

Assessment of Developmental Toxicity of *Moringa oleifera* (Seed Extract) in Pregnant Wistar rats

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Abstract

In North central Nigeria, pregnant women use *Moringa* seeds as food supplement due to its milk production property. The purpose of this preliminary study was to determine if oral administration of ethanolic seed extract of *Moringa oleifera* to on presumably pregnant Wistar rats was developmentally toxic. Female rats, eight weeks old, were separated into four groups (n=10): the control group received distilled water *ad libitum* while the treatment groups received 50, 200 and 800mg/kg extract from gestation days (GD) 1-19 respectively. Maternal body weight, food and water consumption were observed daily. Laparohysterectomies were performed on day 19, a day before expected parturition of half of the dams, Implantations, uterine weight, foetal resorptions foetal death, viability and body weights were recorded. In addition placental weights, crown-rump, tail lengths and some sex hormone levels were determined. The remaining pregnant rats were allowed to litter and offspring parameters were assessed. No maternal endpoints were affected by the test article at doses of 50 and 200mg/kg. However, there was a much lower viability index at 800 mg/kg compared to all other groups and the Lactation indices in the controls and 200 and 800 mg/kg groups respectively. Also a marked decrease in FSH in all the treated groups and an increase in estrogen level at 800mg/kg were observed. This suggests that the extract may not be safe during organogenic period of pregnancy at 800mg/kg.

Keyword: *Moringa oleifera*, Reproductive Toxicity, Sex Hormones

Introduction

The use of complementary and alternative medicines (CAMs) is increasingly common worldwide. Herbal medicines are one of the oldest forms of treatment for diverse ailments and have become increasingly popular due to their affordability, accessibility, and its impact on the socio-cultural well-being of the people (Tinet *et al.*, 2013; Butani and Verma, 1981).

Moringa oleifera Lam belongs to the family Moringaceae that contains a single genus “Moringa” with thirteen species (Morton, 1991); among these, the *M. oleifera* Lam tree is the most common. The plant is known locally as Ewe-Awogba in Yoruba, Southwest Nigeria and the seeds are chewed raw or soaked in alcohol and used traditionally by pregnant women to aid delivery and improve breast milk production.

The plant is cited for its vast nutritional and medicinal use (Fahey, 2005; Ashfaq *et al.*, 2012; Njan *et al.*, 2014). In the Philippines, the plant is used to increase milk production in nursing mothers and is also prescribed for anaemia (D’Souza and Kulkarni, 1993; Estrella *et al.*, 2000). *M. oleifera* noodles have been reported to increase breast milk productivity and lactiferous gland enlargement of female rats (Titi and Nunung, 2014). Various parts of the plant have been reported to be a rich source of natural antioxidants such as β -carotene, Vitamin C and tocopherol (Faizi *et al.*, 1998). The root has been reported to be used in folk medicine as emmenagogue and abortifacient (Hsu *et al.*, 2006).

Alcoholic extract of *Moringa oleifera* stem bark produced adverse effect on fertility index and number of implantation in the uterine horns of the female rats by virtue of an increase in the percentage of the post-implantation embryonic loss; abortifacient activity was evident in the decrease in the percentage of live fetuses (Varsha and Dinesh, 2014). The root is also known for its sex hormone-related properties like in treatment of thyroid disease (Bose, 2007). Jabeen and colleagues (Jabeen *et al.*, 2008) reported antimicrobial activity of the crude seed extracts against, *B. subtilis*, *S. aureus* and fungi species such as *F. solani*. A polypeptide from *Moringa* seed has been found to exert bactericidal action by inducing membrane destabilization and cytoplasmic leakage in susceptible microorganisms including antibiotic-resistant isolates of *Staphylococcus*, *Streptococcus*, and *Legionella* species (Suarez *et al.*, 2003). In addition,

the anti-asthmatic effects of the seed of *Moringa oleifera* in patients with bronchial asthma has been reported. Likewise, the administration of the seed extract resulted in improvement against breathlessness, wheezing, chest tightness and coughin patients (Agrawal *et al.*, 2008). Acute toxicity studies have also shown that *Moringa oleifera* seed has a wide margin of safety (Songpol *et al.*, 2012). However, there is minimal information available, about the safety of using the seed extracts of *M. oleifera* during pregnancy and beyond. Therefore, our preliminary study was designed to evaluate the pre- and post-natal effects of ethanolic seed extract of *Moringa oleifera* on presumably pregnant Wistar rats.

