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Luffa operculata at a late period of gestation dysregulates melatonin and cytokines interfering with weight of dams and their male offspring

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A B S T R A C T

Ethnopharmacological relevance: The tea made with the fruits of *Luffa operculata* (L.) Cogn. (Cucurbitaceae; EBN) is popularly used as abortive.
Aim of the study: The present work aimed at accessing how the exposition of female Wistar rats to 1.0 mg/kg of EBN (experimental group, EG), or distilled water (control group, CG), by gavage, at gestational days (GD) 17–21 interfered with the reproductive performance, and with dams' behavior after weaning.
Materials and methods: At post-natal day 2 (PND2), the number of male and female pups was evaluated, as well as their weight. After weaning (PND21), dams were euthanized, and their liver and kidneys were removed for histological and biochemical analyses, while the blood was used in the evaluation of cytokines IL-1α, IL-1β, IL-6 and TNF-α, corticosterone, adrenocorticotrophic hormone, melatonin, AST, ALT and creatinine levels.
Results and Discussion: Dams that were treated with EBN showed an anxiety-like behavior, weight loss at the end of gestation and weight gain at weaning, accompanied with a significant decrease in pro-inflammatory cytokines and in the melatonin level. No significant histological or biochemical alterations have occurred in the liver or kidneys. The number of female pups was significantly higher in the EG. The male pups showed weight gain at PND60.
Conclusion: The presence of cucurbitacins is probably involved in the dysregulations that were found, due to their polycyclic steroid triterpene structure.

1. Introduction

The dried fruit of *Luffa operculata* (L.) Cogn., Cucurbitaceae, is popularly known as *buchinha-do-norte*, in Brazil, and it is inadvertently and popularly used in the abortion induction (Revilla, 2002). The basionym of *L. operculata* is *Momordica operculata* L. The plant is a liana, native to Brazil, although not endemic. The plant can be found in Cerrado and in Mata Atlântica forests, and there is register of the occurrence of the species in the states of Amazonas, Bahia, Paraíba, Pernambuco, Minas Gerais and Rio de Janeiro (Flora do Brasil, 2019).

Previous work (Barilli et al., 2007) has described how the oral administration of 3.0 mg/kg of the aqueous extract obtained from the fruits of *L. operculata* in different pregnancy stages can lead to fetal resorption or malformation in rodents. Also, it was reported that the oral administration of 1.0 mg/kg of the aqueous extract obtained from the fruits of *L. operculata* (EBN), for five consecutive days, have disrupted normal behaviors of adult male Wistar rats (Alves et al., 2018). Furthermore, the vertical administration of EBN impaired behavior and

Abbreviations: EBN, *buchinha-do-norte* aqueous extract; **ECAU**, Ethics Committee for Animal Use; **GD**, gestation day; **PND**, post-natal day; **CG**, control group; **EG**, experimental group; **OF**, open field; **LDB**, light-dark box; **ACTH**, adrenocorticotropic hormone; IL, interleukine; **AST**, aspartate transaminase; **ALT**, alanine transaminase; **TNF**, tumor necrosis factor.

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neurochemicals in young adult male rats (Frias et al., 2020). Reports describing the pharmacological response to homeopathic remedies made with *L. operculata* to be used against sinusitis are found in the literature (Menon-Miyake et al., 2005a). Nonetheless, there is a gap related to the information regarding the influence of the oral administration of EBN extracts on the pregnant female and on its litter.

In order to fulfill this gap, the present study aimed at prospecting the changes caused in the dams, after weaning, after receiving a dose of 1.0 mg/kg for five consecutive days during gestation, and also to evaluate how the vertical administration of EBN influenced their offspring.

2. Materials and methods

2.1. Plant extract

Dried fruits of *L. operculata* were obtained from Santos Flora (batch # BUCHO 01/0914, collect date 09/24/2014, validity: 09/24/2017, origin Brazil). The fruits were prepared as a decoct, according to its popular use. Seventeen dried fruits, weighing 34.68 g were added to 5100 mL of boiling water, for 10 min. Then, the infusion was lyophilized, and resulted in the dried aqueous extract, EBN (14.82 g or 42.73% yield).

2.2. Animals

2.2.1. Ethics

The present study was developed in accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The project was approved by the UNIP-Ethics Committee for Animal Use (ECAU), under protocol #043/2016). All efforts made to minimize animal suffering and to maximize animal well-being were conducted under the adoption of the 3Rs concept and under guides suggested by the National Institutes of Health - USA.

2.2.2. Maternal treatments and experimental design

Rat dams of the experimental group (EG) received a daily dose of 1.0 mg/kg of EBN, which was administered by gavage (Alves et al., 2018), from GD17 (gestation day 17) to GD21, while the dams from the control group (CG) received water. The CG received a volume of water correspondent to 100 mL/kg, as vehicle control. The volume of 1 mL to be administered by gavage cannot be exceeded due to animal welfare. The period of gestation spanning from GD17 to GD21 has been chosen because at these days the rat embryos reach the first stage of development. The dams gave birth and the reproductive performance was made at post-natal day 2, or PND2. After weaning, the dams were subjected to behavioral evaluation in the open field (OF) and in the light-dark box (LDB) apparatuses. Then, the dams were euthanized, blood, liver and kidneys were collected for biochemical and histological analyses. In addition, the female and male pups were weighed at PND60. Fig. 1 reports the experimental design that was proposed for the present investigation. Male and female pups (F1 generation) were divided into two groups, according to the treatment received by their mothers (F0 generation) during pregnancy (CG or EG; Fig. 1).

2.2.3. Mating and birth protocol

Female rats were donated from the School of Veterinary Medicine, University of São Paulo, Brazil. Female Wistar rats ($n_{total} = 38$), 12 to 14 weeks-old, weighing 200 g–250 g, were distributed in groups of four in propylene cages (45.5 × 34.5 × 20 cm), allocated in a controlled environment under a temperature of 22 °C ± 2 °C, controlled relative humidity of 55–65% and under artificial lighting (12 h/12 h light/dark regulation, lights on at 7:00 a.m.). Habituation to the lab conditions was done for ten days before the beginning of the experiments. Male Wistar rats aged 20–23 weeks ($n_{total} = 11$), weighing approximately 350 g, sexually experienced, were kept under the same conditions. The animals had free access to filtered water and irradiated food (BioBase®, Águas Frias, Brazil) during the study.

