



Prenatal developmental toxicity study of an alkaloid-free *Ageratum conyzoides* extract powder in rats by oral administration

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ABSTRACT

A prenatal developmental toxicological study was conducted to evaluate the safety of an alkaloid-free *Ageratum conyzoides* extract powder administration on pregnant female Wistar rats and on the development of the conceptus in accordance with OECD test guideline (no. 414). Pyrrolizidine alkaloids (PAs) naturally present in *A. conyzoides* have been shown to induce toxicity in past studies, particularly towards hepatic cells. Therefore our test item preparation of *A. conyzoides* extract (aerial part of the plant) consisted of the removal of PAs. There were no treatment related adverse effects found during maternal examinations (body weights, food consumption, numbers of pregnant and non-pregnant female rats, endocrine evaluation, gravid uterine weights, and number of corpora lutea), maternal/fetal examinations (numbers of implantation sites, pre- and post-implantation loss (%), dead and live fetuses (%), resorption sites), or fetal examinations (litter size and weights, number of fetuses, sex ratio, or external, visceral, and skeletal variations and malformations) in the *Ageratum conyzoides* extract powder groups at doses of 500, 1000 and 2000 mg/kg bw/day compared to vehicle control group. The no observed adverse effect level (NOAEL) determined for both maternal and developmental toxicity was 2000 mg/kg bw/day, which was the highest dose tested.

1. Introduction

Recently, the therapeutic use of botanical supplements has generated heightened interest among the scientific community. Consequently, Health and Pharmaceutical world leaders such as the U.S Food and Drug Administration (USFDA) and World Health Organization (WHO) have made considerable research awareness investments in natural and botanical supplements for better living (WHO, 2002). *Ageratum conyzoides* Linn., commonly known as Billy Goat Weed belongs to the family Asteraceae and tribe Eupatorieae. Being an annual herb, *A. conyzoides* has a long history of traditional and folkloric use in several parts of the world including Africa, Asia and South America due to its documented antimicrobial, anti-inflammatory, anti-oxidant, analgesic, antipyretic and gastro-protective properties (Adebayo et al., 2010; Galati et al., 2001;

Okunade, 2002). Bioactive compounds isolated from this plant include flavonoids, pyrrolizidine alkaloids (PAs), coumarins, chromenes, benzofurans, sterols and terpenoids (Kamboj and Saluja, 2008). The compositional amount of all these phytochemicals vary from region to region and from different parts within the plant, accounting for the multifarious use of the plant globally (Bosi et al., 2013).

There is currently limited information on the effect of *A. conyzoides* in humans. Nevertheless, there have been a number of *A. conyzoides* toxicity studies conducted in animals (Palmer et al., 2019). Additionally, studies investigating the functional properties of *A. conyzoides* have also investigated its toxicity (Adebayo et al., 2010; Diallo et al., 2014). Among the bioactive compounds present in *A. conyzoides*, PAs, such as 1, 2-desifropirrolizidinic and lycopsamine have shown to cause hepatotoxicity in animal models (Adebayo et al., 2010; Diallo et al., 2010). PAs are found in many flowering plants around the world and are known to

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Abbreviations

OECD	Organization for economic co-operation and development
NOAEL	No Observed Adverse Effect Level
PA	Pyrrolizidine alkaloids
ALP	alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate transaminase
GD	Gestational day
FT3	Free triiodothyronine
FT4	Free thyroxine
TSH	Thyroid stimulating hormones
RTS	Retrorsine.

produce toxic effects including hepatotoxicity, genotoxicity and carcinogenesis in animals and humans (Couet et al., 1996; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011; Guo et al., 2019; Moreira et al., 2018; Wiedenfeld, 2011). Studies have also shown prenatal PA administration to be causative of developmental toxicity in animal models. Administration of purified PAs such as heliotrine, lasiocarpine and retrorsine (RTS) were found to cause liver lesions and malformation in the developing rat fetuses, with no apparent effects on the dams (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011; Schoental, 1959). It was suggested that PA may induce infantile liver disease in humans through the contamination of human or cow's milk (Green and Christie, 1961).

In 2010, Adebayo et al. conducted a 14-day safety profile study of a 95% ethanolic leaf extract of *A. conyzoides* Linn. via gastric intubation in rats and found that though markers of liver injury were decreased at higher dosages (1000 and 1500 mg/kg bw/day); all treatment groups exhibited some level of hepatocellular necrosis at histopathological examination as well as significant increases in liver and spleen weights (Adebayo et al., 2010). Additionally, they found an elevated white blood cell count, mean platelet volume and % platelet distribution width associated with treatment. Moreover, Diallo et al., in 2014, conducted a 90-day repeated-dose oral toxicity study of a hydroalcoholic extract (90:10 ethanol-water) of *A. conyzoides* in Wistar rats (Diallo et al., 2014) dosed at either 500 or 1000 mg/kg bw/day. They found that treatment with *A. conyzoides* significantly increased liver, spleen and kidney weights as well as elevating markers of liver stress, namely alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate transaminase (AST) compared to control.

In 2019 Palmer et al. investigated the toxicity of an alkaloid-free 90% ethanolic extract of *A. conyzoides* through a battery of toxicological tests in rats (Palmer et al., 2019). The reported results showed no evidence of mutagenicity, clastogenicity or in vivo genetic toxicity. Moreover, in the 90-day repeated-dose oral toxicity study, the no observed adverse effect level (NOAEL) was determined to be 2000 mg/kg bw/day, the highest dose administered, for both male and female rats. This indicates that the toxic effects of *A. conyzoides* could indeed be largely dependent on its alkaloid fraction.