Materials and methods

Identification and Preparation of Seeds Extract:

Moringa oleifera seed was bought from Avuco farms company Ltd, in Kaduna State, Northwest Nigeria, in February (2015) and authenticated by a plant taxonomist Professor F.A Oladele of the Department of Plant Biology, University of Ilorin. The plant material was stored in the herbarium of the same department with a voucher number UIH/011/1011.

The seed husk was removed to get the seeds that were then crushed with a clean mortar. One kilogram of the crushed seeds of *Moringa oleifera* was defatted by macerating with 5L of Petroleum ether (BDH) at room temperature and allowed to extract for 72h with intermittent shaking (model 3017 rotary shaker. GFL, Burgwedel, Germany). The solution was filtered and the defatted seed was then re-extracted with absolute ethanol (BDH) for another 72hrs. The resultant extract was then freeze-dried to yield 32.3 g of semi-solid residue. The residue was reconstituted in distilled water and appropriate concentrations were used in the experiments

Preliminary Phytochemical Screening

The qualitative analyses of the plant constituents were carried out using the methods described by Harbone (Harbone, 1973; Bargah, 2015). The presence of alkaloids, glycosides, tannins, saponins, terpenoids, flavonoids, glycosides, anthraquinones, volatile oil, and steroids were tested.

Animals

Virgin female wistar rats (*Rattus norvergicus*) were obtained from the Animal Housing facility of the Biological Garden of the University of Ilorin, Ilorin, Kwara State, Nigeria. Male rats of the same source and strain were used as breeders. Rats were acclimated for 13 days prior to initiation of cohabitation. The rats were housed in polycarbonate cages (TECHNIPLAST cages, Italy) in the Animal House facility of the Faculty of Basic Medical Sciences, University of Ilorin, and were maintained under standard conditions with a 12-hour light/dark cycle and temperature between 24-27°C. A certified rodent chow (Oluwagbemisola feeds) and drinking quality water were available *ad libitum*. Animal use procedures were approved by the Animal Care and Use Committee of the University of Ilorin. Animal care and use was conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and in accordance with all applicable regulations. The study was performed in accordance with National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH, 1978).

Hormonal assay kits and other reagents

Sex steroid hormones (LH, FSH, progesterone, estradiol and prolactin) kits were purchased from Monobind Inc., 100 North Pontine Drive, Lake Forest, CA 92630, USA. The assays were carried out according to the standard test procedure by the manufacturer.

Acute toxicity study

Lorke's method (Lorke, 1983) was used to evaluate the acute toxicity of *Moringa oleifera* Ethanolic Seed Extract. The method involves two phases. A total of 12 healthy and mature male mice, without food for 3-4 hours, were used for the study. In the first phase, 9 of the mice were equally distributed into three groups and administered 10mg/kg, 10, 100 and 1000mg/kg of *Moringa oleifera* Ethanolic Seed Extract IP, respectively. Mice were observed for signs of toxicity and behavioural changes (sedation, hyperactivity, diarrhea, writhing, restlessness etc.) for 2h post-dose and mortality for 24h post-dose. In the second phase, three mice were given different doses of 1600, 2900, and 5400 mg/kg IP, respectively. They were also observed for symptoms of toxicity and mortality within 24h post-dose.

Developmental toxicity study

After acclimation, 50 virgin female rats were cohabited with 25 breeder male rats (one male per 2 female). The cohabitation period consisted of a maximum of 5 days; mating was evaluated daily during this period. Female rats with spermatozoa observed in a smear of vaginal contents and/or a copulatory plug observed *in situ* were considered to be at day 0 of presumed gestation (DG 0). Healthy, mated females (10 per group) were randomly assigned to three test and one control group using body weight assessment from DG 0.