The mating procedure was conducted by introducing one male in a cage containing three female rats, where they were kept together up to

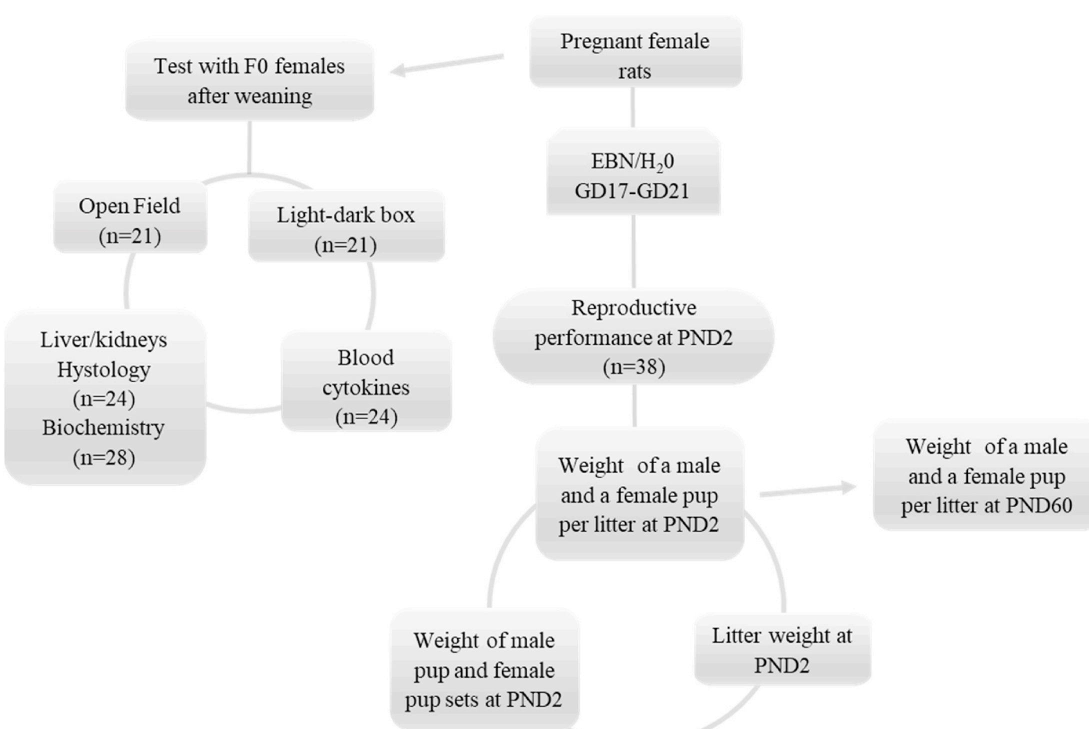

Fig. 1. Experimental design proposed for the evaluation of the oral administration of the aqueous extract obtained from the fruits of *Luffa operculata* to dam rats and their litters. EBN = *buchinha-do-norte* aqueous extract; GD = gestation day; PND = post-natal day.

the moment that the occurrence of pregnancy was verified by the presence of spermatozoids in the external vaginal smear. As soon as the females were identified as pregnant, they were again re-united, and remained together with their original companions up to GD17. At GD17, each dam was allocated to individual cages attached to a microisolator system (Tecniplast®, Buguggiate, Italy), under the same previous environmental conditions. After mating, the male rats were kept to participate in some other assays.

2.2.4. Reproductive performance at PND2

Birth occurred at GD21 ± 1. At post-natal day 1 (PND1), male and female pups (F1 generation) were not manipulated to avoid maternal rejection or cannibalism (DeSantis and Schmaltz, 1984). The weight and sex ratio were verified at PND2 to all pups. They were manipulated over a heat bed at a physiological temperature, and the gloves of the manipulator were impregnated with the cage smell by rubbing them in the shaving bed. Sex identification of each pup was made to all individuals in the litters, and was based on the distance between the sex organ and the anus. Female pups have sex organs closer to the anus than male pups (JHU, 2020; Whitschi, 1962). The number of male and female pups to each litter was also recorded. After that, one male and one female pup were individually weighed, and the litter was also weighed. Then, both female and male groups were separately weighed. At PND2, the litters were standardized in 4 males and 4 females each (Udo et al., 2014), as possible, in order to avoid differences in the litters development. At PND21, the pups were weaned and separated into males and females, in different cages, four animals in each cage. They were identified according to their mother's groups, as CG and EG, (Fig. 1).

2.2.5. Open field evaluation

OF was used in order to evaluate the influence of EBN over the locomotion and anxiety-like behavior of the animals (Hunsberger and Duman, 2007). Briefly, each animal was placed in the central circle of the arena and was evaluated for 3 min according to the following parameters: frequency of locomotion, immobility time, rearing frequency, number and time of grooming, time spent in the center of the apparatus, time spent on the borders of the apparatus and number of fecal *boli*; time in seconds (Alves et al., 2018; Estork et al., 2014, 2016; Frias et al., 2020; Gusmão et al., 2013a, 2013b; Suffredini et al., 2017).

2.2.6. Light-dark box evaluation

The animals were placed in the LDB for evaluation of anxiety immediately after the evaluation in the OF. The model is based on the aversion of rodents to illuminated areas. The LDB consists in an acrylic box unequally divided in two sides, where there is a smaller and dark side and another larger and illuminated, separated by a wall with a hole in which the animals cross. Rodents tend to feel safe in the small, dark side, but also feel a compulsion to explore the light side, which creates a conflict between remaining in the dark side or succumb to the exploratory instinct which leads to the light side.

The animals were allocated in the dark side of the box and remained under experimental conditions for 5 min. The parameters evaluated during this period were the latency to transit to the light side; the number of attempts to enter the light side; the time spent in the dark side and in the light side; the locomotion frequency in the light and dark sides; rearing frequency in light and dark sides; the immobility time in both light and dark sides; side crosses, grooming in both sides; and number of fecal *boli* in the dark and in light sides; time in seconds (Alves et al., 2018; Crawley and Goodwin, 1980).

2.2.7. Biochemical and histological studies

Blood was collected from the dams after euthanasia, at weaning. Blood was centrifuged for 15 min to remove serum, which was fractionated in three vials containing approximately 200 μL each, and then stored at −80 °C until use. Serum was used in the quantification of the inflammatory cytokines, IL-1α, IL-1β, IL-6 and TNF-α (Magpix RECYTMAG 65 K; Corrêa et al., 2018), and stress hormones as corticosterone, adrenocorticotropic hormone (ATCH) and melatonin (Magpix RESHMAG 59 K; Faria et al., 2013).

Liver and kidney samples were harvested and processed as follows. The tissues were fixed in 10% buffered formalin for 24 h at least. The histological procedures were made according to the conventional paraffin embedding and hematoxylin-eosin (HE) staining methods. An evaluation based on scores was performed considering the following parameters: a) for liver degeneration: nucleus morphology (pyknosis, karyorrhexis and karyolysis) and cytoplasm vacuolization; b) for the kidneys: glomerular hypercellularity and tubular vacuolization. All the parameters were scored from 0 to 2, as described as absence (0), moderate (1) and severe (2) (Frias et al., 2020).

Colorimetric assays were conducted to perform evaluations of aspartate aminotransferase (AST), alanin aminotrasferase (ALT) and creatinine in a biochemical analyzer Cobas C111® (Roche® Diagnostics, Basel, Switzerland; Borzouie et al., 2020), using, respectively, Cobas C111® commercial reagents AST IFCC (code: 04657543190), ALTL, (code: 04718569190) and CREJ2 (code: 04718569190).

