



Acute Toxicity Test of Alkaloid Fraction of *Moringa oleifera* Leaf and its Effect on Reproductive Hormones of Pregnant Wistar Rats

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ABSTRACT: Alkaloids occur naturally in plants but are not established nutrients and are responsible for bioactive functions in the plants. This study evaluated the acute toxicity of alkaloid extract of *Moringa oleifera* leaves (AMOL) and its effect on reproductive hormones of pregnant wistar rats using standard techniques. Results showed that in both phases of the oral acute toxicity test, no death occurred within 24 h of post-treatment and 14 days after AMOL administration. Rejection of food, increased salivation and decreased mobility were only observed immediately after treatment. Pregnancy was maintained in all groups, with significant ($P < 0.05$) increase in progesterone and follicle-stimulating hormone (FSH) levels in animals in Group 3. AMOL had no effect on estrogen and luteinizing hormone (LH) levels. In conclusion, AMOL may be relatively safe as pregnancy was maintained in AMOL treated groups and had significant effect on some of the reproductive hormones.

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Alkaloids are basic substances, which contain one or more nitrogen atom(s) in a ring and because of their physiological activities, they are widely employed in medicine. The presence of alkaloids and other secondary metabolites in plants enhances plant reproductive rates, either by improving defenses against biotic and abiotic stresses or by affecting pollinators and seed/fruit disperser visitation. Defensive strategies include predator repellence by toxicity or bitterness taste or damage repair by antioxidant system (Matsuura and Fett-Neto, 2013). Alkaloids extracted from plants has shown to have marked effect on the reproductive hormones. A study by Musa and Isa (2012) showed that alkaloids from *Senna alata* leaves exhibited anti-gonadotropic and anti-progesteronic activities in pregnant rats. Browning *et al.* (1998) demonstrated in steers

receiving injections of ergotamine tartrate (23.8 µg/kg body weight) that luteinizing hormone (LH) concentrations were reduced. Acute ergot alkaloid exposure (19 µg/kg body weight) altered LH and follicle-stimulating hormone (FSH) in primiparous cows during the late luteal phase (day 15 or 16 post-estrus). LH concentrations were reduced following 4 h after injection, although there was no variance in FSH concentrations between of the cows receiving ergotamine tartrate and the saline control (Browning *et al.*, 1998). In another study, alkaloids was seen to have minimal effect during reproductive cyclicity and a greater effect during pregnancy and post-partum in sheep and mares, it caused prolonged gestation, thickened placentas, late-term foal loss, dystocia, and agalactia (Pertz and Eich, 1999). Alkaloids have been shown to induce a vasoconstrictive response via

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interaction with biogenic amine receptors including serotonergic and adrenergic receptors (Klotz *et al.*, 2017). Induced vasoconstriction of the utero-ovarian vessels would eventually decrease nutrients essential to ovarian function, consequently altering sex steroid synthesis, follicular, and/or luteal development along with dysregulation of the estrous cycle (Poole and Daniel, 2019). Estrogen (E₂) production is a critical component of a healthy developing follicle and crucial for reproductive success. Follicular development and E₂ secretion are impaired by alkaloids; however, research is yet to show if alkaloids directly impact granulosa and thecal cell function or indirectly alter folliculogenesis via decreased ovarian blood flow (Poole and Daniel, 2019).

Herbal usage in therapy is well established especially amongst Africans and Asians as it is easily assessed and less cost-effective (WHO, 2013; Moke *et al.*, 2021). *Moringa oleifera* plant is a very popular plant in Africa and Asia eaten by many including women in their reproductive age, because of its nutritional and therapeutic benefits (Attah *et al.*, 2020). The phytochemical study of aqueous, chloroform and petroleum ether extracts of *Moringa oleifera* leaves were investigated by Malliga *et al.* (2014). The phytochemical analysis revealed the presence of alkaloids, flavonoids, steroids, tannins, saponins and glycosides as major components. The different parts of the plant have been shown by previous studies to have effect on the female reproductive system (Varsha *et al.*, 2010; Ijioma *et al.*, 2014; Ekhaton and Osifo, 2015; Agarwal *et al.*, 2018;). They also suggested that these effects may be as a result of some phytochemicals such as alkaloids present in the *Moringa oleifera* plant. Hence, the objective of this study was to assess the acute toxicity test of alkaloid fraction of *Moringa oleifera* leaf and its effect on reproductive hormones of pregnant wistar rats

MATERIALS AND METHODS

Reagents/Materials: Some of the materials used for the present study included; cotton wool, syringes, pipette, normal saline, beakers, chloroform, gloves, dissection materials, electronic scale, *Moringa oleifera* leaves, Alcohol (Ethanol), Base (NaOH, NH₄OH), Acid (HCl, Acetic acid) lipophilic organic solvent (Chloroform).