Female rats were administered, by oral gavage, 50, 200 and 800mg/kg of ethanolic seed extract of *Moringa oleifera*, respectively from GD 1-19. The Control group was administered distilled water on the same treatment regimen. Dosages were adjusted based on the most recently recorded body weight and administered at approximately the same time each day.

Clinical observations

Rats were observed for general appearance at least weekly during the acclimatisation period, on DG 0 (pre-dosage period), daily before dosage administration, and once on the day of scheduled euthanasia (DG 19). During DG 1-19, post-dosage observations were recorded at approximately hourly intervals for the first four hours and at the end of the normal working day for the first 4 days of the dosage period. Subsequent post-dosage observations were recorded between 1–2 h after dosage administration and at the end of the normal working day. Body weights were recorded at least weekly during the acclimation period, on DG 0 (pre-dosage day), and then once daily during the dosage and post-dosage periods. Food and water consumption values were recorded on DG 0 (pre-dosage period), and then once daily during the dosage period. Remaining food was recorded on the day of scheduled euthanasia.

Pre-natal evaluation of maternal and foetal parameters

On day 20, half of the females (n=5) per group were anaesthetized with ether in a chamber and parameters considered were: implantations, uterine weight, foetal resorptions (early and late), death, viability and body weight, crown-rump and tail length. Uterine horns of each rat were carefully dissected, rinsed and kept in phosphate-buffered saline in Petri dishes to prevent drying. Foetal and placental weights were determined after the live foetuses and their placenta were excised. Numbers of live

and dead fetuses were recorded. Placentae and ovaries were observed for abnormalities. Live fetuses were examined for external malformations only. Vital organs (liver, kidney, lungs, heart, spleen, and ovaries) of the rats were excised and weighed.

Post-natal development evaluation of the offspring

The remaining pregnant rats (n=5) were allowed to complete parturition normally. Foetal parameters assessed were: litter size, offspring viability, body weight, crown-rump and tail length, and weight gain on days 0, 5, 10, 15, 20, 25 and 50 of development.

Determination of serum hormone concentration

Maternal blood was collected from the descending aorta on day 21 from each euthanized animal. The blood was left to clot for 10 min at room temperature. The blood samples were centrifuged at 1000G for 10 minutes using an IEC Clinical Centrifuge (Needham, U.S.A) to separate blood cells from the plasma after which the sera were collected using a Pasteur pipette into duplicate clean, dry sample bottles, and refrigerated at -20°C and used within three days of preparation for hormonal assay. The sera were separated for each sample and analysed for sex steroid hormones (LH, FSH, progesterone, estradiol and prolactin). The serum hormone concentration was carried out using a RT-2100C microplate reader (Shanghai international Holding Corp. GmbH, Hamburg, Germany) as described for follicle-stimulating hormone, luteinising hormone (Braustein et al., 1976), progesterone, prolactin, and estradiol (Radwanska et al., 1978).

Statistical analysis

All data were expressed as means $\pm$ standard error of mean (mean $\pm$ SEM). Statistical group analysis was performed with SPSS (version 16) statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups and means were compared using LSD test. Values of $p \leq 0.05$ were considered significant (Mahajan, 1997).

Results

Figures 1, 2 and 3 show the mean ($\pm$ SD) body weight during gestation, food consumption and water ingestion *ad libitum*, there were no significant difference in the extract treated group compared to control.