Therefore further in vivo toxicological research on *A. conyzoides* should be undertaken to get a clearer guideline for its preparation and safe dosage as a therapeutic supplement in humans. This prenatal developmental study was conducted to detect adverse effects on female pregnant Wistar rats and on the development of the conceptuses consequent to the exposure of the female with an alkaloid-free *A. conyzoides* extract powder by gavage administration from gestation day 5–19. As per our knowledge, this study is the first to explore the presence or absence of prenatal developmental toxicity of *A. conyzoides* extract powder, rendering it toxicologically relevant for the safety assessment of *A. conyzoides*.

2. Materials and methodology

The study was performed in compliance with Principles of Good Laboratory Practice as specified by OECD GLP, ENV/MC/CHEM (98)17) (OECD, 1998), except that analytical control of the test item was not performed, and under the permission of the Institutional Animal Care and Use Committee of Toxi-Coop Zrt. according to the National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and in compliance with the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) and Government Decree 40/2013 regulating animal protection. The study followed the procedures indicated by the internationally accepted guidelines and recommendations of OECD Test Guideline No. 414, Prenatal Developmental Toxicity Study (OECD, 2018).

2.1. Animals

One hundred thirty healthy, young adult nulliparous non-pregnant female SPF Han: WIST rats (8–9 weeks) were procured from the breeding facility, TOXI-COOP ZRT (Budapest, Hungary). Eighty healthy, experienced male Han: WIST rats (29–30 weeks) were used for mating. Six days post acclimation in the animal house, on each day, the females were paired to males in the mornings for two to 4 h (male:female ratio of 1:3) until the number of sperm positive females per group (4 different groups) were at least 22 rats. The day of finding sperm in the vaginal smear was considered day '0' of pregnancy (Gestation Day (GD) 0) and thereafter, sperm positive females were separated and caged in groups of a maximum of 2 animals. Group allocation was randomized on each day of mating such that group averages of GD 0 body weight were as similar as possible; females paired with the same male were allocated to different groups. The animal cages were type II polypropylene/poly-carbonate with facilities for food and water. The bedding was produced by J. Rettenmaier® & Sohne GmbH + Co.KG (Lignocel® Safe ¾-S-Fasern Bedding). The animal housing rooms were with central air conditioning and were kept between 19.1 and 23.9 °C and relative humidity of 38–68% with 10 air exchanges/hour, and the daily illumination cycle was set to 12 h of light and dark. The animals received ssniff® SM R/M-Z + H "Autoclavable complete feed for rats and mice - breeding and maintenance" (ssniff Spezialdiäten GmbH, Germany) and tap water from municipal supply, as for human consumption from 500 mL bottles *ad libitum*.

2.2. Test item

The test item, *A. conyzoides* extract (of the aerial part of the plant) (Batch No. AGE/18008), with certificate of analysis and safety data sheet was supplied by the Sponsor (Gencor Pacific Limited, Hong Kong). To manufacture the test item, the aerial parts of *A. conyzoides* were heated in ethanol:alcohol (90:10) and subjected to mild pressure. Then it was followed by distillation, filtration and concentration. To remove the alkaloids the residues were precipitated at room temperature. Absence of PAs were confirmed through an external HPTLC method as compared to reference standard (crotaline) and to raw plant material. The typical compositional analysis of the test item was described previously (Palmer et al., 2019). The powder was formulated in the vehicle (distilled water, Batch No. 190101; Magilab Kft., Budapest, Hungary) in three different concentrations: 50, 100 and 200 mg/mL for the dose levels of 500, 1000 and 2000 mg/kg body weight/day respectively. Each formulation to be administered to the animals was prepared by careful weight measurement just prior to administration daily and was used up within 4 h after preparation.

2.3. Main study

The dose levels used in this study were based on a 90-Day repeated-dose oral toxicity study of *A. Conyzoides* extract powder in rats in which

the NOAEL was determined as 2000 mg/kg bw/day in male and female Han: WIST rats (Palmer et al., 2019). The sperm-positive female rats were divided into 4 groups of 25, 22, 26, and 23 dams/group as follows: Group 1 (Vehicle-Control; G1), Group 2 (Low; G2), Group 3 (Mid; G3) and Group 4 (High; G4), respectively. The dams were treated by gavage administration at doses of 0 (vehicle-control), 500 (low), 1000 (mid) and 2000 (high) mg/kg bw/day from GDs 5 to 19. The treated dose volume in all four groups was 10 mL/kg bw based on the most recent individual body weights.

During the study period dams were checked twice daily for mortality, clinical signs of ill health, and adverse reactions to treatment and behavioral changes. Body weight (GDs 0, 3, 5, 8, 11, 14, 17 and 20) and food consumption (between GDs 0 to 3, 3 to 5, 5 to 8, 8 to 11, 11 to 14, 14 to 17 and 17 to 20) of the dams were recorded until prior to their terminal sacrifice. On the day of sacrifice (GD 20), blood samples from the sublingual vein were collected from all surviving dams for determination of thyroid hormones free triiodothyronine (FT3), free thyroxine (FT4) and thyroid stimulating hormone (TSH). The quantitative determination of these hormones in serum samples were performed by electrochemiluminescence immunoassay with COBAS e411 immunochemistry analyzer.

