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The effect of *Cecropia glazioui* SNETHLAGE on the physical and neurobehavioral development of rats

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This study aimed to determine the LD50, toxic effects on organogenesis/fetal-development, physical aspects, and developmental/neurobehavioral reflexes of litters previously exposed to *Cecropia glazioui* SNETHL (Cg) extract during the entire gestational period. Swiss mice were submitted to doses of 0.5, 1.0, 2.5 and 5.0 g/kg/p.o. Female rats received 1.0 g/kg/day of Cg extract (G1, $n = 10$) or 1.0 mL/kg/day of deionized water (G2, $n = 10$) during pregnancy. The number of successful gestations, pregnant females weight and born/dead-born offsprings were evaluated. Physical development (offspring weight; fluff and hair appearing; ear unsticking and opening; incisor teeth eruption; eyes opening; testis descent; vagina opening; rearing frequency; uprightness latency and negative geotaxis) and the sleeping time (30 mg/kg/i.p. sodium pentobarbital assay) were also observed. Open field assay evaluated the developmental/neurobehavioral reflexes of pups. LD50 was higher than 5.0 g/kg. The extract did not affect the gestation number, born/dead-born offspring number and the female weight during pregnancy. The weight and the physical development of both genders pups were not affected ($p > 0.05$), but the uprightness latency and the negative geotaxis reflexes were enhanced and the rearing frequency decreased ($p < 0.05$). Ambulation, cleaning activity, sleeping time, and immobility were not affected ($p > 0.05$). We concluded that Cg extract showed low toxicity to pregnant rats and their litters.

1. Introduction

Cecropia glazioui SNETHLAGE (Cg), Cecropiaceae family, which is usually called "embauba-vermelha" in Brazil, has been used in popular medicine of many Latin American countries due to its antihypertensive, cardiotonic, antiasthmatic and anxiolytic-like properties (Rocha et al. 2002). Other *Cecropia* species, such as *C. obtusifolia,* have shown peripheric analgesic and anti-inflammatory effects (Perez-Guerrero et al. 2001).

The administration route for Cg preparations is usually the oral ingestion of an infusion obtained from leaves (Nicasio et al. 2005). The environmental parameters that could affect Cg's pharmacological activity were previously studied (Valio and Sacarpa 2001; Klumpp et al. 2002). In addition, the methodology to obtain the infusion (Heberle et al. 2000) and some of Cg's pharmacological activities (Lapa et al. 1999; Rocha et al. 2002) were also reported. The individual phytochemical constituents of Cg were previously determined and include glycosides, lipids, alkaloids, flavonoids (isoorientin and chlorogenic acid), tannins, cardenolides, triterpenes, polyphenols, steroids, and resins (Taylor 2003).

There is little information about the toxicity of Cg on gestation. In fact, few medicinal plants have been properly studied to prove their safety and efficacy during pregnancy, in spite of the large use of medicinal plants to treat a range of diseases in pregnant women (Oliveira and Akisue 1998; Calixto 2000). Their natural origin of herbs does not guarantee absence of adverse effects (De Smet 2004). The current Brazilian legislation (Brazil 2004) requires research regarding phytotherapies safety, including embryotoxicity (or prenatal toxicity) studies. The use of plants during pregnancy and breastfeeding demands a critical evaluation regarding exposure period, dose and fetal/neonatal susceptibility (Gerenutti et al. 1991).

Some medicinal plants, such as *Symphytum officinale* L., *Ruta graveolens* L., *Peumus boldus* Mol., *Luffa* ssp., *Aristolochia triangularis* Cham., *Artemisia absinthium* L. and *Momordica charantia* L., have been recognized as toxic and must not be used during gestation (Mengue et al. 2001).

The aim of the present investigation was to determine the LD_{50}, of *C. glazioui* and to study the possible toxic effects of Cg aqueous extract on organogenesis and fetal development of rats. Physical aspects and developmental-neurobehavioral reflexes were also determined.

