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Potential of *Croton menyharthii* and *Uvariodendron kirkii* aqueous extracts as female Novel contraceptives.

Catherine K. Kaingu,[a*] Jemimah A. Oduma[a]

ABSTRACT

Objective: The aim of the study was to validate the traditional fertility regulating claims by investigating the effect of root bark extract of both plants on ovarian and uterine structures.

Materials and Methods: Twenty five mature normocyclic female winstar rats were used. Group 1 consisted of 5 animals that acted as control. Group 2 and 3 with 5 animals each; received 500 and 800mg/ Kg *Croton menyharthii* respectively on alternative days for 28 days through intra- abdominal gavage. Group 4 and 5 were treated in a similar manner but received *Uvariodendron kirkii* aqueous extract. Left ovaries and uterus were harvested and processed for histomorphology.

Results: *Croton menyharthii* and *Uvariodendron kirkii* extracts caused a reduction in primordial and antral follicles, disrupted granulosa and theca cells with significant degeneration of ova. *Croton menyharthii* caused a disruption of uterine endothelial structure and loss of villi. *Uvariodendron kirkii* aqueous extract caused a significant uterine gland vacuolation and a thickened endothelial lining with an intact endothelial cell layer and its invaginations. Disrupted ovarian and uterine structure possibly led to compromised fertility and implantation.

Conclusion: The study validates the traditional use of the plant in fertility regulation. We suggest further investigation on these potential plants to address the call for novel contraceptive drugs.

Keywords: *croton menyharthii*, *Uvariodendron kirkii,* disrupted zona pelucida, loss of ovum, disrupted endometrium, pyknotic cells.

*Correspondence to:
Catherine K. Kaingu, Department of Veterinary Anatomy and Physiology, University of Nairobi, P.O Box 30197-00100, Nairobi, Kenya
ckaluwa@uonbi.ac.ke kaingucatherine@gmail.com

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INTRODUCTION

Nearly half of all pregnancies globally are unintended. Furthermore, current contraceptive options do not adequately meet the needs of users. The rapidly increasing population has put a strain on the limited resources and poses serious challenge to national planning. *Uvariodendron kirkii* and *Croton menyharthii* are used as fertility regulators in Kenya. Female fertility is driven by the developmental competence of the oocyte in its ability to undergo meiosis, be fertilized and give rise to a viable embryo. Ovarian folliculogenesis, oogenesis and ovulation are regulated by pituitary gonadotropins; Follicular stimulating hormone (FSH) and luteinizing hormone (LH). The recruitment, growth and maturation of pre antral follicles are independent of pituitary gonadotropins. However the development of the antral follicle is dependent on FSH. As the antral follicles grow, theca cells under the influence of LH secrete androgens which are converted within granulosa cells into estradiol. Organization and functioning of the ovary is dependent on very close interactions between oocyte and surrounding follicle cells.[1] Communication between the oocyte and surrounding follicles is bi-directional, follicle cells regulate oocyte growth and oocyte regulates follicular development. It therefore follows that a disruption of the bidirectional communication between the oocyte and surrounding somatic cells; a disruption of either the stroma cells or the oocyte integrity will interfere with folliculogenesis and oogenesis and compromise fertility. Estradiol plays a key role in the cyclic growth of the endometrium layer; which undergoes differentiation under the influence of progesterone hormone and undergoes a short period of receptivity to embryo implantation. Successful implantation requires coordinated interactions between the blastocyst and uterus. A compromised endothelium will interfere with implantation and lead to infertility. The root barks of *Croton menyharthii* and *Uvariodendron kirkii* are traditionally used as fertility regulators in Tana River County, Kenya. Kenya has the highest induced abortion rate in East Africa mostly resulting from unintended pregnancy.[3] In 2012; 465000 unsafe abortions were carried out contributing to one of the highest maternal mortality rates in the region. The present study investigates the effect of graded doses of *Croton menyharthii* and *Uvariodendron kirkii* aqueous extract on Winstar rat ovarian and uterine endothelial lining histomorphology.

[a] Department of Veterinary Anatomy and Physiology, University of Nairobi, P.O Box 30197- 00100, Nairobi, Kenya.

