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Exploratory developmental toxicity study of *Andrographis paniculata* standardized extract in Wistar rats

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The herb *Andrographis paniculata* (Burm.f.) Nees has long been integrated into diverse medicinal preparations for its reported analgesic, antipyretic, anti-inflammatory, antidiabetic, antiviral, and anticancer properties. These remedies are also administered to women of reproductive age, including during pregnancy, though information on fetal safety remains limited. A study on developmental toxicity was undertaken in rats to determine the reproductive safety profile of *A. paniculata* extract for pregnant women. Pregnant Wistar rats were treated orally with *A. paniculata* extract suspended in 0.25% carboxymethylcellulose at daily doses of 0, 30, 100, or 300 mg/kg from gestation day 7 to day 17. Daily body weight and feed intake were recorded. On day 20, the rats were euthanized to record dam and fetal parameters. The pups were euthanized and processed for skeletal and visceral examinations as per the standard protocol. The body weight gain and total feed intake of rats during the gestation period were unaffected by the treatments. Dam and fetal parameters did not vary significantly in the treated groups compared with the control group. Skeletal evaluations revealed delayed ossification of the sternebrae and ischium, along with occasional rib number anomalies, particularly in the high-dose group. These findings were interpreted as transient developmental delays rather than true teratogenic outcomes. Overall, the preliminary rat study indicated that *A. paniculata* extract did not produce teratogenic effects.

Keywords: *Andrographis paniculata*, ICHS5(R3), Preclinical developmental toxicity, Teratogenicity

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The species *Andrographis paniculata* (Burm.f.) Nees, a member of the Acanthaceae family, is native to India and also found across several Asian regions including Thailand, Vietnam, and China. This plant is frequently incorporated into traditional systems of medicine such as Ayurveda, Siddha, Unani, and Homeopathy[1]. Preparations containing *A. paniculata* are often prescribed to women of reproductive age for managing conditions like viral illnesses (including dengue, influenza, and COVID-19), fever, respiratory infections, as well as certain cardiac and hepatic disorders[2-5].

In a 90-day sub-chronic oral toxicity study, standardized *A. paniculata* extract exhibited no adverse effects in rats at doses up to 900 mg/kg/day[6]. Administration of *A. paniculata* extract containing 10% andrographolide at doses up to 1000 mg/kg/day did not produce adverse effects on gametogenesis, androgen production, or fertility in rats[7]. However, Ogundola *et al*.[8] reported that *A. paniculata* extract could impair male fertility by lowering testosterone levels. Likewise, the combination of andrographolide with curcumin has been shown to exert antifertility effects in rats, likely mediated through anti-estrogenic activity and reduced ovarian follicle counts[9]. Findings regarding the reproductive effects of *A. paniculata* extract remain inconsistent, and data concerning its impact on embryonic development are largely absent. Clarifying the developmental toxicity potential of *A. paniculata* extract and its active constituent, andrographolide, in rats is essential for evaluating risks associated with its use during pregnancy. Given the paucity of data on teratogenic effects of *A. paniculata* in rats, a preliminary developmental toxicity study was performed in accordance with the ICH S5(R3) (2020) guidelines on reproductive and developmental toxicity testing for human pharmaceuticals[10].

Materials and Methods

Chemicals

A standardized extract of *Andrographis paniculata* Wall. Ex. Nees (APE; batch no. RD19560, product

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code NRAPE30, manufactured August 2019) was kindly provided by Natural Remedies Pvt. Ltd., Bangalore, for use in this study. Field's stain A and B for vaginal smear staining were procured from HiMedia. Formoaceto alcohol (FAA) was prepared by adding 50 mL of 95% ethanol, 2.5 mL of acetic acid, 5.5 mL of 37% formaldehyde, and 42 mL of distilled water. FAA was used to preserve the pups for visceral examination. Alizarin Red staining was prepared by mixing 0.025% of alizarin stain in 1% potassium hydroxide. A 0.6% thymol solution and clearing solution were prepared by mixing 40 mL of 70% ethanol, 40 mL of 85% glycerin, and 20 mL of benzyl alcohol.