2. Investigations and results

The *Cecropia glazioui* SNETHL specimen were collected in Tapirai city (State of São Paulo, Brazil). A voucher speci-

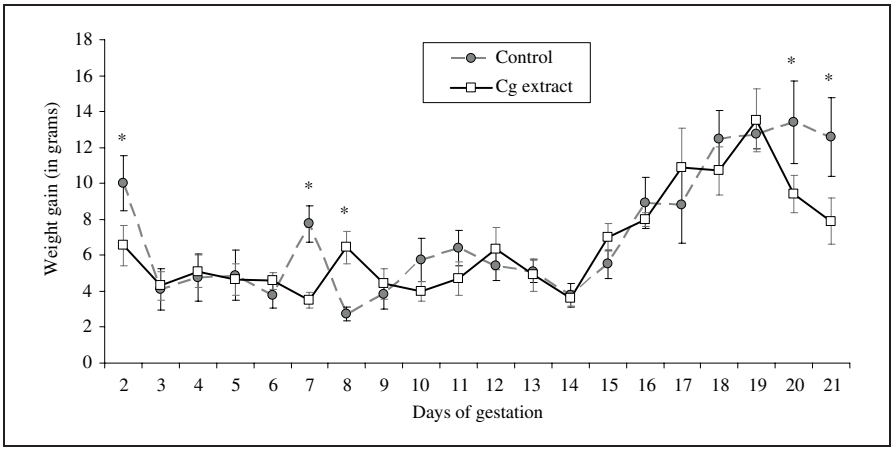

Fig. 1:
Effect of the *Cecropia glazioui* SNETHL aqueous extract on the weight gain of pregnant rats (data are presented as mean ± SD, * $-p < 0.05$, t-test).

men has been deposited on the herbarium at University of Sorocaba (Uniso) after identification was carried out in the Botanic Institute of São Paulo (Brazil) as authenticated by Dr. Sérgio Romaniuc Neto (PQC IV, Especialidade Florística de Mata Atlântica/Taxonomia de Moraceae).
The results of the acute toxicity assay show that the general activity of mice was slightly reduced with a 5.0 g/kg dose of aqueous extract of Cg. A cyanosis of small proportion was observed in 10% of mice (10% of male and 10% of female) with the same dose. No death was observed with any of the tested doses.

2.1. Reproductive ability evaluation and offspring survival

The total number (mean ± SD) of alive-born pups were 121 (12.1 ± 0.74) and 123 (12.3 ± 0.48) pups for Cg and control groups, respectively. Only two pups of the Cg group born died. There is no statistically significant differences (Mann-Whitney, $p > 0.05$) between the number of alive-born pups of both groups.

2.2. Weight gain of pregnant females

No differences (t-test, $p > 0.05$) considering water ingestion and food consumption (data not shown) were observed between dams of groups 1 and 2. Figure 1 shows the mean (± SD) weight gain of the pregnant rat females during the gestation period. Despite the statistically significant differences between both groups in some periods, in general, Cg extract did not affect the weight gain of females during pregnancy.

2.3. Evaluation of pups physical development

2.3.1. Offspring weight

All alive-born pups remained alive until the end of assays. Figure 2 shows the weight gain of both male and female pups previously exposed to aqueous Cg extract during pregnancy. Despite some statistically significant differences between the groups in both genders, in general Cg extract did not affect the weight gain of both male and female offsprings.

2.3.2. Physical development parameters

Table 1 shows the results of physical development parameters analyzed considering the effective time (in days) necessary to verify the initialization of each parameter. There were no statistically significant differences ($p > 0.05$, Litchfield test) between groups 1 and 2, which indicated that Cg extract did not interfere the with physical development of both male and female pups.

2.4. Neurobehavioral development assay

2.4.1. Latency for uprightness

Figure 3 shows the latency for uprightness for both male and female pups. There were statistically significant differences ($p < 0.05$, t-test) considering both genders, which indicated that the Cg extract enhanced the uprightness reflex in both genders during a short period.

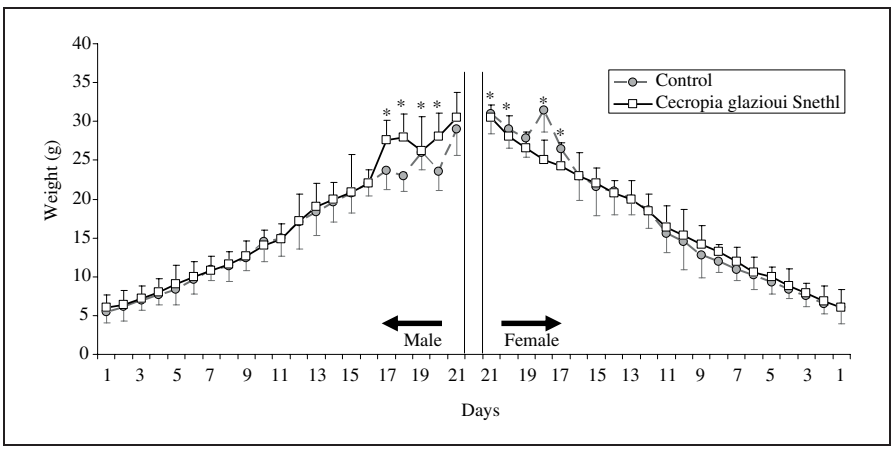

Fig. 2:
Weight gain of male (40 animals per group) and female (40 animals per group) pups exposed to Cg aqueous extract (white squares) or deionized water (Control – grey circles) during the pregnancy (data are presented as mean ± SD, * $-p < 0.05$, t-test).

