JOURNAL OF VETERINARY AND APPLIED SCIENCES
VOLUME 15, ISSUE 1: 937 – 947 (2025)
Published by: Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria
ISSN: 2315-6856; e-ISSN: 2636-5553; Website: www.jvasonline.com

Oral administration of methanolic leaf extract of *Newbouldia laevis* to rabbit does before breeding and during pregnancy: effects on their serum levels of oestrogen and progesterone

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Abstract

In this study, methanolic leaf extract of *Newbouldia laevis* was orally administered to rabbit does before breeding and during pregnancy, and the effects on their serum levels of oestrogen and progesterone was evaluated. Twenty New Zealand rabbit does, aged 6 ± 2 months and of a weight range 1.40 – 2.00 kg each, were used for the study. They were randomly assigned to four groups (A, B, C and D) of five each. Rabbits in Groups A, B and C received the methanolic leaf extract of *Newbouldia laevis* at doses of 1000, 500 and 250 mg/kg, respectively, while those in Group D were the untreated control that were given a distilled water placebo. Rabbits in all the groups were provided with feed and drinking water freely for the 48 days of the study. The varied doses of the extract and the distilled water placebo were administered orally daily for the 48 days of the experiment. The does were mated on day 27 of administration of the extract. Blood samples were collected from the does on days 0, 6, 21, 27, 34, 41 and 48 of the experiment, and serum levels of estradiol (E2) and progesterone (P4) were determined following standard enzyme-linked immunosorbent assay (ELISA) techniques and procedures. Results showed that the serum oestrogen levels were significantly higher ($p < 0.05$) in the extract treated groups in a dose dependent manner when compared to the untreated control on days 21 and 34, while the mean serum progesterone levels significantly varied ($p < 0.05$) among the groups on days 6, 21 and 27. It was concluded that administration of the *Newbouldia laevis* leaf extract as used in the study significantly affected serum oestrogen levels before breeding and during pregnancy, and serum progesterone levels only before breeding. Further studies evaluating the effects of the extract on other reproductive hormones were recommended and proposed.

Keywords: *Newbouldia laevis;* Methanolic leaf extract; Serum Oestrogen and Progesterone; Rabbit does; Breeding; Pregnancy.

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Article History: Initial manuscript submission received – June 26, 2024; Final revised form received – April 14, 2025; Accepted for publication – April 18, 2025; Published – April 23, 2025.

Introduction

Parts of various plants are used as medicine traditionally and in modern orthodox medical practice (Edeoga *et al*., 2005). *Newbouldia laevis* is a plant in Southeastern Nigeria that is used to speed up childbirth and remove the placenta post-delivery (Obute, 2002). It is commonly referred to as the Boundary tree, and is called Ogilisi or Egbo by the Igbo, Aduruku by Hausa, Ewe Akoko by Yoruba and Oniok by Efik people. Uterine contraction-stimulating agents are used in clinical settings to help with labor induction, labor augmentation, and managing the third stage of labor (Goldenberg, 2002). Traditional healers commonly use *Newbouldia laevis* as oxytocics, but this usage is yet to be scientifically verified. According to Odunbaku and Amusa (2012), dysentery, malaria, elephantiasis, migraines, and seizures can be treated using the roots and leaves of *Newbouldia laevis*. Akerele *et al* (2011) reported the utilization of the bark and twigs of *Newbouldia laevis* to address conditions like women's pelvic pain, peptic ulcer disease, earache, skin ulcer, epilepsy, hemorrhoids, and constipation. The flowers of *Newbouldia laevis* are recognized for their anti-inflammatory properties as well (Tanko *et al.,* 2008).