Animals

Adult female Wistar rats (8-10 weeks old, nulliparous) and proven breeder males were procured from the Laboratory Animal Medicine Unit, Centre for Animal Health Studies, TANUVAS, Madhavaram, Chennai, following approval by the Institutional Animal Ethics Committee, (IAEC No.08/SA/IAEC/2020, dt. 21.11.2020), and used in this study. All rats were acclimatized for at least seven days in an experimental animal room at the Central Laboratory Animal Facility at Madras Veterinary College, Chennai. Environmental conditions, such as temperature (24-26^{o}C), relative humidity (50±20%), were provided. Food and water were provided *ad libitum*. This study was performed according to the ICH harmonized guidelines on the detection of reproductive and developmental toxicity for human pharmaceuticals, S5(R3), adopted on Feb 18, 2020. The work was designed as a preliminary investigation under the ICH guidelines, employing a minimum of six pregnant rats per treatment group, which included control, low dose (30 mg of *A. paniculata* extract (APE) / kg of body weight), mid-dose (100 mg/kg), and high dose (300 mg/kg). The dose used in this study was based on the results of a 90-day toxicity study of APE at the same doses, wherein no adverse effects on the female estrous cycle or maternal toxicity were observed up to 300 mg/kg/day in a separate study (unpublished data, Srinivasan).

Study design and procedure

Estrous cyclicity in females was assessed through vaginal cytology with Field's staining[11]. Female rats that showed regularity in the estrous cycle were selected for the study and allowed to mate with male rats at a female-to-male ratio of 2:1 (female: male). Vaginal smears of co-habituated females were checked for the presence of sperm and stage of the estrous cycle. The first day of confirmed pregnancy (GD1) was designated based on detection of spermatozoa in vaginal smears or post-estrus diestrus staging. Oral administration of APE was performed from GD7 through GD17 at 10 mL/kg dosing volume to achieve target doses of 30, 100, and 300 mg/kg/day. Body weight and feed consumption were recorded daily until the day of necropsy (GD20). Dams were deeply anesthetized with 2-3% isoflurane in oxygen prior to necropsy. A midline incision was made on the abdomen and underlying muscles. Gravid uterine horns were removed and weighed, and the number of pups in each horn and resorption (partial or complete), if any, was counted. Subsequently, the dam was exsanguinated by puncturing its abdominal aorta. The gravid uterine weight was recorded. The uterus was cut open to expose the fetus and its corresponding placenta. Fetuses were kept individually in separate Petri dishes containing normal saline. The ovaries were removed from the dam, weighed, and counted for corpus luteum in each ovary, and the same was recorded. Each fetus was assessed for gross external malformations, and measurements of body weight and crown-rump length (CRL) were documented. Pup examination for teratology was performed as procedure described[12]. Each pup was identified individually by tying a colored thread on its limbs and was examined for any external defects.

Euthanasia of pups

Fetuses were humanely euthanized *via*., immersion in chilled physiological saline (10-20°C), inducing hypothermia and anesthesia. After confirming anesthesia in the absence of a touch stimulus, the vital organs were removed and further subjected to skeletal staining or immersion in FAA solution for visceral examination[13].

Visceral examination of pups

The pups were stored in Formo Aceto Alcohol (FAA) after examination for any gross abnormalities or defects till their examination. Before performing the visceral examination, the pups were immersed in an Industrial Methylated spirit for 24 h to remove formalin. The following day, various sections were made through the fetal body, which were observed grossly under appropriate magnification for any kind

of defect. The head sectioning was done with the slight modification of Critchell (2013)[14], as shown in Figure 1. Visceral organs were examined using the microdissection method for any abnormality[15].

Skeletal staining and examination of pups

Skeletal evaluations were carried out using alizarin red staining following standard protocol[16]. Euthanized pups were eviscerated and stored in thymol solution (6 g/L). The skeletal staining procedure for rat pups is presented in a flow diagram below.

