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Effects of Ethanolic Leaf Extract of *Phyllanthus Amarus* (Schum Aand Thonn) on Ovarian Morphology and Reproductive Parameters in Wistar Rats

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ABSTRACT: The study was conducted for eight weeks to investigate the effects of 250mg/kg body weight of ethanolic leaf extract of *Phyllanthus amarus* on the morphology and reproductive functions of the ovary. Twenty-eight (28) adult female Wistar rats were categorized into four groups of seven rats each (n=7) with A (control), B (to assess implantation and resorption), C (to assess the litters), and D (to assess the effects on ovarian features and functions. Male adult rats of proven virility were mated with the female rats of groups B and C. Collected blood samples were used for assay of the following hormones, oestrogen, follicle-stimulating hormone, luteinizing hormone, progesterone and prolactin. The uterus was assessed for resorption, implantation and litters and the ovary was processed for morphological changes. The results revealed no significant difference ($p>0.05$) in the body and organ weight between the experimental groups and the control. None of the rats in groups B and C treated with the extract of *P. amarus* got pregnant; hence no implantation, resorption and litters were seen. There were significant difference ($p<0.05$) in the levels of oestrogen, FSH, LH and progesterone between the treatment groups and the control, but there was no significant difference ($p>0.05$) in the levels of prolactin between the groups. The histoarchitectural pattern of the extract-treated ovary revealed impaired folliculogenesis evidenced by scanty ovarian follicles with atretic changes and degenerative changes in the ovarian stroma. The findings generally confirm the anti-fertility effects of *P. amarus* on ovarian morphology and functions.
Keywords: *P. amarus,* Ovary, Morphology, Functions, Wistar rats

Introduction

The discovery and use of plants products for medicinal purposes has been through all the ages. Quite a number of such plant products have been formulated successfully to control female fertility (Soladoye *et al*., 2014; Tsobou *et al*., 2006). *Phyllanthus amarus (P. amarus),* which belongs to the family of euphorbiaciae and commonly known as kidney beans seed, is a tropical annual herbal shrub found in Nigeria, some West African countries and Southern India whose stem has green capsule, and grows up to 10-50cm high, blooms with flowers and has 5 white sepals and apical acute anther (Adjahloun and Ake Assi, 1972; Burkill, 1985). It has wide acceptability in traditional medical practice from ancient times with its usefulness as anti-hypertensive, diuretic, anti-oxidative, hypoglycaemic and anti-inflammatory effects earning it the name 'miracle plant' (Srividya and Periwal, 1995; Raphael *et al.,* 2002). The phytochemical composition has been elucidated (Kumaran and Karunakaran, 2007; Obianime and Uche, 2009) with flavonoids, tannins, alkaloids terpenoids, steroids, saponins, cardiac glycosides lignins and polyphenol compounds identified. It has been reported to have a wide safety margin by oral route as much as 5 g/kg body weight (Lawson Evi *et al.,* 2008).
Literature has reported the anti-fertility effects of alcohol-extract of this herb affecting cyclicity and pregnancy rate (Rao and Alice, 2001) and reversal of female related problems such as leucorrhoea, menorrhagia and mammary abscess (Patel *et al.,* 2011). This study seeks to elucidate the veracity of these submissions histo-morphologically on the role of *Phyllanthus amarus* in the ovary of adult Wistar rats.