It is widely believed that the phytochemical constituents of most plants, in their varied combinations, are responsible for their biological activity and their traditional clinical applications. Alkaloids, tannins, flavonoids, and phenolic compounds are the most significant phytochemicals (Iwu, 2000). Leaves of *Newbouldia laevis* have been reported to contain various phytochemicals such as flavonoid, terpenoid, tannin, alkaloid, phytic acid, trypsin inhibitor, phenol, antioxidants, carotenoid, oxalate and cyanide (Ayoola *et al.,* 2016). Reports by other researchers have shown the presence of phytochemicals in plants that may be harmful or have a negative impact on conception and reproduction (Edeoga *et al.,* 2005; Yakubu *et al.,* 2005).

The majority of people worldwide rely on food grown on small farms, which have been shrinking due to the increasing human population (McIntire *et al.,* 1992). This has resulted in a necessity to find cheaper protein sources that are easily accessible and do not compete heavily with the human food supply (Akinmutimi, 2007). Characteristics such as the rabbit's ability to reproduce quickly, grow fast, be genetically selected easily, convert feed efficiently, and utilize space economically make them a feasible choice for a low-cost protein source (Lebas, 1997; Hassan *et al.,* 2012). One important factor for this enhancement in the use of rabbits as a protein source is the understanding of their mating habits and the skill to choose for increased reproduction rates (Fayeye and Ayorinde, 2003; 2010).

Infertility is a significant issue in both human and veterinary medicine (Akomolafe, 2012). There is a growing trend towards using plant extracts to enhance fertility in humans and animals due to a shift in focus from synthetic drugs to natural plant products (Dada and Ajilore, 2009). It has been recognized that maintaining an equilibrium of hormones is crucial for fertility in the reproductive systems of both humans and animals. Many natural chemicals from plants that affect hormone levels in humans and animals have been widely studied for their potential advantages and drawbacks (Gamache and Acworth, 1998). There is a dearth of information in available literature on the hormonal profiles of females given leaf extracts of *Newbouldia laevis*, despite its common usage in traditional medicine. In the present study rabbit does were orally given varied doses of methanolic leaf extract of *Newbouldia laevis* before breeding and during pregnancy, and the effects on their serum levels of oestrogen and progesterone were evaluated.

Materials and Methods

Experimental animals and their management: This study utilized twenty healthy female New Zealand white breed of rabbits (*Oryctolagus cuniculus*) that were six months old and had an average body weight ranging from 1400 – 2000 g. The rabbits were housed in the Animal House of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University (ABU), Zaria, Nigeria. The study took place at a regular room temperature of 27.8 ± 3.2°C, with every female rabbit kept in standard cages designed for rabbits. On procurement, the rabbits were screened for illnesses and treated for external parasites using Kepromec® (Kepro, Holland). They were further acclimatized for six weeks before the study commenced. Drinking water and feed were made freely available to the rabbits all through the study. Approval for the study protocol was obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC); Approval number – ABUCAUC/2022/019.

Experimental plant and processing: The *Newbouldia laevis* leaves used for the study were collected from a plantation at ABU Silver Jubilee Quarters in Zango, Zaria, Nigeria. The leaves were brought to the Herbarium at the Botany unit, Department of Biological Science, Faculty of Life Science, ABU Zaria, Nigeria, for identification and verification. A voucher with the number 2881 was provided, and the plant was identified as *Newbouldia laevis* belonging to the Bignoniaceae family. The *Newbouldia laevis* leaves were cleaned to get rid of dirt and debris, air-dried for five days, and crushed into a fine powder. A part of the powder was soaked in 70% methanol for 48 hours. The extract was decanted, filtered and concentrated in a vacuum evaporator at 60°C, followed by drying in an oven at 40°C. The extract was then kept in a sealed container in a refrigerator until the time of use.

Study design: The New Zealand white rabbits does were randomly assigned to four groups (A, B, C and D), with each group consisting of five does. Groups A, B and C received the methanolic leaf extract of *Newbouldia laevis* at doses of 1000mg/kg, 500mg/kg, and 250mg/kg for 48 days, while group D served as the untreated control (given distilled water placebo). The does were mated on the twenty-seventh day of administration of the extract. Blood samples (2 ml) were collected from each of the rabbit does on days 0, 6, 21, 27, 34, 41 and 48 of the experiment for the hormonal assays.