The stained skeletons of the rat pups were examined individually for any abnormalities and the ossified sites were counted.

Statistical analysis

To verify uniformity of variance, the Brown-Forsythe test[17] was applied to the quantitative data. In cases where homogeneity was confirmed, one-way ANOVA with Dunnett's multiple comparison test was subsequently employed[18]. If the variance was not homogenous, the data were transformed to its reciprocal and again subjected to the BFT to check the homogeneity of variance. When variance homogeneity was achieved after transformation, the dataset was analyzed using one-way ANOVA with Dunnett's post hoc comparisons; in cases where the assumption was still not met, the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons was applied.

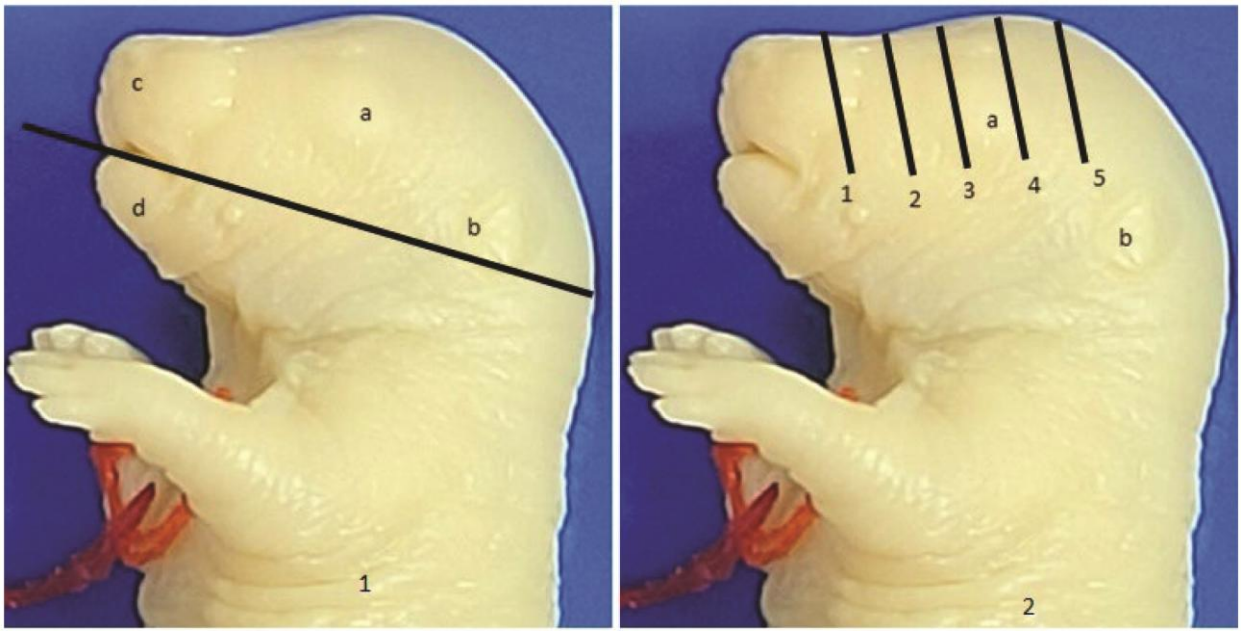

Fig. 1 — Schema of head sectioning to observe for the developmental defects in the parts of the head (1) Normal head of rat fetus (a) Eyelids (b) Ear (pinna) (c) Nares (d) Lower jaw. The line shows where section is made to examine the palantine shelf to examine the presence of cleft palate (2) Normal head of the rat fetus (a) Eye lids (b) Ear (pinna). Lines 1-5 show where sections are made from dorsum of the head to examine the cranial abnormality

I Bath; Thymol solution (0.6%) – Min. 7 days

⇩

II Bath; KOH (1%) – 24-36 h

⇩

III Bath; Alizarin stain (0.025% alizarin stain in KOH (1%) – 24 h

⇩

IV Bath; Clearing solution (70% ethanol + 85% glycerol + Benzyl alcohol in 4:4:2) – 24 h

⇩

IV Bath; 85% glycerol (for preservation until analysis)

Categorical outcomes, such as sex distribution, were assessed using the chi-square test. All statistical analyses were conducted with GraphPad Prism (version 5.02).