2.3. Statistical analysis

Data were obtained based on independency, randomness and normality. All data from female F0 generation and their litters were analyzed by Shapiro-Wilk test for normality check. After that, Student's *t*-test or Mann-Whitney test was adopted to compare means resulted from the effects of the oral administration of the aqueous extract (EG) and of water (CG) to pregnant rats and their litters. Levene's test and Mauchly test were used to verify homogeneity and sphericity of data, respectively, and data are available as Supplementary Material. Eventually effect size for Student's *t*-test, Cohen's d, was calculated to some of the parameter factors, and test power reported for all calculations. Sample size was estimated based on the formula $n = 1+[2C^*(s/d)^2]$, $C= (z\alpha+z\beta)^2$, considering confidence interval of 0,95/2 (0.475), $z = 1.96$, and test power of 90% ($z\beta = 1.282$), maximum deviation of 0.2 (20%) and difference between groups of 0.5 (50%; Eng, 2003). For the reproductive performance, $N_{CG} = 20$, $N_{EG} = 18$; for behavior experiments $N_{CG} = 12$, $N_{EG} = 9$; for cytokine testing $N_{CG} = 13$, $N_{EG} = 11$; for kidneys and lungs histological analyses $N_{CG} = 15$, $N_{EG} = 9$ and biochemical analyses $N_{CG} = 16$, $N_{EG} = 12$. All tests were made in Excel® or in GraphPad Prism7.0 and significances were considered as $\alpha = 0.05$ for all analyses (Zar, 1999).

3. Results

3.1. Reproductive performance at PND2

Dams from the EG group showed diminished weight gain compared to the CG group (Fig. 2A). The dam's weight after weaning was augmented in the EG group (Fig. 2B). The dam's weight before mating was higher in the EG group (Fig. 2C) and their weight at GD21 was not different from that observed in the CG (Fig. 2D). In relation to the litters, the female pups' number (Fig. 2E) was higher in the EG, while the number of males in EG did not differ from CG (Fig. 2F). The percentage of females in the EG was higher (Fig. 2G) and the percentage of males was lower in EG (Fig. 2H).

Fig. 3 shows some more of the reproductive performance stats, as follows. No differences were observed in the female pups' weight at PND2 (Fig. 2A) and in the male pups' weight at PND2 (Fig. 2B). No differences were observed in the female pups' weight at PND60 (Fig. 2C), but the EG male pups showed more weight at PND60 than that of the males of CG (Fig. 2D). No statistical differences in the female pups' weight gain at PND60 were seen (Fig. 2E), but there was an improvement in the male pups' weight gain at PND60 in the EG, in relation to CG (Fig. 2F).

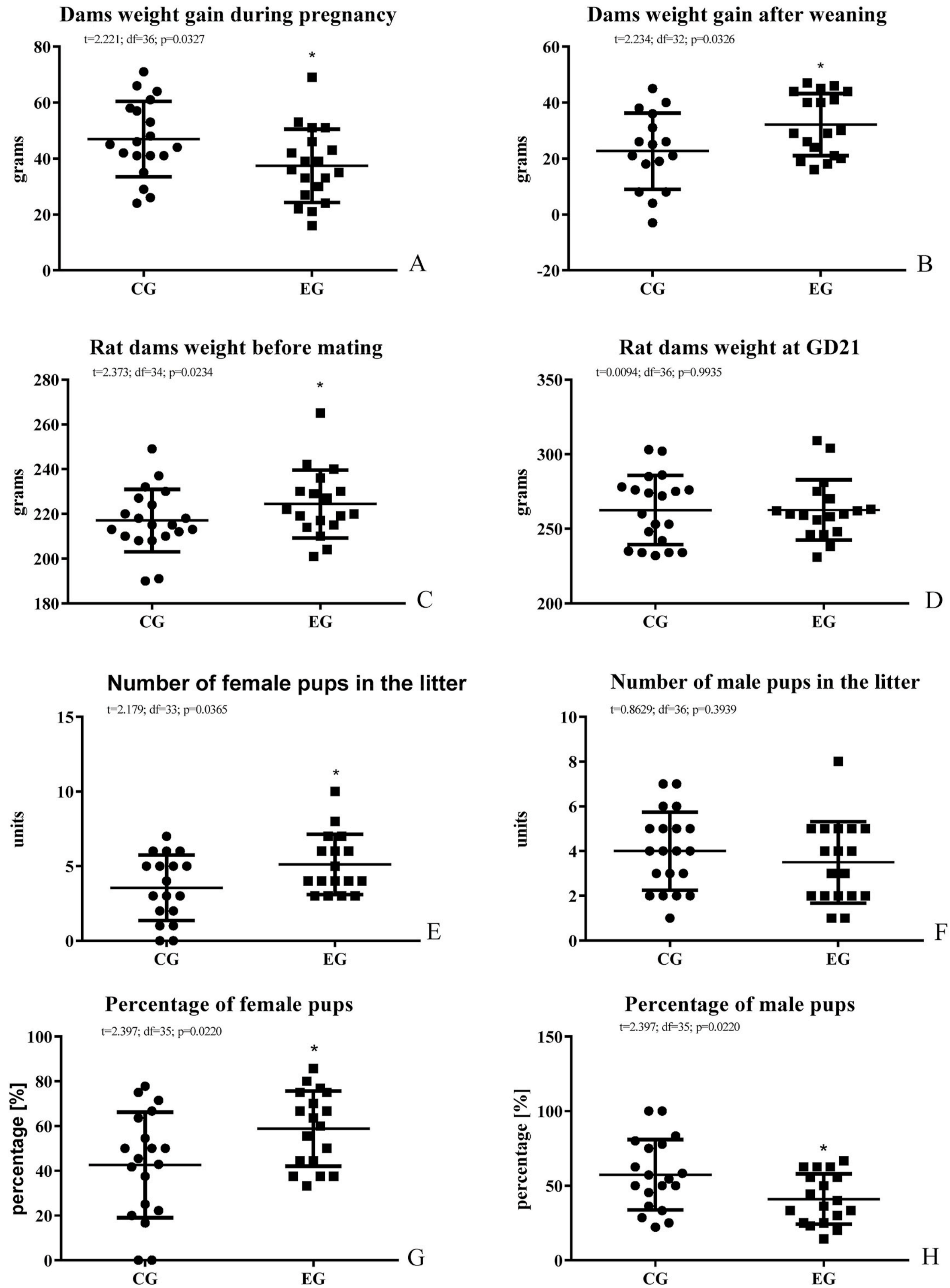

Fig. 2. Results related to the analyses at post-natal day 2, or PND2, obtained after the administration of *Luffa operculata* to the female Wistar rats from mating to weaning, and data on litter and sexing of the pups. **A**. Dams weight gain during pregnancy (CG S–W = 0.9676, p = 0.7279 and EG S–W = 0.9735, p = 0.8429); **B**. Dams weight after weaning (CG S–W = 0.9724, p = 0.8764 and EG S–W = 0.895, p = 0.0385); **C**. Rat dams weight before mating (CG S–W = 0.9568, p = 0.4825 and EG S–W = 0.9446, p = 0.3471); **D**. Rat dams weight at GD21 (CG S–W = 0.9176, p = 0.2724 and EG S–W = 0.9128, p = 0.1000); **E**. Female pup number in the litter (CG S–W = 0.935, p = 0.2373 and EG S–W = 0.8858, p = 0.0395); **F**. Male pup in the litter; **G**. Percentage of female pups in the litter (CG S–W = 0.9528, p = 0.4412 and EG S–W = 0.9329, p = 0.2182); **H**. Percentage of male pups in the litter (CG S–W = 0.9528, p = 0.4412 and EG S–W = 0.9329, p = 0.2182). Data is described as mean ± SE for all measurements. SE = standard error. The Student's *t*-test was used after Shapiro-Wilk normality analyses were performed, considering the significance at $\alpha < 0.05$ for all analyses. SE = standard error. * = $p < 0.05$.