Table 1 shows maternal effects of pregnant rats exposed to (50, 200, and 800mg/kg) during organogenesis. No mortality or treatment-related overt signs of maternal toxicity stress or abnormal behavioural changes were observed. None of the pregnant rats showed vaginal bleeding. Gravid uterine weight differences may be linked to the number of dams that did not abort. Maternal organ weights in the treated groups were similar to control groups. Body weight gain is statistically significantly ($p \leq 0.05$) decreased from GD 0-7 at 50, 200 and 800 mg/kg respectively, from the control group. However, from GD 8-14 body weight gain is statistically significantly ($\geq P0.05$) increased from the control group at 800 mg/kg and statistically significantly ($\leq P0.05$) decreased. Figure 4 shows the result of effect of the extract on reproductive hormones. The extract at given doses shows a statistically significant decrease ($\leq P 0.05$) in serum level of follicle stimulating hormone (FSH) and increase estrogen in all treated groups compared to the control; however levels LH, Prolactin and Progesterone were not affected.

The number of implantation, resorption and foetal tail length did not differ from that of the control group. However there was a statistically significant decrease ($p \leq 0.05$) in the number of fetuses per dam in all dose levels when compared with the control group. Foetal body weight increased significantly ($p \leq 0.05$) at 200 and 800mg/kg when compared with control. Crown-rump length and placental weight increased significantly ($p \leq 0.05$) in all groups when compared with control ([Table 2](#)). No increase in foetal external malformations was seen in this study.

As shown in [Table 3A](#), duration of pregnancy and delivery index are similar to the control group, Crown-rump length at day 0 and PND 50, and pups weight at day 0, PND 5 and 15 of the treated groups are not different when compared with controls (fig. 5). Nonetheless, a minor reduction ($p \leq 0.05$) in pup tail length (fig. 6) was observed at PND day 10 (200mg/kg alone), day 20 (200 and 800mg/kg), day 50 (200 and 800mg/kg) when compared with control. There is a much lower viability index at 800 mg/kg compared to all other groups and the Lactation indices in the controls and 200 and 800 mg/kg groups, respectively. The offspring did not show any sign of developmental delays ([Table 3B](#)).

Discussion

Exposure of pregnant women to herbs and supplements during pregnancy could be harmful leading to maternal and foetal morbidity and mortality (Wilkinson, 2000).

Our preliminary study was undertaken to assess the reproductive and developmental effects of ethanolic seed extract of *Moringa oleifera* when administered orally to gravid Wistar rats. During the period of organogenesis, the effects of chemical or drugs may be manifested as abortions, embtu-fetal deaths or resorptions, foetal malformations or delays in development (Oliveira *et al.*, 2010). Moreover, maternal parameters (body weight gain during pregnancy, vaginal bleeding, food and water intake) can be used to evaluate the integrity of maternal homeostasis (Shukla *et al.*, 1988). The ethanolic seed extract of *Moringa oleifera* treated rats during organogenesis had no significant changes in maternal body and organs weight, body weight gain, food and water consumption and vaginal bleeding, however there was decrease in number of implants and number of live fetuses when compared to control which could have been due to a significant increase in the foetal body weight. This contrasts with the findings of (Varsha and Dinesh, 2014) on antifertility effects of root and stem back and leaves of *Moringa oleifera*.

There were no significant effect on the serum hormone levels of LH, Prolactin and Progesterone concentration in female rats observed with the extract at all the doses investigated, though, there was a significant decrease in follicle stimulating hormones (FSH) level in all groups treated with the extract and increase in estrogen level observed in the 800mg/kg group. *Moringa oleifera* is rich in phytosterols, like stigmasterol, sitosterol and kampesterol which are precursors for hormones. These compounds increase estrogen production, which in turn stimulates the proliferation of mammary gland ducts to produce milk (Plant, 2008) which could be what is responsible for the increase in estrogen levels. Although there was no simultaneous increase in prolactin levels.