Results

The daily mean body weight of dams in all treatment groups is shown in Figure 2. Maternal body weight gain across treatment groups did not differ significantly from controls. Feed intake from GD8 to GD18 remained comparable between treated and control groups (Fig. 3).

The parameters of the dam, such as body weight gain, gravid uterus weight, litter size, percentage of live fetuses, number of corpora lutea, loss of implantation, early or late, and the parameters of the fetus, such as pup weight, pup crown-rump length, placental weight, and percentage of female pups, are presented in Table 1.

No statistically significant differences were observed in fetal endpoints between treatment and control groups. Various skeletal abnormalities were observed in these pups (Table 2). The most frequent skeletal variations in the high-dose group included increased incidence of incomplete ossification in sternebrae and ischium, along with asymmetry in rib numbers (Fig. 4). In visceral examination of the pups, the only abnormality observed was hemorrhage in various organs. However, the incidence of hemorrhage did not vary between the treated and control groups. Notably, a higher incidence of hemorrhage was observed in the lungs of all treatment groups. This could be more of a procedural effect than an abnormality due to the treatment, as this change was also observed in the control group pups[19]. There was no incidence of cleft palate in either the control or the treated groups (Fig. 5).

The images show normal observations of the head and the visceral organs. (1) Comparison of the size of

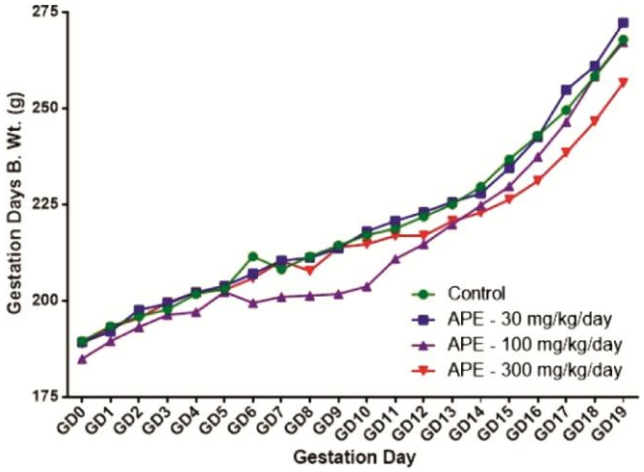

Fig. 2 — Bodyweight of dams on gestation days of teratogenicity study with *Andrographis paniculata* extract (APE). NS - p>0.05 in Two-way ANOVA

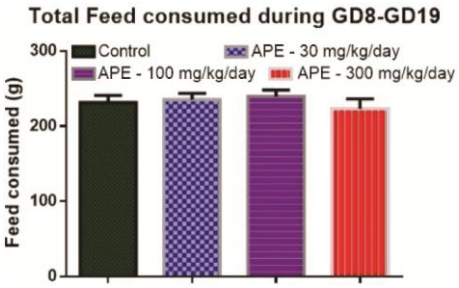

Fig. 3 — Feed consumption of dams on Gestation Days in Teratology study with *Andrographis paniculata* extract (APE) Each bar represents Mean ± SEM; p>0.05

Table 1 — Dam and fetal parameters of teratology study with *Andrographis paniculata* extract (APE) in Wistar rats