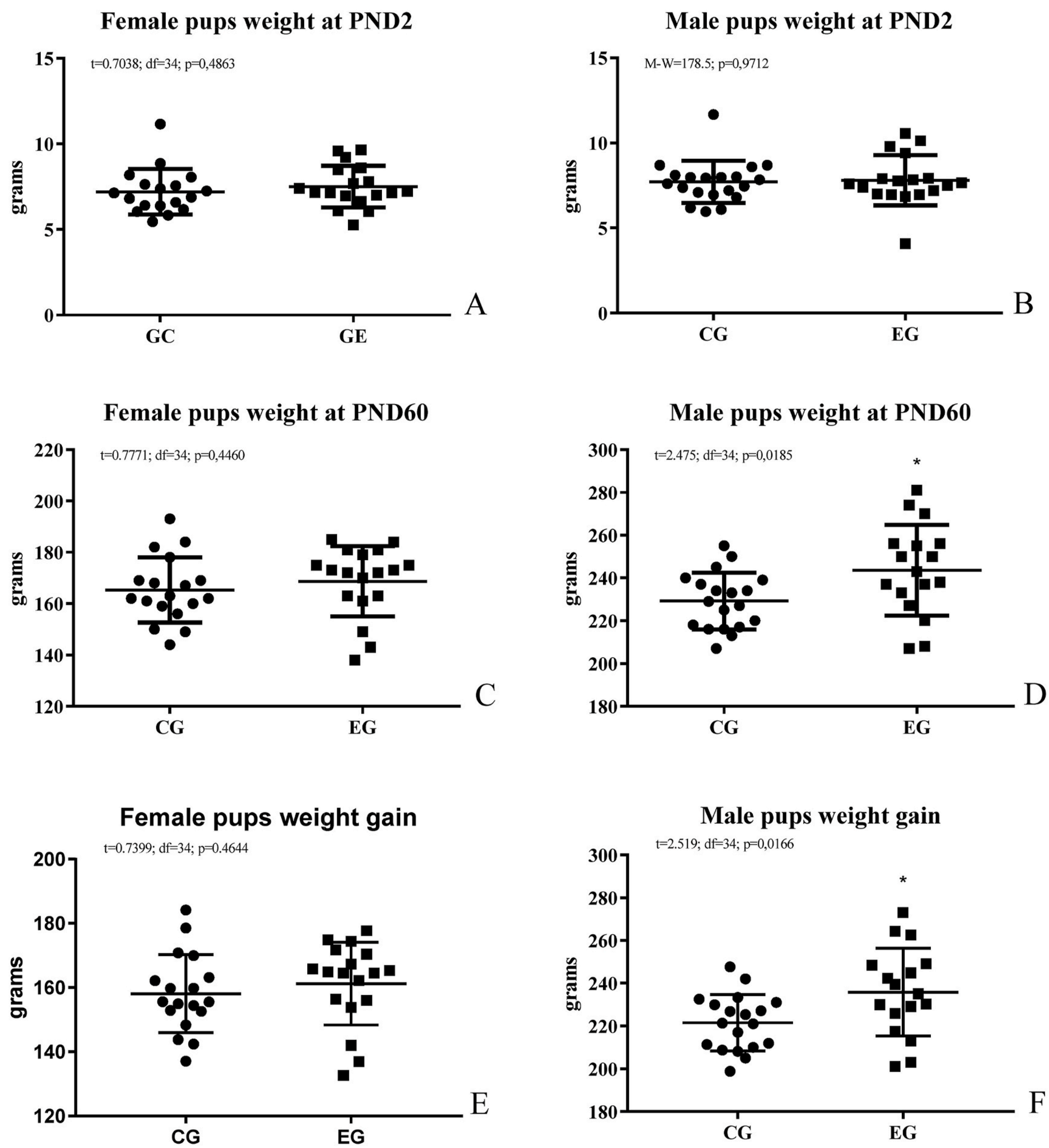

Fig. 3. Results expressing the weight of the pups from PND2 to PND60. **A.** Female pups' weight at PND2 (CG S–W = 0.8849, p = 0.0315 and EG S–W = 0.9521, p = 0.4596); **B.** Male pups' weight at PND2 (CG S–W = 0.865, p = 0.0096 and EG S–W = 0.8875, p = 0.0349); **C.** Female pups' weight at PND60 (CG S–W = 0.9637, p = 0.6744 and EG S–W = 0.8873, p = 0.0346); **D.** Male pups' weight at PND60 (CG S–W = 0.9714, p = 0.8048 and EG S–W = 0.9717, p = 0.8478); **E.** Female pups' weight gain from PND2 to PND60 (CG S–W = 0.9695, p = 0.7880 and EG S–W = 0.8943, p = 0.0321); **F.** Male pups' weight gain from PND2 to PND60 (CG S–W = 0.9737, p = 0.8468 and EG S–W = 0.9762, p = 0.9480). Data is described as mean ± SE for all measurements. SE = standard error. The Student's *t*-test was used after Shapiro-Wilk normality analyses were performed, otherwise, the two-tailed Mann-Whitney test was applied, considering the significance at $\alpha < 0.05$ for all analyses. SE = standard error. * = $p < 0.05$.

3.2. Behavioral studies for the dams

Behavioral studies results are shown in Table 1. No significant differences between groups were noted in exploratory parameters of the OF.

In the LDB, the EG rats showed more attempts to enter the light side and a higher latency to enter in the light side than rats from, although the number of side crosses were the same. In relation to the exploratory parameters, no differences in locomotion frequency, immobility time or rearing frequency were observed, as previously seen in the OF apparatus. Nonetheless, the time that rats from the EG spent performing grooming was significantly diminished in the dark side of the apparatus.

3.3. Serological studies on the dams

3.3.1. Inflammatory cytokines

In relation to CG group, IL-1α (Fig. 4A), IL-1β (Fig. 4B), IL-6 (Fig. 4C) and TNF-α (Fig. 4D) were significantly reduced.

3.3.2. Stress hormones

No statistical differences were observed in the serum levels of corticosterone (Fig. 5A), or in ACTH (Fig. 5B) in the EG, but a significant decrease in melatonin was observed in the EG, in relation to CG (Fig. 5C).

Table 1

Results obtained from the behavioral evaluation of female Wistar rats exposed to 1.0 mg/kg of the aqueous extract of *Luffa operculata* during gestation, after litter weaning. Behavioral parameters were observed in the open field and light-dark box apparatuses. Student's *t*-test or Mann-Whitney test was used after Shapiro-Wilk normality test analyses, significance if α < 0.05 considered for all tests. Data is described as mean ± SE for all measurements. CG-control group; EG-experimental group; sec.-seconds; SE−standard error; M-W-Mann-Whitney; S–W -Shapiro-Wilk.