Following blood collection, the dams of all groups were anesthetized with Isoflurane CP® (Medicus Partner Kft. (Biatorbágy, Hungary) and exsanguinated from the abdominal aorta, after which, a caesarean section was performed for the immediate removal of the fetuses, followed by gross pathological examination of the dams' viscera. Thyroid glands (together with parathyroids) of all dams were weighed and prepared for histological examination. The corrected body weight (body weight minus the weight of the gravid uterus) was calculated for GD 20. The removed uteri and left ovary were weighed and all uteri and both ovaries were examined to determine the number corpora lutea, implantation sites, early and late resorptions, and the number of live and dead fetuses. Animals in which unambiguous implantation, but no fetuses were found, were considered pregnant. Uteri that appeared empty were stained with 2% potassium hydroxide solution (Yamada et al., 1988) to determine the presence of any implantation sites which may have resorbed very early.

Following the removal of fetuses from the opened uteri, euthanasia was performed by hypothermia. Each live fetus and its placenta were weighed individually and subjected to external examination. The sex of the fetuses was determined by the anogenital distance. About half the fetuses of each litter were subjected to visceral examination by means of a dissecting microscope after fixation in Sanomiya mixture (920 mL 100%-os isopropyl alcohol, 30 g sulfosalicylic acid hydrate, and 50 mL acetic acid (95 or 99%)). The heads were examined by Wilson's free-hand razor blade method. The cadavers (with viscera and skin removed) of the remaining half of each litter were fixed in alcian blue-acetic acid-ethanol mixture for staining of cartilage (Peters, 1977) and the skeletons were stained by the KOH-Alizarin red-S method (Staples and Schnell, 1964) after fixation in isopropanol. Skeletons were examined by means of dissecting microscope.

2.4. Statistical analysis

The parameters evaluated in the main study were subjected to means and standard deviation and/or percentage calculations. Statistical analysis was performed with SPSS PC + software version 4 (SPSS, Inc., Chicago, IL, USA). Dams or litters were the unit for statistical analyses and a *P*-value <0.05 was considered statistically significant. The heterogeneity of variance between groups was checked by Bartlett's homogeneity variance test. For the data with no detected heterogeneity, a one-way analysis of variance was carried out. On obtaining positive results of the said analysis, Duncan's Multiple Range test was used to assess the significance of inter-group differences.

In detecting significant heterogeneity, the normal distribution of the data was examined by the Kolmogorov-Smirnov test. In case of a non-normal distribution, the non-parametric method of Kruskal-Wallis

One-Way analysis of variance was used. Upon receiving a positive result, the inter-group comparison was performed using the Mann-Whitney *U* test.

3. Results and discussion

3.1. Maternal observations

One dam in the 1000 mg/kg bw/day group died on GD 9. Clinical signs of hunched back, piloerection, and red discharge on the nose were observed in this animal on GDs 7 and 8. At necropsy, the esophagus, stomach, and duodenum were filled with blood, which was clotted in the latter two compartments. No other clinical signs leading up to the death or macroscopic lesions at necropsy were observed, and the death was ruled as due to mis-gavage. Implantation sites and corpora lutea were counted, and microscopic examination of the thyroid was performed, and the results were added to the individual data.

No other mortalities were observed during the study. Observations of the remaining dams during the 20 days prior to termination revealed no clinical signs of systemic toxicity (Table 1) in any of the treatment groups. There were no statistically significant or dose-dependent differences in dam body weights in any of the treatment groups at any time points during the study. Some transient, slight, but statistically significant, differences in body weight gain in the low- and mid-dose groups and food consumption in the mid- and high-dose groups were observed

Table 1
Effect of AC extract powder on maternal parameters in pregnant female rats.

Parameters	AC extract treatment			
	G1	G2	G3	G4
	VC	500 mg/kg	1000 mg/kg	2000 mg/kg
Pregnancy data				
Number of evaluated litters	18	21	17	17
Confirmed pregnancy at necropsy	20	22	18	18
Pregnancy Rate (%)	80%	100%	69%	78%
Maternal Data				
Mean body weight (g)	205.6 ± 18.07	201.0 ± 13.47	208.4 ± 15.14	201.4 ± 12.58
Mean body weight (g) GD05	228.8 ± 19.11	227.8 ± 13.66	231.2 ± 14.72	226.5 ± 11.69
Mean body weight (g) GD 20	326.8 ± 20.58	322.3 ± 25.29	319.7 ± 20.16	322.6 ± 16.14
Gravid uterus weights (g)	57.6 ± 9.07	57.0 ± 10.77	55.5 ± 9.43	58.5 ± 6.17
Mean corrected body weight (g)	269.2 ± 19.56	265.4 ± 17.24	264.2 ± 17.78	264.1 ± 14.84
Mean body weight change (g) during GD5 to GD20	97.9 ± 12.21	94.6 ± 17.71	88.5 ± 19.91	96.2 ± 15.24
Food consumption (g/day) during GD5 to GD20	24.4 ± 1.92	24.0 ± 1.89	22.0 ± 2.64	22.5 ± 1.99
FT3 (ng/dL)	0.3136 ± 0.0356	0.2979 ± 0.0308	0.2855 ± 0.0301*	0.2894 ± 0.0275*
FT4 (ng/dL)	1.6867 ± 0.2084	1.6210 ± 0.2032	1.5312 ± 0.2148*	1.5047 ± 0.1962*
TSH (μIU/mL)	–	–	–	–
Dilated renal pelvis (%)	5	9	12	11
Thyroid weight (g)	0.0223 ± 0.0017	0.0220 ± 0.0018	0.0225 ± 0.0018	0.0217 ± 0.0015
Thyroid histopathology (no.)	0	0	0	0
Placental weight (mg)	696.0 ± 71.70	669.6 ± 62.83	654.1 ± 63.33	659.2 ± 53.51
Relative placental weight (mg/g)	236.0 ± 23.33	224.3 ± 20.27	237.4 ± 27.63	223.7 ± 20.54

Values are expressed as means ± standard deviation (SD). –, data were below the limit of detection (0.005 μIU/mL); VC, Vehicle Control; GD, Gestation Day. * = *p* < 0.05, ** = *p* < 0.01.

without dose relationships and did not affect overall body weight development and, therefore, were deemed to be unrelated to treatment with the test item.