Table 1: Mean time (in days) necessary to development of each physical parameter considering male and female pups exposed to aqueous Cg extract or deionized water (control) during pregnancy

Parameters	Male		Female	
	Control (n = 40)	Cg extract (n = 40)	Control (n = 40)	Cg extract (n = 40)
Fluff appearing	3.5	3.5	3.5	3.5
Hair appearing	5	5	5	5
Incisor teeth eruption	9.5	10.5	9.5	10.5
Ear unsticking/ ears opening	14.5	15	14.5	14.5
Eyes opening	15.5	16	15.5	15.5
Adult gait	14	14	13.5	13.5
Testis descent	20	20.5	–	–
Vagina opening	–	–	43	44

2.4.2. Negative geotaxis

Figure 4 shows the negative geotaxis for both male and female pups. There were statistically significant differences ($p < 0.05$, t-test) considering both genders, which indicated that the Cg extract also enhanced the negative geotaxis reflex in both genders during a short period.

2.4.3. Open field assay

Figures 5 and 6 shows the results on the general physical activity (ambulation, rearing frequency, cleaning activity, time of immobility and adult gait) of male and female pups, respectively, during the open-field assay. Despite statistically significant differences observed sporadically between groups considering ambulation (Mann-Whitney test), cleaning activity (Mann-Whitney test) and immobility (t-test), only the rearing frequency (Mann-Whitney test) showed substantial evidence of Cg extract interference ($p < 0.05$) mainly on female pups.

Table 2: Pentobarbital sleep inducing time and sleeping time of both male and female pups exposed to Cg aqueous extract or deionized water (control) during pregnancy

		Sleep inducing time (min)	Sleeping time (min)
Female	Control (n = 10)	8.00 ± 1.73	83.45 ± 8.29
	Cg extract (n = 10)	9.00 ± 1.35	72.22 ± 6.00
Male	Control (n = 10)	6.72 ± 3.87	72.81 ± 13.54
	Cg extract (n = 10)	5.50 ± 1.71	77.80 ± 14.26

data are presented as mean ± SD; * $-p < 0.05$, t-test

2.4.4. Pentobarbital sleeping time assay

Table 2 shows the effect of Cg extract on pentobarbital effect. There were no statistically significant differences ($p > 0.05$, t-test) between groups regarding both sleep inducing time and sleeping time after 30 mg/kg/i.p. sodium pentobarbital injection.

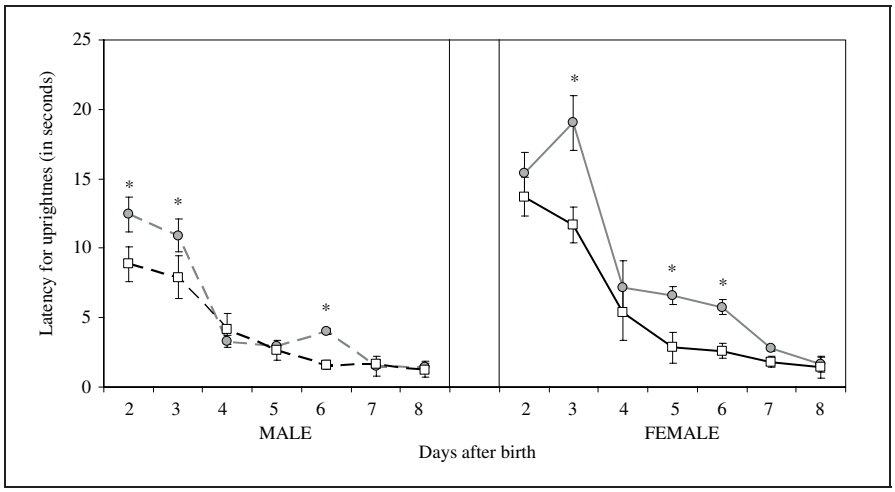

Fig. 3:
Latency for uprightness of male (slashed lines) and female (continuous lines) pups exposed to Cg aqueous extract (white squares) and deionized water (gray circles) during the pregnancy (data are presented as mean ± SD, * $-p < 0.05$, t-test).