OPEN FIELD	mean/median CG	SE/Min,Max	S–W/P	mean/median EG	SE/Min, Max	S–W/P	t/M-W	df	P
Locomotion frequency	61.08	5.26	0.9529/0.6799	68.00	16.54	0.8584/0.0920	0.4476	19	0.6595
Immobility time (sec.)	1	(0; 5.5)	0.5457/ <0.0001	0	(0; 0)	0.6199/ <0.0001	52		0.9088
Rearing frequency	10.42	1.35	0.9541/0.6973	9.78	1.94	0.1080/0.9474	0.279	19	0.7833
Grooming (sec.)	2	(0,15)	0.7441/0.0023	0	(0,12)	0.7153/0.0021	42.5		0.4106
LIGHT-DARK BOX	**mean/median CG**	**SE(Min, Max)**	**S–W/P**	**mean/median EG**	**SE/Min, Max**	**S–W/P**	**t/M-W**	**df**	**P**
Attempts to the light side	2.167	0.787	0.7798/0.0056	8.333	2.587	0.8993/0.2479	2.568	19	0.0188
Latency to the light side	31.1	7.868	0.8725/0.1069	124.3	42.09	0.846/0.0674	2.293	17	0.0349
Locomotion frequency in the light side	19.5	5.095	0.8674/0.0606	13.22	6.264	0.743/0.0045	0.7848	19	0.4422
Locomotion frequency in the dark side	57.58	7.496	0.9376/0.7863	77.11	12.73	0.7965/0.0060	1.398	19	0.1783
Immobility in the light side (sec.)	0	(0,0)	0.3269/ <0.0001	0	(0,0)	0.3898/ <0.0001	53		>0.9999
Immobility in the dark side (sec.)	0	(0,0)	0.352/<0.0001	0	(0,0)	0.6798/ <0.0001	48		>0.9999
Side cross	5	1.206	0.8692/0.0639	5	1.927	0.8169/0.0432	0	18	>0.9999
Time in the light side (sec.)	15	(2,62.5)	0.814/0.0136	7	(0,55.5)	0.7989/0.0198	47		0.6341
Time in the dark side (sec.)	227.5	(167.5, 197)	0.854/0.0412	283	(197,297.5)	0.8211/0.0354	36		0.2181
Grooming in the dark side (sec.)	6	(0,13.5)	0.6384/0.0002	0	(0,0)	0.5703/ <0.0001	22		0.0394
Fecal *boli* in the dark side	0	(0,0)	0.3269/ <0.0001	0	(0,0)	0.3898/ <0.0001	52		0.7429

3.4. Pathology of liver and kidneys of F0 females

3.4.1. Gross pathology and microscopical alterations of kidneys and liver

No histological differences were observed in any of the liver parameters. Regarding to kidneys histopathology tubular vacuolization was augmented in the EG. Fig. 6 shows that tubular vacuolization scores were significantly higher in EG animals, when compared to those from CG.

3.5. Biochemical studies of liver and kidneys functions in the dams

3.5.1. Biochemical parameters of kidneys and liver

No biochemical alterations were observed for AST, ALT or creatinine levels in the dams. Fig. 7 shows the graphics related to the statistical analyses.

4. Discussion

The present work reports novel findings related to the administration of a sub-abortive dose of 1.0 mg/kg, by gavage, daily, between days GD17 and GD21, as a result from the attempt to prospect for alterations in the dams and their litters, after weaning.

The EBN has prevented the pregnant rats from gaining weight during the last period of gestation, which is usually when they gain more weight, due to the need of supplying nutrients to the effective development and maturation of the fetuses. The weight loss during pregnancy that was observed in the EG group can be related to the influence of the EBN in the hypothalamic-pituitary-adrenal (HPA) axis, a condition that has been previously observed by our group (Frias et al., 2020). In the study, we observed that there was an improvement of 5-HIAA, a 5-HT metabolite, in the hypothalamus, in the frontal cortex and striatum of young male rats under EBN vertical treatment provoking the anxiety-like behavior. As the dams were treated for 5 consecutive days during GD17 up to GD21, we understood that the weight loss was a consequence of the anorexia that is associated to a HPA axis stimuli (Raghavendra et al., 2000). We propose that the five-day administration of EBN has somehow induced the HPA via serotonin stimuli, causing the weight loss in the dams.

At weaning, the dams gained weight. This finding is not in accordance with the diminished levels of melatonin that were also observed. Melatonin is a compound told to be antagonist to serotonin receptors (Eison and Mullins, 1996). It is expected that a decrease in melatonin levels would provoke anorexia, as a counteraction of the elevated levels of melatonin provoking suppression of the HPA axis activation and a consequent decrease in body temperature, and elevated food intake via the superquiasmatic nuclei (Raghavendra et al., 2000).

However, previous reports described that physiological and behavioral changes occurs in the maternal brain during pregnancy and lactation, in the rat, which ensures maternal care. Lactating rat dams are less anxious when compared with virgin females and this reduced anxiety in lactation is a result of the adaptations in the brain that are necessary to the survival of the offspring (Lonstein, 2007). The observed reduced emotional responsiveness during lactation can be partially explained by the occurrence of an enhanced activity of the brain's oxytocin and prolactin system (Neumann, 2008), as both have anxiolytic properties, particularly in the female rats during peripartum. As previously explained, the down regulation of oxytocin and prolactin can be mediated by the presence of glucocorticoids (Vilela et al., 2012). In the present work, the lack of statistical differences in the corticosterone levels of both the EG and CG dams are in fact explained by the plant extract interference over the oxytocin and prolactin system, as cucurbitacins, which are steroid triterpenes, are present in the plant extract. In our previous work, we have characterized the presence of cucurbitacins in EBN (Frias et al., 2020).

The evaluation made at PND2 in the litters showed that the EG had the same number of pups of the CG. An interesting finding of the present work is related to the male/female ratio. The number and the percentage of female pups were higher in the litters of dams receiving treatment, while the percentage of males was lower in the same group. A previous study has been done and the evaluation of the administration of