Experimental Animals: The experimental animals were healthy pure breed female *Wistar* albino rats with weights between 200-250g. All animals used in the course of this study were obtained from the animal house of the Department of Physiology, School of Basic Medical Sciences, University of Benin. The animals were kept in cages in a well-ventilated room

and provided with food and water *ad libitum*. The animals were acclimatized for 2 weeks before commencement of the experimental procedure.

Mating: Two mature female rats were mated for four (4) days with one male rat (2:1). Pregnancy was established using the vaginal smear method (Hafez, 1970), presence of vaginal plug, weight gain as well as other behavioral patterns observed in the animals. The day a positive smear of spermatozoa was observed was designated as 'Day 1' of pregnancy for that particular animal.

Collection of Plant Materials: The *Moringa oleifera* leaves (MOL) were freshly collected from University of Ilorin farm, Ilorin, Nigeria. The plant was authenticated by the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Edo State. The collected leaves were air-dried and pulverized to powder form.

Extraction of Alkaloids from MOL: The pulverized leaves were subjected to alkaloid method of Smita and Sushma, (2010) with slight modification. Pulverized leaves were macerated in 70% ethanol (1:5 w/v) and filtered after 24 hours. The filtrate was concentrated using rotary evaporator (RE-2000E LABFREEZ, China). The concentrated extract was dissolved in equal volume of distilled water and chloroform. The mixture was refluxed in a separating funnel and allowed to separate into two phases. The lower phase was collected as the crude alkaloidal fraction. The fractions were concentrated using rotary evaporator (RE-2000E LABFREEZ, China), freeze-dried and stored for subsequent use.

Determination of Acute Toxicity Test (LD₅₀): The acute toxicity test was carried out in accordance with Lorke's method (Moke *et al.*, 2015; Miediegha *et al.*, 2022) for alkaloid extracted from *Moringa oleifera* leaf (AMOL) and this method has two phases. In phase I, nine rats were randomly assigned into 3 groups of 3 rats each. Animals in group 1, 2 and 3 received oral administration of 10, 100 and 1000 mg/kg of the phytochemical extracted from MOL respectively and they were monitored frequently for 24 hours for observable signs of toxicity or mortality. In phase II, three rats were used, and they were orally administered higher doses of the phytochemical extracted from MOL (1600, 2900 and 5000 mg/kg body weight per rat). The rats were also monitored frequently for 24 hours and then daily for 2 weeks for any observable signs of acute toxicity or mortality.

Then the acute toxicity (LD₅₀) of AMOL as calculated using the equation below

$$LD_{50} = \sqrt{D_0 * D_{100}}$$

Where D_0 = Highest dose that gave no mortality; D_{100} = Lowest dose that produce mortality

Experimental Design: Fifteen (15) pregnant animals were randomized into 3 groups of 5 animals each to evaluate repeated toxicity subacute test on reproduction for 2 weeks (Erhirhie and Moke, 2022). Group 1 served as control, Group 2 was the low dose group, animals in this group were treated with 60 mg/kg body weight of AMOL (Attah *et al.*, 2020) and Group 3 served as high dose group, animals were treated with 120 mg/kg body weight of AMOL (Agrawal *et al.*, 2018) from Day 7 to Day 14 of pregnancy. On Day 15 of pregnancy all animals were euthanized. Blood was collected via cardiac puncture for hormonal analysis, the uterus was harvested and the number of pups were counted.

Hormonal Analysis: Serum was obtained from blood sample collected and used for estrogen, progesterone, follicle stimulating hormone and luteinizing hormone

assays. The analysis was carried out using an enzyme-linked immunoassay (EIA) technique. The EIA kits which contained the different EIA enzyme label, substrate reagents and quality control sample were obtained from Fortress Inc. (UK).