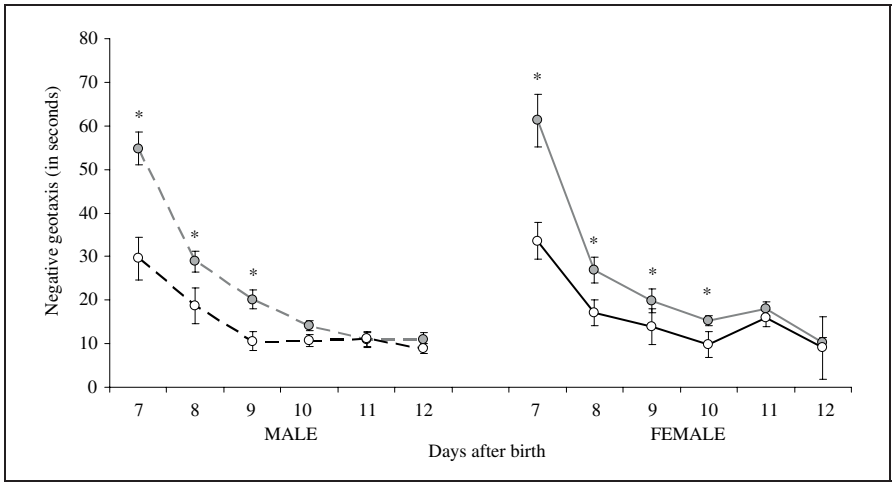

Fig. 4:
Negative geotaxis of male (slashed lines) and female (continuous lines) pups exposed to Cg aqueous extract (white squares) and deionized water (gray circles) during the pregnancy (data are presented as mean ± SD, * $-p < 0.05$, t-test).