Materials and methods

Harvesting and preparation of extract: The stem and leaves of *Phyllanthus amarus* were collected from the premises of University of Benin¸ Benin City, Nigeria, The plant was identified using standard text and authenticated in the Department of Pharmacognosy, University of Benin, Benin city. The leaves of the plant were washed, cleared of debris and then air-dried prior to oven-dried at 50-60 °C. The sample was pulverized to powdered form and subjected to ethanolic extraction using Soxhlet apparatus to obtain 8.20 kg yield for use.
Experimental animals: Twenty-eight (28) Adult female Wistar rats were bred at the animal house in the Department of Anatomy, University of Benin, Benin-city and kept in the same location for the experiment, in cages at room temperature (25 ºC). The animals were fed on livestock grower mash and water *ad libitum.* The animals were categorized into four groups A, B, C and D of seven rats each (n=7) and placed on treatment regimen (Table 1).
Experimental procedure/mating of animals: Experimental procedure was in compliance with the guidelines for the use of experimental animals (Canadian Council of Animal Care, 1985). At oestrus, male adult Wistar rats of proven virility were deployed to mate overnight with the female rats that had regular four day pro-oestrous cycle. Pregnancy was confirmed with vaginal smears or evidence of cervical mucus plug. Such, when noted was earmarked as day one of the pregnancy.
The body weight of the experimental animals was noted at the beginning of the experiment weekly, and at the end using using an electronic analytical and precision balance (Mettler Pm 4800 Delta Range^{R}). The total duration of treatment was 8 weeks. After completion of treatment the animals were sacrificed under anaesthesia. Blood samples from the lateral tail vein was collected into sterile containers and the serum was used for assay of the following hormones, oestrogen, FSH, LH, progesterone and prolactin levels using the ELISA kits, products of DRG Instruments GmbH, Germany.
Histopathology: Abdomen incision was performed to harvest the ovary and uterus. The harvested organs were fixed 10 % buffered formal saline for 24 hrs, after which they were processed using the automated processor and stained with haematoxylin and eosin (Drury and Wallington, 1980). The mounted slides were placed under light microscopes and photomicrographs were taken at magnification x40 and x100.
Data analysis: Data were presented as Mean ± SEM. Means separation and significant differences between the means (Duncan, 1957) were determined at ($P<0.05$) using ANOVA.

Table 1: Grouping and Treatment Regimen of Experimental animals

Groups	Treatment Regimen
Group A (Control female rats, non-mated)	Not mated but received normal feed and water *ad libitum* without administration of extract for 60 days.
Group B (*P.amarus* -treated female rats, mated)	Received 250 mg/kg body weight of extract for 60 days before mating. Sacrifice was done at day 19 post coitus to assess effect of extract on implantation and resorption.
Group C (*P.amarus* -treated female rats, mated.)	Received 250 mg/kg body weight of extract for 60 days before mating. They were thereafter left for another 30 days to assess effect of extract on litters.
Group D (*P.amarus* -treated female rats, non - mated)	Not mated but received 250 mg/kg of extract for 60 days before sacrifice to assess effect of extract on ovarian morphology.

Results

From the results on the mean weight values (Table 2), there were changes in the weight of the animals between the groups. In Group A, the mean value of the initial weight was 235±5.18 g, while the final mean weight value was 235±3.54 g. In Group B, the initial mean weight value was 211±2.92 g, while the final mean weight value was 211±3.67 g. In Group C, the initial mean weight value was 230±5.24 g, while the final mean weight value was 221±6.00 g. In Group D, the initial mean weight value was 241±2.92 g, while the final mean weight value was 240±2.24 g. The body weight across the groups was not significantly ($p>0.05$) different when compared with the control.
The results on the average ovarian weight (Table 3) of the control group was 0.33±0.01 g, 0.32±0.01 g in group B, 0.40±0.03 g in group C and 0.45±0.02 g in group D. There was no significant difference ($p>0.05$) between the mean organ weight across the groups compared to the control. As pregnancy could not be confirmed in the mated animals, zero percent pregnancy rate was thus found with *P. amarus*. Hence, implantation, resorption and litters were not seen in the uteri.

The results of the hormonal assay of the experimental rats (Table 4) revealed the levels of oestrogen in (ng/ml) as 13.02±0.01 for group B, 12.11±0.15 for group C and 16.12±0.11 for group D respectively. Compared with the value of 17.10±1.03 ng/ml in group A (control), the oestrogen values in groups B and C were significantly different ($p<0.05$). Similarly, the levels of FSH were 1.18±0.31 IU/ml in group A (control), 0.09±0.22 IU/ml in group B, 0.12±0.14 IU/ml in group C and 1.15±0.02 IU/ml in group D. Compared to the control, the values of FSH were significantly different ($P<0.05$) in groups B and C. The value of LH for the control group was 1.11±0.13 IU/ml, 0.01±0.22 IU/ml for group B, 0.03±0.25 IU/ml for group C and 1.13±0.33 IU/ml for group D. Also the values of LH were significantly different in groups B and C compared to control ($p<0.05$). The assay for prolactin level was 0.09±0.01 ng/ml in group A, 0.08±0.20 ng/ml in group B, 0.06±0.14 ng/ml in group C and 0.10±0.32 ng/ml in group D, respectively. The values across the groups were not significantly different ($p>0.05$) from control. The progesterone level was 1.07±0.05 ng/ml in control, 0.09±0.02 ng/ml in group B, 0.15±0.15 ng/ml in group C and 1.04±0.24 ng/ml in group D, respectively. There was significant difference ($p<0.05$) in the levels of this hormone in the treatment groups compared to control.