MATERIAL AND METHODS

Laboratory animals

Twenty five mature female winstar rats, eight weeks old weighing between 170-210g were used for the study. The animals were purchased from the Department of Biochemistry and kept in the animal house, Anatomy and Physiology Department, University of Nairobi, Kenya. They were caged in groups of five and were maintained under standard environmental conditions of 12 hours light and 12 hours darkness at 24-25°C. The rats were fed on commercially obtained diet pellets and tap water was provided *ad libitum*. They were monitored daily for the first 20 days using vaginal smears to ascertain cyclicity. Only those with regular 4-5 day estrous cycles were used for the experiment.

Plant collection, identification

Medicinal plants used as fertility regulators in Tana River County were harvested and brought to the University of Nairobi, School of Biological Sciences for botanical identification. Voucher specimens were preserved for future reference. *Croton menyharthii* (CK021) and *Uvariodendron kirkii* (CK008) fresh roots were cut into small pieces using a knife. The roots were then kept under shade and dried at room temperature for a period of two weeks. The roots were ground into powder in a fume chamber using a Cunningham grinder.[2] The plant powder was packed in 300g satchets and stored in cool and airy cupboards away from direct sunlight.

Extract preparation

300g of *Croton menyharthii* root bark powder was weighed using (Lark digital weighing balance LP502A, 500G/0.01g). The root bark powder was macerated in distilled water at a ratio of 1 to 6 (w/v) in a volumetric flask. The suspension was rotated on a shaker for 24 hours at room temperatures and left to soak for 48 hours. Filtration was carried out using whatman filter paper (number 4). The filtrate was freeze dried for 48 hours and the extract weighed to determine yield. *Uvariodendron kirkii* aqueous extract was prepared in a similar manner. The aqueous extract yield for *Croton menyharthii* and *Uvariodendron kirkii* was 83.89 and 118.93 grams respectively.

Effect of *Croton menyharthii* and *Uvariodendron kirkii* extracts on ovarian and uterine endometrium histology.

The rats were divided into five groups (5 rats per group). Group 1 and 2 received 500 and 800 mg/Kg *Croton menyharthii* while group 3 and 4 received 500 and 800 mg/Kg *Uvariodendron kirkii* aqueous extract respectively daily for 28 days through intra-abdominal gavage. Five control animals received 0.5 ml physiological saline for 28 days. All rats were humanely sacrificed after the 28th day using diethyl ether. Physiological saline was used to flush the body of all rats and immediately thereafter left ovaries and uterine horns were harvested and processed for histomorphology. Ovaries and uterine horns were fixed, cut in sections of 8 micron thickness and stained with Hematoxylin & Eosin and observed under a light microscope.

Tissue Processing Protocol

Tissue processing of both ovaries and uterine horns were carried out as per the protocol described.[3]

RESULTS

Effect of *Croton menyharthii* aqueous extract on ovaries and uterine endometrium

Croton menyharthii aqueous extract at 500mg/Kg caused a significant reduction in primordial and pre antral follicles (Figure 1B). The primary follicles were showing degenerative changes compared to the control (Figure 1A) that had structurally intact pre and antral follicles. The cytoplasm was condensed and there was a disruption in arrangement of granulosa cells, shrinkage of ooplasm and zona pelucida disruption (Figure 2B) compared to control (Figure 2A). The theca cell layer of the primary follicle was disrupted. The major degenerative changes were however observed within pre antral follicles (Figure 2B) compared to the control that had a well demarcated granulosa cell layer, zona pelucida and viable oocyte (Figure 2A). There was lack of an oocyte and zona pelucida (Figure 2B). Granulosa cells were condensed giving an appearance of loss of cytoplasm. There was presence of pyknotic cells in the inner lining of the granulosa cell layer and a disrupted theca layer (Figure 3B) compared to the control (Figure 3A). In treated ovaries, the follicles were undergoing degenerative changes and they had lost their normal shape and arrangement of granulosa cells. There was total absence of zona pelucida (4B, 5B). It was a conspicuous finding that all the ovarian follicles including primordial follicles had undergone degenerative changes. In histological study of treated ovaries the principal observation was degeneration of ova in all the follicles simultaneously, which were in different stages of their development. The first sign noticed during the atresia in a follicle was pyknosis and fragmentation of the inner granulosa cells (Figure. 3B). None of the follicles could be seen with intact ovum and its normal nucleus (Figures. 2B, 3B). *Croton menyharthii* caused a disruption of