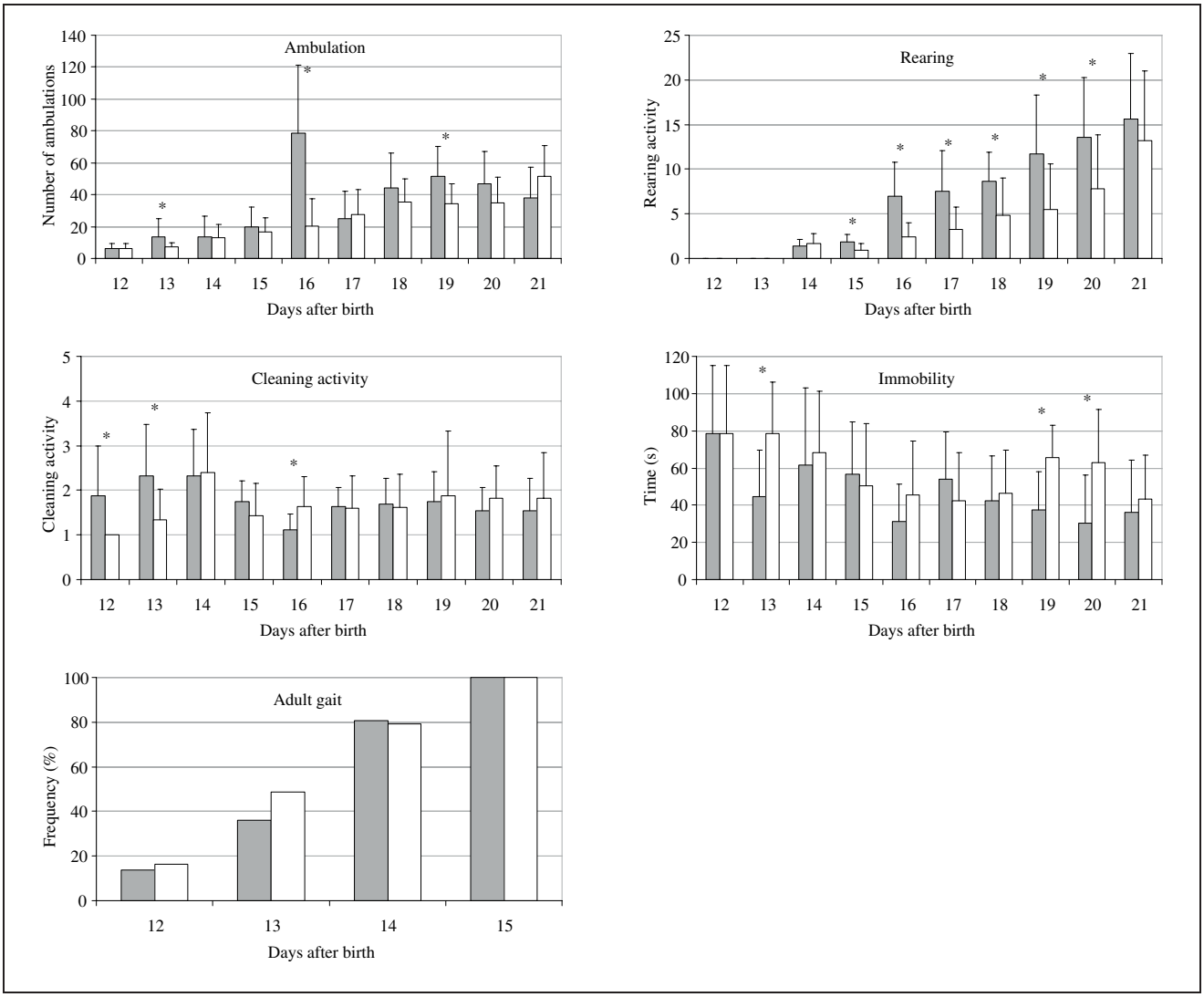

Fig. 5: General activity of female pups exposed to Cg aqueous extract (white bars) or deionized water (grey bars) during the pregnancy (data are presented as mean ± SD, * $-p < 0.05$, t-test or Mann-Whitney test).

3. Discussion

The methodology used in the present study was similar to that of other studies that observed the acute toxicity (Perez-Guerrero et al. 2001), the reproductive ability (Rayburn et al. 2000; Gerenutti et al. 2005), the physical development of rat offsprings (Gerenutti et al. 1992), and the neurobehavioral development, which included rearing (Rocha et al. 2002), negative geotaxis (Schwarz et al. 2003), open field (Batatinha et al. 1995), and pentobarbital sleeping time (Batatinha et al. 1995; Amos et al. 2001).

The weight gain of the animals exposed to any chemical agent during a specific period, is one of the most used parameters used to determine toxic effects (Gerenutti et al. 1992, 2005). The weight gain of the pregnant rat females in the present study was not affected by Cg extract during the gestational period, probably because the water ingestion and the food consumption did not differ between both groups.

The toxicity of Cg extract could be considered not significant since only a dose five-times higher than the dose administered to the pregnant-rat females was able to slightly reduce the animals general activity and no death was observed. This observation is not valid for other *Cecropia* species. *Cecropia obtusifolia* Bertol showed a median lethal dose (LD50) of 1450 mg/kg (Perez-Guerrero et al. 2001).

Pregnant females were treated with 1.0 g/kg, which is the dose also mentioned in other pharmacological reports (Rocha et al. 2002; Lapa et al. 1999). Nevertheless, in the present study, the females were submitted to treatment with Cg extract for twenty-one days, which is far more than the three doses used in previous studies.

Both, alteration of physical parameters of litter and the number of postnatal deaths are important indicators of perinatal toxicity (Leonard 1982). The reproductive ability of females was not affected by Cg extract, but the two died-born pups (approximately 1.67% of the total number of mice) belonged to the Cg group, which could indicate a possible toxic effect of the 5.0 g/kg Cg extract. However, all live-born pups of both groups remained alive and, in general, the weight gain of pups, irrespectively of genders, were not affected by Cg extract.

The exposure to chemical agents during pregnancy is one of the main factors that could interfere in the offspring physical development (Del Fiol et al. 2005) and cause behavioral abnormalities (Lazarini et al. 2004). The Cg extract did not interfere with any of the physical development parameters of both male and female pups observed in the present study. This fact could indicate that the ex-

