Human Pharmaceuticals has implemented the requirement of preclinical safety pharmacology testing guidelines and must be tested before human administration, and FOB is a method to study the CNS adverse effects (Gauvin et al., 2016; Zaio et al., 2019).

A large number of CNS safety assessment studies using standard FOB are conducted each year in contract research organizations around the world (Olivier & Karanth, 2020). In addition, the effort, cost and time required for a FOB study are much less than for chronic studies, although economic constraints and the time required for behavioural FOB studies still limit the number of studies that can be performed.

First of all, it was a 15-dose-day Cinn, without significant physiological and behavioural changes. Nevertheless, Gowder & Halagowder (2010) described that rats treated with Cinn by gavage at a dose of 73.5 mg/kg body weight/day for 90 days showed changes in olfactory discrimination, auditory startle response and negative geotaxis behaviour.

The subacute toxicity study showed that Cinn administration did not result in treatment-related mortality, abnormal preclinical signs or changes in FOB. On the other hand, in our previous report, we showed that the same concentration of Cinn and with a single dose did not show acute toxicity (Zaio et al., 2018).

Concerning spontaneous locomotor activity, Etaee et al. (2019) describe the effect of intragastric administration of Cinn (20 mg kg⁻¹) in acute and chronic stressed mice. They found that Cinn had anxiolytic effects assessed by open field and the elevated plus maze tests and no changes were found in locomotor activity as in our study. The negative values observed in our results on the variation in distance could be attributed to the tendency of mice to display decreased locomotor activity over time due to habituation to the testing environment. It is common for the total distance traveled to decline across repeated sessions, even in the absence of experimental intervention, as a result of increased familiarity with the environment or reduced initial anxiety (Pernold et al., 2023).

It is important to highlight that large amounts of Cinn can be applied and tolerated using intra-rectal administration as observed in our results evaluated by FOB. Currently, the number of applications of Cinn in medicine is growing exponentially and there is no pharmaceutical preparation of Cinn for rectal administration. When interpreting the results of the FOB study, it is important to keep in mind that these considerations should not be evaluated as single parameters, but rather as a complex system, since many non-

specific measures, such as age, sex, use of different mouse strains, circadian rhythm influence the FOB.

Regarding the route of administration for essential oils or secondary metabolites, Dundar et al. (2008) evaluated the effect of intrarectal administration of *Origanum onites* L. in colitis induced by 2,4,6-trinitrobenzenesulfonic acid in rats, showing that the administration of a single dose had a significant protective effect on the colonic lesion. Moreover, Arruda et al. (2009) have demonstrated that intrarectal treatment with limonene in mice infected with *Leishmania* decreased the parasite load, improved the established lesions and succeeded in suppressing the dissemination of ulcers, without observing toxic effects in mice. It is also important to consider that intragastric administration in small experimental animals generates stress and there is a risk of aspiration and lung injury (Walker et al., 2012).

The liver is involved in maintaining homeostasis, such as protein synthesis, storage and metabolism of fats and carbohydrates. AST or ALT levels are a valuable aid primarily in the diagnosis of liver disease.

Cinn has been reported to induce protection against oxidative stress in mice and rats, and excessive production of reactive oxygen species leads to an oxidant-antioxidant imbalance, making membrane lipids susceptible to oxidative damage (Subash-Babu et al., 2014). Nevertheless, Mereto et al. (1994) describe that oral administration of Cinn at a dose of 500 mg/kg for 14 days induced genetic alterations at the chromosomal level in the liver of rats. Indeed, in our experiments, the effects of Cinn did not produce hepatotoxicity in a mouse model by rectal administration with Cinn concentration. Furthermore, in a carcinogenesis model in Swiss albino mice, oral administration of 0.25% Cinnamon decreased the level of lipid peroxidation in the colon (Bhattacharjee et al., 2007).

Moreover, mice subjected to the Cinn regimen did not develop significant hepatocellular damage, as evidenced by serum activities of GOT, GPT, ALP and bilirubin concentrations with the subacute administration of Cinn, indicating normal cellular redox homeostasis. However, we did find binucleated hepatocytes, but hepatocytes commonly have double nuclei and polyploidy and sinusoidal congestion, and the histological appearance of the control and Cinn mouse liver is typical of this species. These incidental findings occurred during the experiment and were therefore interpreted as biological variability normally observed in mice (French & Kristan, 1994; Zaio et al., 2018).

Furthermore, according to the Central Evaluation Group on Pesticide Residues, World Health Organization (FAO & WHO, 2025), liver hypertrophy is one of the most common effects in toxicity tests. However, in many cases, it is an adaptive rather than an adverse effect, although the distinction is not always easy to make. The guideline states that in the absence of histopathological signs of damage (such as necrosis, inflammation or fibrosis) and relevant changes in clinical chemistry (AST; ALT; FA bilirubin), hypertrophy should not be identified as an adverse event at the dose level that only induces hepatocellular hypertrophy and increased liver size (FAO & WHO, 2025). In line with the above, our results suggest that this variation is due to an adaptive phenomenon and not an adverse event.