Methods and Procedures: The blood samples were collected in plain labeled tubes with no anticoagulant. Every blood sample was allowed to coagulate, spun at 3000 × g for 15 minutes, and the serum was collected into a labeled serum container. Then, the serum was kept at -20°C until it was used for the assay of levels of estradiol and progesterone.

The serum estradiol assay was performed with an Accu-bind® estradiol enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's guidelines. This test was conducted at the Biotechnology Laboratory of the National Animal Production Research Institute (NAPRI), ABU, Zaria. The microtitre plate wells were arranged so that each serum reference calibrator, control and serum sample was analyzed in duplicates. Twenty-five microlitre (25 µL) of the serum reference calibrator, control, and serum samples were pipetted into the designated wells, then 50 µL of the Estradiol Biotin Reagent was added to each well. The microplate was gently rotated for 30 seconds, then closed and left to incubate for 30 minutes at room temperature. Afterwards, to each well was added 50 µL of Estradiol Enzyme Reagent, followed by swirling of the plate for 30 seconds, covering it, and incubating for 90 minutes at room temperature. The plate's contents were poured out, and the plate was dried using absorbent paper. Following that 350µL of

wash buffer was added to every well, decanted, and the washing was repeated twice. Afterwards, 100 µL of substrate solution was added to every well; the plate was left at room temperature for 20 minutes, and afterwards, 50 µL of stop solution was added to each well with gentle mixing for 15 seconds. The absorbance values were measured at a wavelength of 450 nm using a microplate reader (Optic System IVYMEN® 2100C, USA).

The progesterone assay was performed with a progesterone ELISA kit (Accu-bind®) following the manufacturer's guidelines. The microtitre plate wells were set up in a way that allowed each serum reference calibrator, control, and serum sample to be analyzed twice. In each labeled well, 25 µL of the serum reference calibrator, control, and serum sample were added, along with 50 µL of the Progesterone Enzyme Reagent. The plate was then swirled for 20 seconds. After this, 50 µL of Progesterone Biotin Reagent was dispensed into each well; the plate was swirled for 20 seconds, covered, and left to incubate at room temperature for 60 minutes. After the incubation, the contents of the microplates were poured off, and were dried with absorbent paper, and each well was washed with 350 µL of wash buffer thrice. After that, 100 µL of substrate solution was added to every well, and the plate was left to incubate at room temperature for 20 minutes. Finally, 50 µL of stop solution was added to each well and mixed gently for 15 seconds. The absorbance values were measured at a wavelength of 450 nm using a microplate reader (Optic System IVYMEN® 2100C, USA).

Statistical Analysis: GraphPad Prism software (Version 5.0, San Diego) was used for the data analysis. Data obtained were subjected to repeated-measure and two-way analysis of variance, followed by a Bonferroni *post-hoc* test for differences between groups. The value of $p \leq 0.05$ was considered significant. Summary of the results (means ± standard error of mean) were presented in tables.

Results

The serum oestrogen (E2) levels of the rabbit groups are shown in Table 1. There were no significant variations ($p > 0.05$) between the groups in their serum E2 levels on days 0 and 6 of the experiment, but on day 21, Group A does had a significantly ($p < 0.05$) higher E2 level, when compared to Groups B, C and D (Table 1). Additionally, Group B does had a significantly ($p < 0.05$) higher mean E2 level compared to Groups C and D, though there was no significant difference ($p > 0.05$) in E2 levels between Groups C and D on day 21 of extract administration. On days 27 and 34 of the experiment, there were no significant ($p > 0.05$) variations between the groups in their serum E2 levels, but on day 34 the mean serum E2 levels of the three extract treated groups (A, B and C) were significantly ($p < 0.05$) higher than that of the untreated control group D (Table 1). On day 41, the mean serum E2 levels of Group C does was significantly ($p < 0.05$) lower than those of Group D, but on day 48, there were no significant variations in the serum E2 levels of all the rabbit groups (Table 1).