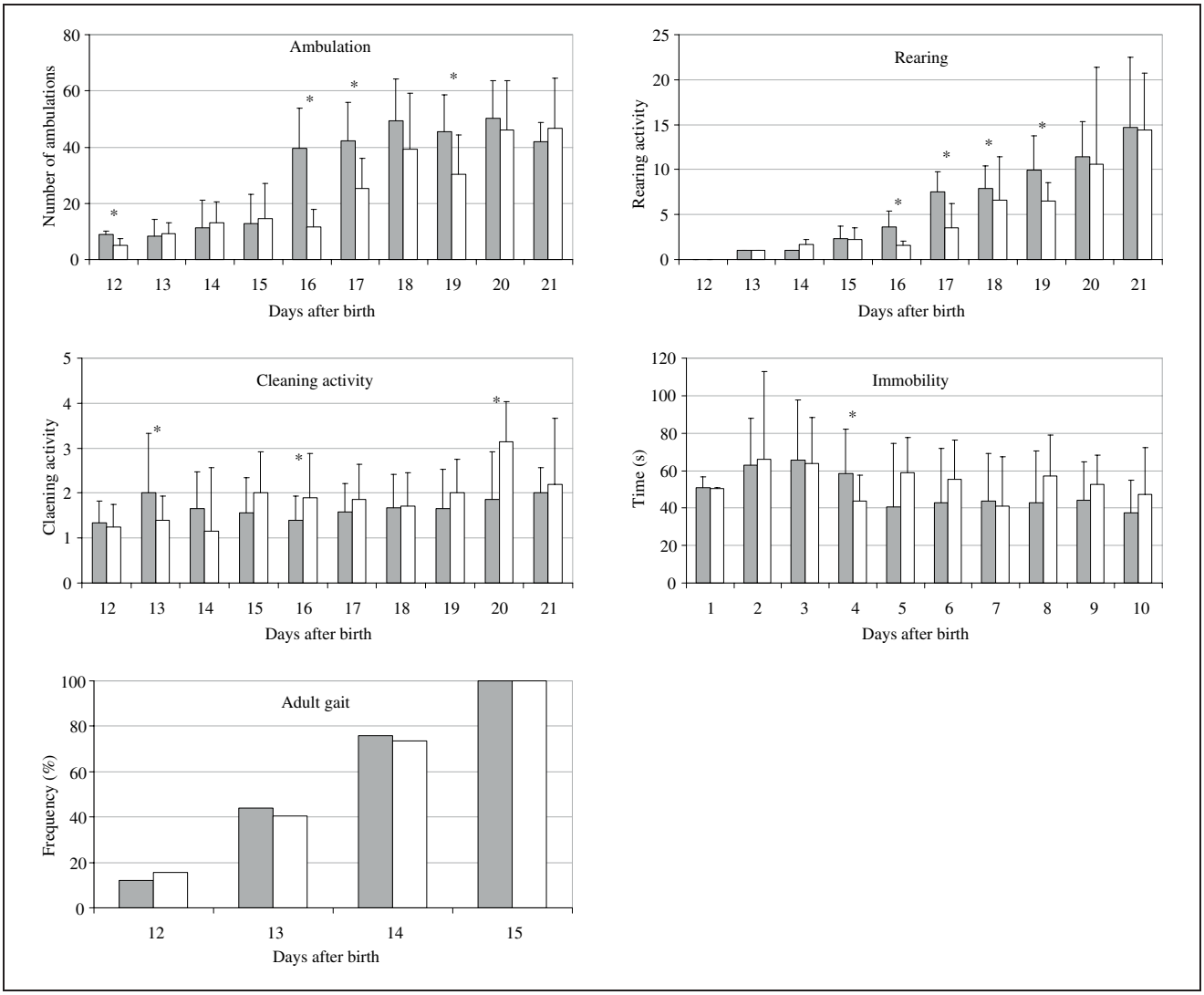

Fig. 6: General activity of male pups exposed to Cg aqueous extract (white bars) or deionized water (grey bars) during the pregnancy (data are presented as mean ± SD, * $-p < 0.05$, t-test or Mann-Whitney test).

tract did not affect the epidermal tissues, which originates fur, ear, incisor teeth and eyes, or the hypothalamic-pituitary-gonadal axis, which is responsible for the testis descent and vaginal opening. The development of fur, the unfold of ears, the eruption of the incisors and the opening of eyes depend on the activity of epidermal growth factor (Cohen 1962; Popliker et al. 1987; Lu et al. 2005), which was probably not affected by Cg extract because none of this parameters was affected in the present study.

The latency for uprightness was higher, considering both male and female pups, in the Cg group. The female pups appeared to be more affected than males, but the uprightness reflex of both genders showed a tendency to match with the time observed in the control animals. The negative geotaxis was also higher in the Cg group, considering male and female pups, but the results of this parameter observed along the time of both genders also showed a tendency to match with the ones observed for the control group. These results together suggest a reduced development of the neuromuscular function in the Cg group.