Also, the coordination of the hypothalamic–pituitary–gonadal (HPG) axis is regulated by gonadotropin-releasing hormone (GnRH). The GnRH neurons from the arcuate nucleus within the hypothalamus secrete GnRH in a pulsatile manner into the hypophyseal portal system, where it acts on

cell surface receptors in the anterior pituitary (Wierman *et al.*, 2004). In turn, pituitary gonadotropes respond by increasing the production of the common α -subunit and specific β -subunits of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These assembled α - β dimers of FSH and LH exert their principal function in the gonads where gametogenesis and folliculogenesis occur. Similarly, any process leading to low FSH simultaneously results in low LH levels, except in rare instances of isolated FSH deficiency or isolated LH deficiency in fertile eunuch syndrome, low levels of the hormone can indicate that the pituitary gland is not functioning correctly which could be due to significant levels of a xenobiotic or stress present (Holson *et al.*, 1994).

Agents known to interfere directly with fetal nutrition, growth factors, and other developmental processes may also induce growth retardation by a direct effect on the fetus (Keller, 2001; Behl *et al.*, 2013). After birth, growth can be affected by alterations in maternal care, milk production, or milk ejection reflexes, or even by direct effects on pup's suckling behavior or continued test material exposure through milk (Repo *et al.*, 2014; Tanaka *et al.*, 2012).

In our study, maternal behavior in terms of gestation and delivery was not affected, the control and test animals were comparable. Additionally, grossly malformed pups were not observed in treated groups compared to control, as eye opening, incisor eruption, hair growth, ear opening, testes descension and vaginal opening of the pups were unaffected by the extract. However, reduced viability and lactation index were observed, these findings generally related to effects on survival of the offspring and may have been mediated through the dams or via direct exposure of the conceptuses to the extract. There is an indication that the extract could have potentials of being toxic and may not be safe during pregnancy at the given dose.

In conclusion, the results of the current study revealed that *Moringa oleifera* ethanolic seed extract may adversely affect pregnancy in the female rats at high doses. More work is on the way in our laboratory to establish this finding.

Acknowledgement

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3. Results

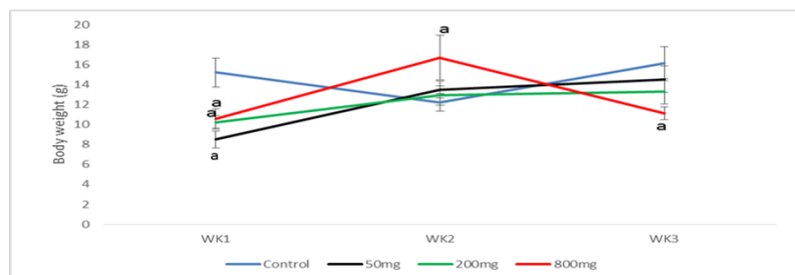
Table 1: Maternal effect of pregnant rats exposed to *Moringa oleifera* ethanolic seed extract

Parameters	Dose (mg/kg b.wt)			
	0	50	200	800
No of dams	5	5	5	5
No. of death dams	0	0	0	0
No. of aborted dams	3	4	3	3
Uterine weight	55.34 ± 4.52	33.17 ± 1.33	58.19 ± 15.11	44.13 ± 12.70
Maternal weight gain				
- Day 0-7	15.2 ± 1.47	8.5 ± 0.83 ^a	10.2 ± 0.70 ^a	10.6 ± 0.99 ^a
- Day 8-14	12.2 ± 0.88	13.5 ± 0.87	12.9 ± 0.94	16.7 ± 2.25 ^a
- Day 15-20	16.1 ± 1.68	14.5 ± 1.33	13.3 ± 1.27	11.1 ± 0.62 ^a
Organ weight (g)				
Lung	2.15 ± 0.51	2.05 ± 0.41	1.70 ± 0.44	1.83 ± 0.16
Liver	8.06 ± 1.11	7.93 ± 0.84	8.61 ± 0.88	8.30 ± 0.78
Heart	0.78 ± 0.10	0.65 ± 0.11	0.73 ± 0.06	0.63 ± 0.01
Kidney	0.72 ± 0.09	0.60 ± 0.65	0.61 ± 0.48	0.58 ± 0.39
Spleen	0.65 ± 0.06	0.79 ± 0.08	0.76 ± 0.13	0.78 ± 0.09
Ovary	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.01

Data are given as mean ± S.E. Data were analyzed by ANOVA followed by LSD test.

