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Synergistic Amelioration of Endocrine Dysregulation by *Vernonia amygdalina* and *Tamarindus indica* in Letrozole and Diet-induced Polyendocrine Metabolic Ovarian Syndrome in Rats

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Abstract

Background: Polyendocrine metabolic ovarian syndrome (PMOS) is a prevalent endocrine disorder characterised by hyperandrogenism, anovulation, and hypothalamic-pituitary-ovarian (HPO) axis dysregulation. Current pharmacological treatments remain limited by incomplete efficacy and accessibility constraints, necessitating the investigation of multi-target botanical alternatives. This study evaluated the synergistic effects of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on HPO axis hormonal parameters in a letrozole and high-fat high-fructose diet-induced PMOS rat model.

Methods: Forty-eight (48) female Wistar rats were randomly assigned to eight groups (n=6): normal control, PMOS untreated, positive control (metformin 10 mg/kg + clomiphene citrate 1 mg/kg), VAWE monotherapy (200 mg/kg), TIME monotherapy (400 mg/kg), low-dose combination (VAWE 100 mg/kg + TIME 200 mg/kg), high-dose combination (VAWE 200 mg/kg + TIME 400 mg/kg), and mid-dose combination (VAWE 200 mg/kg + TIME 200 mg/kg). PMOS was induced via letrozole (1 mg/kg) and HF-HFr diet for 21 days. All treatments were administered orally for 21 days. Serum hormones were quantified by ELISA. Estrous cyclicity was monitored by daily vaginal smear cytology.

Results: PMOS induction produced persistent diestrus by Day 12, elevated LH, increased LH/FSH ratio, hyperandrogenism, and significantly reduced progesterone. The mid-dose combination demonstrated the most comprehensive HPO axis restoration, reducing testosterone below control levels, restoring SHBG, normalising the LH/FSH ratio, and demonstrating the most organised estrous cycle progression among all treatment groups.

Conclusions: These findings provide pharmacological justification for the combined ethnomedicinal use of *Vernonia amygdalina* and *Tamarindus indica* in reproductive health and warrant further mechanistic and clinical investigation.

Keywords: Polyendocrine metabolic ovarian syndrome, Hypothalamic-pituitary-ovarian axis, Letrozole-induced PMOS, *Vernonia amygdalina*, *Tamarindus indica*

Introduction

Polyendocrine metabolic ovarian syndrome (PMOS) is one of the most prevalent endocrine disorders affecting women of reproductive age, with an estimated global prevalence of 6–21% depending on diagnostic criteria applied [1]. Characterised by hyperandrogenism, chronic anovulation, and polycystic ovarian morphology, PMOS is associated with significant reproductive, metabolic, and psychological morbidity [2,3]. Diagnosis of PMOS is established using the Rotterdam criteria, which require the presence of at least two of three features: oligo-ovulation or anovulation, clinical or biochemical hyperandrogenism, and polycystic ovarian morphology on ultrasound [4]. The burden of PMOS is particularly significant in sub-Saharan Africa, where limited access to specialist reproductive healthcare and pharmacological treatments amplifies its impact on affected women [5].

The pathophysiology of PMOS is characterised by increased gonadotropin releasing hormone (GnRH) pulse frequency, which selectively drives luteinizing hormone (LH) hypersecretion while suppressing follicle stimulating hormone (FSH), impairing follicular recruitment and perpetuating androgen excess [2,6,7]. Elevated testosterone and reduced sex hormone binding globulin (SHBG) further amplify androgenic burden, contributing to anovulation and infertility [8,9]. This hormonal cascade collectively disrupts folliculogenesis, impairs corpus luteum function, and perpetuates the chronic anovulatory state that underlies PMOS-associated infertility [10].

Current pharmacological management including metformin and clomiphene citrate targets individual aspects of this dysregulation but is associated with significant side effects, limited efficacy in resistant cases, and restricted accessibility in resource-limited settings [11]. These limitations underscore the need for multi-target therapeutic alternatives that address the complexity of HPO axis dysregulation in PMOS.

Medicinal plants represent a rich and largely underexplored reservoir of multi-target bioactive compounds with potential for addressing the complex hormonal dysregulation of PMOS [12,13]. *Vernonia amygdalina*, commonly known as bitter leaf, is widely distributed across sub-Saharan Africa and has been used in traditional medicine for the management of metabolic and reproductive disorders [14,15]. Its bioactive constituents including sesquiterpene lactones, flavonoids, and saponins have been reported to exert antiandrogenic, anti-inflammatory, and insulin-sensitising effects in experimental models [16,17,18]. Similarly, *Tamarindus indica*, widely cultivated across Africa and Asia, has an established ethnomedicinal history in reproductive and gynaecological health [19,20]. Phytochemical investigations have identified tartaric acid, phenolic acids, flavonoids, and procyanidins as its principal bioactive constituents, with reported activity on hormonal and metabolic parameters [21,22]. Despite the individual pharmacological potential of both plants, their combined effects on HPO axis dysregulation in PMOS have not been previously investigated. Given the complementary phytochemical profiles of both plants and their individual activity on overlapping hormonal pathways, their combination may produce synergistic multi-target modulation of HPO axis dysregulation that neither plant achieves independently.

The present study therefore investigated the synergistic effects of combined *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on HPO axis hormonal parameters in a letrozole and high-fat high-fructose (HF-HFr) diet-induced PMOS rat model. The dual induction protocol was employed to simultaneously recapitulate the hormonal and metabolic dimensions of PMOS, providing a more clinically representative experimental phenotype than single-induction approaches. The specific aim of this study was to evaluate the effects of individual and combined administration of VAWE and TIME on serum gonadotropin, sex hormone, SHBG concentrations, and estrous cyclicity in PMOS rats, with particular focus on identifying the optimal combination dose for HPO axis restoration.

2. Methodology

2.1 Plant Material and Extract Preparation

The fruit of *Tamarindus indica* was collected from the Adeoyo area in Saki, Oyo State, Nigeria, while fresh leaves of *Vernonia amygdalina* were obtained from the Gwojo area, also in Saki, Oyo State. Both plant materials were authenticated at the Forest Herbarium Unit of the Forestry Research Institute of Nigeria (FRIN), Ibadan, and voucher specimens were deposited. The assigned voucher numbers were FIH/1142128 for *Tamarindus indica* and FIH/1142121 for *Vernonia amygdalina*.

The *Vernonia amygdalina* leaves were washed to remove dirt, shade-dried for two weeks, and ground into fine powder using a mechanical grinder. The powdered leaves were stored in airtight containers until extraction. 150g of the powdered leaves was soaked in 1000ml of distilled water. The mixture was left to stand for 72 hours with intermittent stirring to enhance solute-solvent interaction. Thereafter the mixture was sieved using muslin cloth followed by filtration with Whatman No.1 filter paper. The resulting filtrate was concentrated using a rotary evaporator at 40–45°C under reduced pressure and final solvent elimination was done using a regulated water bath. The concentrated extract was stored in airtight containers at 4°C until use.

The *Tamarindus indica* fruit pulp was manually separated from the seeds and fibrous strands, thoroughly rinsed with clean water, and air-dried at room temperature for one week. The dried pulp was then pulverized using a blender and stored in an airtight container until extraction. The extraction procedure for *Tamarindus indica* fruit pulp followed the same protocol as described for *Vernonia amygdalina*, with the exception that methanol was used as the solvent in place of distilled water.

2.2 Experimental Animals

Healthy female Wistar rats, aged 8–10 weeks and weighing between 120–150 g, were procured from a reliable animal breeder in Ogbomosho, Oyo State, Nigeria. Animals were housed in groups of six per cage in standard plastic cages with wood shaving bedding. The animals were acclimatized for 14 days under standard laboratory conditions: a 12-hour light/dark cycle, room temperature of 22–25°C, and *ad libitum* access to water and standard pellet diet.

2.3 PMOS Induction

Letrozole was administered orally at a dose of 1 mg/kg body weight once daily for 21 consecutive days[23]. The drug was suspended in 0.5% carboxymethyl cellulose (CMC) to ensure uniform dispersion and ease of administration via oral gavage. The control group received 0.5% CMC orally without letrozole or dietary modification, to account for any effect of the vehicle.