Hormonal evaluation revealed statistically significant reductions in mean FT3 and FT4 levels in the 1000 and 2000 mg/kg bw/day groups when compared to the control. However, since the values in these groups were well within the historical control ranges of the laboratory and had no correlating histopathology or alterations in thyroid weights, they were not considered as adverse. TSH levels in all groups were below the detection limit of the assay and, therefore, provided no insight into thyroid function in the dams.

Necropsy examination yielded no significant abnormalities. The presence of one-sided dilated renal pelvis was observed at a low incidence in all groups, including the control group, and hydrometra was observed in a single non-pregnant animal in each of the control and mid-dose groups, suggesting these findings were not treatment related. No statistically significant differences compared to controls were observed in gravid uterine weights, corrected body weights, corrected body weight gain, or placental weights (absolute and relative) of the treated groups (Table 1). No statistically significant differences were observed in numbers or percent of pregnant rats. Thus, the maternal examinations revealed no adverse events caused as a result of treatment with *A. conyzoides* extract.

3.2. Maternal/fetal observations

Treatment with *A. conyzoides* extract did not cause statistically significant differences in the mean numbers of corpora lutea, mean percentage of pre- and post-implantation loss, mean number of implantations, mean percentages of early and late resorptions and dead fetuses, mean percentage of total and intrauterine mortality, or mean number of viable fetuses when compared to control. A summary of these results is seen in Table 2.

3.3. Fetal observations

There were no significant differences in sex ratio, fetal weight, or ano-genital distance (absolute and normalized) per litter between treatment groups and control (see Table 3). The number of litters with malformed fetuses (three in control, one in 500 and two in the 1000 mg/kg bw/day groups respectively) did not show a dose-dependent relationship. Furthermore, there were no incidences of litters with malformed fetuses in the 2000 mg/kg bw/day group.

Treatment with *A. conyzoides* with doses of 500, 1000 and 2000 mg/kg bw/day did not cause any external malformations. The number of fetuses (litters) examined for external abnormalities were 202 (18), 231 (21), 190 (17) and 195 (17) in the control, 500, 1000 and 2000 mg/kg bw/day groups, respectively. The fetal incidence of total external abnormalities was statistically significantly increased compared to controls in both the 500 and 1000 mg/kg bw/day groups; however, the incidence per litter differed statistically significantly only at 1000 mg/kg bw/day, which was due to an increased incidence of body weight retardation (limit: below 2.20g (2 standard deviations from control) for males and for females). Four fetuses from two litters of the 500 mg/kg bw/day group and 15 fetuses from five litters of the 1000 mg/kg bw/day group showed body weight retardation. In the 1000 mg/kg bw/day group, 60% occurred within a single litter. As the incidence was low and confined largely to a single litter and was without a dose response (there was no incidence of external variations in the highest dose group (2000 mg/kg bw/day)), it was not considered to be related to administration of the test item. One fetus from one litter in the 500 mg/kg bw/day group displayed a variation in the form of a slightly flatter vertex but this was not proved at skeletal examination and was considered likely a technical artifact due to processing. External abnormality data is presented in Table 3.

In terms of visceral examinations (see Table 3), 103 (18), 116 (21),

Table 2

Effect of AC extract powder on ovarian and intrauterine parameters in pregnant female rats.

Parameter	AC Extract (mg/kg)			
	VC	500	1000	2000
	G1	G2	G3	G4
All litters ^a	20	22	18	18
Corpora lutea (no.)	13.1 ± 1.99	11.8 ± 2.84	12.1 ± 2.14	12.9 ± 1.23
Total implantation (no.)	11.4 ± 3.91	11.1 ± 3.14	11.1 ± 3.37	12.1 ± 1.84
Pre-implantation loss (%) ^b	13.6 ± 25.84	5.9 ± 10.93	9.7 ± 23.73	6.0 ± 11.83
Post-implantation loss (%) ^c	14.8 ± 23.36	10.0 ± 21.12	4.3 ± 5.89	13.1 ± 22.59
Early resorptions (%)	11.9 ± 22.9	4.4 ± 6.27	2.3 ± 3.75	9.5 ± 19.15
Late resorptions (%)	2.9 ± 4.45	5.3 ± 21.45	1.4 ± 3.19	3.3 ± 5.09
Litters available for evaluation ^d	18	21	17	17
Viable fetuses (no.)	10.1 ± 4.18	10.5 ± 3.32	10.6 ± 3.22	10.8 ± 2.96
Dead fetuses (%)	0.0 ± 0.00	0.3 ± 1.64	0.5 ± 2.02	0.4 ± 1.57
Total intrauterine mortality (%)	23.5 ± 29.60	15.3 ± 23.03	13.6 ± 23.55	16.1 ± 22.10

Values (with the exception of number of litters) are expressed as means ± standard deviation per litter. a, Females with confirmed pregnancy (excluding dams that died unrelated to treatment (e.g., mis-gavage)) expressed as absolute count). b, [(Number of Corpora Lutea – Number of implantations)/Number of Corpora Lutea]X100. c, [(Number of implantations – Number of viable fetuses)/Number of implantations]X100 (%). d, All litters – Total litter loss (note, Total litter loss = Number of dams with total pre-implementation loss + Number of dams with total post-implantation loss).