Results of the determination of the serum progesterone (P4) levels are shown in Table 2. There were no significant variations ($p > 0.05$) in the serum P4 levels of all the rabbit groups on day 0, but on day 6 of the experiment, the serum P4 levels of Group A and C rabbits were significantly ($p < 0.05$) lower than that of the Group B rabbits (Table 2). On day 21, mean serum P4 levels of Group A and B does were significantly ($p < 0.05$) lower than those of the Groups C and D does, but on day 27, the mean serum P4 levels of the Group A does was significantly ($p < 0.05$) higher than those of the other three groups (B, C and D). On days 34, 41 and 48, however, there were no significant ($p > 0.05$) variations in the mean serum P4 levels of all the rabbit groups (Table 2).

Table 1. Serum oestrogen levels (pg/mL) of rabbit does that were given graded oral doses of methanolic leaf extract of *Newbouldia laevis* (MLENL) before breeding and during pregnancy. [Results are presented as mean ± standard error of mean]

Days of extract administration	*Mean serum oestrogen levels (pg/mL) ± SEM*			
	Group A (1000 mg/kg MLENL)	***Group B (500 mg/kg MLENL)***	***Group C (250 mg/kg MLENL)***	***Group D (Untreated Control)***
Day 0	167.15 ± 14.29 [a]	156.78 ± 90.20 [a]	206.25 ± 52.78 [a]	231.34 ± 21.92 [a]
Day 6	219.68 ± 23.10 [a]	202.01 ± 111.47 [a]	250.39 ± 55.03 [a]	282.45 ± 31.46 [a]
Day 21	261.18 ± 60.98 [a]	50.33 ± 50.19 [b]	0.19 ± 0.03 [c]	0.14 ± 0.02 [c]
Day 27	0.16 ± 0.05 [a]	0.14 ± 0.02 [a]	0.17 ± 0.02 [a]	0.14 ± 0.02 [a]
Day 34	16.75 ± 5.11 [a]	14.42 ± 1.68 [a]	12.18 ± 1.71 [a]	0.12 ± 0.02 [b]
Day 41	12.38 ± 3.22 [ab]	14.49 ± 1.70 [ab]	11.55 ± 1.45 [a]	16.86 ± 0.44 [b]
Day 48	14.03 ± 1.98 [a]	18.07 ± 2.47 [a]	14.43 ± 2.91 [a]	15.88 ± 1.07 [a]

Mean (± SEM) values with different superscript alphabets along the same row differ significantly ($p < 0.05$).

Table 2. Serum progesterone levels (ng/mL) of rabbit does that were given graded oral doses of methanolic leaf extract of *Newbouldia laevis* (MLENL) before breeding and during pregnancy. [Results are presented as mean ± standard error of mean]

Days of extract administration	*Mean serum progesterone levels (ng/mL) ± SEM*			
	Group A (1000 mg/kg MLENL)	***Group B (500 mg/kg MLENL)***	***Group C (250 mg/kg MLENL)***	***Group D (Untreated Control)***
Day 0	4.78 ± 1.00 [a]	5.66 ± 0.94 [a]	5.67 ± 0.99 [a]	4.42 ± 0.53 [a]
Day 6	4.90 ± 1.19 [a]	9.07 ± 1.92 [b]	5.66 ± 1.24 [a]	7.97 ± 1.59 [ab]
Day 21	10.87 ± 0.41 [a]	9.87 ± 1.54 [a]	14.11 ± 2.08 [b]	16.09 ± 1.07 [b]
Day 27	19.95 ± 0.38 [a]	13.58 ± 2.16 [b]	12.31 ± 0.63 [b]	13.83 ± 2.07 [b]
Day 34	9.59 ± 2.32 [a]	12.91 ± 3.52 [a]	12.00 ± 3.30 [a]	10.28 ± 1.48 [a]
Day 41	13.65 ± 1.77 [a]	15.32 ± 1.95 [a]	14.80 ± 1.66 [a]	14.73 ± 2.19 [a]
Day 48	13.25 ± 3.46 [a]	14.39 ± 1.78 [a]	15.30 ± 1.80 [a]	16.05 ± 2.41 [a]

Mean (± SEM) values with different superscript alphabets along the same row differ significantly ($p < 0.05$).

