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Action of the hydroethanolic extract of the flowers of *Acmella oleracea* (L.) R.K. Jansen on the reproductive performance of Wistar females rats: A popular female aphrodisiac from the Amazon

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ABSTRACT

Ethnopharmacological relevance: The species *Acmella oleracea* (L.) R.K. Jansen (Asteraceae), popularly known as jambú, is marketed in fairs as a female aphrodisiac and has several pharmacological activities already confirmed, among them the sexual stimulant action. The objective of this study was to evaluate the effects of the oral administration of the hydroethanolic extract of *A. oleracea* flowers (EHAo) on wistar rats during the pre-mating, mating, and pre-implantation period.

Material and methods: During the treatment period, measurements of feed intake, water intake, weight, estrous cycle, behavior, reproductive parameters, biochemical parameters, hematological parameters, and histopathology of ovaries were performed daily.

Results: In the gas chromatography analysis – mass spectrometry characterization, the compound (2*E*, 6*Z*, 8*E*)-N-isobutyldeca-2,6,8-trienamide (spilanthol) was detected as the majority compound at the 84% concentration. In the conditions of this study, EHAo did not cause maternal toxicity. However, in the estrous cycle, the frequency of the Proestrous (P) and Estrous (E) phase was significantly increased with the doses of 88.91 and 444.57 mg/kg of the EHAo in relation to the control. On the other hand, the metaestrous (M) and diestrous (D) phases showed a significant reduction in their frequency in the groups treated with EHAo. Water intake increased significantly ($p < 0.01$), as well as the triglyceride levels, the total cholesterol and fractions ($p < 0.05$), and the percentage of neutrophils ($p < 0.05$).

Conclusion: It is concluded, therefore, that the treatment with EHAo, which is one of the forms popularly used, is safe in the concentrations and time of treatment studied as it is able to influence the estrous cycle without altering folliculogenesis and fertility.

1. Introduction

The *Acmella oleracea* (L.) R.K. Jansen species is popularly known as jambu, agrião-do-Pará, agrião-do-norte, agrião-bravo, agrião-do-Brasil, and botão-de-ouro, native of South America, very cultivated, and used as a seasoning in the cuisine of the northern region of Brazil. At the Ver-o-Peso fair in the city of Belém, Pará, Brazil, the largest city from the Amazon region, it is marketed as a female aphrodisiac and is sought by

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many women (Favoreto and Gilbert, 2010; Lorenzi and Matos, 2002; Prachayasittikul et al., 2013). Chemically, it consists mainly of alkylamides and the compound (2*E*, 6*Z*, 8*E*)-N-isobutyldeca-2,6,8-trienamide (spilanthol), which is described as one of those responsible for the pharmacological activities attributed to this species (Boonen et al., 2010; Jacobson, 1957; Prachayasittikul et al., 2013).

Among the pharmacological activities described, the ones that stand out are the following: Anesthetic (Andrade et al., 2013; Freitas-Blanco et al., 2016), insecticide and larvicide (Moreno et al., 2012; Ramsewak and Erickson, 1999), anti-inflammatory and analgesic (Chakraborty et al., 2004; Ratnasooriya and Pieris, 2005; Wu et al., 2008), diuretic (Gerbino et al., 2016; Ratnasooriya et al., 2004; Yadav et al., 2011), and antioxidant (Hossain et al., 2012; Tania et al., 2015; Wongsawatkul et al., 2008).

A clinical trial with cream with *A. oleracea* was shown to improve sexual response and described to be promising in the treatment of male and female sexual dysfunctions (Regadas, 2008; Sharma et al., 2011). Sexual dysfunction is considered to be any change in one or more phases of the sexual response cycle that may be persistent or recurrent, and there are few effective therapeutic resources for the treatment of patients with these disorders (Clayton and Hamilton, 2009; Mendonça and Amaral, 2011; Ohl, 2007).

There are reports on plants that present toxic potential on the reproductive system, with the ability to interfere with fertilization and implantation (Almeida and Lemonica, 2000; Goonasekera et al., 1995; Leite et al., 2004). *Drynaria quercifolia* (L.) J. Smith (Das et al., 2014; Rajendran and Rajan, 1996), *Pouzolzia mixta* (Sewani-Rusike, 2013), *Lepidium meyenii* (Mummenhoff et al., 2009; Taylor, 2005; Wang et al., 2007), *Ruta graveolens* L. (Ciganda and Laborde, 2008; Freitas et al., 2005; Kong et al., 1989) and *Coleus barbatus* are examples of these plants (Almeida and Lemonica, 2000).

Reproductive toxicology studies by specific tests the substances that can interfere in the phases of the reproduction due to the high susceptibility to adverse effects (EMA, 2008; ICH, 2005). The most used studies for these investigations are those recommended in guides of international regulatory agencies such as Organization for Economic Cooperation and Development (OECD), Food and Drug Administration (FDA), European Medicines Agency (EMA), and International Conference on Harmonisation (ICH) (Bernardi et al., 2006; Spinosa et al., 2014).

From the embryo to the formation of the blastocyst, successive mitoses occur, and their implantation corresponds to a complex process, including the proliferation of endometrial cells, decidualization, and the increase of blood flow (Dimitriadis and Menkhort, 2010; Mourik et al., 2009). There are cytotoxic substances that act on the reproductive system and can interrupt gestation by interfering with mitotic division. Such substances may interfere before and after the implantation process, resulting in pre- and post-implantation embryo loss (Yakubu and Bukoye, 2009).

