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Influence on some female fertility hormonal response in wistar albino rats: Possible contraceptive role for methanol leaf extract of *Ocimum gratissimum* ?

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ABSTRACT

Ocimum gratissimum is a well-known medicinal plant. Folklore claims its use in management of headache, fever, diarrhea, pneumonia etc. Results of several research works support most of the claims. There is however scanty information on its effect on female fertility hormones production. This study was therefore designed to investigate the effect of its methanol extract on some fertility hormones in female albino rats. The animals were divided into three groups 1,2 and 3 representing those that received higher dose, lower dose of the extract and feed only which served as control. The extract was administered orally for 21 days at the end of which the animals were sacrificed and blood collected for the assay. The serum level of prolactin, progesterone, luteinizing hormone, estradiol, follicle stimulating hormone and testosterone were determined using enzyme-linked immunosorbent assay (ELISA) technique. Data from the study were analyzed using ANOVA at 95% confidence level. Results showed that administration of methanol extract of Ocimum gratissimum caused non-significant (P > 0.05) difference in estradiol concentration in group1(1.84± 0.16) and a non-significant (P > 0.05) increase in group 2 (2.06±0.24) compared to the control(1.74±0.05).The result also showed a dose dependent significant (P <0.05) decrease in serum testosterone level in the test groups (34.29±3.32) (30.46±0.50) compared to the control (35.18±2.31). Level of follicle stimulating hormone decreased non-significantly (P > 0.05) (9.25±0.93), (10.22±2.31) compared to the control(11.83±2.14). Equally a non-significant decrease was seen in the level of luteinizing hormone in both test groups (9.11± 0.94) (8.64±0.72) compared to the control group (9.68± 1.26). Non-significant increases were also observed in prolactin (7.69± 1.17), (7.42± 3.64) and progesterone (7.66± 0.96), (7.19±0.96) levels in the test groups compared to the control control group (6.64±2.33) (6.91± 0.60).The findings in this study have important implications for female contraceptive development.

Key words: Hormones, Elisa, Albino Rats, *Ocimum gratissimum*

INTRODUCTION

Fertility hormones regulate the reproductive cycle and are used to test for various associated conditions including infertility, menopause, early or delayed puberty as well as non-reproductive disorder. Female fertility is a biological process regulated by female hormones. The brain uses hormones to send signals to the body to trigger events. The body returns feedbacks to the brain to help regulate these events. This process repeats every cycle in women of child bearing age.

The most common causes of female infertility are hormones commonly associated with ovulation, polycystic ovarian syndrome, pre-mature ovarian failure, damage to the fallopian tube or uterus or problem with cervix.

Endocrine disorder results from excessive production of hormones or insufficient production of one or more hormones or the lack of the tissues responses to normal circulating hormones (21).

The female reproductive cycles functions primarily by the interplay between the lutenizing hormone, follicle-stimulating hormone, progesterone, estradiol and testosterone. Also the female reproductive organs can be assayed by the serum level of these hormones.

A large number of plant species have been screened for their anti-fertility efficacy. A review of reported that 577 plant species have been used traditionally in fertility regulation in females. (18)

Reports have shown that women are increasingly using herbs amongst other things to combat the negative effect of industrial pollutants of fertility (20). Medicinal herbs have an effect on female reproductive system and these herbs include: *Ocimum sanctum* and *Hibiscus Cosasinensis* (4), *Xylopia aethiopica* (African pepper) (23).

Ocimum gratissmum is an herbaceous shrub notably found in tropical countries including Nigeria, where it is commonly called clove basil, teabrush, scent leaf or fever plant; but it is also popularly known with different local name in Nigeria (Nupe; Tanmotsung-wawagi, Hausa; Daidoya tagida. Yoruba; Efinrin ajase: Ibo; nchanwu (9).

A recent research on the antifertility effects of aqueous crude extracts of *Ocimum gratissimum* leaves in male mice revealed that *Ocimum gratissimum* has an antifertility effect on male mice (23). *Ocimum gratissimum* extract reduced the level of testosterone in the male mice which may be due to the oil altering the androgen hormones synthesis of leydig cell (28).

Moreover a study indicated that administered orally aqueous extract of *Ocimum gratissimum* leaf could reduce activities of hepatic antioxidant enzymes in rats (2).