Discussion and Conclusion

Estrogen (E2) is the hormone that is in charge of the growth of the female reproductive organs, getting the reproductive tract ready for pregnancy and mating actions, and it is crucial during pregnancy in female rabbits (Gadsby *et al.,* 1983; Mustafa and Elhanbaly, 2020). In this present study, the rabbit does given methanolic leaf extract of *Newbouldia laevis* showed differences in E2 levels before mating and throughout gestation. On days 0 and 6 of administration of the extract, E2 levels were elevated in all does, with a slightly higher level in the group receiving 250 mg/kg extract, although not significantly. Elevated E2 levels have been reported to signal the presence of oestrus and preparedness for mating (Ermayanti *et al*., 2019; Marcondes *et al.,* 2002; Ajayi and Akhigbe, 2020). This indicates that the rabbit does used for the study were in estrus and were prepared for breeding. On day 21, there was a notably increased E2 level in the rabbit does that were given the extract at doses of 1000 mg/kg and 500 mg/kg, followed on day 27 by sharp decreases in all groups. This sharp variation in the E2 levels, which is not uncommon is rabbits is thought to be as a result of the fact that rabbits do not have a regular estrous cycle, as they are induced or reflex ovulators, meaning ovulation mainly occurs after mating (Hoffman *et al.,* 2010; Lone *et al.,* 2017). This reflex ovulatory trait is linked to the occurrence of alternating cycles of receptive and non-receptive periods, causing fluctuations in estrogen levels, which were seen in all the rabbit does used for the study. Immediately after mating (day 34), it was noted that the mean serum levels of E2 were significantly higher in the extract-treated groups when compared to the untreated control; this was followed by days 41 and 48, when the mean serum E2 levels of the untreated control group were relatively high. Klaus and Uwe (1988) found that E2 levels were lower in non-pregnant or lactating rabbits than when they were pregnant, with the highest levels observed in the first week of pregnancy. Another study by Challis *et al* (1973) showed a small rise in E2 levels from day 21 to 30 of pregnancy, whereas Lau *et al.* (1982) reported a reduction in serum E2 levels during the same period. In the second part of pregnancy, Bostanci *et al* (2012) found an E2 level of 98.1 pg/mL, while Kirat *et al* (2015) reported a level of 5.5 pg/mL on the 10th day of gestation in female rabbits. Gonzalez-Mariscal *et al*. (2009) recorded an E2 level of 24±6 pg/mL on day 21 of gestation, whereas Kirat *et al* (2012) reported a level of 10.5 pg/mL. In their study, Ashour and Abdel Rahman (2019) found E2 levels of 47.31 ± 4.99 pg/mL and 56.33± 5.34 pg/mL on day 14 and day 21 of gestation, which matched the findings of Al-Atawi *et al* (2004). The differences in the E2 levels pattern seen in our study compared to others may be attributed to the effect of the extract used for the study. It is thought to be a result of the presence of alkaloids and flavonoids found in the *Newbouldia laevis* leaf extract, as previous research reports have indicated that these components may decrease plasma levels of certain fertility hormones (Browning *et al.,* 1998; Bianco *et al.,* 2006). Variations in the E2 testing techniques, doe breeds, regions, and feed types could also contribute to the differences seen in the present study when compared to previous studies.