Because of the vast interest in the species in question, which is used in the aqueous form and mainly ethanolic preparations, the objective of this study was to investigate the effect of the hydroethanolic extract of the *Acmella oleracea* species on the reproductive performance and estrous cycle of female Wistar rats.

2. Material and methods

2.1. Plant material

The samples of *Acmella oleracea* (L.) R. K. Jansen was collected in September 2016 in the district of Fazendinha (S 0°02′30.40″/W 5106′37.5″), Macapá City, Amapá State, Brazil. An exsiccata of the species was deposited in the IAN Herbarium (Embrapa Amazônia Oriental- Bélem, Pará- Brazil), under identification number 196011.

2.2. Obtaining the hydro-ethanolic extract of Acmella oleracea (L.) R. K. Jansen (EHAo)

Previously selected fresh flowers were ground to obtain a fine powder, then a cold (temperature at 20 °C) maceration for seven days in 70% hydroethanolic solution (1:5, raw material: solvent) was realized. The resulting extractive solution was filtered and then concentrated in a rotary evaporator (Quimis Model Q 218.2) at 40 °C. Subsequently, the concentrate was lyophilized, yielding a yield of 2.5%. One microliter of the sample was dissolved in dichloromethane (1:100 mg/μL) for gas chromatography analysis – mass spectrometry (GC-MS).

The phytochemical characterization of EHAo was based in method described by Franca et al. (2016), then was performed by gas chromatography coupled to mass spectrometry (GCMSQP2010-Shimadzu). The conditions of the analysis were: Temperature of the injector, 210 °C; Detector temperature, 290 °C with electron impact ionization (70 eV); Gas, Helium; Flow rate, 1 mL/min and injection rate 1:20. The initial temperature was 120 °C for 2 min, at a rate of 6 °C/min to 290 °C, remaining for 8 min, the total analysis time was 35 min. The column used was DB-5 (0,25 mm × 30 m × 0,25 μm). Identification of the compounds and percentage (%), was identified by comparison of the fragmentation spectrum with those contained in the CG-MS library (MS database NIST 5.0).

2.3. Animals

Wistar rats (*Rattus norvegicus albinus*) (female), approximately 12 weeks of age and with an average weight of 250 g, were kept on temperature-controlled racks (22 ± 2 °C), at a relative humidity of 55 ± 10%, and a light/dark cycle of 12 h. They received adequate ration for rodents and filtered water ad libitum.

The experimental protocol was approved by the Ethics Committee on the Use of Animals of the Federal University of Amapá – CEUA-UNIFAP under Registration No. 0020/2016.

2.4. Experimental design

Animals after the adaptation period were randomly divided into three groups (n = 5/group, quantity approved by the CEUA-UNIFAP). The animals were treated orally, through gavage, with a daily dose for 21 consecutive days, in the pre-mating, mating, and pre-implantation periods. Doses were established (10% and 50% of lethal dose) from the study of Nomura et al. (2013), that estimated LD_{50} value was 889.14 mg/kg (hydroethanolic extract from the flowers by intraperitoneal route) in mouse.

The weight, water intake, feed intake, and estrus cycle were evaluated daily (Marcondes et al., 2002; OECD, 2015). The following groups were established:

G1 - Control Group (0,5 mL of distilled water);
G2 - 10% of the DL_{50} of EHAo Group (88,91 mg/kg, diluted in 0.5 mL of distilled water);
G3 - 50% of the DL_{50} of EHAo Group (444,57 mg/kg, diluted in 0.5 mL of distilled water).

2.5. Biochemical and hematological analysis

On the thirteenth day of pregnancy, blood was collected from the retroorbital plexus for biochemical measurements of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total cholesterol and fractions, triglycerides, glucose, creatinine, and urea, using Labtest® kits (colorimetric enzymatic assays and colorimetric kinetics) and the semi-automatic biochemical analyzer model BIO-200. For complete hematological analysis, the HumaCount Plus hematology automatic blood count was used (Human GmbH).

2.6. Macroscopic and histopathological analyses

On the 13th day of gestation, the rats were euthanized, and the ovaries, uterine horns, vaginal canal, heart, lung, liver, kidneys, and spleen were analyzed macroscopically. The mass of these organs was then measured. The ovaries were separated for luteal body count and histopathological analysis, fixed in 10% buffered formaldehyde for 24 h, and then dehydrated in alcohol solution, clarified in xylol, impregnated and paraffin-embedded at 60 °C. The slides were sectioned semi-serially in a CUT 5062 semiautomatic rotating microtome (SLEE) in sections of 5 μm and stained with hematoxylin-eosin (H/E) method. The microscopic analyses were performed under an Olympus microscope coupled with image software. The following structures were analyzed: developing follicles, antral follicles, luteal bodies, and interstitial cells in the medullary region (Luna, 1986).

2.7. Statistical analysis

To analyze the data of the different groups, the analysis of variance (one-way ANOVA) was applied, followed by the Tukey test. The significance level considered was 5% ($p < 0.05$). All results were expressed as the mean ± standard deviation (mean ± SD). The statistical program GraphPad Prism® (version 5.03) was used.

3. Results and discussion

Alkylamides are a group of compounds constituted by the union of a fatty acid of medium chain length with large, eight to eighteen, generally aliphatic, chains and an amine (Nakatani and Nagashima, 1992). The most important plant families in which alkylamides are present are the *Asteraceae*, *Solanaceae*, and *Piperaceae* (Torres and Chávez, 2001).