In 2004 researchers carried out an *in-vitro* study on the ileum of guinea pig. The effect of *Ocimum gratissimum* extract on intestinal motility, determined by the magnitude of contraction of isolated guinea pig ileum. Results shows that *Ocimum gratissimum* extract mimicked the action of adrenalin and nor-adrenaline on the isolated guinea pig ileum abolishing the acetyle choline-induced contraction of the smooth muscle of ileum (10).

A study analyzed the antimicrobial effect of aqueous and ethanolic fraction of two species, *Ocimum gratissimum* and *Xylopia aethiopica* against five pathogenic organisms: *Staphylococcus aureus, Escherichia coli, Streptococcus fecalis, Pseudomonas aeruginosa* and *Lactobacilli*. The result indicate that ethanolic extract of *Ocimum gratissimum* had a minimum inhibitory concentration (MIC) of 30µg/ml) against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* and *Lactobacilli* while *Streptococcus fecalis* had an MIC of 15µg/ml (15).

The findings suggest that the antimicrobial activity of these spices reside in their aqueous functions and also indicate that very low concentration are required to achieve antimicrobial effect and justifies the ethnomedicinal use of *Ocimum gratissimum* leaves as a plaster to cover wound surface and baby cord. Results show that extract of *Ocimum gratissimum* are active in-vitro against human pathogenic dermatophytes (32).

EXPERIMENTAL SECTION

Collection and identification of plant materials

Fresh leaves of *Ocimum gratissimum* (scent leaf) were purchased from Umuahia market (Good morning market) Abia State. The leaves were identified as *Ocimum gratissium* leaves by Dr. Garuba Omosun a taxonomist at the Department of Plant Science and Biotechnology, College of Natural Science, Michael Okpara University of Agriculture, Umudike.

Preparation of *Ocimum gratissium* (scent leaf) extract

Fresh leaves of *Ocimumgratissium*were shade dried and then ground with an electric blender.

The dried powdered plant material leaves (250g) was dissolved in 1,500ml of methanol and allowed to stand for 48hrs then filtered using Whatman no 2 filter paper. The filtrate was evaporated to dryness using water bath at 60^0C. The yield was calculated and the dry extract was stored until use for the experiment, during the experiment.

THE EXPERIMENTAL ANIMALS

The animals used in this study were female wistar albino rats weighing 120-140g (12 – 13 weeks old). The animals were obtained from the animal house of the Department of Zoology, University of Nigeria Nsukka, Enugu State. The animals were randomly distributed into cages and allowed to aclimatize for two weeks in a well ventilated animal house at a room temperature of 28.0 ± 2.0^0C under natural lighting condition. The animals were fed with normal rat chow and allowed free access to water daily. All animals used in this study were handled in accordance with the international, national and institutional guidelines for care and use of laboratory animals in Biomedical Research as promulgated by the Canadian Council of Animal Care (2009).

EXPERIMENTAL PROTOCOL

Animals were divided into three main groups each having four rats
Group I received 200 mg/kg body weight of plant extract
Group II received 200 mg/kg body weight of plant extract
Group III (control) received standard feed and distilled water
The administration lasted for 21 days (3 weeks) at the end of which blood was collected through the ocular puncture into plain sample bottles. This centrifuged for 2000rpm for 10mins to obtain clear sera for hormonal assay.

ASSAY OF HORMONAL PARAMETERS (HORMONAL ASSAY)

Blood was collected into plain sample bottles and assayed for testosterone, prolactin, estradiol, lutenizing hormone, follicle stimulating hormone and progesterone using enzyme linked immunoassay method according to the principle highlighted by (35) for prolactin, estradiol, testosterone and progesterone while that of Uotila *et al*. (36) was used for luteinizing and follicle stimulating hormones.

Determination of testosterone concentration

Assay procedure:

The desired number of coated wells of microtiter was secured in the holder. 10µl of the specimens were dispensed into appropriate wells. 100µl of testosterone HRP conjugate reagent was dispensed into each well. It was thoroughly mixed for 30 seconds, and was incubated at 37^0C for 90mins.

The incubation mixture was removed by flicking plate contents into a waste container. The microtites wells was rinsed and flicked 5 times with distilled water. The wells were struck sharply onto absorbent paper to remove all residual water droplets. 100µl of TMB reagent was dispensed into each well and gently mixed for 5 seconds, and was incubated at room temperature for 20 minutes. The reaction was stopped by adding 100µl of stop solution to each well, and then gently mixed for 30 seconds to ensure that all the blue colour changes to yellow completely.