The HF-HFr diet was formulated by the authors based on established macronutrient ratios known to induce metabolic dysfunction in rodents. The diet consisted of 50% standard rat pellets, 30% lard as the fat source, and 20% fructose powder as the simple sugar source. This composition was designed to simultaneously induce insulin resistance and hyperandrogenaemia consistent with the metabolic phenotype of PMOS. The HF-HFr was administered *ad libitum* for the duration of the induction phase, typically lasting 21 days.

Letrozole administration and HF-HFr diet were initiated concurrently on Day 1 of the induction phase and continued simultaneously for 21 days. The induction period lasted for three weeks, during which vaginal smear monitoring was conducted daily to track disruptions in the estrous cycle. Successful PMOS induction was confirmed by the presence of persistent diestrus on vaginal smear examination by Day 21 of induction. Only animals confirmed to be in persistent diestrus were included in the treatment phase.

2.4 Experimental Design and Treatment

A total of Forty-eight Wistar rats were included in this study and were randomly assigned to eight groups, each consisting of six animals. Following the 21-day induction period, treatment was administered orally via gavage twice daily for 21 days as described below.

Group 1 served as the normal control and received 0.5% carboxymethylcellulose (CMC) vehicle only, without induction or treatment, throughout the experimental period. Group 2 served as the PMOS untreated group and received letrozole (1 mg/kg) combined with the HF-HFr diet for 21 days but received no therapeutic intervention thereafter. Group 3 served as the positive control and received letrozole and HF-HFr diet for 21 days, followed by concurrent administration of metformin (10 mg/kg) and clomiphene citrate (1 mg/kg) for 21 days. Group 4 received letrozole and HF-HFr diet for 21 days, followed by *Vernonia amygdalina* water extract (VAWE) at 200 mg/kg for 21 days. Group 5 received letrozole and HF-HFr diet for 21 days, followed by *Tamarindus indica* methanol extract (TIME) at 400 mg/kg for 21 days. Group 6 received letrozole and HF-HFr diet for 21 days, followed by a low-dose combination of VAWE (100 mg/kg) and TIME (200 mg/kg) for 21 days. Group 7 received letrozole and HF-HFr diet for 21 days, followed by a high-dose combination of VAWE (200 mg/kg) and TIME (400 mg/kg) for 21 days. Group 8 received letrozole and HF-HFr diet for 21 days, followed by a mid-dose combination of VAWE (200 mg/kg) and TIME (200 mg/kg) for 21 days.

2.5. Sample Collection

At the end of the treatment period, animals were anesthetized, and blood samples were collected via cardiac puncture into plain (non-heparinized) tubes. The blood was allowed to clot at room temperature for about 30 minutes and then centrifuged at 3,000 rpm for 10 minutes at 4°C. The clear serum obtained was carefully transferred into clean Eppendorf tubes and stored at -20°C until biochemical analysis.

2.6. Hormonal Assays

Estrogen, Progesterone, Prolactin, Luteinizing Hormone(LH), follicle stimulating hormone(FSH), Testosterone and sex hormone binding globulin was quantified using enzyme-linked immunosorbent assay (ELISA). Serum hormone concentrations were quantified using commercially available kits from Calbiotech (Calbiotech, Spring Valley, CA, USA). All assays were performed according to the manufacturer's instructions. The LH/FSH ratio was calculated by dividing mean serum LH concentration by mean serum FSH concentration for each group.

2.7 Vaginal Smear Cytology

Daily vaginal smears were taken during the induction and treatment phases using standard procedures. Using a sterile pipette, a small volume of normal saline (0.9% NaCl) was gently flushed into the vaginal canal of each rat and then aspirated. The fluid was transferred onto a clean microscope slide and stained with Giemsa to improve cell visibility. The slides were examined under a light microscope at X10 magnification. The stages of the estrous cycle (Proestrus, Estrous, Metestrus and diestrus) were identified based on the predominant cell types observed (Figure 1). To determine these stages of the estrous cycle, the method by Long and Evans (1922) was adopted [24]. The proestrus stage consists of nucleated epithelial cells predominated with a small number of leukocytes. The estrus stage of the estrous cycle comprises cornified epithelial cells. Metestrus stages are embraced with leukocytes, cornified epithelial cells, and nucleated cornified cells while the diestrus stage consists of mostly leukocytes with more mucus.

Estrous cycle stage data were recorded daily for each animal and compiled into a colour-coded chart in which each phase was represented by a distinct colour, providing a visual map of estrous cycle progression across the treatment period.

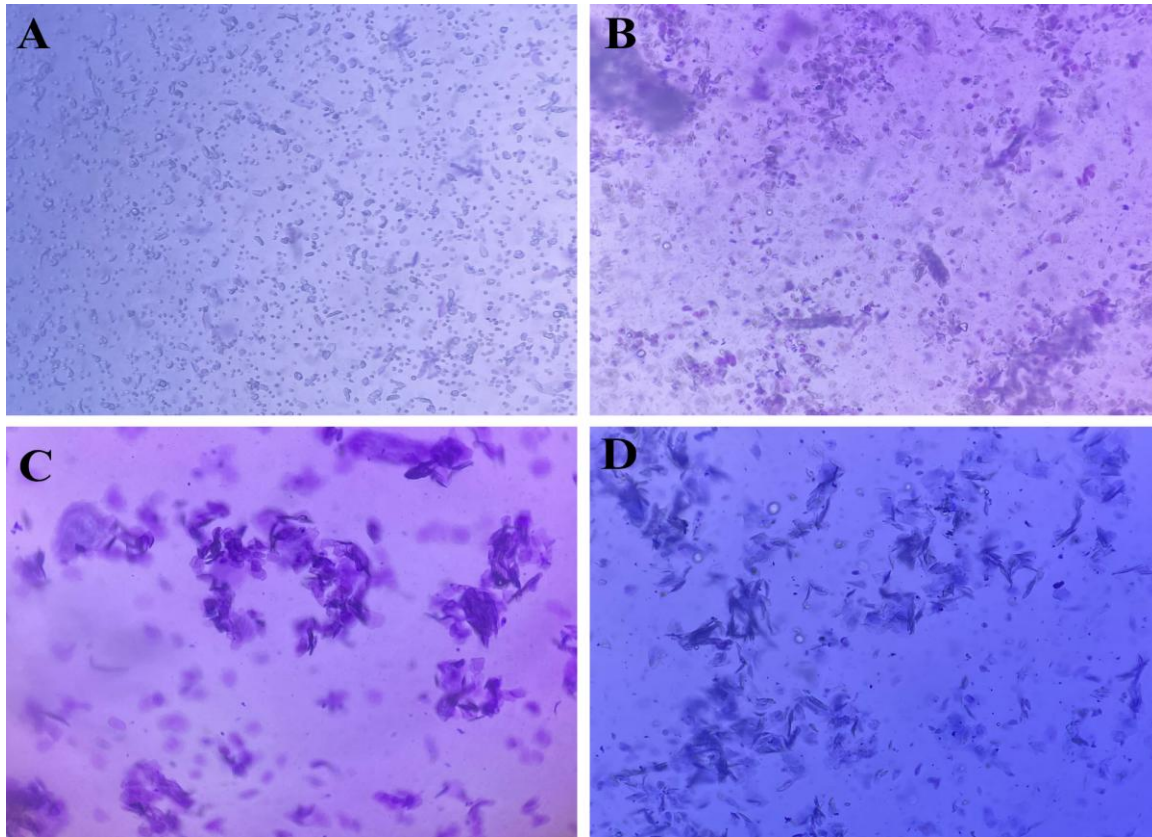


Figure 1: Photomicrographs of estrous cycle phases in normal control Wistar rats.

Representative vagina smear cytology images showing the four phases of the estrous cycle. (A) Diestrus predominantly leucocytes with increased mucus (B) proestrus predominantly nucleated epithelial cells with few leukocytes. (C) Estrus predominantly cornified epithelial cells. (D) Metestrus Mixture of cornified epithelial cells, nucleated epithelial cells, and leukocytes. Slides were stained with GIEMSA and examined at x10 magnification.

2.8. Statistical Analysis

The data obtained were analyzed using the Graphpad prism software version 10.5.2. The statistical significance of difference between groups was performed using the one-way analysis of variance (ANOVA) followed by Tukey's multiple test at different confidence levels ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$). The results obtained from this study were expressed as mean $\pm$ standard error of mean of four replicate determinations.