Dam and Pups Parameters	Control	APE 30 mg/kg	APE 100 mg/kg	APE 300 mg/kg
No. of dams	8	8	7	7
Mean Body weight gain(g) GD20 Vs GD1	91.3±4.8	101.6±3.7	89.7±6.1	95.6±7.8
Mean weight of Gravid uterus (g)	61.2±8.2	68.5±4.8	65.7±7.4	57.8±5.5
Mean no. of pups/litter	10.8±2.1	11.1±2.1	10.7±2.5	12.2±1.9
% of Live fetuses	100	100	100	100
Mean no. of Corpus lutea/dam	13.2±2.3	12.3±1.9	11.7±1.4	12.8±2.2
Loss of Implantation	2	1	1	1
Early /Late Resorption	0	0	0	0
No. of dams with no pups (%)	2/8 (25)	1/8 (12.5)	0/7 (0)	2/7 (28.6)
Weight of pup (g)	3.0±0.2	3.5±0.2	3.1±0.2	2.9±0.2
Crown-rump length of pups (cm)	3.4±0.09	3.7±0.09	3.7±0.10	3.4±0.08
Weight of placenta (g)	0.41±0.02	0.40±0.01	0.41±0.01	0.40±0.01
% Female pups	46.4±11.9	48.2±6.5	47.2±6.5	49.1±7.3

Values are expressed as means SEM or numbers or percentages, p>0.05 by one way ANOVA for mean data, by Kruskal-Wallis test for median data, or by Chi-square test for percentage of female pups

Table 2 — Skeletal examination of pups of the dams treated with *Andrographis paniculata* extract (APE) from GD6 to GD17

Parameters	Control	APE 30 mg/kg	APE 100 mg/kg	APE 300 mg/kg
Total no. of pups examined	33	33	40	39
Head: Incomplete ossification(%)				
Nasal	6.1	0	0	0
Premaxilla	6.1	0	0	0
Frontal	21.2	0	17.5	17.9
parietal	21.2	12.1	17.5	17.9
Interparietal	21.2	12.1	17.5	20.5
supraoccipital	18.2	12.1	17.5	20.5
Mandible	3.0	0	0	0
Zygomatic	3.0	0	17.5	2.6
Squamosal	3.0	0	7.5	0
Thoracic Cavity: Abnormality (%)				
Unequal right and left ribs	3.0	3.0	0	12.8*
No. of Sternabrae ossified ≤5	27.3	21.2	32.5	51.3*
Pelvic Cavity: % Incomplete ossification of				
Ilium	0	0	0	0
Ischium	0	0	12.5	15.4*
Pubis	9.1	0	17.5	15.4
Vertebral Bones Ossified (%)				
Cervical (<7)	0	0	0	0
Thoracic (<13)	0	18.2	0	12.8
Lumbar (<6)	0	0	0	0
Sacral (<4)	15.2	0	12.5	15.4
Caudal (<5)	66.7	75.8	72.5	89.7
No. of Phalanges (Metacarpals/Metatarsals) Ossified< 3 (%)	21.2	0	17.5	12.8