The absorbance was read at 450nm with a microtiter well within 15 minutes using microplate reader machine (MR-9620A).

Determination of estradiol concentration

Assay procedure:

The desired number of microtiter coated wells was secured in the holder. 25µl of specimens were dispensed into the appropriate wells. 100µl of esstradiol – HRP conjugate reagent was dispensed into each well. 50µl of rabbit anti-estradiol (E2) reagent was dispensed into each well and was thoroughly mixed for 30 seconds, and then incubated at room temperature for 30 seconds, and then incubated at room temperature for 90 minutes.

The micro wells was rinsed and flicked 5 times with distilled water 100µl of TMB reagent was dispensed into each well and gently mixed for 10 seconds, then incubated at room temperature for 20 minutes. The reaction was stopped by adding 100µl of stop solution into each well and then mixed gently for 30 seconds to ensure that all blue colour changed to yellow completely.

Absorbance was read at 450nm with a microtiter well reader within 15 minutes using microplate Reader machine (MR – 9620A)

Determination of Prolactin Concentration
Assay Procedure:
The desired number of coated wells of microtiter was secured in the holder. 50µl of specimens were dispensed into appropriate wells. 100µl of enzyme conjugate reagent was dispensed into each well and was gently mixed 10 seconds to have a complete mixing set up.

The mixture was incubated for 45 minutes after which it was removed by flicking plate contents into sink. The microtiter wells was rinsed and flicked 5 times with distilled water and the wells were sharply striked into absorbent paper to remove all residual water droplets 100µl of TMB reagent was dispensed into each well and gently mixed for 10 seconds, then incubated at room temperature in the dark for 20 minutes, after which the reaction was stopped by adding 100ul of stop solution to each well and gently mixed for 30 seconds to ensure that all blue colour changed to yellow completely. The absorbance was read at 450 nm with a microplate reader within 15 minutes.

Determination of progesterone concentration
Assay procedure:
The desired number of coated wells of microtiter was secured in the holder. 25µl of specimens were dispensed into appropriate wells. 100µl of working progesterone – HRD conjugate reagent was dispensed into each well. 50µl of rabbit anti-progesterone reagent was dispensed into each well and was thoroughly mixed for 30 seconds. It was incubated at room temperature for 90 minutes. The microtiter wells were rinsed and flicked 5 times with distilled water, 100µl of TMB reagent was dispensed into each well and was gently mixed for 10 seconds. It was again incubated at room temperature for 30 minutes, after which the reaction was stopped by added 100µl of stop solution to each well. It was gently mixed for 30 seconds and was ensured that all blue colour changed to yellow completely. The absorbance was read at 450 nm with a microwell reader within 15 minutes (MR – 9620A).

Determination of Luteinizing Hormone Concentration
The desired number of coated wells of microtiter was secured in the holder. 50µl of specimens were dispensed into appropriate wells. 100µl of enzymes conjugate reagent was dispensed into each well, and then mixed gently for 30 seconds. The mixture was incubated at room temperature for 45 minutes. The incubation mixture was removed by flicking the contents into sink, the microtiter wells were rinsed and flicked 5 times with distilled water, and the wells were stricked sharply onto absorbent paper to remove residual droplets. 100µl of TMB reagent was dispensed into each well and gently mixed for 10 seconds. It was incubated at room temperature in the dark for 20 minutes.

The reaction was stopped by adding 100µl of stop solution to each well and gently mixed for 30 seconds to ensure that all blue colour changed to yellow completely.

The optical density was read at 450nm with a microplate reader within 15 minutes.

Determination of follicle stimulating hormone concentration
The desired number of coated wells of microtiter was secured the holder. 50ul of specimens were dispensed into each well. 100µl of enzyme conjugate reagents was dispensed into each well and mixed thoroughly for 30 seconds to have a complete mixing in the step.