3. Results

3.1. Vaginal Smear Cytology

Vaginal smear cytology was monitored daily throughout the induction and treatment phases to assess estrous cycle integrity across all experimental groups. During the induction phase, the normal control group maintained regular estrous cyclicity, progressing through all four phases proestrus, estrus, metestrus, and diestrus in an organised and sequential manner throughout the 21-day period. In contrast, all PMOS-induced groups exhibited progressive disruption of estrous cyclicity, culminating in persistent diestrus by Day 12 of induction. This was characterised cytologically by a predominance of leukocytes with increased mucus, consistent with the anovulatory state associated with PMOS. Persistent diestrus on Day 12 across all induced groups confirmed successful PMOS induction prior to the commencement of treatment (Figure 2).

Following the commencement of treatment, the normal control group continued to cycle regularly throughout the 21-day treatment period. The PMOS untreated group remained predominantly in diestrus, with at least one animal showing a first non-diestrus phase transition on Day 7, though no organised or sequential progression through the estrous cycle phases was observed. The positive control group, which received metformin and clomiphene citrate, showed the earliest resumption of non-diestrus phase activity, with at least one animal transitioning out of diestrus by Day 3 of treatment; however cycling remained irregular throughout the treatment period without consistent phase progression. Among the plant extract treatment groups, all five groups VAWE monotherapy, TIME monotherapy, and the low-dose, mid-dose, and high-dose combinations showed first non-diestrus phase activity by Day 6 of treatment. Of these, the mid-dose combination group receiving VAWE (200 mg/kg) and TIME (200 mg/kg) demonstrated the most organised estrous cycle progression, showing greater regularity in phase transitions compared to all other treatment groups, though complete normalisation of cyclicity was not achieved in any treatment group within the 21-day observation period (Figure 3).

3.2. Luteinizing Hormone (LH)

Serum LH concentration was significantly elevated in the PMOS untreated group compared to the normal control, confirming successful hormonal induction of the PMOS phenotype. Among treatment groups, only the positive control group receiving metformin and clomiphene citrate showed a statistically significant reduction in LH compared to the PMOS untreated group. The VAWE monotherapy, TIME monotherapy, low-dose, mid-dose, and high-dose combination groups all showed a downward trend in LH concentration compared to the PMOS untreated group; however these reductions did not reach statistical significance. The high-dose combination group (200VAWE+400TIME) showed the least reduction among treatment groups, though this difference was not statistically significant from the PMOS untreated group (Figure 4).

3.3 Follicle stimulating Hormone (FSH)

The PMOS untreated group showed a marked decrease when compared to control although not significant. Among treatment groups, the TIME monotherapy showed the most pronounced increase even surpassing control. The positive control group, the low dose group and the medium dose group showed slight increase when compared to the PMOS untreated. The VAW monotherapy, and high-dose combination showed marginal changes. Although, there were no statistical differences among groups. (Figure 5).

3.4. LH/FSH Ratio

The LH/FSH ratio was significantly elevated in the PMOS untreated group compared to the normal control, reflecting the characteristic gonadotropin imbalance associated with PMOS. Among treatment groups, all groups demonstrated a reduction in LH/FSH ratio compared to the PMOS untreated group, with most groups showing levels comparable to the normal control. The exception was the high-dose combination group (200VAWE+400TIME), which showed a significantly elevated LH/FSH ratio compared to the normal control, indicating incomplete restoration of gonadotropin balance at this dose. No treatment group showed a statistically significant reduction when compared directly to the PMOS untreated group (Figure 6).

3.5 Testosterone

The PMOS untreated group was significantly higher than control. The positive control group and the high dose group (200VAWE+400TIME) were not statistically different from the PMOS untreated. The VAW monotherapy, TIME monotherapy, low dose group (100VAWE+200TIME) and mid dose group (200TIME+200VAWE) were significantly lower than the PMOS untreated group. The most pronounced reduction was observed in the mid dose group (200TIME+200VAWE) with levels even lower than the control group (Figure 7).

3.6 Sex Hormone Binding Globulin (SHBG)

The PMOS untreated group was significantly lower than the control group. Treatment groups showed significant increase in SHBG levels when compared to PMOS untreated except in the high dose group (200VAWE+400TIME) which showed no statistical difference when compared to PMOS untreated (Figure 8).

3.7 Estradiol

The PMOS untreated group showed no significant difference when compared to control. All treatment groups except the TIME monotherapy and the mid dose group showed similar levels to control and PMOS untreated. However, the most pronounced elevation was observed in the mid dose group (200VAWE+200TIME) and this was statistically different from all groups including control and PMOS untreated (Figure 9).

3.8 Progesterone

Serum progesterone concentration was significantly reduced in the PMOS untreated group compared to the normal control. The positive control group, TIME monotherapy, and high-dose combination (200VAWE+400TIME) similarly showed progesterone concentrations that were significantly lower than the normal control. The VAW monotherapy and low-dose combination (100VAWE+200TIME) showed significant progesterone recovery compared to the PMOS

untreated group, with levels that did not differ significantly from the normal control. The mid-dose combination (200VAWE+200TIME) showed concentrations that were not significantly different from either the normal control or the PMOS untreated group (Figure 10).

3.9 Prolactin

Prolactin levels did not differ significantly across all experimental groups (Figure 11).

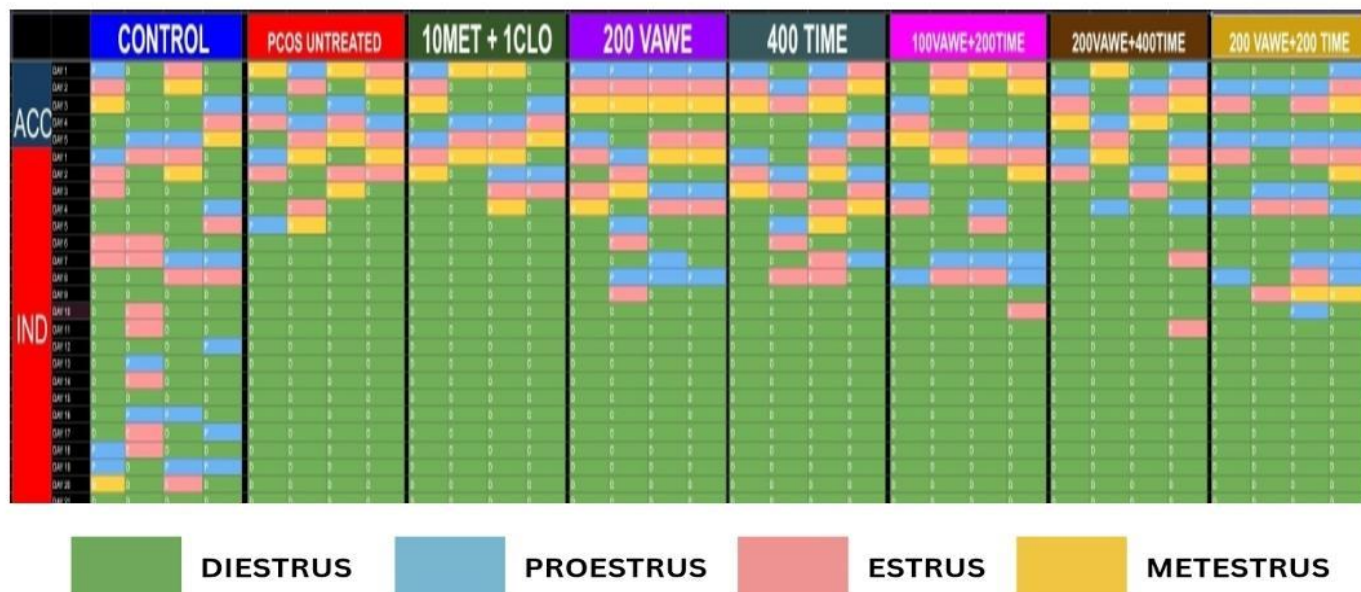


Figure 2: Colour map of estrous cycle phase progression during the induction phase across all experimental groups.

Daily vaginal smear cytology was recorded for all 48 animals over the 21-day induction period. Each cell represents the predominant estrous phase observed per animal per day. Colour coding: Green = Diestrus; Blue = Proestrus; Red = Estrus; Yellow = Metestrus. Key: ACC = Acclimatization period; IND = Induction phase; 10MET+1CLO= positive control group taking 10 mg/kg Metformin and 1 mg/kg Clomiphene citrate; VAWE: *Vernonia amygdalina* water extract; TIME: *Tamarindus indica* methanol extract.