Histopathological findings

The histological findings in the control slides of the ovary showed ovarian follicles (f) at different stages of maturation developing across the germinal epithelium, with normal ovarian stroma (s). The medulla was well defined, richly supplied by nerves, vessels and lymphatics. The ovary of the treated rats (group D) showed scanty ovarian follicles (s) with atretic changes (f) and ovarian stroma with degenerative changes. The germinal epithelium was well-delineated and the medulla contained normal neurovascular distribution.

Table 2: Effect of *Phyllanthus amarus* on the body weight of experimental animals

Groups	Initial Mean Weight and SEM (g)	Final Mean Weight and SEM (g)
A	235±5.18	235± 3.54
B	211±2.92	211±3.67
C	230±5.24	221±6.00
D	241±2.92	240±2.24

Table 3: Effect of *Phyllanthus amarus* on the ovarian weight of the experimental animals

Groups	Group A	Group B	Group C	Group D
Ovary Weight and Standard Error	0.33 ± 0.01	0.32 ± 0.01	0.40 ± 0.03	0.45 ± 0.02

***** Values are Mean ± SEM. Means with alphabetic remarks are significantly different from control at *($p<0.05$).*

Table 4: Mean hormonal profile of experimental animals

Groups	Oestrogen (ng/ml)	FSH (IU/ml)	LH (IU/ml)	Prolactin (ng/ml)	Progesterone (ng/ml)
A:	17.10±1.03	1.18±0.31	1.11±0.13	0.09±0.01	1.07±0.05
B:	13.02±0.01^{c}	0.09±0.22^{b}	0.01±0.22^{b}	0.08±0.20	0.09±0.02^{b}
C:	12.11±0.15^{c}	0.12±0.14^{b}	0.03±0.25^{b}	0.06±0.14	0.15±0.15^{b}
D:	16.12±0.11	1.15±0.02	1.13±0.33	0.10±0.32	1.04±0.24

***** Values are Mean ± SEM. Means with alphabetic remarks are significantly different from control at *($p<0.05$)*

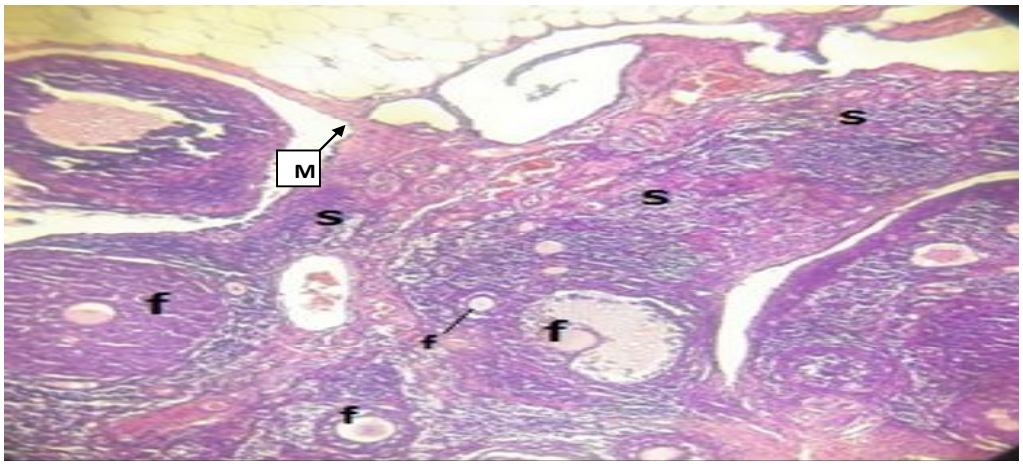

Figure 1: Group A (Control) Slide of the ovary showing ovarian follicles at different stages of maturation (f), and normal ovarian Stroma(S). The Medulla (M) is well delineated in outline with normal blood vessels, nerves and lymphatics. (H&E) x40.