^a p≤0.05 is significant vs control.

Figure 1: Effect of *Moringa oleifera* ethanolic seed extract on maternal body weight.



Data are given as mean $\pm$ S.E.M. Data were analyzed by ANOVA followed by LSD test.

^a $p \leq 0.05$ is significant vs control.

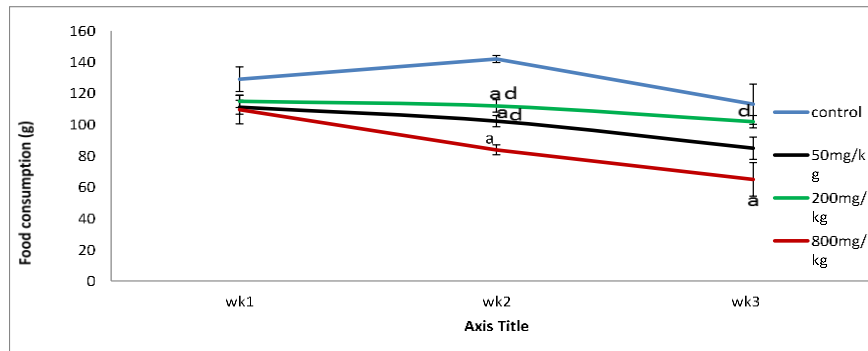
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Kidney	0.72 $\pm$ 0.09	0.60 $\pm$ 0.65	0.61 $\pm$ 0.48	0.58 $\pm$ 0.39
Spleen	0.65 $\pm$ 0.06	0.79 $\pm$ 0.08	0.76 $\pm$ 0.13	0.78 $\pm$ 0.09
Ovary	0.09 $\pm$ 0.01	0.10 $\pm$ 0.01	0.10 $\pm$ 0.01	0.12 $\pm$ 0.01

Data are given as mean $\pm$ S.E. Data were analyzed by ANOVA followed by LSD test.

^a $p \leq 0.05$ is significant vs control.

Figure 2: Effect of *Moringa oleifera* ethanolic seed extract on food consumption.

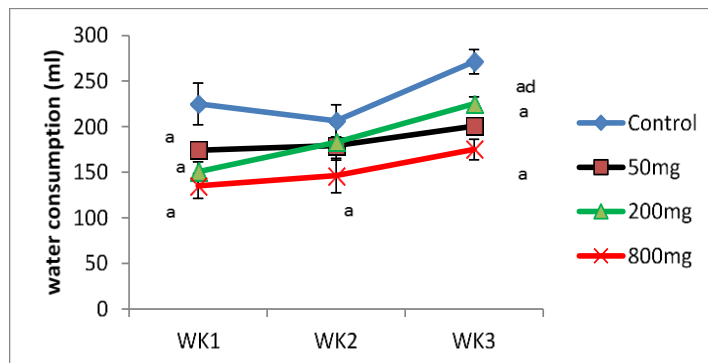


Data are given as mean $\pm$ S.E.M. Data were analyzed by ANOVA followed by LSD test.

^a $p \leq 0.05$ is significant vs control,

^d $p \leq 0.05$ is significant vs 800mg/kg.

Figure 3: Effect of *Moringa oleifera* ethanolic seed extract on water intake.

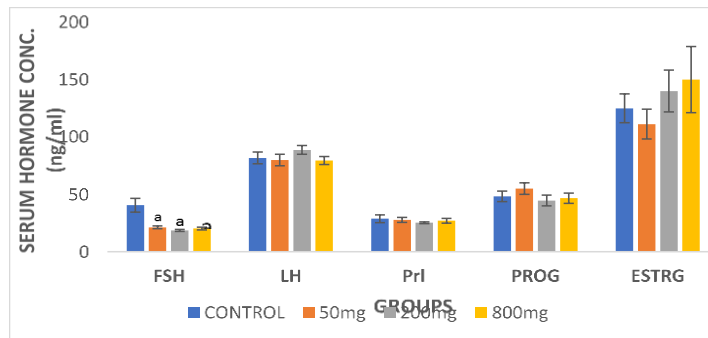


Data are given as mean $\pm$ S.E.M Data were analyzed by ANOVA followed by LSD test.