Identification of the (2*E*, 6*Z*, 8*E*)-N-isobutyldeca-2,6,8-trienamide (spilanthol) alkylamide by gas chromatography showed the recording of two characteristic fragmentation signals resulting from the homolytic cleavage of the C-C bond in $m/z = 81$ (100%) and $m/z = 141$ (72%) (Fig. 1) (Hiserodt et al., 2004). In the quantification of spilanthol in EHAo, a percentage of 84% was found and was considered as the majority substance present in EHAo. The relative purity of spilanthol after peak integration was 82%.

This result is similar to those described in other studies, where spilanthol variations between 84% and 90% were found (Anholeto et al., 2017; Boonen et al., 2010; Cheng et al., 2015). There is much variability in the detection of spilanthol in the parts of *A. oleracea*, with a higher concentration in the inflorescences, followed by the leaves (Ramsewak and Erickson, 1999).

The systemic toxicity of substances can be evaluated not only by behavioral changes but mainly by variations in food consumption, mass gain, and organ evaluation (Volpato et al., 2015).

The clinical parameters of the animals treated with EHAo were not different from those of the control group (G1). However, the animals treated with EHAo showed excess salivation of rapid occurrence, but remaining throughout the treatment period, including interfering in water consumption ($p < 0,05$) (Fig. 2). There was no significant difference in body mass gain (Fig. 3) and food consumption (Fig. 4).

The significant difference in water consumption between the groups treated with EHAo (G2 and G3) in relation to the control group (G1), may be directly related to the high percentage of spilanthol in EHAo because studies have shown that spilanthol can modify the palate by

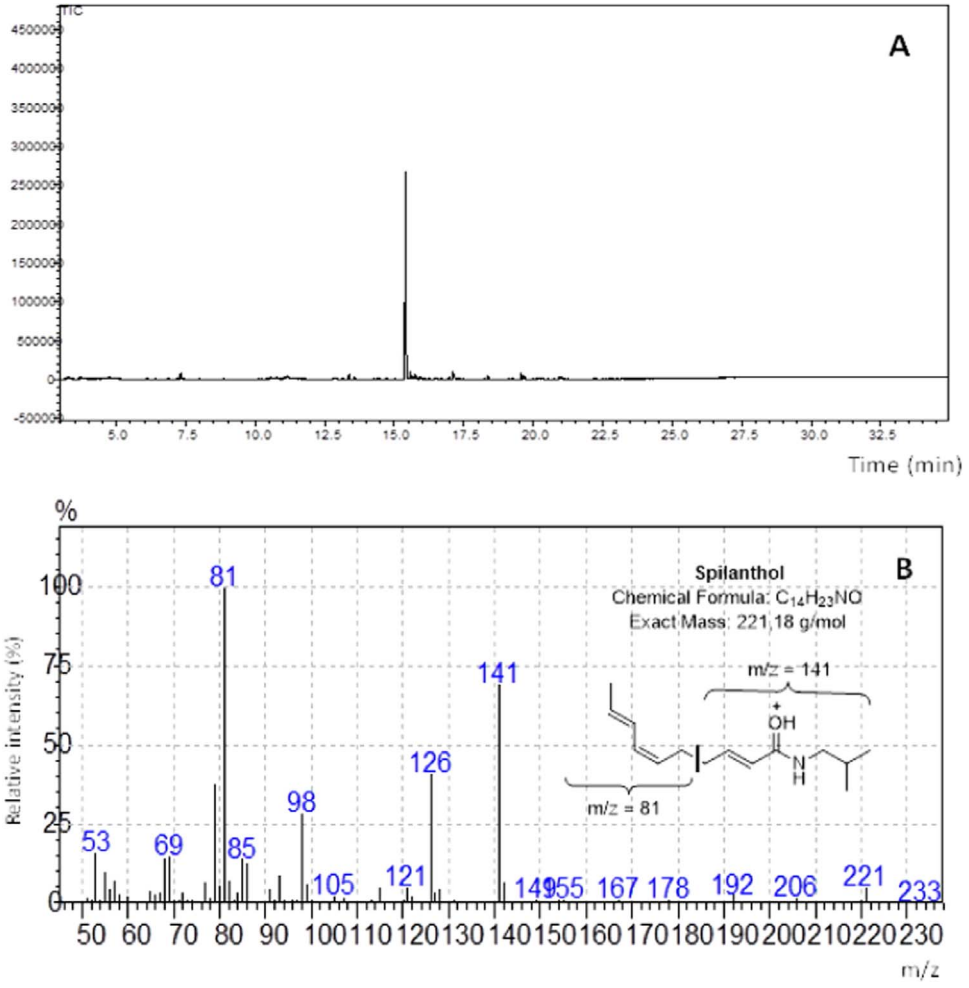


Fig. 1. Chromatogram (**A**) and Mass Spectrum (**B**) for the hydroethanolic extract of the flowers of *Acmella oleracea* (L.) R.K. Jansen. Highlight the (2*E*, 6*Z*, 8*E*)-N-isobutyldeca-2,6,8-trienamide (spilanthol) majority compound.