According to reports by Szendro *et al* (2010), it is widely recognized that progesterone (P4) levels in rabbits are consistently elevated during the entire pregnancy term. This is because P4 plays a crucial role in the initiation of mammary gland development (lactogenesis) during mid-gestation and the growth of alveoli (Neville *et al.,* 2002). The increase in P4 levels further supports foetal implantation and ensures that pregnancy continues (Kelden *et al.,* 2017), making it important for maintenance of uterine calmness to prevent early labor (Kirat *et al.,*

2015). Challis *et al* (1973), along with Lau *et al.* (1982) found that the highest P4 levels were reached on the 12th day of pregnancy. Klaus and Uwe (1988) reported higher P4 levels during the initial week of pregnancy compared to non-pregnant, non-lactating does and a peak P4 level during the 2nd week. Challis *et al* (1973) and Lau *et al* (1982) similarly found that the highest P4 level occurred on day 12 of pregnancy. According to Merkelbach and Emmens (1977), during a typical pregnancy, the average P4 levels peaked at 10 ng/mL by day 7, remained steady at 14 ng/mL from day 10 to 20, and then decreased to very low levels during childbirth. In pregnant does who had their ovaries removed, receiving exogenous P4 with/without E2 led to plasma P4 levels of 10 ng/mL by day 6, which were maintained at 10 to 13 ng/mL until day 20 before decreasing. The present study recorded no notable variation in plasma P4 plateau levels among the treated and control groups, as well as among the treated groups. In the first week of gestation, Szendro *et al* (2010) found a P4 level of 9.4 ng/mL, whereas Bostanci *et al* (2012) observed a P4 level of 1.5 ng/mL in pregnant rabbits. In the second week of pregnancy, Kirat *et al* (2015) found a P4 level of 9.9 ng/mL on day 10, Gonzalez-Mariscal *et al* (2009) found a level of 11 ± 3 ng/mL on day 14, and Kelden *et al* (2017) and Ashour *et al* (2018) found P4 levels ranging from 3.16 – 4.00 ng/mL. During the third week of gestation, Haneda *et al* (2010) noted a P4 level of 8.8 ± 1.0 ng/mL on day 18, Kirat *et al* (2015) observed 5.3 ng/mL on day 20, and Alfonso (2016) reported a P4 level of 10 ng/mL on day 21. Differences in P4 levels in our study compared to reports of previous studies may be attributed to the effects of constituents of the *Newbouldia laevis* leaf extract. This could be linked to the alkaloid and flavonoid content of the extract, as mentioned before (Browning *et al.,* 1998; Bianco *et al.,* 2006). Other potential factors could include variations in P4 assay methods, sample type, sample collection time, doe characteristics (such as age, health, breed, and other intrinsic factors), location, and feed type.

Egba *et al* (2014) reported that the aqueous leaf extract of *Newbouldia laevis* was found to regulate fertility hormones like testosterone, follicle-stimulating, and luteinizing hormones in male albino rats, indicating its potential to modulate the hormonal profile at specific doses. Oladimeji and Aroyehun (2015) proposed that the leaf extract of *Newbouldia laevis* could have positive effects on fertility and help remedy hormone-induced pathologies in female albino rats.

Based on the results of the study, it was concluded that oral administration of *Newbouldia laevis* leaf extract as used in the study led to significant alterations in the serum levels of oestrogen and progesterone at specific days before breeding and during pregnancy, and this was thought to be as a result of the effects of the phytochemical constituents of the extract. Further research on the effects of the *Newbouldia laevis* extract on other reproductive hormones is recommended.

Acknowledgments

This study would not have been possible without the technical support of the entire staff of the Department of Theriogenology and Production. We also thank the Staff of the Animal House of the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, ABU Zaria, for the collective work and support. We are grateful to Dr. Felix Uchenna for his assistance during the hormonal profiling and to Dr. Ochuko Orakpoghenor for assisting with the statistical analysis. Finally, we appreciate families, colleagues, and friends for their support.

Funding

This study was partly supported through a study leave to the first author by the Faculty of Veterinary Medicine, Bayero University Kano State, Nigeria.

Conflict of interest

The authors declare no conflict of interest.

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