96 (17) and 99 (17) fetuses (litters) were examined in the control, 500, 1000 and 2000 mg/kg bw/day groups respectively. One fetus from one litter in the control and one fetus from one litter in the 1000 mg/kg bw/day group exhibited situs inversus totalis, a malformation not attributed to the treatment. Observed visceral variations included bilateral hydroureter and hydroureter with dilated renal pelvis, which were present in all experimental groups. These results were not statistically significant, did not exhibit a dose relation, and also occurred in the control litters and, therefore, were not considered as a consequence of treatment.

Ninety-nine (18), 115 (21), 94 (17), and 96 (17) fetuses (litters) were examined for skeletal abnormalities in the control, 500, 1000 and 2000 mg/kg bw/day groups, respectively (see Table 3). The skeletal examination revealed malformations in two fetuses from two litters in the control group and one each from one litter in the 500 and 1000 mg/kg bw/day groups respectively. In the 1000 mg/kg bw/day group, the sternal cartilage was split in one fetus from one litter. One fetus from one litter in the 500 mg/kg bw/day group exhibited bifurcate and fused ribs in addition to multiple malformed vertebrae (Th VIII half absent, Th VII inclined, 12 thoracic present). One fetus in the control group displayed hemicentric ossification of a thoracic vertebrae and a different fetus in the control group displayed a bent scapula. The low incidence and the diversity in the type of malformations as well as the fact that malformations were present in the control group suggested they were not a result of treatment. Moreover, no fetus in the highest dose group exhibited any malformations. Variations found in the skeletal examination included retarded ossification of the skull, incompletely ossified or unossified skull bones, bipartite supra occipital, unossified hyoid bone, less than three ossified or bipartite and/or asymmetric sternbrae, wavy ribs, dumb-bell shaped or bipartite and/or asymmetric vertebrae, unossified or incompletely ossified thoracic, lumbar or sacral centra and/or arches, unossified pubis or ischii, and less than 2.5/3 or asymmetrically ossified metacarpal/metatarsal. The overall incidence of

Table 3
Effect of AC extract powder on fetal development.

Parameter	AC Extract (mg/kg)			
	VC	500	1000	2000
	G1	G2	G3	G4
Total no. of Fetuses (Litters) examined	202 (18)	231 (21)	190 (17)	195 (17)
Sex ratio (%) ^a	50.5 ± 14.10	48.1 ± 14.35	46.2 ± 17.44	51.4 ± 10.84
Fetal weight (g) ^b	3.0 ± 0.34	3.0 ± 0.35	2.8 ± 0.34	3.0 ± 0.21
Anogenital distance absolute (mm) (M)	4.5 ± 0.37	4.7 ± 0.38	4.5 ± 0.44	4.6 ± 0.40
Anogenital distance normalized (AGD/3 √bw) (M)	3.1 ± 0.17	3.2 ± 0.18	3.2 ± 0.21	3.2 ± 0.25
Anogenital distance absolute (mm) (F)	3.0 ± 0.04	3.0 ± 0.11	3.0 ± 0.16	3.0 ± 0.14
Anogenital distance normalized (AGD/3 √bw) (F)	2.1 ± 0.08	2.1 ± 0.10	2.1 ± 0.09	2.1 ± 0.10
Anogenital distance absolute (mm) (M + F)	3.8 ± 0.24	3.8 ± 0.34	3.7 ± 0.37	3.8 ± 0.27
Anogenital distance normalized (AGD/3 √bw) (M + F)	2.6 ± 0.15	2.6 ± 0.21	2.6 ± 0.20	2.7 ± 0.15
External Examination				
Total no. of Fetuses (Litters) examined	202 (18)	231 (21)	190 (17)	195 (17)
External abnormalities, no. Fetuses (Litters)	0 (0)	6* (3)	15** (5*)	0 (0)
% per Litter Incidence ±SD	0.0 ± 0.00	2.4 ± 6.80	7.2 ± 15.76*	0.0 ± 0.00
External malformations, no. Fetuses (Litters)	0 (0)	0 (0)	0 (0)	0 (0)
% per Litter Incidence ±SD	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
External variations, no. Fetuses (Litters)	0 (0)	6* (3)	15** (5*)	0 (0)
% per Litter Incidence ± SD	0.0 ± 0.00	2.4 ± 6.80	7.2 ± 15.76*	0.0 ± 0.00
Retardation in body weight ^c , no. Fetuses (Litters)	0 (0)	4 (2)	15** (5*)	0 (0)
% per Litter Incidence ± SD	0.0 ± 0.00	1.7 ± 6.14	7.2 ± 15.76*	0.0 ± 0.00
Head (vertex): flatter (slight), no. Fetuses (Litters)	0 (0)	1 ^d (1)	0 (0)	0 (0)
% per Litter Incidence ± SD	0.0 ± 0.00	0.4 ± 1.68	0.0 ± 0.00	0.0 ± 0.00
General: pale, no. Fetuses (Litters)	0 (0)	2 (1)	0 (0)	0 (0)
% per Litter Incidence ± SD	0.0 ± 0.00	0.7 ± 3.36	0.0 ± 0.00	0.0 ± 0.00
Visceral Examination				
Total no. of Fetuses (Litters) examined	103 (18)	116 (21)	96 (17)	99 (17)
Visceral abnormalities, no. Fetuses (Litters)	6 (5)	4 (1*)	2 (2)	9 (6)
% per Litter Incidence ±SD	5.3 ± 9.42	3.2 ± 14.55	2.2 ± 6.12	9.3 ± 16.37
Visceral malformations, no. Fetuses (Litters)	1 (1)	0 (0)	1 (1)	0 (0)
% per Litter Incidence ±SD	0.8 ± 3.37	0.0 ± 0.00	1.2 ± 4.85	0.0 ± 0.00
General: situs inversus totalis, no. Fetuses (Litters)	1 (1)	0 (0)	1 (1)	0 (0)
% per Litter Incidence ± SD	0.8 ± 3.37	0.0 ± 0.00	1.2 ± 4.85	0.0 ± 0.00
Visceral variations, no. Fetuses (Litters)	5 (4)	4 (1)	1 (1)	9 (6)
% per Litter Incidence ± SD	4.5 ± 9.21	3.2 ± 14.55	1.0 ± 4.04	9.3 ± 16.37
Ureters and/or kidneys:				
Hydroureter (bilateral), no. Fetuses (Litters)	1 (1)	1 (1)	0 (0)	0 (0)
% per Litter Incidence ± SD	1.4 ± 5.89	0.8 ± 3.64	0.0 ± 0.00	0.0 ± 0.00
Hydroureter and dilated renal pelvis, no. Fetuses (Litters)	4 (3)	3 (1)	1 (1)	9 (6)