^a $p \leq 0.05$ is significant vs control,

^d $p \leq 0.05$ is significant vs 800mg/kg.

Figure 4: Effect of *Moringa oleifera* ethanolic seed extract on reproductive Hormones.



^a $p \leq 0.05$ is significant vs control.

Table 2: Fetotoxicity from dams' exposure to *Moringa oleifera* ethanolic seed extract during organogenesis

Parameters	Dose (mg/kg bwt)			
	0	50	200	800
No. of pregnant rats	5	5	5	5
No. of aborted dams	0	0	0	0
No. of implantations/pregnant rat	14.3 ± 0.44	10.4 ± 0.32	11.9 ± 1.26	10.3 ± 1.03
No. of resorption/ pregnant dams	3.0 ± 0.00	2.7 ± 0.3	2.3 ± 1.2	3.0 ± 0.6
No. of foetal death/ pregnant dam	0	0	0	0
No. of foetus/ pregnant dam	11.3 ± 0.88	7.66 ± 0.33 ^a	9.66 ± 1.33	7.33 ± 1.45 ^a
Foetal body weight (g)	3.92 ± 0.01	3.86 ± 0.04	4.19 ± 0.08 ^{a,b}	4.76 ± 0.04 ^{a,b,c}
Placenta weight	0.39 ± 0.13	0.33 ± 0.01 ^{a,d}	0.62 ± 0.02 ^{a,b}	0.62 ± 0.02 ^a
Crown- rump length (cm)	3.62 ± 0.05	3.79 ± 0.04 ^a	3.91 ± 0.06 ^a	3.91 ± 0.03 ^a
Tail length (cm)	1.40 ± 0.02	1.37 ± 0.02	1.37 ± 0.02	1.40 ± 1.34

Data are given as mean ± S.E.M. Data were analyzed by ANOVA followed by LSD test.

^a $p \leq 0.05$ is significant vs control

^b $p \leq 0.05$ is significant vs 50mg/kg

^c $p \leq 0.05$ is significant vs 200mg/kg

^d $p \leq 0.05$ is significant vs 800mg/kg.

Table 3A: Developmental effect on Pups following exposure to *Moringa oleifera* ethanolic seed extract during organogenesis

Parameters	Dose (mg/kg)			
	0	50	200	800
No. of dams	5	5	5	5
Duration of the pregnancy	21-23	21-23	21-23	21-23
No. of pups/litter	8.20 ± 1.53	7.00 ± 0.71	5.60 ± 0.93	5.20 ± 0.92
Delivery index ^x	100	100	100	100
Viability index ^y	95.1	97.1	92.9	61.5
Lactation index ^z	60.98	97.14	57.14	57.69
Crown-rump length (cm)				
- Day 0	4.31 ± 0.01	4.32 ± 0.02	4.34 ± 0.03	4.33 ± 0.02
- Day 20	6.24 ± 0.07	6.38 ± 0.05	6.25 ± 0.75 ^d	6.57 ± 0.07 ^a
- Day 50	10.26 ± 0.12	10.15 ± 0.15	10.20 ± 0.13	10.15 ± 0.17

Data are given as mean ± S.E.M. Data were analyzed by ANOVA followed by LSD test.