Cecropia species contain many flavonoids, which are capable to depress the CNS. Previous studies showed that these substances have affinity for the central benzodiazepine receptors (Paladini et al. 1999; Rocha et al. 2002). Lapa et al. (1999) showed increasing levels of 5-HT (probable receptor for buspirone, an anxiolytic agent) in the hippocampus of rats treated with Cg aqueous extract. This benzodiazepine-like activity could explain the CNS depression, which could be characterized by the increased latency for uprightness and negative geotaxis, observed in the pups of the present study. However, diazepam causes the immediate-evasion reflex missing in rats, but it had no effect on the litter size and in the negative geotaxis in three-week pups, which mothers received 10 mg/kg/day diazepam from day 7 to 21 of pregnancy (Laitinen et al. 1986). Similarly as observed in the present study, these authors classified the changes caused by diazepam in the motor development of pups (aging 2-to-3-weeks) as transient. In addition, Cg extract did not influence the pentobarbital sleeping time confirming that the extract is not a general CNS depressant.

Some of the general physical activity (ambulation, rearing frequency, cleaning activity, time of immobility and adult gait) of male and female pups showed sporadically differences between groups, all differences have a tendency to disappear with animal aging.

Considering all results together, the use of an aqueous extract of *Cecropia glazioui* SNETHL during pregnancy slightly interfered with the physical and neurobehavioral development of the offspring and the physical parameters

of their mothers. These results reinforce the importance of studies in this area, in order to clarify possible toxic effects of this commonly used medicinal plant.

4. Experimental

4.1. Preparation of the aqueous fraction extract

Fresh leaves (450 g) without petiole were dried, powdered and a hydroalcoholic (70%) extract was obtained by percolation. The extract was concentrated under reduced pressure and lyophilized providing 102.3 g of powder (22.7% efficiency). It was stored at room temperature without light and humidity until the toxicological assays were performed. The aqueous extract was freshly prepared in distillated/deionizated water before oral admonistration.

4.2. Animals

Adult Swiss mice weighing 25 g to 30 g and Wistar rats weighing 160 g to 200 g, of both gender, were obtained and kept in the UNISO/Pharmacy School facilities according to "The Guide for the Care and Use of Laboratory Animal" (National Research Council 1996) and "European Community guidelines" (EEC Directive of 1986; 86/609/EEC). All animals were maintained in groups (10 mice or 5 rats per cage) with food and water *ad libitum*, except during the experimental days. A twelve hour light/dark cycle and constant temperature ($23 \pm 1\,^{\circ}C$) was maintained. All animals were previously adapted to laboratory conditions during one week before the experiments. The study design was approved by the UNISO Ethical Committee for Experiments.

4.3. Acute toxicity assay (LD_{50})

This assay was carried out according to previous studies (Perez-Guerrero et al. 2001). Fifty mice (50% of each gender) were distributed into five groups (one control and four experimental) of five animals of each gender. Experimental groups received 0.5, 1.0, 2.5 and 5.0 g/kg/p.o. of Cg aqueous extract (w/w). Animals in the control group received the vehicle (deionized water).

4.4. Reproductive ability evaluation

Twenty sexually-naive rat females were mated with males, which were previously tested as fertile (two females and one male per cage). After mating, the pregnancy of female rats was confirmed through the presence of spermatozoids in vaginal-washing rubbing observed by microscopy analysis (Vickery and Bennett 1970). The presence of spermatozoids was considered as the first day of pregnancy.

Pregnant females were kept in separate cages. Water and food were supplied *ad libitum* during all the experiment and the consumption of both was monitored daily. Right after the confirmation of pregnancy, ten females received 1.0 g/kg/day of Cg extract (group 1) by gavage and other ten animals received 1.0 mL/kg/day of deionized water (group 2) from days 0 to 20 of pregnancy. The following parameters were evaluated in order to observe the reproductive performance of rats:

a) Number of gestations carried out considering the number of matings;
b) Weight gain of pregnant females: the daily weight gain of females among the second and the twenty-first days of gestation was measured;
c) Total number of born offsprings;
d) Number of dead- and alive-born offsprings;

The number of offsprings was standardized, being each female allowed to keep only eight pups and the others pups were discarded (Rayburn et al. 2000). The number of male and female offsprings was equally distributed for each rat dam.