Table 3 (continued)

Parameter	AC Extract (mg/kg)			
	VC	500	1000	2000
	G1	G2	G3	G4
% per Litter Incidence ± SD	3.1 ± 7.69	2.4 ± 10.91	1.0 ± 4.04	9.3 ± 16.37
Skeletal Examination				
Total no. of Fetuses (Litters) examined	99 (18)	115 (21)	94 (17)	96 (17)
Skeletal abnormalities, no. Fetuses (Litters)	21 (12)	24 (13)	30 (12)	23 (13)
% per Litter Incidence ±SD	21.4 ± 20.62	19.3 ± 19.16	32.3 ± 33.07	23.5 ± 17.96
Skeletal malformations, no. Fetuses (Litters)	2 (2)	1 (1)	1 (1)	0 (0)
% per Litter Incidence ±SD	1.9 ± 5.63	0.8 ± 3.64	1.0 ± 4.04	0.0 ± 0.00
Sternabra:				
Split in cartilage, no. Fetuses (Litters)	0 (0)	0 (0)	1 (1)	0 (0)
% per Litter Incidence ±SD	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 4.04	0.0 ± 0.00
Ribs and vertebrae:				
Fused and bifurcate ribs, multiple malformed vertebrae, no. Fetuses (Litters)	0 (0)	1 (1)	0 (0)	0 (0)
% per Litter Incidence ± SD	0.0 ± 0.00	0.8 ± 3.64	0.0 ± 0.00	0.0 ± 0.00
Vertebrae:				
Thoracic centrum-hemicentric ossification, no. Fetuses (Litters)	1 (1)	0 (0)	0 (0)	0 (0)
% per Litter Incidence ± SD	1.1 ± 4.71	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Pectoral girdle:				
Scapula bent, no. Fetuses (Litters)	1 (1)	0 (0)	0 (0)	0 (0)
% per Litter Incidence ± SD	0.8 ± 3.37	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Skeletal variations, no. Fetuses (Litters)	19 (11)	23 (13)	29 (11)	23 (13)
% per Litter Incidence ± SD	19.5 ± 20.62	18.5 ± 18.89	31.3 ± 33.80	23.5 ± 17.96
Skull:				
Incomplete ossification (≥3 bones), no. Fetuses (Litters)	0 (0)	1 (1)	0 (0)	2 (2)
% per Litter Incidence ± SD	0.0 ± 0.00	0.6 ± 2.73	0.0 ± 0.00	2.4 ± 6.64
Incomplete ossification, marked/not ossified, no. Fetuses (Litters)	2 (2)	6 (5)	7 (5)	3 (2)
% per Litter Incidence ± SD	1.7 ± 5.02	4.5 ± 8.45	6.8 ± 13.24	3.1 ± 9.16
Retarded, no. Fetuses (Litters)	0 (0)	0 (0)	0 (0)	1 (1)
% per Litter Incidence ± SD	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.2 ± 4.85
Supra occipital bipartite, no. Fetuses (Litters)	1 (1)	1 (1)	5 (2)	1 (1)
% per Litter Incidence ± SD	1.1 ± 4.71	0.6 ± 2.73	5.9 ± 16.98	1.2 ± 4.85
Hyoid not ossified, no. Fetuses (Litters)	1 (1)	4 (2)	4 (4)	3 (3)
% per Litter Incidence ± SD	0.8 ± 3.37	2.8 ± 8.87	3.8 ± 7.23	3.1 ± 7.02
Sternabra:				
2 or 2.5 ossified, no. Fetuses (Litters)	5 (4)	2 (2)	6 (5)	5 (4)
% per Litter Incidence ± SD	5.1 ± 10.91	1.4 ± 4.44	6.0 ± 9.88	5.0 ± 9.57
1 ossified, no. Fetuses (Litters)	5 (4)	2 (2)	6 (4)	4 (4)
% per Litter Incidence ± SD	5.4 ± 12.67	1.4 ± 4.44	7.4 ± 14.37	4.2 ± 7.85
0 ossified, no. Fetuses (Litters)	7 (3)	3 (2)	4 (3)	1* (1)
% per Litter Incidence ± SD	5.8 ± 15.28	2.4 ± 7.97	4.0 ± 9.15	1.2 ± 4.85
Bipartite and/or asymmetric, no. Fetuses (Litters)	0 (0)	0 (0)	0 (0)	2 (2)
% per Litter Incidence ± SD	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	2.0 ± 5.78