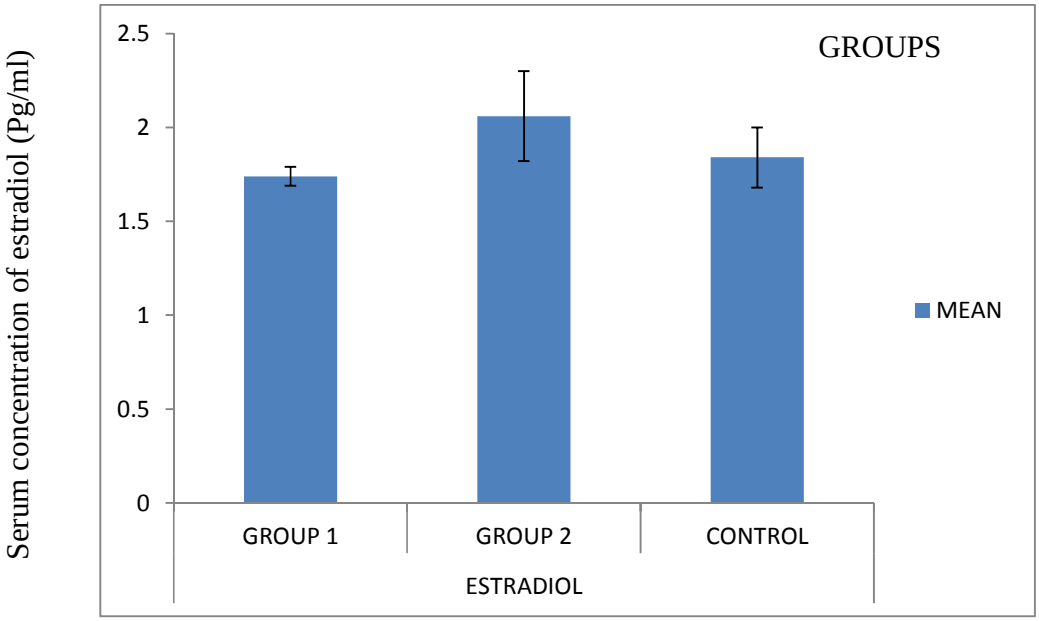
It was incubated at room temperature for 45 minutes. The incubation mixture was removed by flicking the plate contents into a waste container. The microtiter wells was rinsed and flicked 5 times with distilled water, the wells were sharply striked onto absorb paper to remove all residual water droplets. 100µl of TMB reagent was dispensed into each well and gently mixed for 10 seconds. It was again incubated at room temperature in the dark for 20 minutes.

The reaction was stopped by adding 100µl of stop solution into each well and mixed gently for 30 seconds to ensure that all blue colour changed to yellow completely. The absorbance was read at 450nm with a microplate reader within 15 minutes.

STATISTICAL ANALYSIS
The data are expressed as mean±standard deviation. Comparisons between animals administered with the extract and the control and between two groups treated with different doses of the extract were performed using the one way ANOVA. Significance was accepted at $P<0.05$.

RESULTS

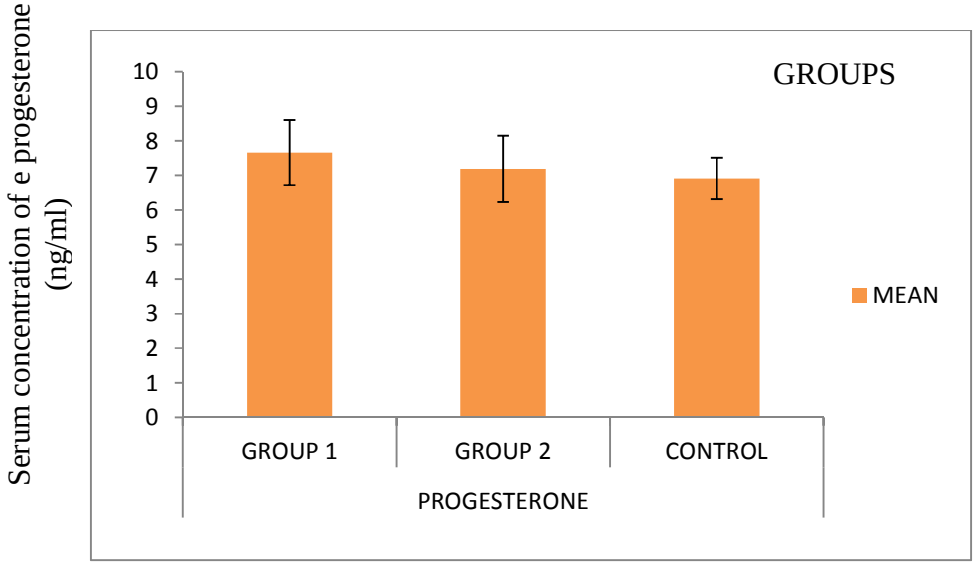
Fig. 1: Effect of methanolic extract of *Ocimum gratissimum* leaves on estradiol