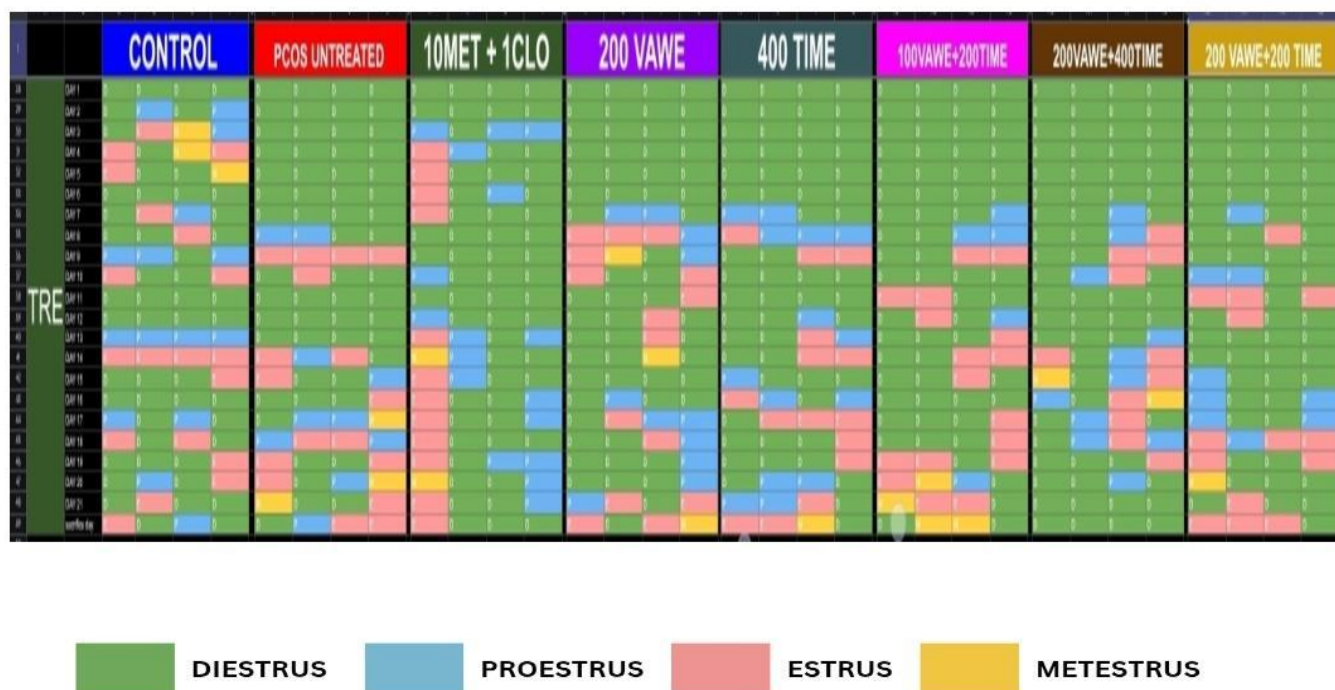


Figure 3: Colour map of estrous cycle phase progression during the treatment phase across all experimental groups.

Daily vaginal smear cytology was recorded for all 48 animals over the 21-day treatment period. Each cell represents the predominant estrous phase observed per animal per day. Colour coding: Green = Diestrus; Blue = Proestrus; Red = Estrus; Yellow = Metestrus. Key: TRE = Treatment phase; 10MET+1CLO= positive control group taking 10 mg/kg metformin and 1 mg/kg Clomiphene citrate; VAWE: *Vernonia amygdalina* water extract; TIME: *Tamarindus indica* methanol extract.

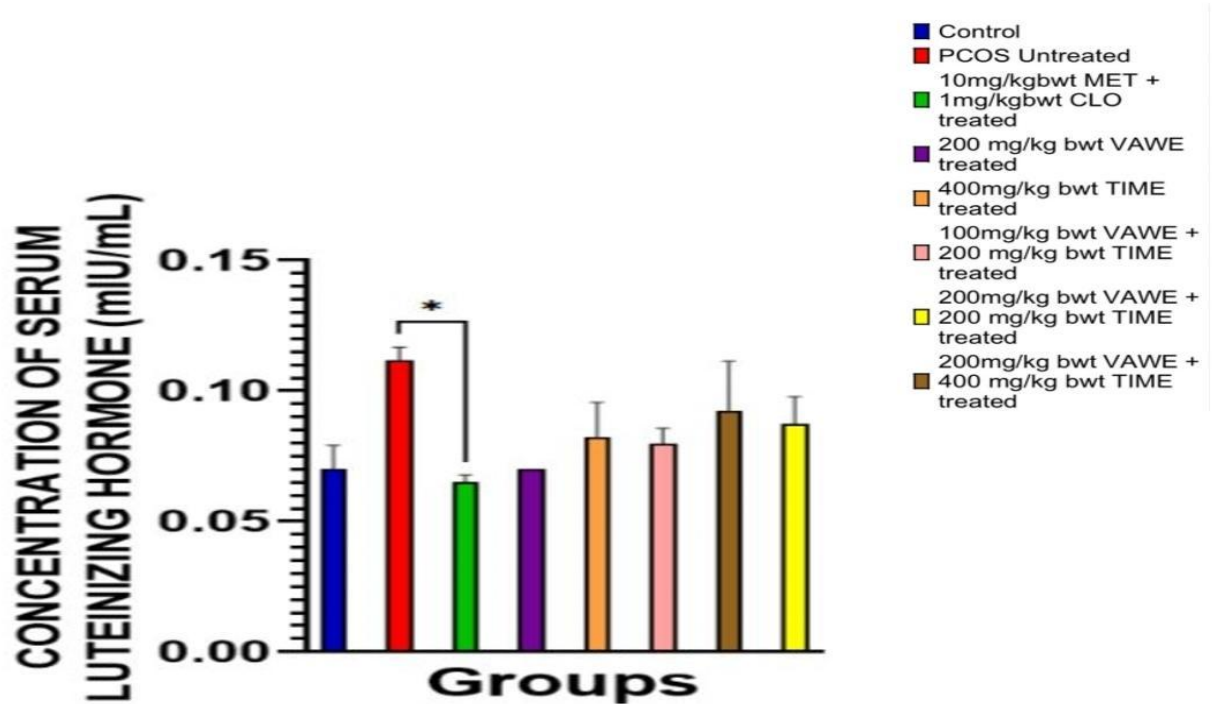


Figure 4: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum luteinizing hormone (LH) concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. * $P < 0.05$ indicates statistically significant differences between the connected groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWE = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

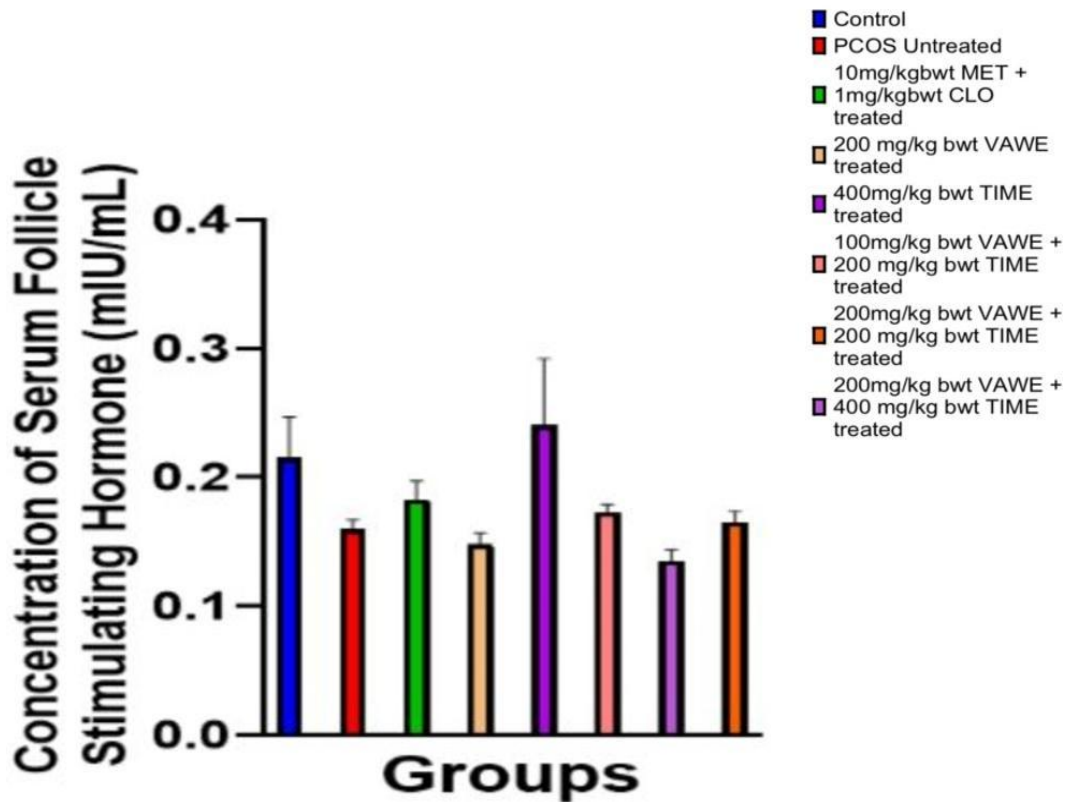


Figure 5: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum follicle stimulating hormone (FSH) concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. No statistically significant differences were observed among groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWE = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