Values are expressed as the percentage incidence. *p<0.05 by Chi-square test

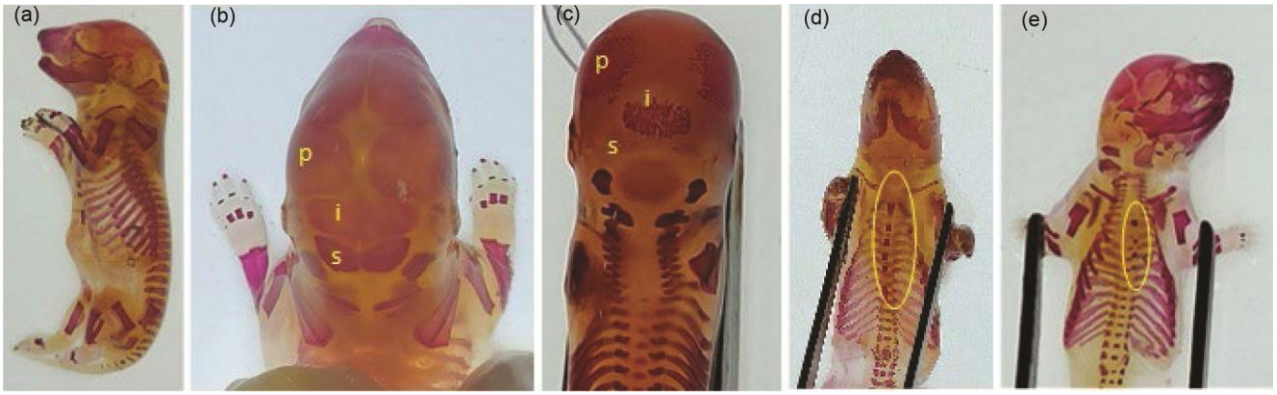

Fig. 4 — Skeletal staining of fetuses with Alizarin Red dye in teratogenicity study in rats with *Andrographis paniculata* extract (a) Normal Skeletal development; (b) complete ossification of the cranium; (c) incomplete ossification of the [p]parietal, [i]interparietal, and [s]supraoccipetal bones; (d) complete ossification of six sternabrae; and (e) incomplete ossification of sternabrae – 3 nos.

the pups in the control and high-dose groups, (2) microdissection of the abdomen showing the normal architecture of the visceral organs, (3) lungs showing the hemorrhagic lesion, (4) serial sectioning of the head illustrating various parts (a) nasal septum, (b) nasal conchae, (c) palantine shelf, (d) olfactory bulb, (e) eyelids, (f) lens, (g) nasal tract, (h) maxilla, (i) superior sagittal sinus, (j) lateral ventricles, (k) third ventricle, (l) indication of the internal ear, and (5) Palantine shelf showing normal palatine ridges (p).

Discussion

Pregnant rats were exposed to standardized *A. paniculata* extract at 30, 100, and 300 mg/kg/day during GD7-17 to evaluate its teratogenic potential. Treatment-associated effects were limited to increased frequencies of incomplete ossification of sternebrae and rib asymmetry in the high-dose group pups. All

