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Tetracarpidium conophorium and Ascorbic Acid Enhances Some Serum Reproductive Hormone Concentrations in Cimetidine and *Citrus aurantifolia* Treated Male Albino Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author OUA designed the study, wrote the protocol and statistical analysis. Author EOE wrote the first draft of the manuscript. Author IOU managed the analyses of the study searches and the literature. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aims to comparatively investigate the effects of Cimetidine, Ascorbic acid (Vit C), *Citrus aurantifolia* and *Tetracarpidium conophorium* on male fertility hormones in adult male albino Wistar rats.
Study Design: 96 Albino rats randomly assigned into 6 groups (A – F), the first 4 groups (A – D) further sub-divided into 3 Subgroups of 6 rats each and the last 2 groups (E –F), sub-divided into 2 Subgroups of 6 rats each.
Place and Duration of Study: Department of Clinical pharmacology Animal house, University of Uyo, Nigeria.

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Methodology: Group A – D had sub-groups$_1$ as control groups, sub-group$_2$ and $_3$ as experimental groups which received medium and high doses of Cimetidine, Vit C, *Citrus aurantifolia* and *Tetracarpidium conophorium* respectively, while Groups E – F also had Control sub-groups and experimental sub-groups treated with medium doses of Cimetidine and Vit C, *Citrus aurantifolia* and *T. conophorium* respectively. Treatment was performed daily for 21 days; serum reproductive hormonal assay was carried out.

Results: Vit C significantly (P<0.05) increased follicle stimulating hormone levels in Vit C high dose sub-group$_3$ compared to control and Vit C low dose sub-group$_2$, significantly (P<0.05) increased testosterone serum levels in Vit C low dose sub-group$_2$ and Vit C high dose sub-group$_3$ compared to control, also significantly (P<0.05) decreased Luteinizing hormone (LH) levels in Vit C low dose group compared to control. Combination treatment of Cimetidine and Vit C significantly (P<0.05) increased follicle stimulating hormone levels when compared to control, also significantly (P<0.05) decreased LH levels in sub-group$_2$ compared to control. Combination treatment of *Citrus aurantifolia* and *T. conophorium* significantly (P<0.05) increased follicle stimulating hormone and testosterone levels when compared to control.

Conclusion: Vitamin C and *T. conophorium* can boost fertility. *Citrus aurantifolia* at high doses affects fertility negatively having a mild effect at medium doses.

Keywords: Ascorbic acid; Tetracarpidium conophorium; Citrus aurantifolia; male reproductive hormones; fertility.

1. INTRODUCTION

Citrus aurantiifolia, commonly known as 'lime' is of the Rutaceae family, an evergreen tree about 5 meters high, fruits are yellow when ripe but usually picked green commercially, globosely measuring four to five centimetres in diameter with thinner rind and very sour [1]. Cultivated extensively in tropical and subtropical countries mainly for its use as a food item or food additive, it is medicinally best known throughout the world as a remedy that relieves fevers, sore throat, coughs, common cold and indigestion [2,3,4]. Lime-juice is highly acidic and has been proven to have medicinal, industrial and cosmetic values. The wide range of bioactive compounds from lime has been found to possess anti-infection and anti-inflammatory activities [5,6]. Under laboratory conditions, lime-juice has shown to immobilise sperm as well as an advocate as a 'natural' spermicide. An in-vitro study done, showed that lime juice destroys both human immunodeficiency virus (HIV) and sperm cells [7,8].

Cimetidine is a hydrogen (H_2) receptor antagonist used initially in the treatment of gastric and duodenal ulcers its regular use has been found to increase prolactin levels which can result in female infertility as well as male fertility problems. In men, the increase in prolactin can cause a decrease in luteinizing hormone (LH) and testosterone levels resulting in a lowered sperm count, decreased libido and decreased sexual functioning, all which can contribute to male infertility [9].

Ascorbic acid also referred to as vitamin C or ascorbate belongs to the water-soluble class of vitamins. It plays a useful role in improving fertility because of its antioxidant property which minimizes free radical/oxidative damage and DNA fragmentation which results in less DNA damage and semen defects. It was also reported that lack of this vitamin in males will result in clumping of semen and the motility of this sperm cells will be inhibited [10,11].

Tetracarpidium conophoruim from the family Euphorbiaceae commonly called the African Walnut and Conophor is a perennial climbing plant found in the moist forest zones of sub-Sahara Africa and India, cultivated principally for the nuts which are cooked and consumed as snacks, along with boiled corn [12,13]. In southern Nigerian ethnomedicine, *Tetracarpidium conophorum* is used as a male fertility agent to improve fertility in males and the leaves are used for the treatment of dysentery, it was also known to have anti-microbial, anti-inflammatory, anti-carcinogenic and anti-mutagenic effect and also plays a role in preventing and controlling high blood pressure [14].