Effect of Methanolic extract of *Ocimum gratissimum* on Estradiol

The fig above shows the effect of *Ocimum gratissimum* on estradiol level. It was observed that the mean value of group 1 (1.74±0.05) shows a significant ($p<0.05$) decrease when compared to the mean value of group 2 (2.06±0.24) but a non-significant ($p>0.05$) decrease when compared to the control group (1.84±0.16) while comparing the mean value of group 2 shows a non-significant ($p>0.05$) increase when compared against the control group.

Fig.2: Effect of *methanolic extract of Ocimum gratissimum leaves on progesterone*



Effect of Methanolic extract of *Ocimum gratissimum* on Progesterone

It was observed from the chart above that the mean value of group 1 and group 2 (7.66±0.96) and (7.19±0.96) respectively shows a non-significant (p>0.05) increase when they were compared against the mean value of the control group (6.91±0.60).

Fig.3: Effect of methanolic extract of *Ocimum gratissimum*leaves on testosterone

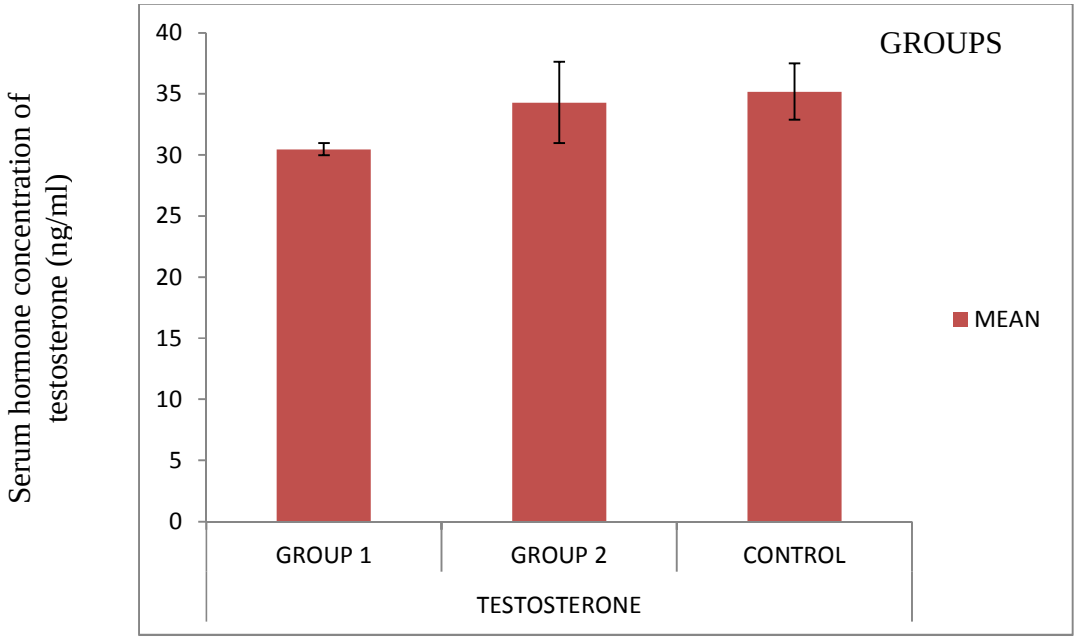
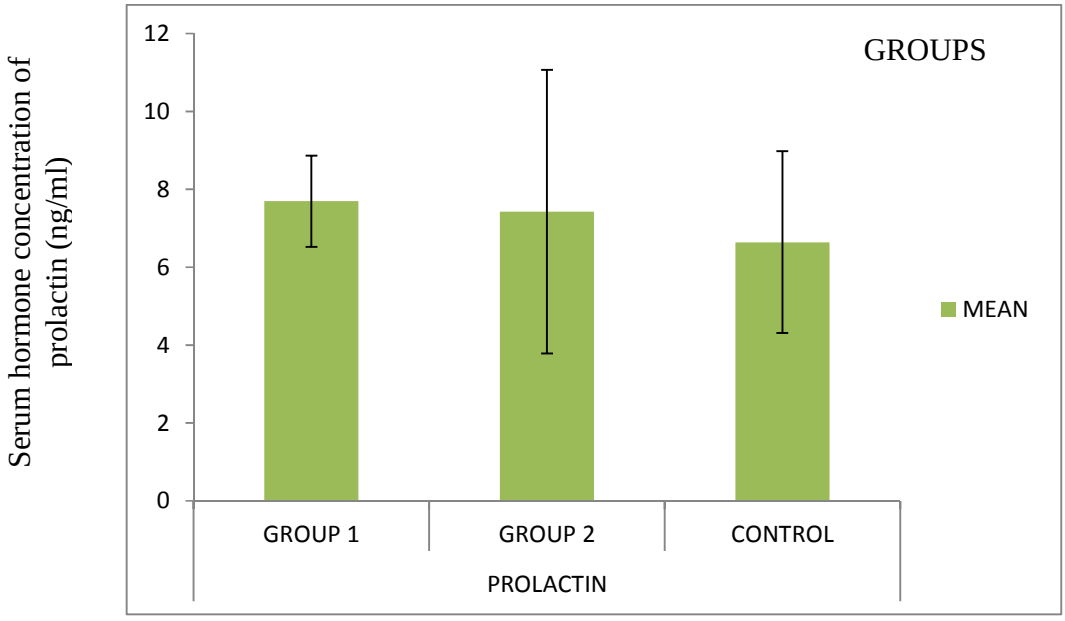