Statistical Analysis: For the *in vivo* study, the data was expressed as mean \pm standard error. It was analyzed using One Way Analyses of Variance (ANOVA) to compare the control and experimental groups, followed by Bonferroni *post hoc* test and p values \leq 0.05 was considered significant.

RESULTS AND DISCUSSIONS

Acute toxicity (LD_{50}) study of AMOL: The result of the oral acute toxicity test of AMOL is shown in Table 1, it was observed in both phases of the oral acute toxicity test that no death occurred within 24 h of post-treatment with AMOL. Similarly, no mortality was observed in all the treatment groups 14 days after the extract AMOL administration. Rejection of food, increased salivation and decreased mobility were only observed immediately after treatment.

Table 1: Evaluation of Oral Acute toxicity of AMOL

Groups	No. of Rats	Dose (mg/kg)	No. of Deaths	Survival	Mortality ratio
Phase I					
1	3	10	0	3	0/3
2	3	100	0	3	0/3
3	3	1000	0	3	0/3
Phase II					
1	1	1600	0	1	0/1
2	1	2900	0	1	0/1
3	1	5000	0	1	0/1

Table 2: Physicochemical Properties of AMOL

Sample	Color	Texture	Solubility
AMOL	Dark Green	Slurry	Insoluble in water

Thin Layer Chromatography Analysis: After developing the alkaloid extract on the TLC plate, it was viewed under the UV lamp hood at visible light (Fig. 1) and ultraviolet (UV) light at 365nm (Fig. 2). Six spots (Rf; 0.12, 0.31, 0.36, 0.38 and 0.93) were observed documented under the visible light while eight spots (Rf; 0.12, 0.31, 0.36, 0.38, 0.73, 0.83, 0.93 and 0.98) were documented under the 365nm UV light. Extract was observed with 0.12, 0.31, 0.36, 0.38, 0.73, 0.83, 0.93 and 0.98 Rf respectively.

Effect of AMOL on Pup Number/Size: In the *in vivo* study pregnant rats were used and on Day 15 of pregnancy, animals were sacrificed and it was observed that pregnancy across all the groups were maintained since there was no significant difference in

the number pups in the uterus of animals in all the treatment groups compared to that of control (Table 3).

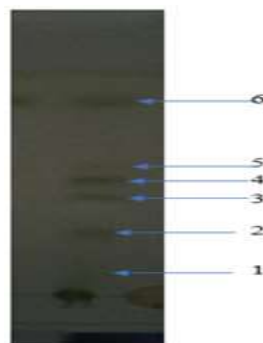


Fig 1: Representative TLC plate showing the compounds (spots) in the crude alkaloid extract of *Moringa oleifera* under visible light

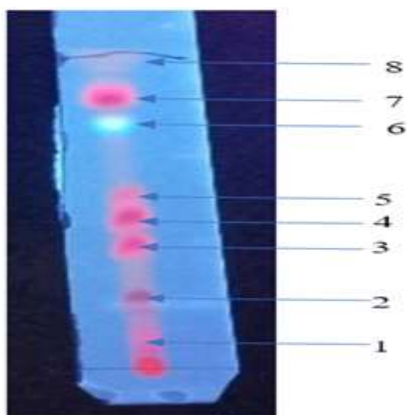


Fig 2: Representative TLC plate showing the compounds (spots) in the crude alkaloid extract of *Moringa oleifera* under ultraviolet (UV) light at 365nm

Table 3: Showing the average number of pups on Day 15 of pregnancy

Group (EXTRACT)	Average number of Pups
1. Control	8
2. AMOL (Low dose)	7
3. AMOL (High dose)	8

Effect of AMOL on Reproductive Hormones: Results show that there is no significant ($P > 0.05$) change in the estrogen level in experimental animals administered with 60 mg/kg and 120 mg/kg of AMOL compared with control (Fig 3). An increase in the progesterone level was observed in experimental animals administered with 120 mg/kg of AMOL compared to control animals (Fig 4). There was a significant ($P < 0.05$) increase in the FSH level in experimental animals treated with 120 mg/kg AMOL and no significant ($P < 0.05$) effect at 60 mg/kg when compared with control (Fig 5). However, the alkaloid extract of *Moringa oleifera* leaves had no significant ($P < 0.05$) effect on luteinizing hormone (Fig 6).