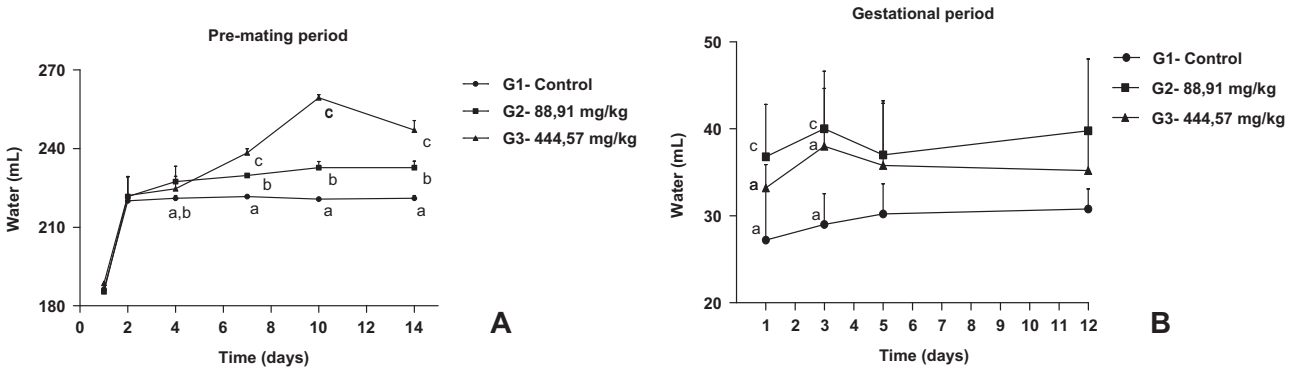


Fig. 2. Effect of oral treatment with EHAo (G2 and G3) on water consumption (mL) of Wistar rats treated during the pre-mating period (A) (14 days) and during the pre-implantation period (d0-d5) and after treatment (d13) (B). In the G1 group, distilled water (0.5 mL/animal) was administered. Values represent mean ± SD, $p < 0.05$. ANOVA test was applied, followed by the Turkey test, n = 5/group. Where: [a]comparison between G1 vs G2 groups, [b]comparison between the G1 vs G3 groups, and [c]comparison between the G2 vs G3 groups.

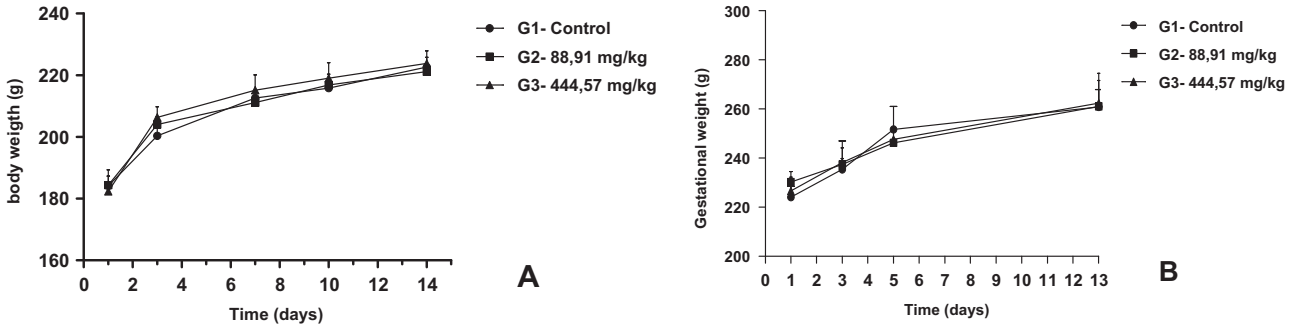


Fig. 3. Effect of oral treatment with EHAo (G2 and G3) on the body weight of Wistar rats treated during the pre-mating period (A) (14 days) and during the pre-implantation period (d0-d5) and after treatment (d13) (B). In the G1 group, distilled water (0.5 mL/animal) was administered. Values represent mean ± SD, $p < 0.05$. ANOVA test was applied, followed by the Turkey test, n = 5/group.

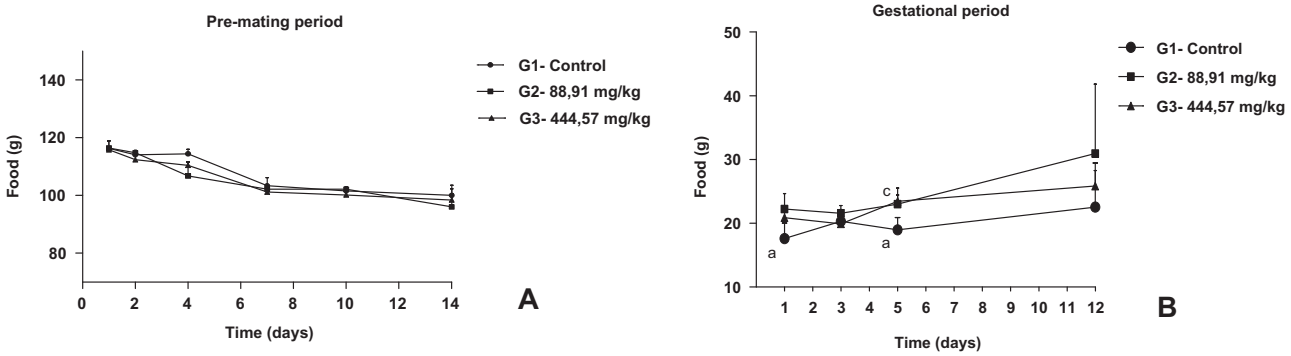


Fig. 4. Effect of oral treatment with EHAo (G2 and G3) on feed consumption of Wistar rats treated during the pre-mating period (A) (14 days) and during the pre-implantation period (d0-d5) and after treatment (d13) (B). In the G1 group, distilled water (0.5 mL/animal) was administered. Values represent mean ± SD, $p < 0.05$. ANOVA test was applied, followed by the Turkey test, n = 5/group. Where: [a]comparison between G1 vs G2 groups, [b]comparison between the G1 vs G3 groups, and [c]comparison between the G2 vs G3 groups.

promoting a tingling sensation, inducing the animals to ingest water (Campbell et al., 2009; Prachayasittikul et al., 2013).