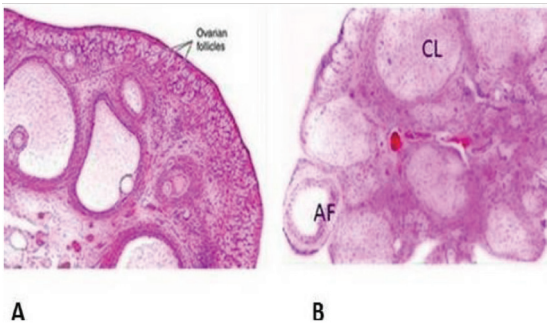

Figure 1 The effect of *Croton menyharthii* extract on ovarian structures. A: Control, presence of primordial, preantral, antral and Graffian follicle. B: Significant reduction in primary follicles and presence of a degenerating antral follicle compared to the control. Magnification ×100 AF-antral follicle CL-corpus luteum

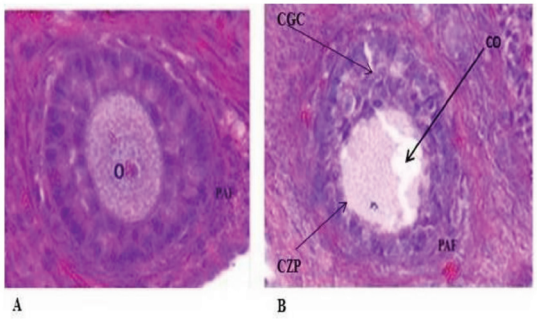

Figure 2 The effect of *Croton menyharthii* extract on pre antral follicle. A: Control, intact oocyte and zona pellucida, intact structural intergrity of granulosa cells and theca cells. B: Pre antral follicle: Structural intergrity of granulosa cell layer disrupted, oocyte compromised, ooplasm shrinkage, zona pellucida and theca cells disrupted. Magnification × 400. Key: CO-compromised oocyte PAF-preantral follicle CGC- compromised granulosa cells CZP- compromised zona pelucida.

endothelial structural integrity (Figure. 4B, 5B); loss of endothelial villi (4B); presence of pyknotic cells within uterine stroma (Figure. 4B) compared to the controls (Figure. 4A, 5A) with an intact endothelial lining structural integrity.

Effect of *Uvariodendron kirkii* aqueous extract on ovaries and uterine endometrium

Figures 6B, 7B and 8B are a selection of slides that show the effect of *Uvariodendron kirkii* on

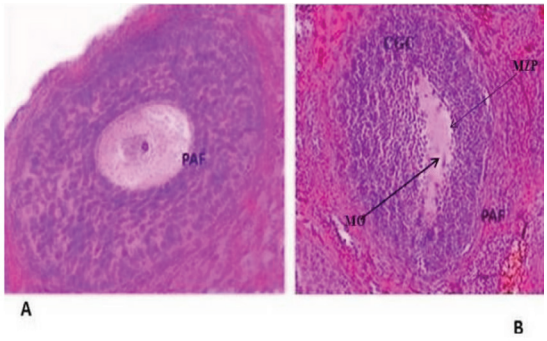

Figure 3 The effect of *Croton menyharthii* extract on pre antral follicle A: Control, the photomicrograph shows a pre antral follicle. Structural intergrity of granulosa cell intact, presence of viable oocyte and zona pellucida. B: Atretic pre-antral follicle, lack of oocyte and zona pelucida. Granulosa cells condensed with loss of cytoplasm. Presence of pyknotic cells in the granulosa inner lining and disrupted theca layer. Magnification × 400. Keys: PAF- preantral follicle CGC-condensed granulosa cell layer MZP-missing zona pelucida MO-missing oocyte