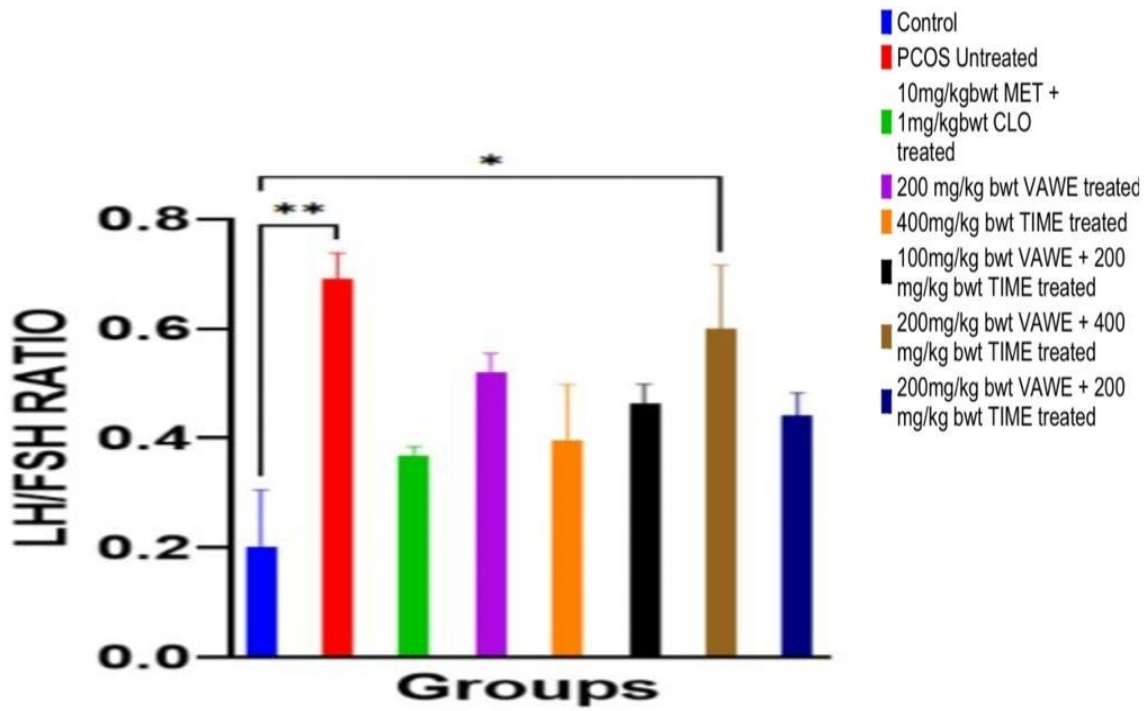


Figure 6: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on LH/FSH ratio in letrozole and HF-HFr diet-induced PMOS rats.

The LH/FSH ratio was calculated by dividing mean serum LH concentration by mean serum FSH concentration for each group. Values are expressed as mean of four replicates $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ indicates statistically significant differences between the connected groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWA = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

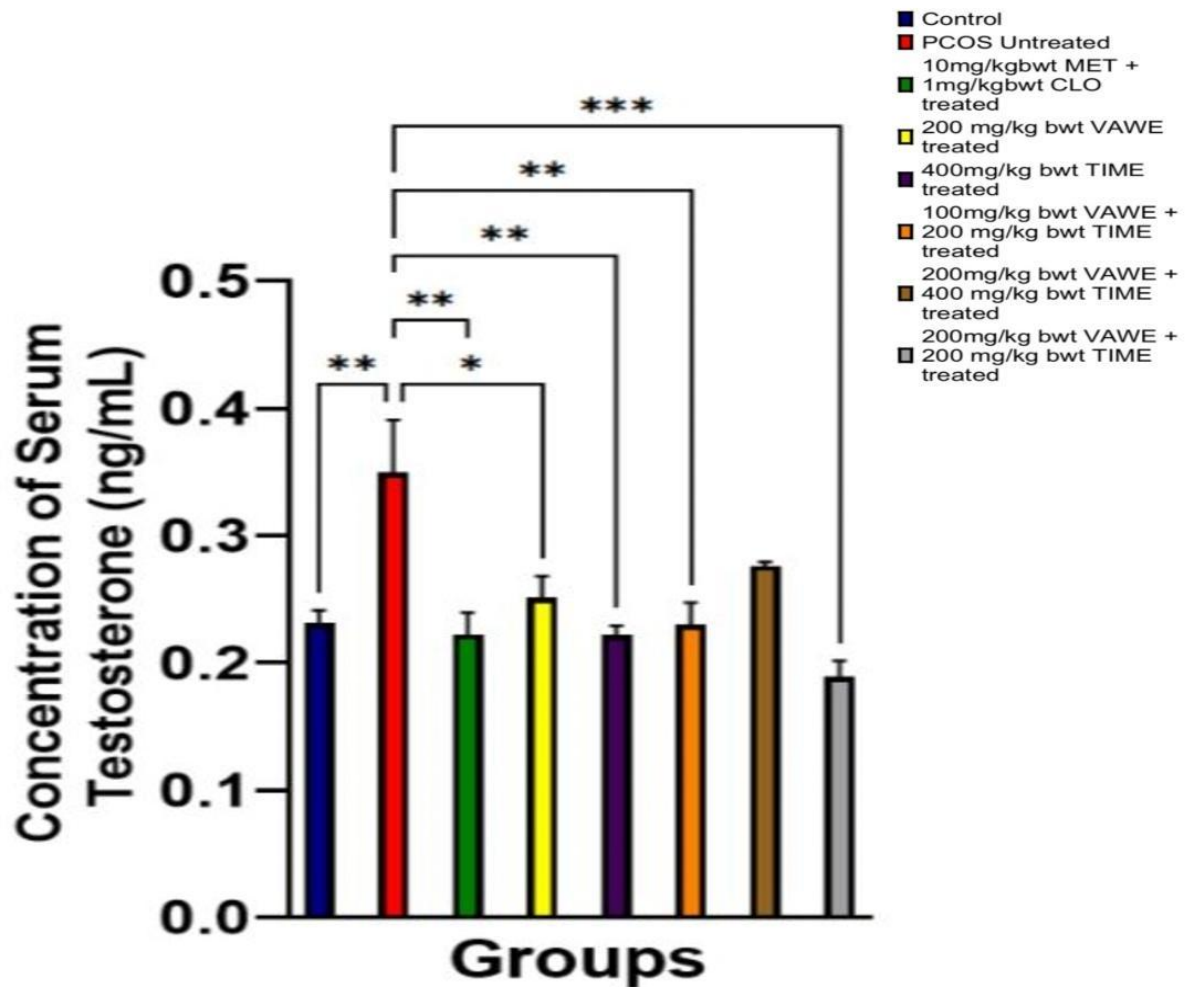


Figure 7: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum testosterone concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicates statistically significant differences between the connected groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWE = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