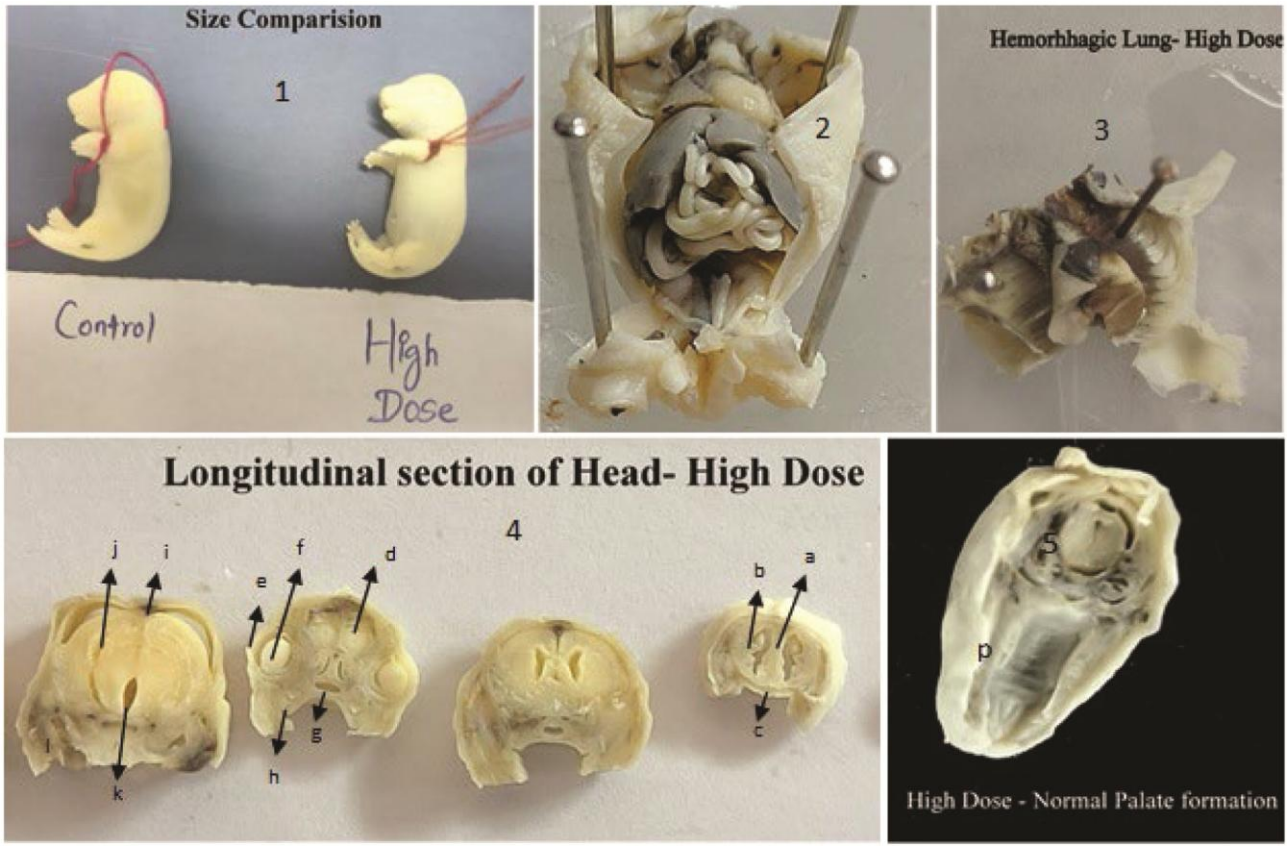

Fig. 5 — Visceral Examination of pups in Teratogenicity study in rats with *Andrographis paniculata* extract

other parameters studied were comparable to control group.In the current study, there was no effects on the litter size, though it was previously reported that a combination of curcumin and andrographolide reduced the number of implants and the size of the litters in rats compared to control rats[9]. It should be noted that the animals were dosed with andrographolide from gestation days 1 to 7 and sacrificed on day 10 of gestation in that study, which enables studying the preimplantation loss; in contrast, in our study, we initiated dosing from day 7 when the implantation would have already completed on day 5[(20)]. Delayed ossification in structures including the sternebrae, ischium, caudal vertebrae, and irregular rib formation indicates slowed fetal development, as these skeletal sites ossify at later stages in pups[21-23]. These alterations are considered developmental delays rather than malformations, as ossification proceeds to completion postnatally[11]. A previous study with a 50% ethanolic extract of APE containing 1.33% andrographolide showed teratogenic effects, such as micrognathia and rhinocephaly, in SD rats[24]. In the present study, no abnormalities were observed in visceral organs. Findings from this preliminary investigation suggest that *A. paniculata* extract (34.34% andrographolide) is non-teratogenic when administered to Wistar rats. However, further studies, such as definitive embryo-fetal developmental toxicity studies in rats and rabbits, as per ICH S5(R3)2020[(10)], are required to confirm the results obtained in this preliminary embryo-fetal developmental toxicity study.

Conclusion

This preliminary embryo-fetal toxicity study indicates that standardized *A. paniculata* extract (34.34% andrographolide) administered during organogenesis in pregnant Wistar rats does not induce teratogenic effects. Skeletal variations at the highest dose were attributable to delayed ossification, not structural malformations, and are anticipated to resolve after birth. In the absence of effects on litter size, visceral development, or key reproductive parameters, the findings provide preliminary evidence supporting developmental safety; however, confirmatory studies in multiple species and with extended evaluations are needed to fully establish its reproductive safety profile.

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Conflict of Interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the manuscript.

Author Contributions

Conceptualization; SMR, RS. Formal analysis; SMR, RTV. Resources; RS, TKG. Supervision; RS, TKG. Roles/Writing - original draft; SMR. Writing - review & editing; RS, TKG.

Ethics Approval

The study was approved by the InstitutionalAnimal Ethics Committee (IAEC No.08/SA/IAEC/2020, dt. 21.11.2020) was used to conduct a teratogenicity study in Wistar rats. Animals were maintained in accordance with the guideline of Committee for the Control and Supervision of Experimentation on Animals (CCSEA), Compendium of CPCSEA, 2018. The experiment was conducted following ICH S5(R3) guideline, 2020.

Data Availability

All the raw data is available with the corresponding author and will be provided on request.

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