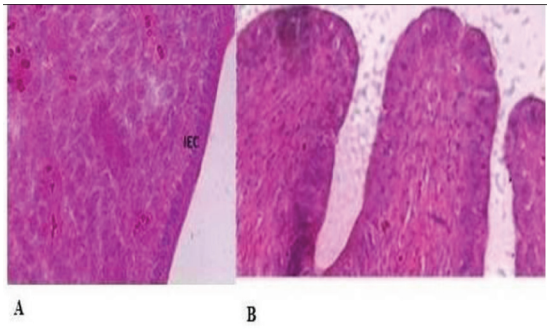

Figure 4 The effect of *Croton menyharthii* extract on uterine endometrium. A: Control: simple columnar endothelium cells, intact structural intergrity. B: loss of structural intergrity of endothelium and villi, loss of invagination of endothelial lining. Compromised endothelium, stroma cells compromised. Magnification × 400.

ovarian tissue. Figure 6B shows a degenerating pre antral follicle, fewer or missing oocytes as well as fewer primary and primordial follicles (Figure 6B) compared to control (6A) which shows structurally intact pre antral follicles. Figure.7B the pre antral follicle is degenerating. The most significant finding being the missing oocyte and zona pelucida. The granulosa cell layer was also condensed

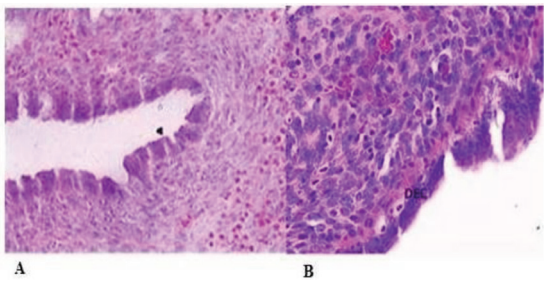

Figure 5 The effect of *Croton menyharthii* extract on uterine endometrium. A: Control: intact simple columnar endothelial cells. B: Endothelial cells structural intergrity disrupted and presence of pyknotic cells within uterine stroma. Magnification × 400. IEC: Intact endothelial cell layer DEC: disrupted endothelial cell layer.

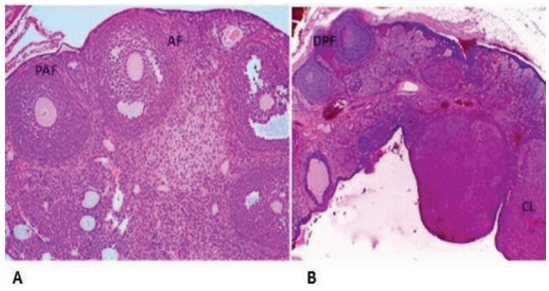

Figure 6 The effect of *Uvariodendron kirkii* extract on pre and antral follicles A: Control, the photomicrograph shows a pre and antral follicles. The structural intergrity of both follicles are intact. The micrograph shows an intact oocyte, zona pelucida and intact structural intergrity of granulosa and theca interna layer. B: The photomicrograph shows degenerating preantral follicles, with missing oocytes and zona pelucida. The granulosa cell layer is condensed. Magnification ×100. Keys: PAF preantral follicle AF- antral follicle DPF-degenerating pre antral follicle. CL-corpus luteum.

compared to the negative control (Figure.7A). Figure 8B shows a degenerating antral follicle. The most significant finding being the loss of the zona pelucida, degenerating oocyte, shrunken ooplasm and presence of pyknotic cells within granulosa cell layer compared to the negative control (Figure 8A) with an intact oocyte and zona pelucida, cumulus oophorus and intact structural integrity of somatic cell layers. *Uvariodendron kirkii* aqueous extract caused a significant uterine gland vacuolation within stroma (Figure. 9B) and a thickened

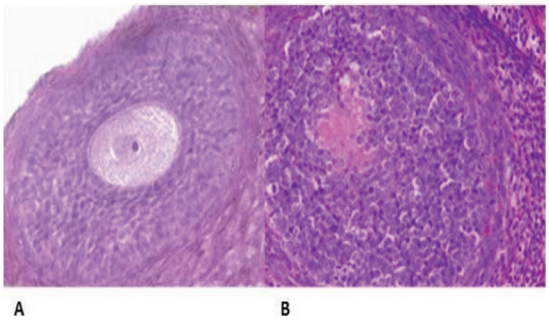

Figure 7 The effect of *Uvariodendron kirkii* extract on secondary follicle A: Control: Secondary follicle, intact structural intergrity of oocyte, zona pelucida, granulosa and theca cell layer. B: Degenerating secondary follicle, oocyte and zona pelucida missing with a compromised granulosa cell layer. Magnification × 400.