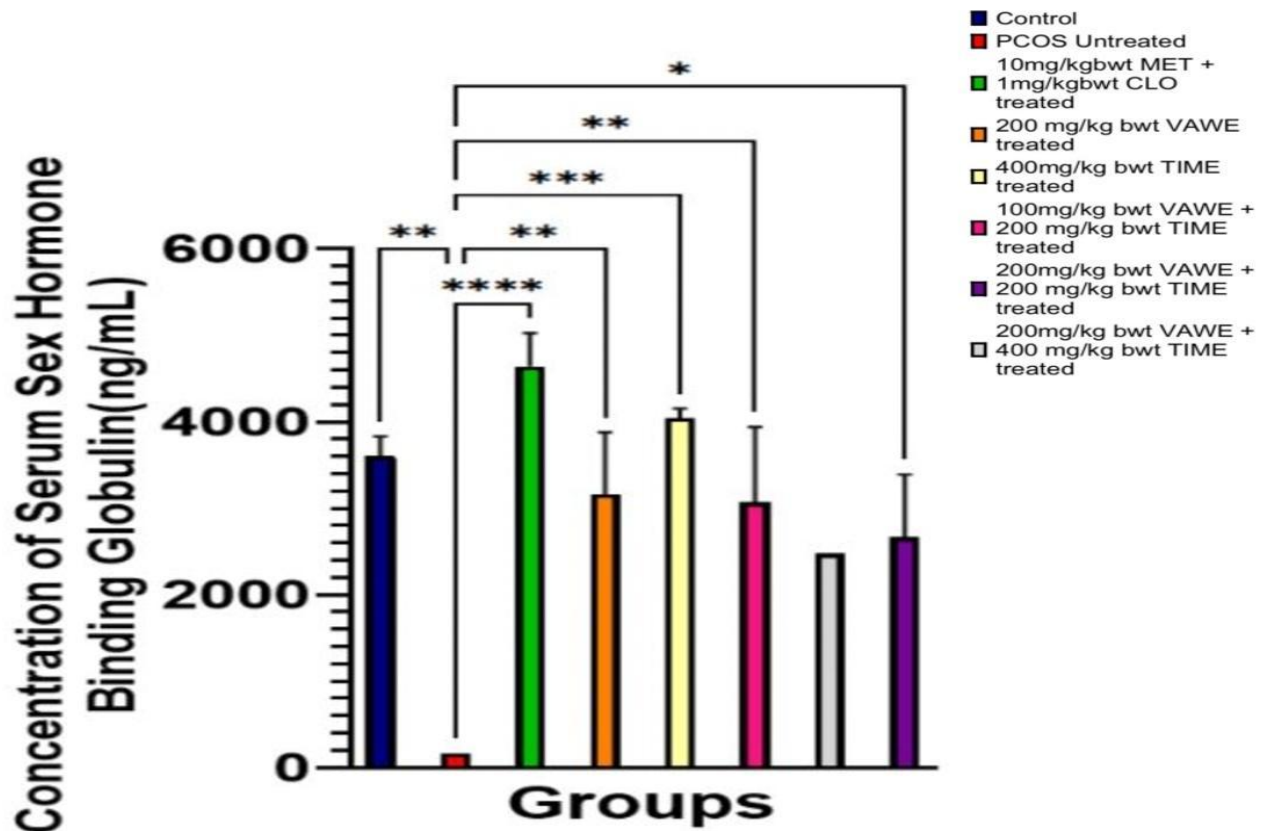


Figure 8: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum sex hormone binding globulin (SHBG) concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ indicates statistically significant differences between the connected groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWE = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

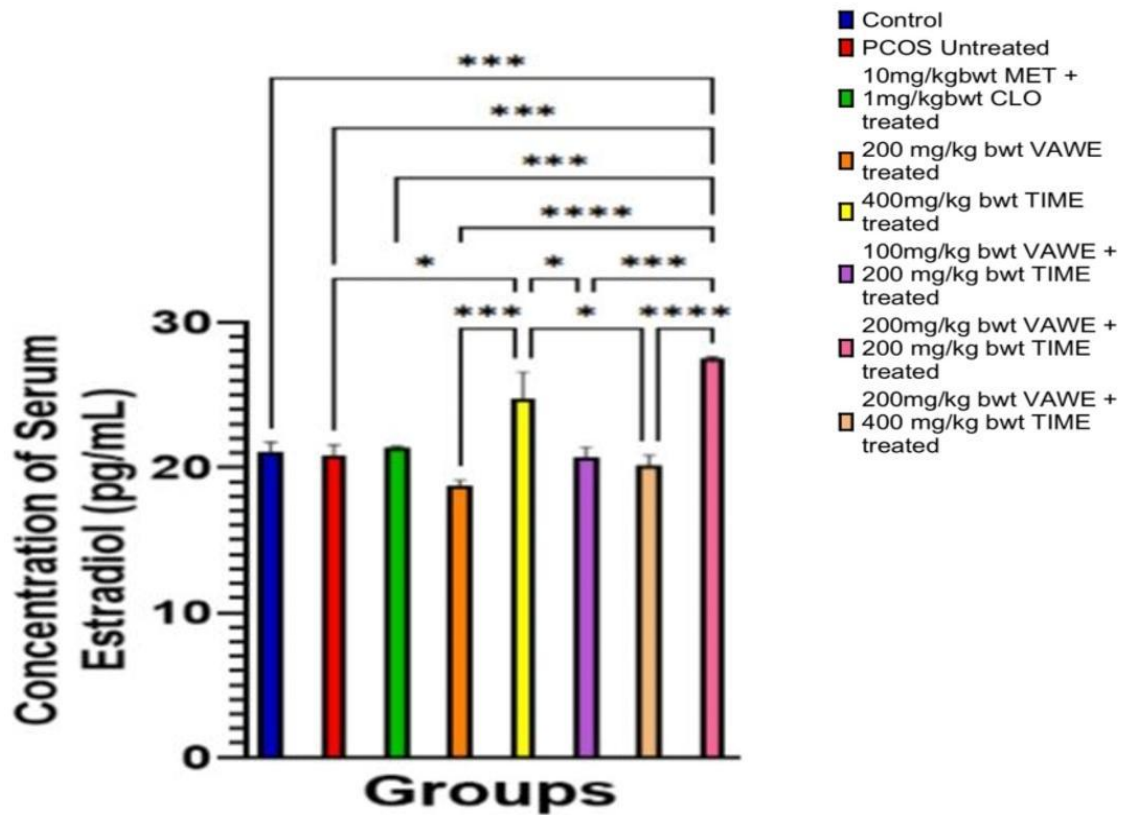


Figure 9: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum estradiol concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$ indicates statistically significant differences between the connected groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWE = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

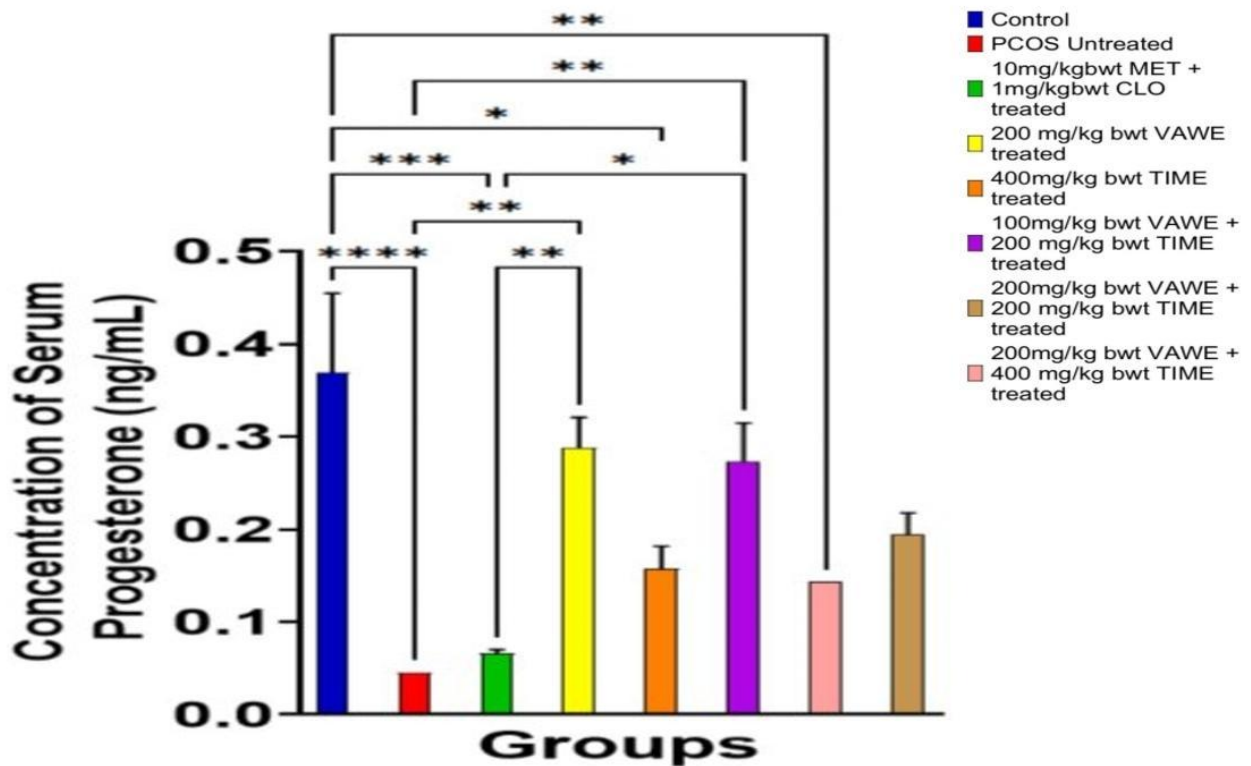


Figure 10: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum progesterone concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ indicates statistically significant differences between the connected groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWA = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