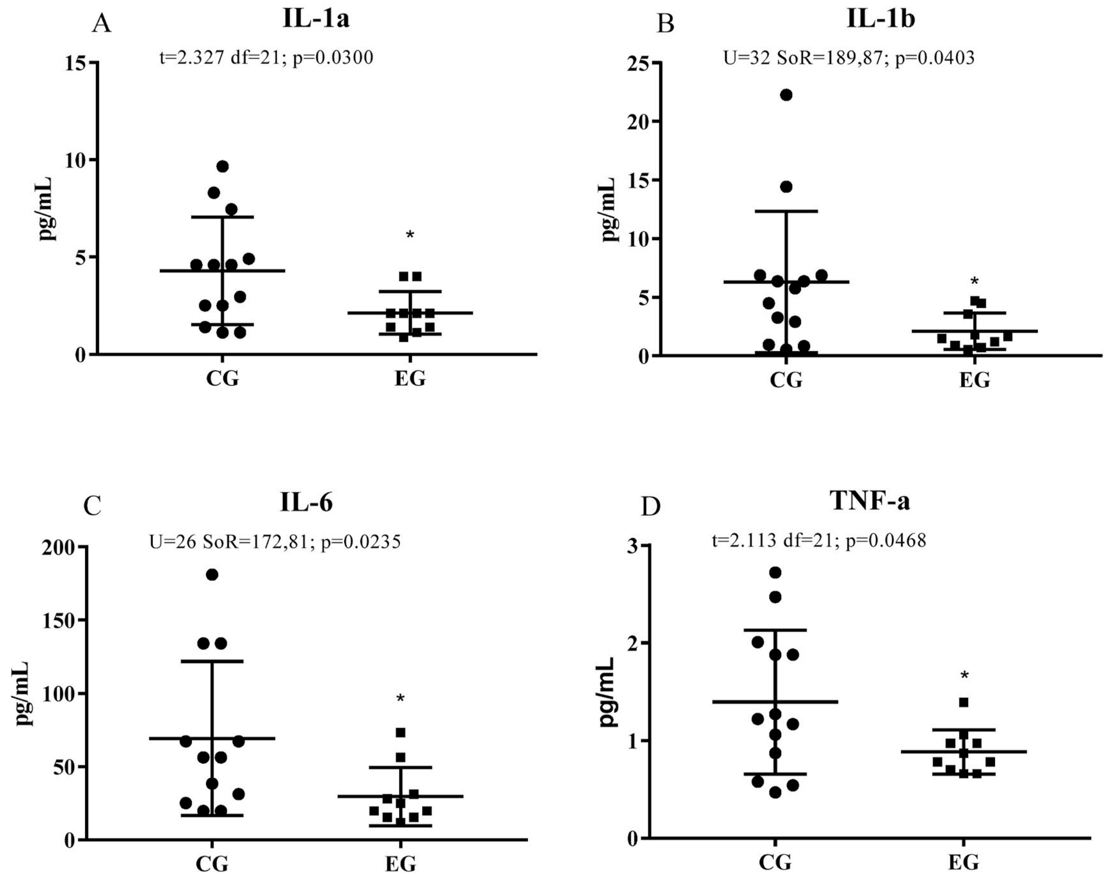

Fig. 4. Pro-inflammatory cytokine levels in the dams' sera. Quantifications are given in pg/mL for all parameters. **A.** Interleukin-1α (CG S–W = 0.9086, p = 0.1757 and EG S–W = 0.831, p = 0.0344); **B.** Interleukin-1β (CG S–W = 0.7999, p = 0.0068 and EG S–W = 0.8395, p = 0.0435); **C.** Interleukin-6 (CG S–W = 0.8422, p = 0.0294 and EG S–W = 0.7996, p = 0.0143); **D.** Tumor necrosis factor-α (CG S–W = 0.9319, p = 0.03606 and EG S–W = 0.8813, p = 0.1351). Data is described as mean ± SE for all measurements. SE = standard error. Student's *t*-test or Mann-Whitney test was used, after Shapiro-Wilk normality test analyses, considering significance at α < 0.05 for all analyses. * = p < 0.05.

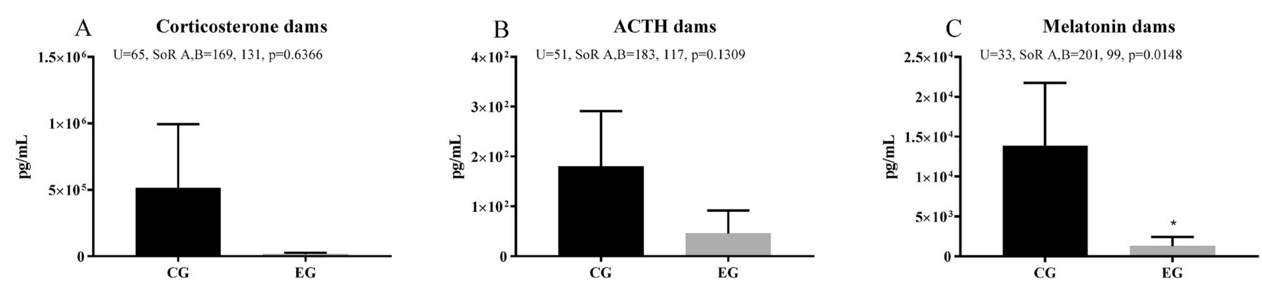

Fig. 5. Stress hormone levels in the serum of dams at PND50. Quantifications are given in pg/mL for all parameters. **A.** Corticosterone (CG S–W = 0.3371, p < 0.0001 and EG S–W = 0.5117, p < 0.0001); **B.** Adrenocorticotrofic hormone (CG S–W = 0.539, p < 0.0001 and EG S–W = 0.345, p < 0.0001); **C.** Melatonin (CG S–W = 0.555, p < 0.0001 and EG S–W = 0.4068, p < 0.0001). Data is described as mean ± SE for all measurements. Mann-Whitney test was used, considering significant differences at level of α < 0.05. * = p < 0.05.

L. operculata decoction at pre-implantation, implantation and organogenesis periods of gestation confirmed a high rate of fetal resorption (Barilli et al., 2007), but the same study did not report any influence of the *L. operculata* administration in a late period of gestation in order to prospect for fetal resorption (Menon-Miyake et al., 2005b), or even to prospect the frequency of female or male pups in the litter, or even a behavior alteration in the pups as the treatment was done in a period of modulation of the central nervous system, in its final development. In order to establish the real reasons of the alteration in the male/female ratio in the litters of the treated dams further analyses must be done. Further evaluation concerning the administration of the EBN in different periods of gestation would be more representative to explain the present finding. EBN administration has also influenced on the weight gain of male pups. We observed that male pups from the EG showed increased weight gain, compared to that of the CG males. The mechanisms underlying the male pup weight gain are uncertain, in the experiment, but may be related to over care of the mother, maybe related to the EBN intake in the late pregnancy, causing somehow a long-term influence over male pup metabolism, but those arguments are yet to be accessed.

At weaning the dams showed a diminish in melatonin and in cytokines levels and increased anxiety-like behavior, although corticosterone was not augmented.

Melatonin evolved other functions, including its multiple receptors which regulate seasonal changes, immunity and has functions as an