Regarding the estrous cycle, it was observed that the frequency of the proestrous (P) and estrous (E) phases was significantly increased with the doses of 88.91 and 444.57 mg/kg in relation to the control group. On the other hand, the metaestrous (M) and diestrous (D) phases showed a significant reduction in their frequency in the treated groups (Fig. 5). In the interestrous interval (G1 [0.82 ± 0.08], G2 [0.74 ± 0.03], and G3 [0.78 ± 0.00]) and diestrous index (G1 [0.28 ± 0.08], G2 [0.28 ± 0.08], and G3 [0.28 ± 0.11]), there was no significant difference.

M and D are non-fertile phases of the cycle, characterized by the predominant presence of leukocytes, mucus, and reduction of the concentration of estradiol, progesterone, FSH, and LH. The P occurs shortly after D, gradually with the estrogen plasma peak that declines rapidly, and E corresponds to the phase in which the female is receptive to the male; however, at this stage, the maximum increase in the degree of cornification of the epithelium is observed, which will result in high estrogen levels, especially if compared to the levels of progesterone (Barha and Galea, 2010).

Treatment with EHAo did not induce acyclicity, characterized by persistence in the estrous, diestrous or irregular patterns, with prolonged duration cycles. The changes possibly occurred due to the presence of active substances in the EHAo that act on the hypothalamic-pituitary axis, inducing the longer stay in the fertile phases, characterized by the permanence in P and E (Goldman et al., 2007).

Fertility is the capacity for reproduction, and fertility is the concretization of reproduction, and the number of new individuals is

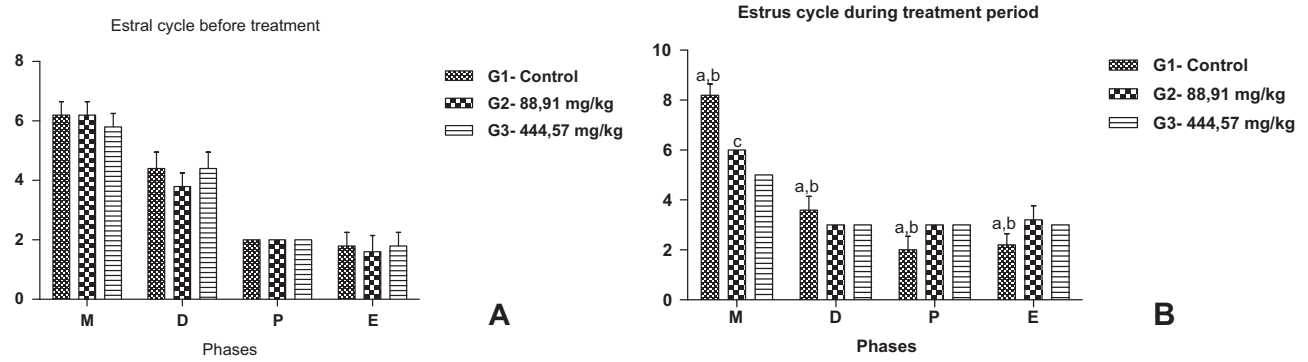


Fig. 5. Effect of oral treatment with EHAo (G2 and G3) on the estrous cycle (Proestrous (**P**), Estrous (**E**), Metaestrous (**M**) and Diestrous (**D**)) of Wistar rats treated during the pre-mating period (14 days) (**A**) and during treatment in the pre-mating period (14 days) (**B**). In the G1 group, distilled water (0.5 mL/animal) was administered. Values represent mean ± SD, $p < 0.05$. ANOVA test was applied, followed by the Turkey test, n = 5/group. Where: [a]comparison between G1 vs G2 groups, [b]comparison between the G1 vs G3 groups, and [c]comparison between the G2 vs G3 groups.

Table 1
Effect of oral treatment with EHAo (G2 and G3) on the reproductive parameters of rats with interrupted pregnancy on the 13th day of gestation.

Parameters	G1-Control (n = 5)	G2- 88, 91 mg/kg (n = 5)	G3- 444,57 mg/kg (n = 5)
Females mating (n)	05	05	05
Females with implants (n)	05/05	05/05	05/05
Mating index (%)	100%	100%	100%
Pregnant uterus (g)	2.95 ± 0.5	3.25 ± 1.4	5.09 ± 1.7
Corpora lutea (n)	9.2 ± 0.4	10.8 ± 1.0	10.6 ± 1.9
Implants (n)	7.3 ± 2.9	7.6 ± 3.8	11.8 ± 1.4
Index of deployment (%)	102.22 ± 32.77	82.47 ± 43.85	100 ± 5.44
Pre-implantation losses (n)	24.44 ± 18.26	37.17 ± 35.32	11.50 ± 16.92

Values represent the mean ± S.D (n = 5/group). One way ANOVA/Tukey ($p < 0.05$). n = number, g = gram.