(continued on next page)

Table 3 (continued)

Parameter	AC Extract (mg/kg)			
	VC	500	1000	2000
	G1	G2	G3	G4
Ribs:				
Wavy, no. Fetuses (Litters)	3 (3)	2 (2)	0 (0)	3 (3)
% per Litter Incidence $\pm$ SD	3.1 $\pm$ 7.41	1.3 $\pm$ 4.04	0.0 $\pm$ 0.00	3.3 $\pm$ 7.45
Vertebrae:				
<i>Thoracic centra</i> - dumbbell shaped, no. Fetuses (Litters)	1 (1)	0 (0)	0 (0)	0 (0)
% per Litter Incidence $\pm$ SD	1.9 $\pm$ 7.86	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00
<i>Thoracic centra</i> -bipartite or bipartite and asymmetric, no. Fetuses (Litters)	2 (2)	5 (4)	3 (2)	2 (2)
% per Litter Incidence $\pm$ SD	1.9 $\pm$ 5.63	4.4 $\pm$ 10.35	3.4 $\pm$ 9.80	2.2 $\pm$ 6.12
<i>Thoracic centra</i> -thoracic centrum not ossified, no. Fetuses (Litters)	1 (1)	0 (0)	2 (1)	0 (0)
% per Litter Incidence $\pm$ SD	0.9 $\pm$ 3.93	0.0 $\pm$ 0.00	1.7 $\pm$ 6.93	0.0 $\pm$ 0.00
<i>Lumbar</i> -centra or arch not ossified, no. Fetuses (Litters)	0 (0)	0 (0)	2 (1)	0 (0)
% per Litter Incidence $\pm$ SD	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	1.7 $\pm$ 6.93	0.0 $\pm$ 0.00
<i>Sacral</i> -from SI centrum not ossified, no. Fetuses (Litters)	1 (1)	2 (2)	1 (1)	0 (0)
% per Litter Incidence $\pm$ SD	0.9 $\pm$ 3.93	1.5 $\pm$ 5.03	0.8 $\pm$ 3.46	0.0 $\pm$ 0.00
<i>Sacral</i> -SII or from SII centra or arches not ossified, no. Fetuses (Litters)	3 (2)	2 (2)	3 (2)	0 (0)
% per Litter Incidence $\pm$ SD	2.6 $\pm$ 8.36	1.7 $\pm$ 5.54	2.7 $\pm$ 7.80	0.0 $\pm$ 0.00
Pelvic girdle:				
<i>Pubis and/or ischii</i> -not ossified, no. Fetuses (Litters)	7 (4)	3 (2)	6 (3)	2 (2)
% per Litter Incidence $\pm$ SD	7.1 $\pm$ 13.84	2.4 $\pm$ 7.97	5.7 $\pm$ 14.81	2.4 $\pm$ 6.64
Fore-and hindlimbs:				
<i>Metacarpal/metatarsal</i> -asymmetric ossification, no. Fetuses (Litters)	0 (0)	3 (2)	2 (2)	3 (2)
% per Litter Incidence $\pm$ SD	0.0 $\pm$ 0.00	2.7 $\pm$ 9.29	2.2 $\pm$ 6.12	2.7 $\pm$ 7.80
<i>Metacarpal/metatarsal</i> -less than 2.5/3 ossified, no. Fetuses (Litters)	5 (2)	4 (2)	13* (5)	9 (6)
% per Litter Incidence $\pm$ SD	4.2 $\pm$ 12.43	2.8 $\pm$ 8.87	14.0 $\pm$ 24.68	9.1 $\pm$ 14.53

Fetuses from litters per group were examined. Litter data are expressed as means $\pm$ standard deviation. VC, Vehicle control; M, Male; F, Female; SD, standard deviation. * $p < 0.05$; * $p < 0.01$. a, (Male fetus/total viable fetuses) $\times 100$; b, Male and Female combined; c, Below 2.20g (2 SD) for males and females; d, This was not proved at skeletal examination.

these variations was not statistically significant compared to controls and did not exhibit a dose response and, as such, was not deemed to be treatment related.

The lack of toxicologically significant findings in our study could be related to the removal of potentially toxic alkaloids in our *A. conyzoides* extract powder. Previously, Palmer et al. also investigated the toxicity of an alkaloid-free ethanolic extract of *A. conyzoides* and demonstrated a lack of genetic toxicity or general oral toxicity (Palmer et al., 2019). These results are in contrast to prior toxicity studies by Diallo et al. and Adebayo et al., where oral administration of *A. conyzoides* extracts at doses 500, 1000 and 1500 mg/kg bw/day correlated with increased hepatotoxicity (Adebayo et al., 2010; Diallo et al., 2014). While these studies did not explicitly state alkaloid levels, it was assumed that they were present due to no mention of their removal.