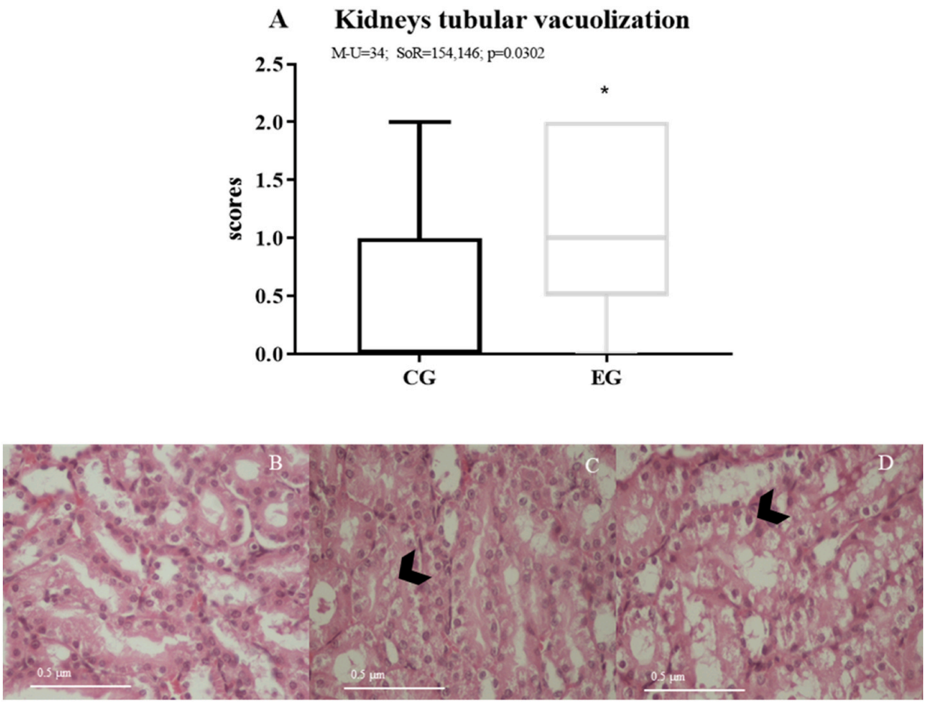

Fig. 6. Histopathological features of the kidneys from dams at PND50, after receiving an oral (by gavage) administration of *Luffa operculata*, and the statistical analysis of tubular vacuolization between control and experimental groups. **A.** Histological alterations (CG S–W = 0.5962, p < 0.0001 and EG S–W = 0.838, p = 0.0548). Mann-Whitney test was used, significance between medians if α < 0.05; **B.** Score 0 for tubular vacuolization, following the control group standards; **C.** Score 1 for tubular vacuolization; **D.** Score 2 for tubular vacuolization. Data is described as mean ± SE for all measurements; SE = standard error. Significant differences between means were considered as α < 0.05. * = p < 0.05.

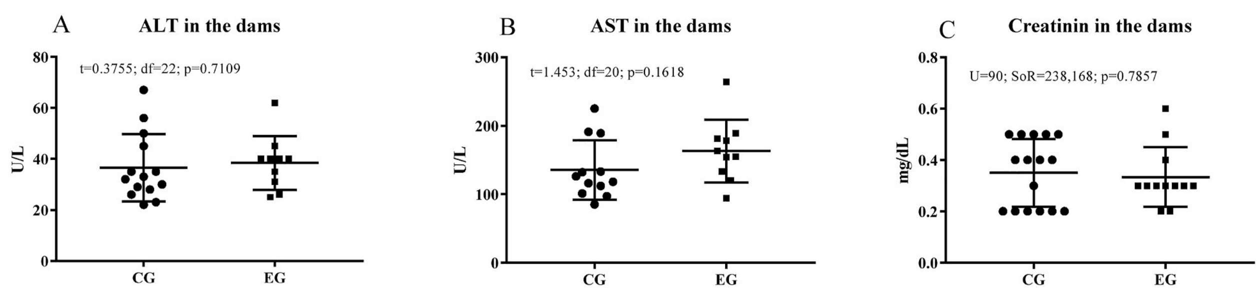

Fig. 7. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST) and creatinine of dams at PND50, after receiving a vertical administration of *Luffa operculata*. **A.** Alanin aminotransferase – ALT (CG S–W = 0.8771, p = 0.0528 and EG S–W = 0.892, p = 0.1785); **B.** Aspartate aminotransferase – AST (CG S–W = 0.8697, p = 0.0648 and EG S–W = 0.9381, p = 0.5316); **C.** Creatinine (CG S–W = 0.7915, p = 0.0021 and EG S–W = 0.7984, p = 0.0090). Data is described as mean ± SE for all measurements; SE = standard error. The Student's *t*-test or the Mann-Whitney test was used after Shapiro-Wilk normality analyses, significance was considered at α < 0.05 for all analyses.

oncostatic agent, besides the ability to reduce oxidative stress by processes that are, in part, receptor-independent. Melatonin acts through two G-protein coupled receptors, MT1 and MT2, and regulates many brain functions, including circadian rhythms, mood, pain, feed and sleep. Melatonin and non-selective MT1/MT2 receptor agonists are clinically used in neuropsychiatric and/or sleep disorders. An agomelatine agonist behaves as an agonist at melatonin receptors and as an antagonist at serotonin (5-HT)2C receptors. Its anxiolytic properties are related to MT1 receptor activation (Millan et al., 2005).

It is known that the unbalance of the melatonin can lead to a depressive behavior and to an anxiety-like behavior in rats (Tapia-Osorio et al., 2013). In an experiment using anxiety-like induction by chronic unpredictable mild stress behavior in rats, the authors verified that the administration of melatonin has diminished the anxiety symptoms and has increased dopamine in the hippocampus (Spasojevic et al., 2016). Thus, some signals of anxiety-like behavior in the dams that were observed in our work could be related to the decreased levels of melatonin, but this fact does not totally explain why the dams had gained weight during lactation (Williamson, 1986).

Melatonin is described to have anti-inflammatory properties by reducing chronic and acute inflammation due to its antioxidant properties (Berkiks et al., 2018; Esposito and Cuzzocrea, 2010) and immunomodulatory activity (Szczepanik, 2007). Besides melatonin capacity to detoxify free radicals generated during the processes of photosynthesis and metabolism (Tan et al., 2015) during organism evolution, it became a pleiotropic molecule that resists oxidation-related stress but also suppresses inflammation (Radogna et al., 2010). Melatonin is a hormone related to sleep induction, and its deregulation is frequently associated to longer periods of sleep absence. In the present study, the decrease in melatonin observed in the EG may have caused the induction of longer sleepless periods in the rats and a consequent increase in the food intake, which justifies the weight gain, but contradicts the antagonist effect over serotonin receptors and the decrease in the pro-inflammatory cytokines. We believe that the presence of some chemical constituents in EBN may have caused the alterations here described, apart from the melatonin decrease.

As we first reported, the administration of EBN to adult male Wistar rats induced stress and increased 5-HIAA (Alves et al., 2018; Frias et al., 2020) in the pregnant rats, and that may be related to the stimulation of the HPA axis. The activity of EBN may have been caused by the presence of cucurbitacins, as previously reported (Frias et al., 2020). Cucurbitacin E has been found to interfere with signal transducers and transcription, or STATs, that are present in fat cells. These molecules are responsible for modulating different steps of fat cell development and biochemical functioning, and directly interfere with fat accumulation (Zhao and Stephens, 2013). Cucurbitacin E has been reported to interfere with STAT signaling pathways as a stimulator, reducing fat deposition within the cells (Murtaza et al., 2017). Also, the STATs signaling pathway, on fat cells is known to be activated by cytokines as interleukin-6 (IL-6; Flower et al., 2003), IL-11, and growth hormones as prolactin and leptin. A dysregulation of the system may lead to metabolic syndrome and unbalance in fat regulation in the body. In our study, the level of IL-6 in the female EG was lower than that observed in the CG, as well as for IL-1α, IL-1β and TNF-α. The lower IL-6 level may have also contributed to the observation of weight gain in the EG. Although the present work has evoked the prospection of the mechanisms involved in the melatonin unbalance, much has to be done in order to explain what exactly is the EBN influence on the body system. Furthermore, the pro-inflammatory cytokines were low, in the EG, which is not quite explained by the decreased melatonin in the system, but may be related to the administration of EBN, which contains cucurbitacins, molecules that have a chemical structure that is similar to corticoids (Fig. 8).