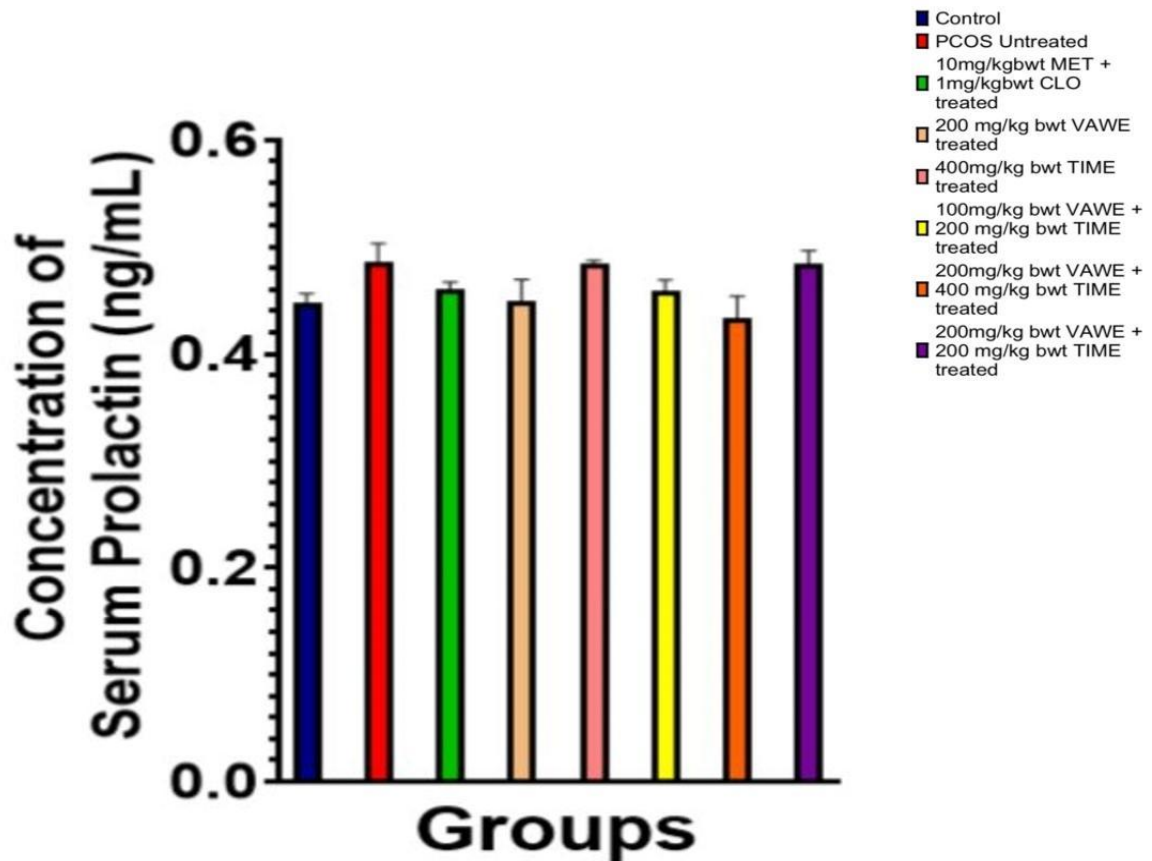


Figure 11: Effect of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) on serum prolactin concentration in letrozole and HF-HFr diet-induced PMOS rats.

Values are expressed as mean of four replicates $\pm$ SEM. No statistically significant differences were observed among groups. Key: MET+CLO = induced with PMOS, treated with 10 mg/kg bodyweight Metformin + 1 Mg/kg bodyweight Clomiphene Citrate.; VAWE = induced with PMOS, treated with water extract of *Vernonia amygdalina*; TIME = induced with PMOS, treated with methanol extract of *Tamarindus indica*; PCOS- polycystic ovarian syndrome; PMOS- polyendocrine metabolic ovarian syndrome; HF-HFr = high-fat high-fructose.

4. Discussion

Polyendocrine metabolic ovarian syndrome (PMOS) is a complex endocrine disorder characterized by hyperandrogenism, ovulatory dysfunction and metabolic disturbances [2]. Despite its high prevalence, effective multi-target therapies remain limited [1, 5,11]. *Vernonia amygdalina* and *Tamarindus indica* are widely used in African and Asian ethnomedicine for metabolic and reproductive health, with bioactive profiles rich in flavonoids, phenolic acids, alkaloids, and terpenoids [15,22] This study evaluated the combined effects of *Vernonia amygdalina* and *Tamarindus indica* in a letrozole and high fat, high-fructose (HF-HFr) Diet-induced PMOS rat model. The dual induction model was employed to simultaneously recapitulate the hormonal and metabolic dimensions of PMOS, providing a more comprehensive experimental phenotype than single-induction protocols.

Estrous cycle monitoring confirmed successful PMOS induction as all untreated PMOS-induced rats displayed persistent diestrus on day 12 of the induction phase. Persistent diestrus phase in letrozole-induced PMOS models reflects anovulation and gonadotropin imbalance, while a regular 4-5 day sequence of proestrus-estrus-metestrus-diestrus denotes normal reproductive function [25]. This finding is consistent with the report of Kurniawati *et al* (2024) who reported that thirty-seven studies demonstrated irregular cycles during the induction phase with Letrozole[26]. During treatment, spontaneous resumption of cycling occurred across all groups, including untreated animals but the pattern was irregular in the latter. The mid dose group (VAWE 200 mg/kg + TIME 200 mg/kg) demonstrated restoration of near-normal stage progression and fewer irregular transitions compared with untreated rats, positive control, monotherapies or other combination groups suggesting a faster resynchronization of the HPO axis. This finding aligns with earlier reports where letrozole induction produced persistent diestrus and estrous cycling resumed after pharmacological or herbal interventions [27,28]. Most reports on letrozole-induced PMOS models in rats either focus exclusively on the induction phase or omit detailed descriptions of estrous cycle progression in untreated animals beyond induction. The cessation of the HF-HFr diet following induction may have partially relieved the metabolic component of HPO axis suppression, contributing to the spontaneous cycling tendency observed in untreated animals. This highlights the reversible nature of diet-mediated reproductive dysfunction and underscores the importance of dietary modification as an adjunct to pharmacological or herbal intervention in PMOS management. Functional restoration of estrous cyclicity in the mid-dose group (VAWE 200 mg/kg + TIME 200 mg/kg) provides cytological evidence that the hormonal improvements observed biochemically translated into meaningful reproductive recovery.

PMOS induction produced the expected neuroendocrine alterations, with untreated rats showing markedly elevated LH, reduced FSH and significantly increased LH/FSH ratio [6]. High LH/FSH ratio has been considered a clinical biomarker of PMOS and is said to be consistent with HPO axis dysregulation [29]. In PMOS, rapid GnRH pulse frequency selectively increases LH release while suppressing FSH, impairing follicular recruitment, reducing aromatase activity and limiting estrogen production[6,30]. Treatment with both monotherapies and combinations generally shifted LH downwards and FSH upwards although non-significant reducing the LH/FSH ratio towards

normal values. Our findings agree with previous reports in letrozole-induced PMOS models showing exaggerated LH secretion, suppressed FSH and normal LH/FSH ratios [28,31,32]. While LH suppression did not reach statistical significance in plant-treated groups, the consistent downward trend across all treatment groups, particularly the mid-dose combination, suggests modulation of GnRH-LH signalling that may achieve significance with extended treatment duration. The relative underperformance of the high-dose combination is consistent with the dose-dependent pattern observed across multiple parameters. The absence of significant FSH dysregulation is consistent with the letrozole model literature and reflects the preferential disruption of LH pulsatility over FSH secretion in aromatase inhibitor-induced PMOS [28,32]. Restoration of LH/FSH ratio to control-comparable levels in the mid-dose combination but not the high-dose combination provides the first direct statistical evidence that dose selection critically determines whether synergistic HPO axis restoration or paradoxical gonadotropin imbalance occurs.