Fig.4: Effect of methanolic extract of *Ocimum gratissimum* leaves on prolactin



Effect of Methanolic extract of *Ocimum gratissimum* on Testosterone

The mean value of group 1 (30.46±0.50) showed a significant ($p<0.05$) decrease when compared with group 2 and the control group (34.29±3.32) and (35.18±2.31) respectively while that of group 2 showed a non-significant ($p>0.05$) decrease when compared to the control group.

Effect of Methanolic extract of *Ocimum gratissimum* on Prolactin

The mean value of group 1 (7.69±1.17) showed a non-significant increase ($p>0.05$) when compared across the mean value of group 2 (7.42±3.64) and the control group (6.64±2.33), while that of group 2 was non-significant ($p>0.05$) when compared with the control group.

Fig.5: Effect of methanolic extract of *Ocimum gratissimum* leaves on follicle stimulating hormone

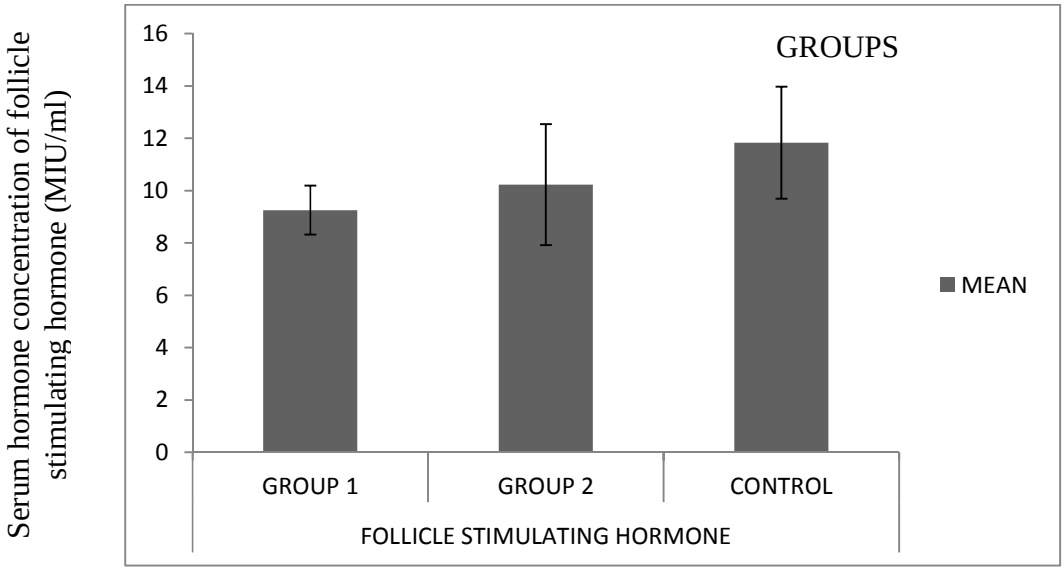
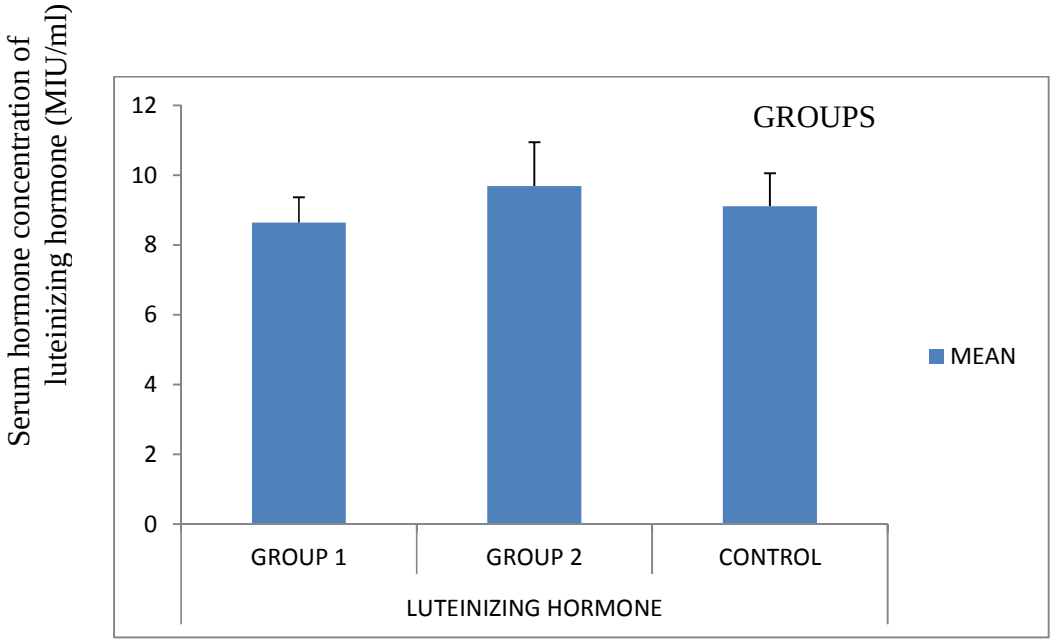


Fig.6: Effect of methanolic extract of *Ocimum gratissimum* leaves on luteinizing hormone



Effect of Methanolic extract of *Ocimum gratissimum* on Follicle Stimulating Hormone
The mean value of group 1 (9.25±0.93) reduced non-significantly (p>0.05) when compared against groups 2 and the control with mean values of (10.22±2.31) and (11.83±2.14) respectively.

Effect of Methanolic extract of *Ocimum gratissimum* on Lutenizing Hormone
The mean value of group 2 (9.68±1.26) was non-significantly (p>0.05) higher when compared against the mean value group 1 (8.64±0.72) as well as mean value of the control (9.11±0.94), while the mean value of group 1 was non-significantly (p>0.05) lower than that of the control group.

DISCUSSION

The use of herbs in the management of ailment has been a regular practice in Africa with considerable therapeutic success.*Ocimum gratissimum* is a plant highly praised for its nutritional and therapeutic benefits. Phytochemical screening has revealed many bioactive as well as toxic agent of plant extract that can affect the regulation of oestrous cycle, conception and reproduction (26, 12, 25). Therefore the presence of these phytochemicals may account for the alterations in the levels of the circulating hormones observed in this study. Administration of exogenous factors such as dietary oil can influence hormonal response in host animals (8)

The result showed that oral administration of methanolic extract of *Ocimum gratissimum* significantly ($p < 0.05$) reduced the level of testosterone in the test groups compared to the control. The result is in agreement with the work of Parandin *et al*.,(28) who reported that administration of oil extract of *Ocimum gratissimum* leaves decreased serum levels testosterone in experimental animals.