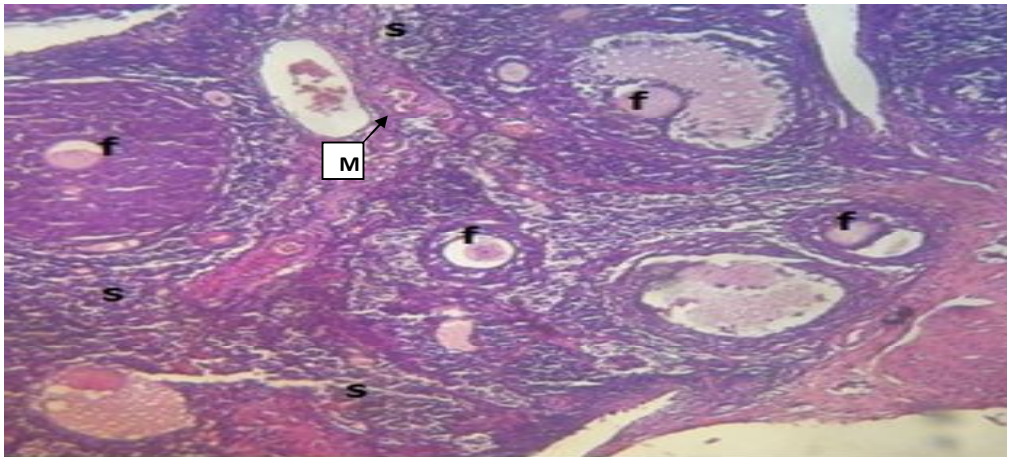

Figure 2: Group A (Control) Slide of the ovary showing numerious ovarian follicles at different stages of maturation (f) and normal ovarian stroma (S). The Medulla (M) is well delineated in outline with normal blood vessels, nerves and lymphatics (H&E) x100.

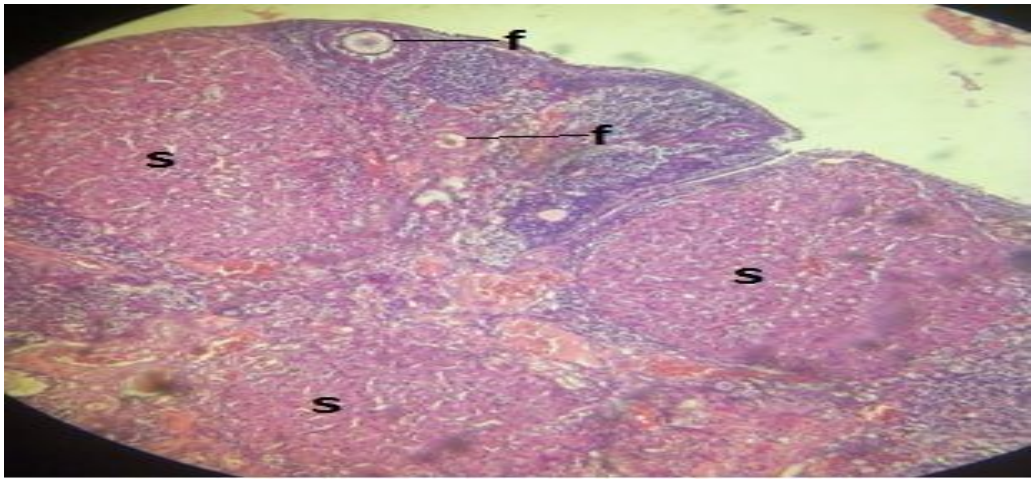

Figure 3: Group D Slide of *P.amarus*-treated ovary for 8 weeks, showing scanty ovarian follicles (f), with atretic changes and ovarian stroma (S) with degenerative changes and impaired folliculogenesis (H&E) x40.