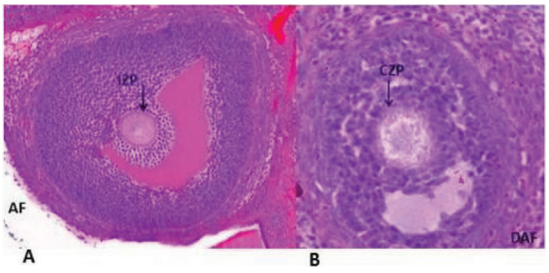

Figure 8 The effect of *Uvariodendron kirkii* extract on antral follicles. A: The photomicrograph shows an antral follicle with an intact zona pelucida, presence of oocyte, cumulus oophorus structural intergrity intact. B: the photomicrograph shows a degenerating antral follicle, with loss of an oocyte, shrunken ooplasm, loss of zona pellucida and pyknotic cells within granulosa cell layer. Magnification × 400. Keys: AF-Antral follicle; DAF-degenerating antral follicle; CZP-compromised zona pelucida. IZP- intact zona pelucida

endothelial lining (Figure. 9B) compared to the control (Figure. 9A) with an intact endothelial cell layer and its invaginations.

DISCUSSION

This study was undertaken to evaluate the effects of *Croton menyharthii* and *Uvariodendron kirkii* aqueous extract on the morphological of ovaries and endometrium of the rat. There has been no study documenting the same so far. Histological studies of ovaries in the control group showed the various types of follicles at all stages of folliculogenesis (primordial,

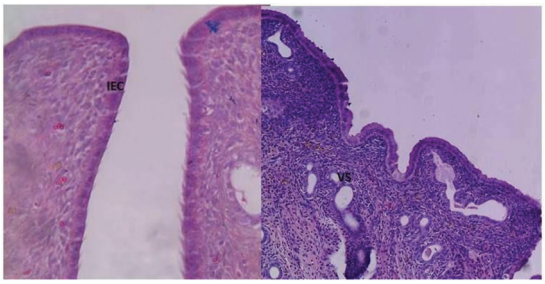

Figure 9 The effect of *Uvariodendron kirkii* extract on uterine endometrium. A: Control; intact endothelial cell layer. Structurally intact endothelial cell layer invagination. B: *Uvariodendron kirkii* condensed endothelial layer, loss of invaginations. Magnification ×400