Table 2
Effect of oral treatment with EHAo (G2 and G3) on the biochemical parameters of rats with interrupted pregnancy on the 13th day of gestation.

Parameters	**G1-Control (n = 5)**	**G2- 88, 91 mg/kg (n = 5)**	**G3- 444,57 mg/kg (n = 5)**
Triglyceride (mg/dL)	72.75 ± 15.16[b]	76.4 ± 18.13[c]	169.4 ± 17,64
Cholesterol (mg/dL)	27.25 ± 5.44[b]	32.6 ± 2.60[c]	44.6 ± 2.60
HDL cholesterol (mg/dL)	18.75 ± 3.30[b]	23.4 ± 1.10[c]	31.8 ± 4.02
LDL cholesterol (mg/dL)	6.45 ± 1.79[b]	5.79 ± 2.88[c]	34.6 ± 3.35
Glucose (mg/dL)	135.25 ± 4.93	136.2 ± 3.67	137.4 ± 3.34
Urea (mg/dL)	35.25 ± 4.64	33.4 ± 7.44	33 ± 8.21
Creatinine (mg/dL)	0.3 ± 0.04	0.4 ± 0.17	0.5 ± 0.19
ALT (U/L)	40.5 ± 24.61	34.8 ± 19.88	33.2 ± 10.40
AST (U/L)	142 ± 10.23[a]	96.2 ± 67.82	102.6 ± 30.12
ALP (U/L)	53 ± 16.95	53.8 ± 17.63	54.4 ± 17.67
GGT (U/L)	3.5 ± 0,57	3.6 ± 3.39	3.8 ± 3.56

Values represent the mean ± S.D (n = 5/group). One way ANOVA/Tukey ($p < 0.05$).
[a] Comparison between G1 vs G2 groups.
[b] Comparison between G1 vs G3 groups.
[c] Comparison between G2 vs G3 groups.

Table 3
Effect of oral treatment with EHAo (G2 and G3) on the hematological parameters of rats with interrupted pregnancy on the 13th day of gestation.

Parameters	**G1-Control (n = 5)**	**G2- 88, 91 mg/kg (n = 5)**	**G3- 444,57 mg/kg (n = 5)**
Leukocyte (10^3/μL)	6.618 ± 1.22	6.464 ± 1.99	6.768 ± 0.50
Neutrophils (%)	28.05 ± 5.02[b]	22.07 ± 13.24[c]	11.15 ± 0.50
Lymphocytes (%)	71.4 ± 4.53	77 ± 12.79	88.75 ± 0.50
Monocytes (%)	0.03 ± 0.42	0.03 ± 0.22	0.02 ± 0.00
Eosinophils (%)	0.11 ± 0.07	0.13 ± 0.22	0.10 ± 0.00
Basophils (%)	0.10 ± 0.14	0.25 ± 0.25[c]	0.00 ± 0.00
RBC (10^6/μL)	8.23 ± 0.85	7.78 ± 0.41	8.69 ± 0.25
HGB (g/dL)	14.94 ± 2.05	14.2 ± 0.70	15.6 ± 0.70
HCT (%)	46.6 ± 6.37	45.35 ± 2.42	47.9 ± 0.57
MCV (fL)	56.55 ± 1.91	57.1 ± 0.94	56.5 ± 0.98
MCH (pg)	18.1 ± 0.57	18.15 ± 0.41	17.95 ± 0.35
MCCH (g/dL)	33.05 ± 0.07	33.23 ± 0.22	33.25 ± 1.21
RDW	13.4 ± 0.85	13 ± 0.54	12.5 ± 0.28
Platelets (10^9/L)	857 ± 28.28[a,b]	1018 ± 28.92	1108 ± 28.29

Values represent the mean ± S.D (n = 5/group). One way ANOVA/Tukey ($p < 0.05$). HGB (Hemoglobin), HCT (Hematocrit), MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin), MCCH (Concentration Hemoglobin Corpuscular Mean), RDW (Coefficient of VCM variation). [a]Comparison between G1 vs G2 groups, [b]Comparison between G1 vs G3 groups, [c]Comparison between G2 vs G3 groups.

Table 4
Effect of oral treatment with EHAo (G2 and G3) on the relative weight of organs of rats with interrupted pregnancy on the 13th day of gestation.

Organ (g)	**G1-Control (n = 5)**	**G2- 88, 91 mg/kg (n = 5)**	**G3- 444,57 mg/kg (n = 5)**
Spleen	0.2997 ± 0.02	0.2896 ± 0.03	0.2960 ± 0.03
Heart	0.4029 ± 0.04	0.3820 ± 0.06	0.3769 ± 0.03
Liver	3.5988 ± 0.74	3.3701 ± 0.38	3.2330 ± 0.36
Lung	0.4836 ± 0.05	0.5187 ± 0.12	0.5230 ± 0.07
Kidneys	0.6841 ± 0.05	0.7119 ± 0.03	0.7252 ± 0.05
Ovaries	1.1408 ± 0.03	1.0565 ± 0.03	1.2066 ± 0.16
Vaginal canal	0.4627 ± 0.09	0.4492 ± 0.08	0.3585 ± 0.03

Values represent the mean ± S.D (n = 5/group). One way ANOVA/Tukey ($p < 0.05$).

evaluated at the end (Almeida et al., 2000). In females, reproductive capacity involves the varied aspects of reproductive physiology, related to the capacity of production of adequate gametes, balanced hormonal production, and the morphological and biochemical alterations of the oviduct and uterus (Mukherjee and Mitra, 2009). However, many studies have already shown that plants may be useful for anti-fertility purposes in both rodents and humans (Kumar and Mishra, 2011).