In addition, one of the most relevant findings of this study is that Cinn has no adverse effects on reproductive physiology, according to other authors (Faddladdeen, 2022). Spermatozoa are also particularly susceptible to peroxidative damage, indicating toxicity (Baiardi et al., 1997; Zaio et al., 2018). The epididymal transit time of spermatozoa in mice is approximately 1 week (Kempinas et al., 1998). The treatment period in our study was set at 2 weeks for maximum effect, therefore long-term administration of Cinn

maintained sperm motility, suggesting that Cinn does not affect the motility of mouse spermatozoa.

Obesity results from an imbalance between energy expenditure and intake, and Cinnamon, used as an herbal medicine and spice, has been used for thousands of years in traditional Chinese medicine for obesity and diabetes. There are numerous reports on the role of Cinn, although little is known about the mechanism of action of this phenylpropanoid intrarectally in the body weight of mice (Camacho et al., 2015; Hul et al., 2018; Mousavi et al., 2020).

The present study shows that Cinn induces body weight loss without affecting food consumption in animals. However, Hébert et al. (1994) described decreased body weight and food consumption in rats treated with Cinn at a dose of 73.5 mg/kg bw/day for 90 days.

Cinnamon has been reported in several studies to contain a reasonable amount of phenolics and flavonoids. Polyphenolic and flavonoid compounds are important secondary metabolites in plants, along with the essential oils. Cinnamon bark essential oil was found to be a unique natural source of aromatic monoterpenes, with Cinn as the major constituent. These components have been previously described to have a probable anti-obesity effect (Wang et al., 2009).

Taken together, these results suggest a potential anti-obesity effect of Cinn. Rectal Cinn administration could modify the intestinal microbiome composition, which will be studied in the future. Further studies are needed to confirm our results and the potential side effects of Cinn intra-rectal administration.

Limitations: This study has several limitations. First, the number of animals per group was relatively small, and the evaluation period was short. The sample size was determined based on the 3R principle (Replacement, Reduction, and Refinement) as described by Hubrecht & Carter (2019) and on previous trials conducted by our group (Zaio et al., 2018), which provided sufficient statistical power to detect significant differences. Second, the treatment period was limited to a subacute regimen, informed by our prior acute administration study (Zaio et al., 2018). Third, it should be noted that no standard pharmacological agent was included as a comparator in the present study, since our primary objective was to evaluate the safety profile and neurological/physiological responses to rectally administered Cinn. Future investigations will address this limitation by incorporating established reference drugs (e.g., diazepam for anxiolytic-like effects or donepezil for memory performance) in order to better contextualize the effects of cinnamaldehyde. Fourth, the physicochemical properties of Cinn (odor, lipophilicity, and hepatic metabolism) influenced the choice of the rectal route, the use of an organic diluent (sunflower oil), and the inclusion of a vehicle control group to account for potential diluent effects. Taken together, these factors may influence the generalizability and reproducibility of our findings. Nevertheless, the consistency of our results with previous reports supports the validity of the data.

5. Conclusion

Subacute intra-rectal administration of Cinn at 2000 mg/kg/day for 15 days in male C57BL/6J mice did not produce neurotoxic, hepatotoxic, or reproductive adverse effects. The treatment was well tolerated and associated with reduced body weight gain without changes in food or water intake.

These results support the safety of Cinn and suggest potential applications in metabolic research, particularly in the context of obesity. Further studies are warranted to elucidate the mechanisms underlying its effects on body weight regulation and to confirm the long-term safety of this administration route.

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Conflict of Interest: Declare any potential conflicts.

There is no conflict of interest in our study

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Author Contributions: Detail the specific contributions of each author.

EB, YPZ: participated in the animal experiment, preclinical observation, semen analysis, locomotor activity and FOB evaluation.

NASL: participated in macroscopic and histological analyses and serum biochemical assay.

VBO: collaborated in methodology writing, figure design and reviewed the manuscript.

MPZ, AAP: conceptualized and organized the experiments, data curation, writing-reviewing and editing the manuscript.

Ethical Information.

All experimental procedures involving animals were performed in accordance with the international guidelines for the care and use of laboratory animals and were approved by the [CICUAL; Animal Care and Use Committee / Ethics Committee]. Male C57BL/6 mice (3–5 months old, 25–35 g) were housed under standard laboratory conditions with free access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Statements and Declarations

The authors declare that the investigation was conducted in the absence of any commercial or financial relationships that could be considered potential conflict.

Data Availability Statement: Indicate the availability of data and supplementary materials.

The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included in the paper.

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