Hyperandrogenism is a central feature of PMOS, both in clinical presentation and experimental models, and is largely driven by LH-mediated stimulation of ovarian theca cells leading to elevated testosterone and low SHBG [33]. Similar patterns have been reported in letrozole-induced PMOS rats, where aromatase inhibition reduces conversion of testosterone to estrogens, resulting in androgen accumulation [32,34,35]. VAWE and TIME monotherapies each produced reductions in testosterone and moderate-to-large increases in SHBG compared with untreated rats. Combination regimens except the high dose group generally achieved similar improvements, with the mid dose group (VAWE 200 mg/kg + TIME 200 mg/kg) showing concurrent lowering of testosterone and robust elevation of SHBG, suggesting complementary modulation of androgen production and hepatic SHBG synthesis. The mid-dose combination achieved the most comprehensive antiandrogenic effect, simultaneously reducing total testosterone below control levels and restoring SHBG, collectively minimising bioavailable androgen. The failure of the high-dose combination to replicate this effect on either parameter despite containing higher concentrations of both extracts is compelling evidence that synergy in this combination is dose-dependent rather than concentration-dependent.

Estradiol levels remained comparable to controls in this study possibly due to the compensatory mechanisms of aromatase activity in letrozole-induced PMOS. Letrozole blocks aromatase activity, however, peripheral aromatisation in adipose tissue and adrenal glands can maintain estradiol [36]. The elevation of estradiol in the mid-dose combination group may reflect restored aromatase activity consequent to the antiandrogenic effects observed in the testosterone analysis, suggesting that testosterone reduction by the mid-dose combination created substrate conditions favourable to estradiol synthesis; a mechanistic consequence of HPO axis restoration rather than a direct estrogenic effect of the extracts though direct measurement of aromatase activity was beyond the scope of this study and warrants investigation in future work.

Progesterone is a marker of luteal function as it is only produced after ovulation by the corpus luteum [37]. Restored progesterone therefore means ovulation occurred, which is the ultimate functional goal of HPO axis restoration. The failure of the positive control to restore progesterone

despite pharmacological intervention, contrasted with the significant progesterone recovery in VAWE monotherapy and low-dose combination, suggests that the plant extracts engage steroidogenic pathways beyond the gonadotropin axis; an effect that may complement rather than simply replicate conventional pharmacological mechanisms. Metformin primarily targets insulin resistance and hepatic glucose production, while clomiphene citrate acts as an estrogen receptor antagonist to stimulate gonadotropin release [38,39,40]. Neither mechanism directly supports corpus luteum function or progesterone biosynthesis, which may explain the failure of the positive control to restore luteal progesterone despite partial gonadotropin normalisation. The progesterone-restorative effect of VAWE monotherapy and the low-dose combination may reflect direct steroidogenic stimulation, as *Vernonia amygdalina* have been reported to protect against steroidogenic pathway alteration [41]. The restoration of progesterone in these groups is further corroborated by the cytological evidence of estrous cycle resumption observed during treatments, collectively suggesting that ovulatory function was partially reinstated.

Prolactin supports luteal function in normal reproductive physiology, but sustained hyperprolactinaemia can inhibit GnRH release, reduce gonadotropin secretion, and impair ovulation [42]. In PMOS, levels are often within the normal range, though mild elevations may occur, typically linked to stress, age, high body mass index, alopecia, altered estrogen status, or pituitary microadenomas [43]. The absence of prolactin dysregulation confirms that the HPO axis disruption observed in this study was driven by letrozole-mediated androgenic mechanisms rather than hyperprolactinaemia, and that the restorative effects of the plant extracts were specific to the androgen-gonadotropin axis.

Taken together, the mid-dose combination of VAWE (200 mg/kg) and TIME (200 mg/kg) emerges from this multi-parameter analysis as the optimal therapeutic dose, demonstrating consistent superiority across gonadotropin, androgenic, estrogenic, and functional endpoints. and the high-dose combination provides multi-parameter evidence of synergistic HPO axis restoration. The paradoxical underperformance of the high-dose combination may reflect competitive receptor interactions or phytochemical antagonism at elevated concentrations, wherein high doses of *Tamarindus indica* phytochemicals may exert pro-estrogenic effects that stimulate rather than suppress GnRH pulsatility, reversing the therapeutic synergy observed at lower doses. These findings provide a pharmacological rationale for the combined ethnomedicinal use of *Vernonia amygdalina* and *Tamarindus indica* in reproductive health, and support further investigation of this combination in mechanistic and clinical studies.

Several limitations of this study warrant acknowledgement. First, synergistic interaction between VAWE and TIME was inferred from comparative multi-parameter biochemical outcomes across treatment groups rather than formally quantified using established methods such as the Chou-Talalay combination index. Future studies should employ formal synergy quantification to mathematically confirm and characterise the nature of the interaction between these extracts. Second, the mechanistic basis of the observed hormonal effects was not directly investigated, as assessments were limited to serum biochemical parameters. The molecular pathways underlying the treatment responses including receptor binding interactions, steroidogenic enzyme modulation,

and gene expression changes at the hypothalamic, pituitary, and ovarian levels remain to be elucidated in subsequent studies. Third, the study employed a fixed 21-day treatment duration, and it remains unclear whether extended treatment would achieve complete restoration of hormonal parameters and estrous cyclicity, particularly in groups showing partial recovery. Finally, as with all rodent-based studies, the translational applicability of these findings to human PMOS requires validation through clinical investigation.

Conclusion

This study demonstrates that combined administration of *Vernonia amygdalina* water extract (VAWE) and *Tamarindus indica* methanol extract (TIME) exerts synergistic ameliorative effects on HPO axis hormonal dysregulation in letrozole and high-fat high-fructose diet-induced PMOS rats, with the mid-dose combination of VAWE (200 mg/kg) and TIME (200 mg/kg) emerging as the optimal therapeutic dose, demonstrating superior restoration of testosterone, SHBG, LH/FSH ratio, estradiol, and progesterone concentrations alongside the most organised estrous cycle progression among all treatment groups. The consistent underperformance of the high-dose combination across multiple hormonal parameters indicates a paradoxical dose-dependent shift from synergism toward antagonism at elevated phytochemical concentrations, underscoring that optimal phytochemical ratios rather than maximum concentrations determine therapeutic efficacy, a finding with important implications for rational herbal combination formulation. These findings provide a pharmacological basis for the combined ethnomedicinal use of *Vernonia amygdalina* and *Tamarindus indica* in reproductive health management and support further mechanistic, phytochemical, and clinical investigation to fully translate these results into evidence-based therapeutic recommendations for PMOS.

List of Abbreviations

ANOVA: Analysis of Variance
CMC: Carboxymethylcellulose
ELISA: Enzyme-Linked Immunosorbent Assay
FRIN: Forestry Research Institute of Nigeria
FSH: Follicle Stimulating Hormone
GnRH: Gonadotropin-Releasing Hormone
HF-HFr: High-Fat High-Fructose
HPO: Hypothalamic-Pituitary-Ovarian
LH: Luteinizing Hormone
PCOS: Polycystic Ovarian Syndrome
PMOS: Polyendocrine Metabolic Ovarian Syndrome
SHBG: Sex Hormone Binding Globulin
TIME: *Tamarindus indica* Methanol Extract
VAWE: *Vernonia amygdalina* Water Extract

Declarations

Ethics approval and consent to participate: Ethical approval for the use of animals in this study was obtained from the Research Ethics Committee of Lead City University, Ibadan, Nigeria (approval number: LCU-REC/25/187). All procedures complied with institutional guidelines for the care and use of laboratory animals. Animals were maintained and handled in accordance with the guidelines of the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author's contributions: OJ conceptualised the study, designed the methodology, conducted the laboratory investigations, performed formal analysis and data curation, and wrote the original manuscript draft. OM supervised the research, contributed to conceptualisation, provided resources, and reviewed and edited the manuscript. AO and BM assisted with laboratory investigations and animal handling procedures. All authors read and approved the final manuscript.

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