In the early 2000's, neocucurbitacins A and B (Kawahara et al., 2001), opercurins A and B (Kawahara et al., 2004), dihydrocucurbitacin B and cucurbitacin B (Lang et al., 2012) were described to occur in the fruits of *L. opereculata*. Dihydrocucurbitacin derivatives, although isolated from different plant species, are reported to show antiproliferative activity against HeLa cell line (Zhang et al., 2018) and HT-29 colon cancer cell line (Tang et al., 2015), and it is also known to induce cell cycle arrest and apoptosis in breast cancer cells (Yang et al., 2007). Dihydrocucurbitacin was also reported to reduce the levels of IL-1β, IL-4 and TNF-α (Escandell et al., 2007).

Such cytokines are produced by activated macrophages and other immune system cell lines, as for our knowledge, some components of

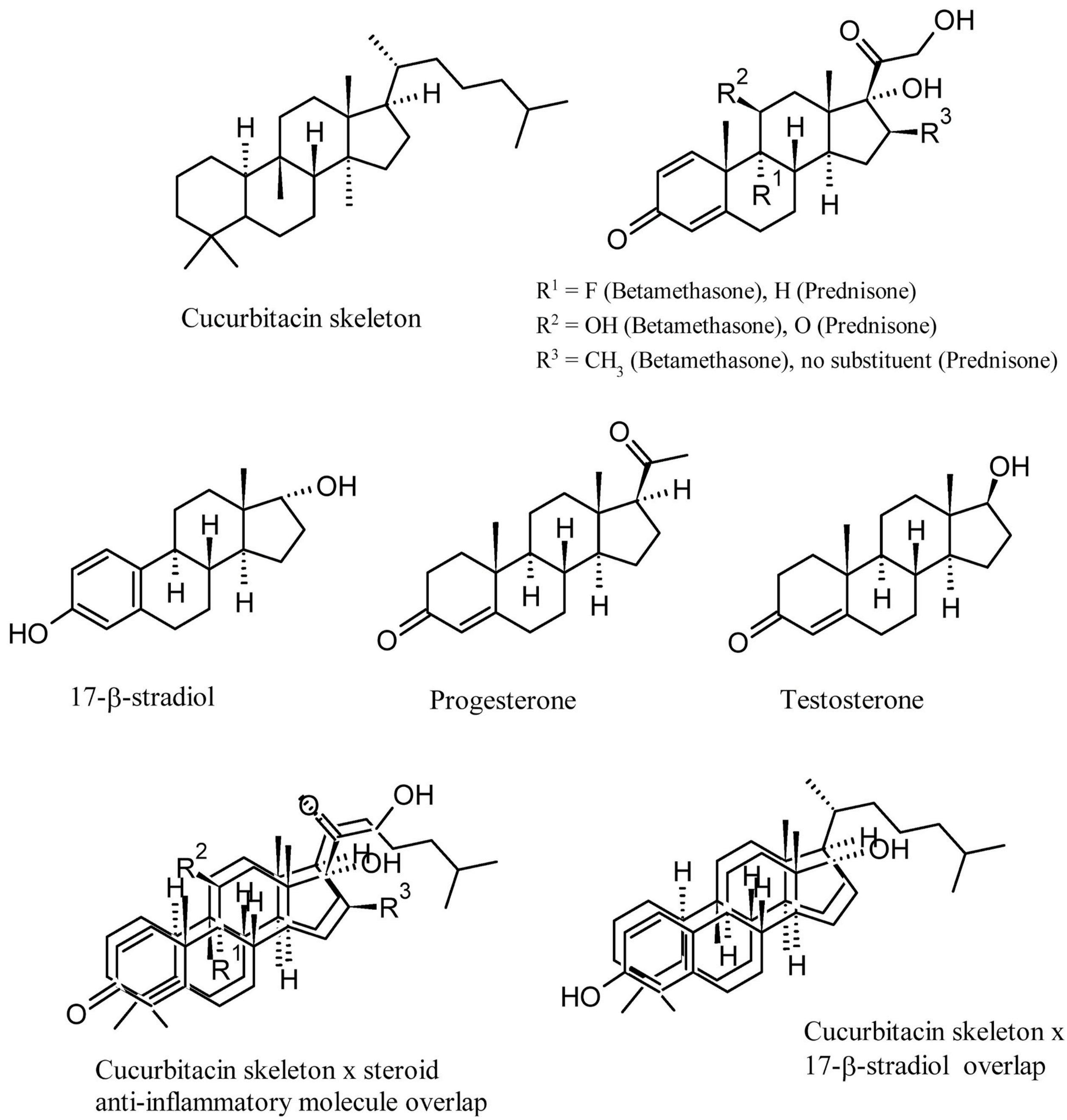

Fig. 8. Chemical structures of cucurbitacin skeleton, corticosteroids and hormones.

EBN can be contributing as anti-inflammatory agents. Dihydrocucurbitacin B was first reported to have inhibited the expression of TNF-α, IL-1β and IL-4 in mice under inflammation induced by oxazolone and other components (Escandell et al., 2007). In the present work, the inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α were decreased in the treated dams. We understand that the reduced serum levels of the cytokines may be associated to the anti-inflammatory activity of some cucurbitacins that are present in the EBN, as previously evaluated by our group (Frias et al., 2020), and to their side effects which resembled those observed with glucocorticoids. Cucurbitacins are tetracyclic terpenes with steroidal structures frequently found among Cucurbitaceae species.

The administration of EBN did not change any of the liver parameters, but in the kidneys, tubular vacuolization was observed in the F0 females that received EBN. Moreover, previous morphological studies in male testis showed changes in the parenchyma and lumen and in the diameter of seminiferous tubules with a decrease in Leydig cell numbers (Alves et al., 2018). Cucurbitacin B is known for its acute toxicity when ingested, and it is classified as a category 2, according to the Globally Harmonized System of Classification (ACS, 2019). Cucurbitacin B is also found in the fruits of *L. operculata* and was reported to have cytotoxic effects against A549 non-small cell lung cancer cells (Lang et al., 2012). Considering that the EBN was orally administered to the pregnant females during 5 days, at a dose of 1.0 mg, the renal toxic effects were likely due to cucurbitacin B. Further analyses are yet to be performed, although no physiological alteration is related to tubular vacuolization changes at that level. The histological findings are in accordance to a lack of differences observed for AST, ALT and creatinine.

5. Conclusions

The oral administration of a sub-abortive dose of the dried fruits of *L. operculata* at a late period of gestation induced maternal anxiety-like behavior and decreased the pro-inflammatory cytokine serum levels. The direct administration of EBN did not cause liver or kidney toxicity. In addition, the number of female pups at birth was higher than that observed for male pups. Weight gain in male pups at PND60 was also observed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.113867.

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