Administration of EHAo did not interfere with the mating of rats and, in all mated rats, spermatozoa were present in the vaginal smear, showing a mating index of 100% and 100% of implants. Such characteristics suggest that EHAo has no contraceptive effects. There was no statistically significant difference in the number of luteal bodies, implantations, and preimplantation losses (Table 1).

When implantation occurs, the embryo can continue its normal development or be absorbed (Mourik et al., 2009; Yakubu and Bukoye, 2009). Certain substances may interfere in the preimplantation period, causing increased losses, developmental delay, fetal death, and malformations (Almeida and Lemonica, 2000; Goonasekera et al., 1995; Leite et al., 2004).

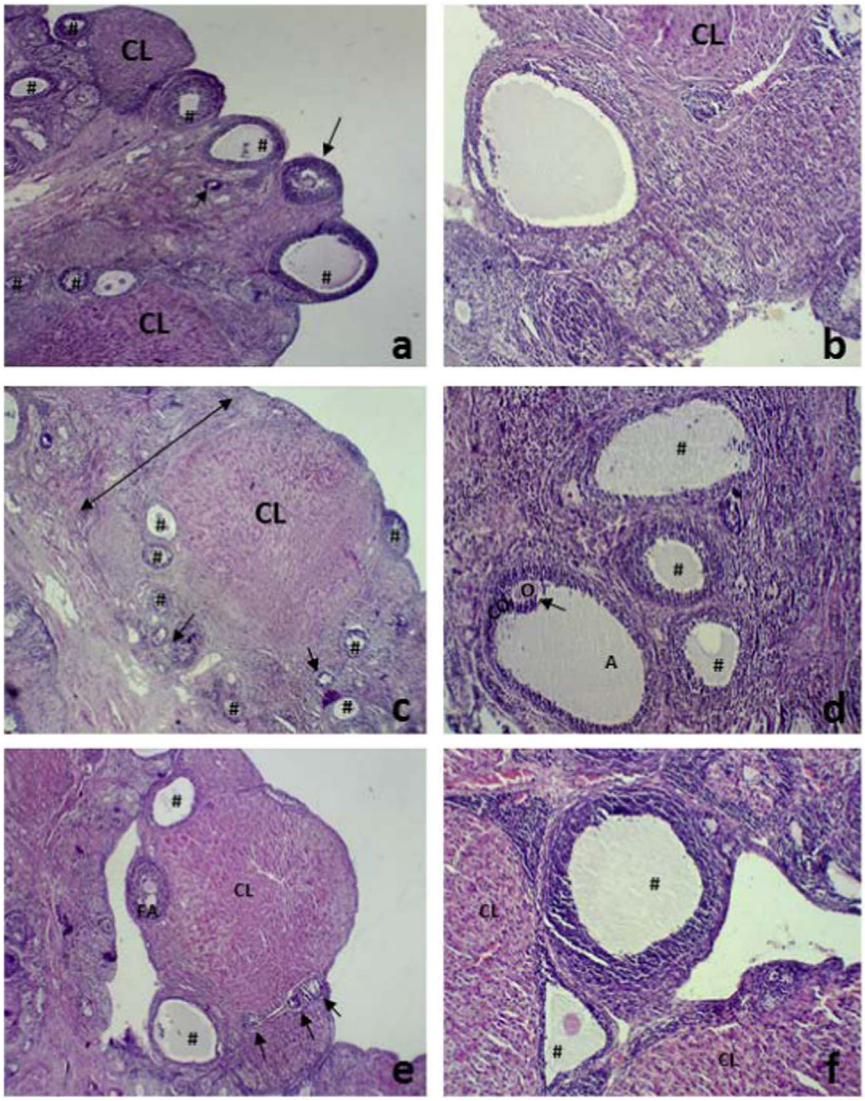


Fig. 6. Effect of oral treatment with distilled water (0.5 mL/animal, **G1** group) and EHAo (**G2** and **G3** groups). Photomicrographs of histological sections of wistar rats ovaries in cross-section, H.E. Note: **a)** Presence of primordial ovarian follicle (small arrow), fully developing follicles (#), presence of developing antral follicle (large arrow) and presence of corpus luteum (CL) (**G1**) (100×). **b)** Observe well preserved region, with presence of corpus luteum (CL) and developing follicle (#) (**G1**) (400×). **c)** In the cortical region (double arrow), observe the presence of primordial follicles (arrows), developing follicles (#) and presence of corpus luteum (CL) (**G2**) (100×). **d)** Presence of well-developed secondary or antral follicles (oocytes (O), *cumulus oophorus* (CO), radiated corona (arrow), follicular antrum (A)) and developing follicles (#) **G2** (400×). **e)** Cortical region and presence of primordial ovarian follicles (arrows), developing follicles (#), antral follicle (FA) and presence of corpus luteum (CL) (**G3**) (100×). **f)** Presence of developing follicles (#) and observe corpus luteum (CL) (G3) (400×).

The implantation index, resorption index, and preimplantation loss are important parameters for evaluating the number of blastocytes implanted in the uterus and of those that did not develop (Almeida and Lemonica, 2000; Chang et al., 2002). In this study, the absence of changes in these indices indicates that EHAo does not present a toxic effect on embryo development before implantation, which may lead to embryo loss, as observed by Kong et al. (1989) in the *Ruta graveolens* L. species.