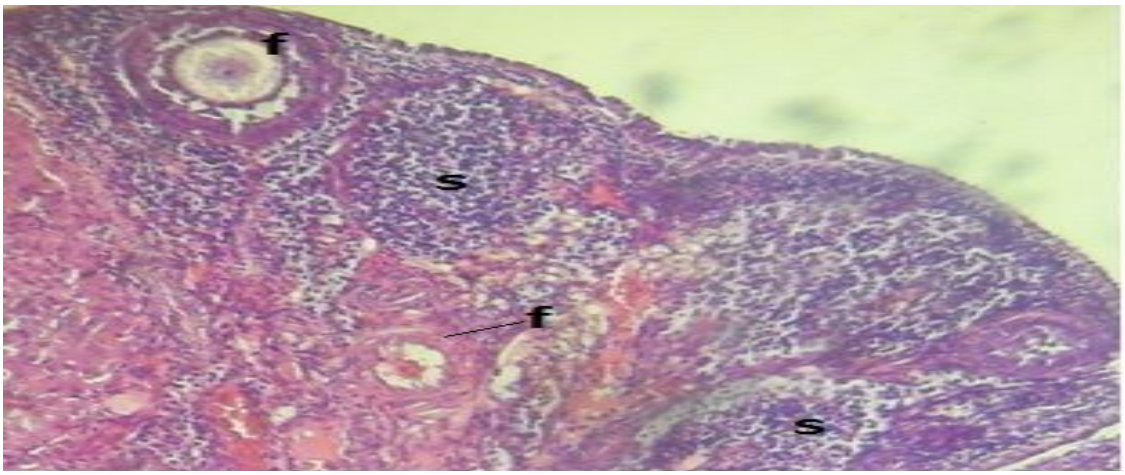

Figure 4: Group D Slide of *P.amarus*-treated ovary for 8 weeks showing scanty ovarian follicles (f) with atretic changes and ovarian stroma (S) with degenerative changes and impaired folliculogenesis (H&E) x100.

Discussion

The study showed that *Phyllanthus amarus* treatment in the rats caused no significant change in body and organ weight ($p<0.05$) compared to control. This finding correlates with the report of (Rao and Alice, 2001). This implies that *P.amarus* does not significantly affect the nutritional status or metabolic activities within the animals as to have been related to the findings.

Also, the study revealed that the mated animals were unable to get pregnant with *P. amarus* treatment; hence implantation rate and effect of extract on resorption and litters could not be ascertained. These observations also support earlier findings (Rao and Alice, 2001; Iranloye *et al*., 2010).

The number of ovarian follicles in the ovary at oestrus is a reflection of the degree of ovarian activity and by implication, the levels of balanced hormonal influence modulating ovarian activity. Hence, Plowchalk *et al.* (1993) reported that the quantitative assessment of follicle number is an indicator of the normal function as well as toxic responses in the ovary. This observation is further supported by Islam *et al.* (2008), who remarked that follicles are the principal functional units of the mammalian ovary with FSH and LH as the controllers of its development. The observation of degenerative changes in the ovarian stroma with reduced folliculogenesis and follicular atresia with *P. amarus* on the ovary in this study may be attributed to several factors, but supported by previous studies. Rao and Alice, 2001 reported that female animals treated with plant extract of *P. amarus* had their cyclicity affected and were unable to become pregnant. Pregnancy involves implantation of the blastocyst in the uterus after successful ovulation and fertilization of the ovum by matured normal spermatozoon (Timarche *et al.,* 2016).

Chandrasekhar *et al.* (1990), emphasized that blastocyst implantation normally takes place in small rodents like rats on day 5 or 6 and involves a basic hormonal sequence composed of 48 hour period of progesterone preparation and presence of oestrogen along with progesterone at the end of this period. This brings the role of hormonal inter-play in moderating the process of reproduction to mind (Raju *et al.,* 2013). In this study, the values of FSH and LH which moderates folliculogenesis and ovulation respectively were found to be significantly reduced compared to control. A depressed ovarian stroma with degenerative changes and non proliferative germinal epithelium would be counter-productive in eliciting appropriate feed-back control communication with the hypothalamus and pituitary gland which modulate these hormones secretion. The findings revealed that *P. amarus* caused almost 80 % degeneration of the ovarian cortex. Hence follicular maturation and release for ovulation becomes impaired, consequently leading to zero percent pregnancy. Furthermore, the ovarian degeneration could result from low oestrogen and progesterone levels which does not favour ovulation, implantation and maintenance of pregnancy as progesterone is known to play key role in regulating granulosa cell functions and follicular rupture during ovulation. Its low levels might then cause follicular atresia and possible corpus luteum regression (Kaipa and Hsueh, 1997; Rolaki *et al.,* 2005). *Phyllanthus amarus* extracts is reported to contain alkaloids, lignins, flavonoids and polyphenol compounds (Kumaran and Karunakaran, 2007). Also, it has been described as a phytooestrogen, i.e. non steroidal plant molecule with oestrogen-like activity capable of interacting with oestrogen receptors showing both agonist and antagonist mode of action (Islam *et al.,* 2008). The two lignan components of the plant, phyllanthin and