4.5. Evaluation of the physical development of rat offsprings

The parturition day was defined as first day of life of the litter. On this first day, the offspring was examined externally (macroscopically) and sexed. The same male and female pups were used for the physical and developmental tests. Pups that had been exposed prenatally either to Cg extract or to the placebo undertook the physical development parameters and the behavioral tasks at the same time during the infancy. The following physical development parameters were observed (Gerenutti et al. 1992):

a) Offspring weight: The weight of each offspring was recorded daily during the entire lactation period, i.e., during 21 days;
b) Fluff and hair appearing: The observation of fluff started on the first day after birth and the hair started from the first fluff observation.
c) Ear unstitching and opening: The ear unstitching was observed from the first day of birth until both ear unstuck. The ears opening day was considered when the external-conduit orifice of ears appeared, the folds of ear pavilion were visible and the response to sound stimulation first occurred. This evaluation started right after the verification of both ears displacement;
d) Incisor teeth eruption: Started from the sixth day after birth, the eruption was considered when the inferior or superior incisor first appeared outside gingival mucosa;
e) Eyes opening: The observation initiated in the tenth day after birth and when the eyelid fissure started it was considered as the first day of eyes opening. The number of eyes opened per group was daily recorded;
f) Testis descent: Each male pup was hold in vertical position and the scrotal pouch was touched in order to feel the testis inside. The observation initiated in the twenty-first day after birth;
g) Vagina opening: The observation initiated in the thirty-fourth day after birth and stopped when the vaginal orifice was first noted.

4.6. Neurobehavioral development assays

4.6.1. Latency for uprightness

Each pup was placed in supine position and the first day they successfully took the ventral position (within a period of the time not exceeding 15 s) over their four paws was recorded as the "latency for uprightness". This parameter was evaluated from the second to the seventh day after birth. Pups were observed daily in the morning (between 8 and 11 h). They were separated from their mother during observation (no more than 3 min) and immediately returned to cages after the test.

4.6.2. Negative geotaxis

This assay was performed in order to test vestibular and postural reflexes requiring motor coordination for successful completion. Each pup was placed in prone position with its head down on a 45° inclined ramp (abrasive surface). The time the pups required to turn their head 180° and to make the first step in this new direction (within a period of time not exceeding 30 s) were recorded. This parameter was evaluated from the seventh to the twelfth day after birth. In this test, pups were also observed daily in the morning (between 8 and 11 h). They were separated from their mother during this assay for not more than 3 minutes and immediately returned to the cages.

4.6.3. Open field assay

This test was used to evaluate the spontaneous locomotive and exploratory activity of both male and female pups (adapted from Broadhurst 1960; Batatinha et al. 1995). The open-field was a cylindrical container made of transparent acrylic (40 cm diameter and 40 cm height walls). The open-field's floor had three concentric circles divided into 25 segments of equal area by lines radiating from the center. During the experiments, light was provided by a 40 W white bulb located 72 cm above the floor providing continuous illumination of the area. The room was also sound-attenuated. Hand-operated counters and an electronic timer were used to score the following parameters:

1) ambulation: number of floor units entered using four paws;
2) rearing frequency: number of times an animal stood on its hind legs;
3) cleaning activity: number of times an animal licked its paws or fur;
4) time of immobility: total time (in seconds) without spontaneous movements;
5) adult gait: the day the animal walked as an adult, i.e., walked over four paws without propping their abdomen on the floor.

The measure of these parameters started when the offspring were 12 days-old and ended at 21 days after birth. The pups were individually placed on the center of the open-field container and the behavioral parameters have been observed for 3 min. Before the introduction of the next pup, the open-field container was cleaned (5% ethanol) in order to avoid possible odors. Both control and experimental animals were intermixed during this assay.

4.6.4. Pentobarbital sleeping time assay

This assay was conducted as previously described by Batatinha et al. (1995) and Amos et al. (2001). Briefly, each animal was injected with 30 mg/kg/i.p. sodium pentobarbital. The time (in minutes) elapsed from the injection to the loss of the uprightness reflex (induction time) and the time from the loss of uprightness reflex to awakening (duration of sleeping) were both registered during four hours.

4.7. Statistical analysis

Data from the different assays were first analyzed regarding distribution and variance homogeneity. Normally distributed data were submitted to comparison between both groups by using Student's *t*-test. Non-normally distributed data were first transformed (log). The Student's *t*-test was also used when the transformation changed the distribution to normal or the Mann-Whitney (Wilcoxon rank sum test) was used if the distribution was still non-normal. The Litchfield and Wilcoxon test (Litchfield 1949) were used for evaluation of physical development parameters. The significance level was set at 5%.

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