Regarding the biochemical results, the treated groups (G2 and G3) presented a significant increase ($p < 0.05$) in the triglycerides, total cholesterol, and fractions (Table 2). However, these changes are within the reference values for the sex and age of the animals (Giknis and Clifford, 2008). However, due to the lack of studies and to the clinical relevance of such findings produced by EHAo, the involvement of alkylamides (spilanthol) in the increase in lipids should be verified with more extensive studies.

Also, according to the evaluation of the biochemical parameters (Table 2), there was no significant difference between the levels of urea and creatinine, as well as for the values of the enzymes transaminases (ALT and AST), ALP and GGT (OECD, 2001; Sá et al., 2015; Vásquez and Guerrero, 2007). These results indicate that treatment with EHAo under the experimental conditions was not able to cause renal and hepatic damage.

The analysis of hematological parameters is of great importance for the evaluation of risk. Changes in the hematological system have a high predictive value for human toxicity when data are converted from animal studies (Olson et al., 2000). In the present study, the statistical difference found in the number of neutrophils (G1 vs. G3) is within the range of references described for the species and age group, which suggests that this variation is not clinically important (Table 3) (Giknis and Clifford, 2008).

In non-clinical reproductive toxicity assays, monitoring the vital organ function of animals that were treated with a particular substance is important because changes in these organs may compromise the reproductive process (Bailey et al., 2004; Wolfsegger et al., 2009; Zenick and Clegg, 1989). No macroscopic alteration of the vital and reproductive organs that could signal any injury caused by EHAo was evidenced, considering the following parameters: color, texture, size, and shape. No significant reduction was observed in the relative weight of these organs (Table 4). The results of the histopathology of the ovaries (Fig. 6) showed that the treatment with EHAo was not able to promote structural changes in this organ, which is responsible for the production of growth factors, hormones, and potentially fertilizable female gametes (Baerwald et al., 2012; Miller and Auchus, 2011; Vickery and Lunenfeld, 2013).

The reproductive function in women requires the integration of hormonal signals from the hypothalamus, pituitary gland, and ovaries (Vanputte et al., 2016). Folliculogenesis is the process of formation,

growth, and follicular maturation. The ovarian follicles consist of an oocyte and a surrounding epithelial layer of teak and granular cells, which develop during intrauterine life until menopause (Hansen, 2015).

In the histological photomicrographs, developing follicles (primordial, secondary, antral, and preovulatory) can be observed in the corpora lutea and the medullary interstitial cells. These observations demonstrate that treatment with EHAo did not interfere in folliculogenesis (Devoto et al., 2009; Hansen, 2015). It is emphasized that although many follicles are activated, only a small percentage is able to develop until ovulation because a large part suffers from atresia (Cortvrindt and Smitz, 2001; Edson et al., 2009; Jahromi et al., 2015).

In this study, there was no ovarian acyclicity and changes in follicular development, suggesting that EHAO did not interfere with the amount and release of androgens (Liu et al., 2015; Walters et al., 2012). There was no follicular damage indicative of temporary infertility (primary, secondary and antral) nor permanent infertility (primordial follicles) (Hirshfield, 1997; Vanputte et al., 2016).

In studies using the extract of *Myracrodruon urundeuva* Allen., results were also observed in which there was no influence on the estrous cycle and on follicular development (Oliveira et al., 2016). Another study with the species *Senecio biafrae*, also from the *Asteraceae* family, used in the African pharmacopeia for the treatment of many diseases, including infertility, demonstrated that the biochemical and physiological parameters of fertility were significantly improved (Lienou et al., 2015). Sharma et al. (2011) studied *Spilanthus oleracea* L., basionym of *Acmella oleracea* (L.) R. K. Jansen, ethanolic extract showed dose dependent effect on the FSH, LH and testosterone serum levels, and in vitro nitric oxide release was significantly higher compared to the control group. The aphrodisiac potential of this ethanolic *Spilanthes acmella* extract was demonstrated in vitro and in vivo. N-Alkylamides were attribute to the improved sexual potential and this study support to the utilization as a sexual stimulating agent.

All studies published so far on the action of *Acmella oleracea* on sexual performance were performed in male rats, therefore, our study contributes to the confirmation of the popular use of this species as female aphrodisiac, even though more studies with hormonal dosages are necessary. It is noteworthy, that the popular preparations used as an aphrodisiac in the Brazilian Amazon region, all are obtained with alcoholic beverages, and in this study a hydroethanolic extract was used, where the presence of a majority alkylamide, spilanthol, was detected. This alkylamide is described as primarily responsible for possible effects on sexual mechanisms (Sharma et al., 2011).

4. Conclusion

From the results obtained, it has been demonstrated that the use of EHAo in the experimental conditions of this study is safe since no signs of maternal toxicity or any gestational or reproductive interference were observed. On the other hand, treatment with EHAo was able to improve the estrous cycle without altering folliculogenicity. This effect may be due to the action of the majority component found in EHAo (Spilanthol). It is suggested to conduct future studies related to the safety of EHAo, including treatment during the other stages of development.

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Authors' contributions

CFR, YMSL, HOC, and RCP carried out in-vivo tests under the supervision of CSL and JCTC. IMF conducted the phytochemical study. ANC and JCTC helped in data analysis, prepared the manuscript and revision of the manuscript.

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