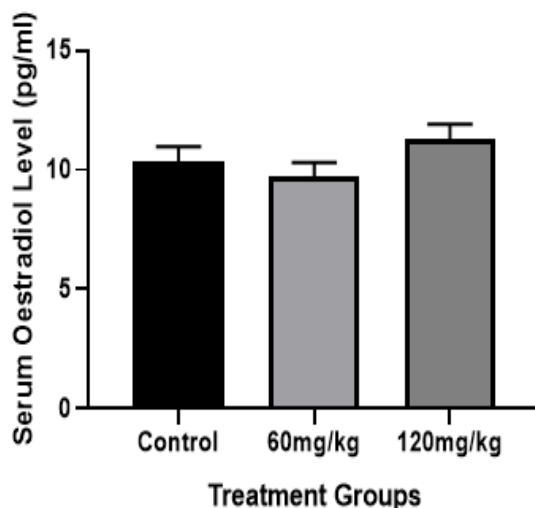


Fig. 3: Effect of Alkaloid extract of *Moringa oleifera* leaves on estrogen in pregnant wistar rats.

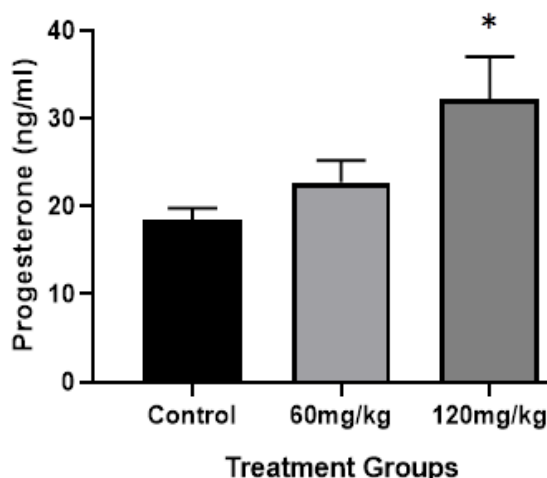


Fig. 4: Effect of Alkaloid extract of *Moringa oleifera* on progesterone in pregnant wistar rats.

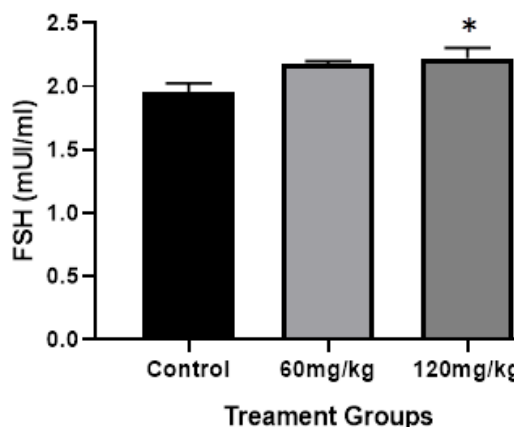


Fig. 5: Effect of alkaloid extract of *Moringa oleifera* leaves on follicle stimulating hormone in pregnant female wistar rats

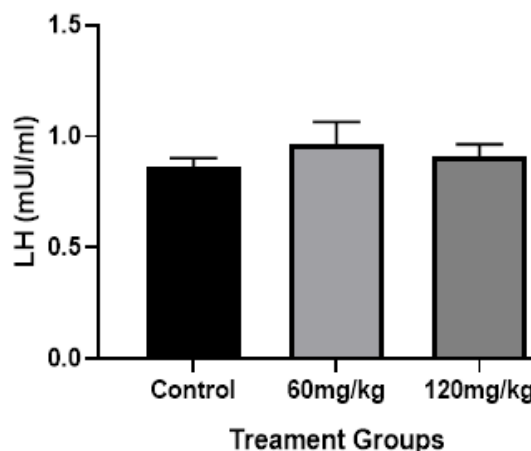


Fig. 6: Effect of alkaloid extract of *Moringa oleifera* leaves on luteinizing hormone in pregnant female wistar rats

There is a growing interest in toxicity of substances isolated from plants to basically determine their safety. This study determined the oral LD50 of alkaloids isolated from *Moringa oleifera* leaf using Wistar rats.