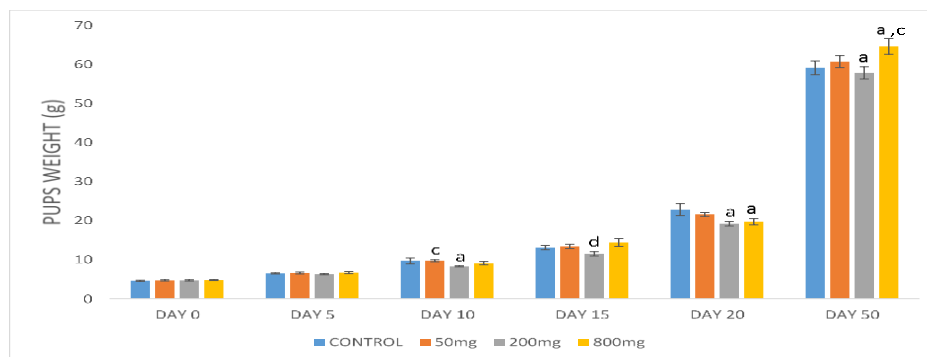
^a p≤0.05 is significant vs control, ^b p≤ 0.05 is significant vs 50mg/kg, ^cp≤0.05 is significant vs 200mg/kg, ^dp≤0.05 is significant vs 800mg/kg.

^x (Number of females delivering/number of pregnant female) ×100

^y (Number of live offspring at lactation day 4/number of live offspring delivered) ×100

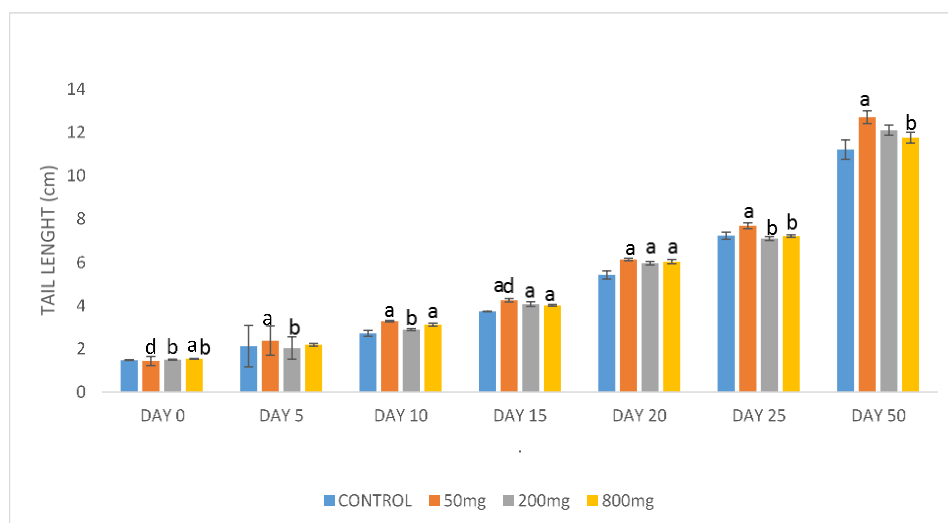
^z (Number of live offspring at day 21/number of live offspring born) ×100

Fig 5. EFFECTS OF MORINGA OLEIFERA ETHANOLIC SEED EXTRACT ON PUPS WEIGHT



^a p≤0.05 is significant vs control, ^b p≤ 0.05 is significant vs 50mg/kg, ^cp≤0.05 is significant vs 200mg/kg, ^dp≤0.05 is significant vs 800mg/kg.

Fig. 6. EFFECTS OF *MORINGA OLEIFERA* ETHANOLIC SEED EXTRACT ON TAIL LENGHT



^a $p \leq 0.05$ is significant vs control, ^b $p \leq 0.05$ is significant vs 50mg/kg, ^c $p \leq 0.05$ is significant vs 200mg/kg, ^d $p \leq 0.05$ is significant vs 800mg/kg.

Table 3B: Developmental effect on Pups following exposure to *Moringa oleifera* ethanolic seed extract during organogenesis

Parameters	Dose (mg/kg)			
	0	50	200	800
Eye opening (day)	14-17	14-17	14-17	14-17
Incisor eruption (day)	8-10	8-10	8-10	8-10
Hair growth (day)	8-11	8-11	8-11	8-11
Ear opening (day)	3-4	3-4	3-4	3-4
Testes descension (day)	26-28	26-28	26-28	26-28
Vaginal opening (day)	33-35	33-35	33-35	33-35