primary, secondary and mature follicles) (Figure. 1A). *Croton menyharthii* and *Uvariodendron kirkii* aqueous extract at 500 and 800 mg/Kg caused a significant increase of atretic pre antral follicles (2B, 3B, 6B and 7B) and a significant reduction in primary follicles numbers. This suggests compromised folliculogenesis and may explain traditional consumption to regulate fertility. The significant reduction in the number of follicles in the presence of *Croton menyharthii* and *Uvariodendron kirkii* at 500 and 800 mg / Kg could probably be due to a reduction in estradiol levels.[4] The reduction in estradiol levels could be due to the disrupted bi-directional communication between granulosa and theca cellular layer as exemplified in figures 2B, 3B, 6B, 7B and 8B) which play an active role in androgen and estradiol synthesis. The reduction in estradiol could also be due to a direct effect of both plant extract on the pituitary gland. An optimal blood level of FSH is a pre requisite for initiation and maintenance of normal ovarian folliculogenesis. Therefore the present histological finding suggests a hypothalamic-pituitary gonadal axis dysfunction after treatment with both plant extracts. Similar studies have reported corroborating results.[5,4,6,7,8,9,10,11,12,13,14,5,16,17] The number of primordial ovarian follicles was not affected by *Croton menyharthii* and *Uvariodendron kirkii* aqueous extract treatment at 500 mg/Kg but was affected at 800mg/Kg. This may be due to the fact that formation, growth and development of primordial follicles to early primary follicular stages are accomplished during perinatal period. Therefore *Croton menyharthii* and *Uvariodendron kirkii* aqueous extract may not have any effect on the population of these follicles especially at lower concentrations. As shown in this study ovarian follicle atresia might have occurred due to the diminution in serum FSH levels which is in line with the present hormonal results.[19] The zona pelucida (ZP) is a relatively thick extra cellular coat that surrounds the plasma membrane of the oocyte. The ZP is laid down during the final stages of oogenesis when growing oocytes enter their growth phase. As oocytes increase diameter (15-80 μm) the ZP also increases in thickness. The ZP is constructed by three glycoproteins referred to as mZP1, mZP2 and mZP3 that are synthesized and secreted by the growing oocyte. Studies[1] have shown that a disruption of mZP1 leads to a severe reduction of both secondary and Graffian follicles thereby leading to a reduction in ovulated oocytes and litter size. Fully grown oocytes and ovulated eggs from rats without mZP2 and mZP3 glycoprotein lack a ZP and are infertile. mZP2 and mZP3 knock-out mice do not construct ZP resulting in deleterious effects on folliculogenesis leading to a reduced number of antral follicles in ovaries and severely reduced number of ovulated eggs in oviducts. Although this study did not scrutinize the ZP in similar details, it however demonstrated a disruption of the ZP in several photomicrographs (Figure. 2B, 3B, 6B, 7B, 8B) compared to intact ZP in Figure. 2A and 3A. Ovarian morphology from mZP2 and mZP3 null females suggests that growing and fully grown oocytes are less intimately associated with follicular and cumulus cells in particular than oocytes from intact females. Notably cells of corona radiata from mZP2 and mZP3 knock-out females are less orderly arrayed around growing oocytes than the control. As stated in the introduction it is possible that a disruption of this bi-directional communication between oocyte and surrounding granulosa cells is responsible for compromised folliculogenesis and oogenesis especially where the structural intergrity of granulosa cells and theca cells was compromised (Figure 2B, 3B, 7B, 8B). Through gap junctions, cumulus cells supply nutrients and energy substrates to the oocyte, including amino acids, glucose, and ribonucleosides.[19] Oocytes are unable to metabolize glucose and can only generate ATP through oxidative phosphorylation. However, cumulus cells consume glucose via aerobic glycolysis, the product pyruvate sent to the oocyte for oxidative phosphorylation via gap junctions and other modes of communication such as paracrine signaling cumulus cells provide developmental assistance to the oocyte in several ways. Paracrine signaling between oocytes and the cells of the follicle is bidirectional and essential to development. In general terms, it has been demonstrated that the disruption of paracrine signaling between mouse oocytes and their cumulus cells *in vitro* reduces oocyte competence[1] and compromises fertility. This may be particularly important during pre-ovulatory development, as the rate of pyruvate consumption in maturing metaphase-I oocytes is significantly higher than that in immature oocytes. Cumulus cells also help the oocyte take in amino acids. As follicular cells are recruited in each estrus cycle for growth and maturation, simultaneously

the oocytes grow and resume meiosis. This complex process involves a close interaction between the oocyte, surrounding granulosa and theca cells. In this study the results Figures 2B, 3B, 6B, 7B, 8B, showed a disruption of the structural intergrity of oocyte and surrounding cellular cells. Possible cause of infertility is probably due to a compromised folliculogenesis and oogenesis. Structural intergrity of uterine endothelial lining is essential for successful implantation and establishment of gestation. In this study the endometrium was compromised Figures 4B, 5B, 9B. Endothelium growth and differentiation during each estrus cycle is modulated by estradiol and progesterone. A disruption of the hormonal balance impedes implantation.[12,20] Significant reduction of estradiol and progesterone[19] supports the uterine lining histomorphology results. The results are further supported by the significant reduction in implantation index.[18] It is possible that endometrial receptivity was compromised leading to failed implantation.

CONCLUSION

This morphological study has revealed a disruption of the structural integrity of the follicular cells, granulosa cells, theca cells and a disruption of ZP at both dose levels of *Croton menyharthii* and *Uvariodendron kirkii* aqueous extract. This strongly points to interference with folliculogenesis and oogenesis that would lead to a compromised fertility of the female. This might therefore explain the traditional consumption of crude plant extract to cause infertility as a method of contraception desired by the women in Tana River County.

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interest

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