The results showed that after 24 hours no death was recorded in both phases. Hence, the LD₅₀ (median lethal dose) of the AMOL in rats was found to be greater than 5000 mg/kg body weight of animals. Similarly, no mortality was observed in all the treatment groups 14 days after the AMOL extract administration. Rejection of food, increased salivation and decreased mobility were only observed immediately after treatment. The major signs of toxicity observed within 24 h were: difficulty in breathing, loss of appetite and general weakness. These results suggest that the alkaloids isolated from *Moringa oleifera* leaf may be relatively safe (Abu *et al.*, 2019). In this study the observation that pregnancy across all the groups was maintained is in variance with Ekhaton and Osifo (2015) whose study recorded 100% abortifacient activities in pregnant animals treated with aqueous extract of MOL. Pregnancy was maintained in this study and this could be as a result of the effect of the alkaloid isolated from MOL on reproductive hormones. AMOL had no significant effect on the serum estradiol level in pregnant *Wistar* rats, an observation in line with reports of Zeng *et al.* (2019) who reported that *Moringa oleifera* leaves did not change serum estradiol level and the expressions of estrogen receptor beta (ER β). Increase in estrogen level significantly could have resulted in pregnancy loss; studies have shown that estrogen causes changes in the myometrium, such as increased production of prostaglandins, enhanced synthesis of prostaglandin and oxytocin receptors, up regulation of proteins responsible for muscle contraction like myosin light chain kinase and calmodulin (Ofulue *et al.*, 2022) and all of these changes increase uterine muscle contraction which can lead to pregnancy loss. Increased estrogen level has also been shown to prevent implantation, because of its ability to suppress both follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Vandana and Varsha, 2014). Follicle Stimulating Hormone acts on the follicles in the ovary and specifically on the granulosa cells which surround the ovum. These cells contain the enzyme aromatase which converts local androgen (from the theca cells) to estrogen (Shivalingappa *et al.*, 2002). FSH level was significantly increased in all animals treated with AMOL, this increase could be as a result of estrogen levels remaining the same in this study. So, because increase estrogen exerts a negative feedback blocking secretion of GnRH, LH and FSH. This result is in line with Ogunsola *et al.* (2017) who reported in a study an increase in FSH levels in animals treated with *Moringa* plant parts. Progesterone is essential for the regulation of normal female reproductive functions. The major physiological actions of progesterone are; in the uterus and ovary: induction of ovulation, facilitation of implantation, and maintenance of early

pregnancy; in the mammary gland. In this study, the observation that progesterone level was significantly increased in treatment groups treated with 120 mg/kg of phytochemicals of *Moringa oleifera* leaves is in contrast to a study carried out by Agrawal *et al.* (2018). For contraction to take place, there are usually changes that must occur, and one of such changes is the decrease in progesterone level, and others include increase in estrogen, up regulation of myometrial oxytocin receptors, increased prostaglandins synthesis, decreased nitric oxide activity and increased influx of calcium into myocytes (Ofulue *et al.*, 2022). Thus, the increased serum progesterone level in this study may be one of the factors that caused pregnancy to be maintained. AMOL had no significant effect on LH in treatment groups compared to the control group and this is in line with a study that found no difference in LH secretion in postpartum beef cows nor in cycling heifers and cows treated with ergot alkaloids (Mizinga *et al.*, 1992). Additional studies further described that ergot alkaloids do not suppress LH or equine chorionic gonadotropin (eCG) in ewes or mares, respectively (Louw *et al.*, 1974; Brendemuehl *et al.*, 1996). Li *et al.* (2017) found minimal differences in pathways utilized for FSH and LH production, secretion, or signaling from pituitary tissue collected from steers grazing.

Conclusion: Alkaloid isolated from *Moringa oleifera* leaf (AMOL) is relatively safe and it maintained pregnancy of adult *Wistar* rats treated with it, increased the serum level of progesterone and follicle stimulating hormone but had no significant effect on the serum level of estrogen and luteinizing hormones.

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