Prolactin helps to initiate breast development by inducing labulo-alveolar growth of the mammary gland. It also stimulates lactogenesis. Dopamine serves as the major-inhibiting factor on prolactin secretion (30). The enhanced level of prolactin observed in this study may be attributed to the effect of the extract probably acting as a dopamine antagonist. High prolactin level tend to suppress the ovulatory cycle by inhibiting the secretion of both follicle stimulating hormone and gonadotropic-releasing hormones (GnRH) (30) which are necessary for ovulation. Such increase in prolactin may inhibit ovulation and promote the loss of menstrual periods which will hinder conception.

Follicle stimulating hormone is the central hormone of mammalian reproduction, essential for gonadal development and maturation at puberty as well as gamete production during the fertile phase of life (33). It stimulates the growth and maturation of ovarian follicles by acting directly on the receptors located on the granulose cells. The reduction in the levels of follicle stimulating hormone by the extract may hamper folliculogenesis and delay maturation of the follicle in the pre-ovulatory phase (18). It is possible that the extract might have exerted its effect on the anterior pituitary or the hypothalamus since the secretion of stimulating hormone is regulated by the gonadotropic releasing hormone secreted by the hypothalamus. The reduction observed in the level of this hormone may adversely affect conception in female animals. Other researchers had earlier observed the inhibitory effect of other plant parts on the release of the gonadotropins (5,3,1,12).

Lutenizing hormone stimulates secretion of sex steroids from the gonads. In females, ovulation of mature follicles in the ovary is induced by a surge of luteinizing hormone secretion during the pre-ovulatory periods. Several authors have demonstrated that lutenizing hormone release surges at the pre-estrous stage are responsible for ovulation (11,13). Any substance capable of inhibiting this release could provoke disruption of ovulation by decreasing the number of mature follicles or induce an oestrous cycle disruption at rest. (12). The reduction in level of serum lutenizing hormone indicates the inhibitory effect of the extract on the release of lutenizing hormone which may trigger disruption of ovulation. This may result in impairment of oestrous cycle, hamper conception and normal reproduction in the females. It is therefore possible that *Ocimum gratissimum* contains anti-gonadotropic substance which may affect the oestrous cycle and hamper reproduction in females.

The result of this study indicates that methanolic extract of *Ocimum gratissimum*non significantly ($p > 0.05$) increased serum progesterone in female rats. Phytochemical constituents of *Ocimum gratissimum* are alkaloids, saponin, tanins, phlobatannins, anthraquinones, steroids, flavonoids and cardiac glycosides (24,29).Yu *et al*.,(38) had reported that saponins lower serum androgens and 17β-estradiol, but elevate progesterone levels, suggesting that saponins modulate steriodogenesis in the ovary. Progesterone has antiestrogenic effect on the myometrial cell, decreasing their excitability, their sensitivity to oxytocin, and their spontaneous electrical activity while increasing

their membrane potential (12). Estradiol stimulates the growth of the uterine lining, causing it to thicken during the pre-ovulatory phase of the cycle. It is well established that estradiol is directly responsible for growth and development of reproductive organs. In synergy with follicle stimulating hormone, estradiol stimulates granulose cell proliferation during follicular development (2). Plants with estrogenic property can directly influence pituitary action by peripheral modulation of lutenizing hormone and follicle stimulating hormone, decreasing secretion of these hormones and blocking ovulation (6) thus, the reduction in the serum concentration of estradiol observed in the low dose group may be attributed to a decreased aromatase activity or substrate supplementation during estrogen synthesis (14). Consequently such decreased in estradiol levels may hamper ovulation, preparation of the reproductive tract for zygote implantation and the subsequent maintenance of pregnancy state (14). Kadohama *et al*.,(17) had reported that several plant alkaloids inhibit aromatase activity.

Thus it is possible that the methanolic extract of *Ocimum gratissimum* contain biologically active phytochemicals which may be endocrine disrupting. Such substances in the plant extract may induce hormonal imbalance or disorders such as anti-fertility and contraception in hormone dependent organs like the ovary and mammary glands. The findings in this study have important implications for female contraceptive development. Plant products as contraceptive will be more acceptable for economic reasons and for the fact that products are associated with less side effects than synthetic agents.

CONCLUSION

The alteration in the female reproductive hormones by the extract is indicative of adverse effect on the maturation and ovulation of follicles. Consequently, the extract may impair fertility and conception in female rats. Thus *Ocimum gratissimum* may be explored as a female contraceptive and control especially in third world countries where population explosion remains a current danger.

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