PAs are present in around 3% of flowering plants. While they possess several beneficial properties including anti-inflammatory, antimicrobial

and antitumor activities among others, they are found to be toxic at high doses (Moreira et al., 2018). This is potentially due to it being produced by plants as a defense mechanism against insect herbivores (Guo et al., 2019). Previous studies have shown PAs to be hepatotoxic, genotoxic and carcinogenic in several animal models (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011; Moreira et al., 2018). Considering bioactivation of PAs occurs largely in the liver, their toxicity mainly affects this organ (Moreira et al., 2018). One pathway of metabolic activation of PAs results in the formation of dehydro pyrrolizidine alkaloids (DHPA). DHPAs are able to bind to DNA and proteins resulting in the formation of adducts, leading to hepatocyte damage (Ma et al., 2018; Moreira et al., 2018). The development of tumors, caused by the PA riddelline in rodents, is attributed to the formation of these DNA adducts. Importantly, given that riddelline is metabolised similarly in human and rodent liver microsomes, it is likely that it could cause tumorigenicity in humans. In addition to the liver, PAs can also cause toxicity to lungs, neurotoxicity and teratogenicity (Moreira et al., 2018).

The ability of PAs to cross the placental barrier and enter the fetal blood circulation enables them to also cause toxicity in embryos, accounting for the numerous studies reporting fetal mortality and teratogenesis with PA administration during pregnancy (Guo et al., 2019). Purified PAs such as heliotrine have reported fetal malformation at doses of 100–300 mg/kg maternal body weight in rats along with decreased fetal weight and length in dams dosed at 50 mg/kg bw/day (Green and Christie, 1961). Another PA, RTS, was found to be more toxic to the pups than the dams when administered via intraperitoneal injection (4–10 mg/kg for up to 14 days) to lactating rats (Schoental, 1959). In 2015, Diallo et al., reported that oral administration of 500 and 1000 mg/kg/bw/day HAE of *A. conyzoides* during late pregnancy in Wistar rats caused a statistically significant reduction in fetal weight ($p < 0.01$) (Diallo et al., 2015). The fetal toxicity was attributed to the presence of PAs in *A. conyzoides*. The proposed mechanism of action was the oxidative stress induced by PA which can affect cell proliferation, differentiation and apoptosis (Brocardo et al., 2011; Dennery, 2007). Additionally, Guo et al., 's 2019 study found that intragastric administration of two PAs, RTS (>95% purity) and monocrotaline (>99% purity) at doses of 20 mg/kg bw/day, to pregnant rats from GD9 - GD20 induced hepatic and pulmonary toxicity in fetuses as seen by the presence of pyrrole metabolites and oxidative injury (Guo et al., 2019). Reduction in body and tail lengths of fetuses further correlated with growth retardation as a consequence of PA administration. The study authors also suggested that fetuses are more susceptible to PA-induced toxicity compared to their mothers due to their decreased ability to eliminate toxic substances.

Our study was conducted with an alkaloid-free test item of *A. conyzoides* extract powder and we were able to demonstrate that this extract has no clinically or statistically significant toxicity on rat pre-natal development nor was any maternal toxicity observed. Therefore, our study further verifies the lack of toxic effects of alkaloid-free *A. conyzoides* extract. This is a step forward in the pursuit of a therapeutic supplement for humans to yield the wide range of benefits of the plant.

4. Conclusion

Our study is to our knowledge, the first that investigated the prenatal developmental toxicity of *A. conyzoides* in the rat model. Therefore, it adds a crucial new dimension to the toxicity studies that were conducted before. Treatment of 22–26 pregnant Wistar rats from GD 5 to 19 by gavage administration of alkaloid-free aqueous *A. conyzoides* extract at varying doses caused no mortality in pregnant Wistar rats. Absence of clinical signs or adverse effects on the body weight, food consumption, FT3 and FT4 hormone levels as well as thyroid weight suggest that the product is devoid of maternal toxicity under the tested conditions. Moreover, the treatment of the dams with the test item under the conditions described in this study caused no adversities on the intrauterine parameters, on fetal and placental weight or upon external, visceral and

skeletal examinations of the fetuses. Based on these observations, alkaloid-free aqueous *A. conyzoides* extract was safe and well tolerated up to 2000 mg/kg bw/day, and the NOAELs for both maternal and developmental toxicity were determined to be 2000 mg/kg bw/day.

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Data availability

The mean data sets generated and utilized for statistical analysis to support the findings of these studies are included within the article. All other raw and processed data used to support the findings of these studies are available from the corresponding author upon request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Authors Silma Subah and Nathasha Bogoda are salaried Research Interns working at Gencor Pacific Limited (the sponsor of the study).

Author Ruchitha Venkatesh worked as a Summer Intern at Gencor Pacific Limited from June 2019 to September 2019 on another research project overseas, not connected to this paper. She participated in the writing of this paper from November 2019.

Author Timothy Murbach is a salaried employee of AIBMR Life Sciences, Inc. (Seattle, WA, USA). AIBMR was contracted by the study sponsor, as an independent third party, to determine appropriate study protocols and dose selection, place the studies, approve the study plans, and monitor the toxicological study herein described and to analyze and interpret the resulting data, contribute to the development of the manuscript, and act as corresponding author for the journal submission.

Author Kornélia Kolep is a salaried employee of Toxi-Coop and Study Director of the reported study and author Róbert Glávits is an independent contractor to Toxi-Coop and Senior Histopathologist on the reported study. Toxi-Coop, Zrt (Balatonfüred, Hungary) was contracted by AIBMR to develop the study plans and conduct, analyze and interpret, and report the results of the toxicological study described.

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