hypophyllanthin which systematically become enterolignans have been considered to be responsible for augmenting oestrous cycle in rats (Islam *et al.,* 2008). It therefore implies that these phytosteroid components of phyllanthus can competitively inhibit the effects of negative feedback stimulation of the ovary to the higher centres via their action on the oestrogen receptors. The granulosa cells of the developing follicles and the gonadotrophin releasing hormone produces oestrogen and progesterone (Iwai *et al.,* 1991). Most of the follicles require steroids and granulose cells for nutritive purpose and its proliferation (Hartshorne, 1997; Drummond, 2006). The suppressed follicular development and maturation therefore, further accounts for these low hormonal levels. Thus *P. amarus* exhibit anti-contraceptive and anti-implantation effects and where pregnant rats were used in study, its anti-deciduous effect resulted in abortions (Iranloye *et al.,* 2010). The finding of significant difference in the levels of prolactin in the extract-treated animals compared to control is partly supported by related study (Obianime and Uche, 2009). The observed scanty ovarian follicles in the cortex with atretic changes and impaired folliculogenesis demonstrated the deleterious effect of *P.amarus* on the ovary. This might also be attributable to its growth inhibition, cell cycle modulation effects and ability to cause apoptotic cell death (Abhyankar and Reddy, 2010; Tang *et al.,* 2010). These possible attributes of *P. amarus* give credence to the pathological observations in the ovary. It is likewise possible that the herb's modulatory role on the cell cycle may have interfered with the paracrine and autocrine factors too, normally associated with the development and function of the gland (Cummings and Kavlock, 2004) apart from the regulatory role of FSH and LH from the anterior pituitary.

Folliculogenesis is the maturation of the ovarian follicle, a densely packed shell of somatic cells that contains an immature oocyte (Molyneaux and Wylie, 2004). It is the progression of a number of small primordial follicles into large pre-ovulatory follicles that enter the menstrual cycle (Komatsu and Masubuchi, 2016). Naturally, folliculogenesis ends when the remaining follicles in the ovaries are incapable of responding to the hormonal cues that previously recruited some follicles to mature. This depletion in follicle supply, signals the beginning of menopause. As women (and mice) age, double-strand breaks accumulate in their primordial follicle reserve. These follicles contain primary oocytes that are arrested in prophase of the first meiotic division. Meiosis, in eukaryotic organisms, is the general process underlying germ cell formation, and it appears to be an adaptation for repairing DNA damages, particularly double-strand breaks in germ line DNA (Grey *et al.,* 2009). Double-strand breaks are accurately repaired during meiosis by the particular process termed "homologous recombinational repair. As humans (and mice) age therefore, expression of four key DNA repair genes necessary for homologous recombinational repair are reported to decline in oocytes (Cohen *et al.,* 2006; Guigon and Magre, 2006).They hypothesized that DNA double-strand break repair is vital for the maintenance of oocyte reserve, and that a decline in efficiency of repair with age plays a key role in the depletion of the ovarian reserve (ovarian aging).

However, some agents are capable of inducing this gradual depletion of follicular reserve apart from the natural apoptotic process (Hartshorne, 1997). The consequence is gradual fertility decline in the female due to the inability to produce eggs that can go through the entire process of follicular maturation from primary oocytes to matured graafian follicle for ovulation. This may occur either by the gonadal suppression to produce oogonia or the repression of their life cycle through atretic degeneration. These factors might have come to play in this study on the observed role of *P. amarus* on the ovary, which can be considered for contraceptive purpose if the observed actions are not permanently deleterious to the organ, but reversible; an indication for its further evaluation in fertility management.

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