Fertility issues have been a major concern of individuals and health caregivers in Nigeria, and various studies have tried to answer some questions such as how to enhance fertility.

However, the use of Cimetidine as the most common/relatively cheap antiulcerative drugs, Vitamin C as remedy for cold, scurvy and some minor injuries, *C. aurantiifolia* as a major source of ingredient in drinks, medications and *T. conophoruim* as snacks increases globally alongside its rapid social and medicinal acceptance with vague knowledge of their effects of consumption on reproductive health. In view of their increased utilization especially in Nigeria, it becomes necessary to ascertain any possible relationship of these substances on male fertility hormones the major determinants of male reproductive processes.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Fruits and Drugs

The Cimetidine and Vitamin C tablets were purchased from Top Care Pharmaceutical Store, Uyo, Akwa-Ibom State, Nigeria. The brand of cimetidine drug used in the cause of this research work had identification records as follows;

CIMEC 400 mg: cimetidine tablets B.P. 400mg is its composition. Manufactured by ZIM LABORATORIES LTD. B-21/22 MIDC area KalmesKalmeshwar, Nagpur 441-501.Manufacturing license no. 1224.Sole agent – Climax Pharmchem Ltd, Nigeria.

Fresh lime fruits were obtained from Uyo metropolis and were identified at the Department of Botany and Ecological studies of the University of Uyo, Nigeria. The fruits were carefully washed, sliced into two halves and then gently squeezed into a container. The obtained lime juice was then filtered through a filter paper. The pH of the lime juice was 1.7.

Tetracarpidium conophorum seeds (walnuts) were obtained from Aba, Abia State of Nigeria in large quantities, fresh and uncooked. The seeds were identified and authenticated in the Department of Botany and Ecological Studies of the University of Uyo, Akwa Ibom State, Nigeria. The fruits were washed, cooked for about two and a half hours, after which they were removed from the water, allowed to cool for some minutes and then the nutshell was cracked to obtain the fruits. Boiling was done to reduce its toxic effect as fresh walnut can be corrosive to the mouth. The edible part was then blended into powder; 200 ml of distilled water was added to it and allowed to stand for 24 hours. After 24 hours, it was filtered and the filtrate obtained was stored in a cork-sealed container and put into the refrigerator for use.

2.2 Experimental Animals and Feeding Protocol

Ninety-six adult male albino rats of Wistar strain weighing 154 -281 g were obtained from the animal house of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria. The animals were kept in well-ventilated wooden cages of 50 x 30 cm dimension in a section of the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Nigeria, and exposed to 12/12 h light/dark cycle. Animals were fed with grower's pellets and water *ad libitum* before commencement and during the experiment. Extract and drugs administration commenced after two weeks of habituation and was done orally twice daily by means of a calibrated syringe with attached rubber cannula for a period of 21 days.

Ethical approval: The study was approved by the University of Uyo Animal Research Ethics Committee in accordance with the internationally accepted principles for laboratory animal use and care in the European Community guidelines [15].

Animals were randomly divided into six (6) groups. Group A – D had eighteen animals per group while Group E and F had twelve animals each. Groups A - D were further divided into 3 Sub - Groups of 6 animals each, with sub-group $_1$ as a control group, sub-group $_2$ and $_3$ as experimental groups. Sub - Groups $_2$ and $_3$ were treated with medium and high doses respectively. Group E and F were divided into two Sub - Groups of six animals each with sub-group$_1$ as control and sub-group$_2$ as the experimental group.

Group A – Administered Cimetidine

Sub-group A_1: this group was treated with 10 ml/kg of distilled water

Sub-group A_2: this group was treated orally with a medium dose of cimetidine (475 mg/kg)

Sub-group A_3: this group was treated orally with a high dose of cimetidine (950 mg/kg).

Group B – Administered Ascorbic Acid

Sub-group B_1: this group was treated with 10 ml/kg of distilled water

Sub-group B_2: this group was treated orally with a medium dose of ascorbic acid (250 mg/kg)

Sub-group B_3: this group of animals was treated orally with a high dose of ascorbic acid (400 mg/kg).

Group C – Administered *Citrus aurantifolia* (Lime Juice)

Sub-group C_1: this group was treated with 10 ml/kg of distilled water

Sub-group C_2: this group received 1000 mg/kg lime juice (medium dose)

Sub-group C_3: this group received 1500 mg/kg lime juice (high dose)

Group D – Administered *Tetracarpidium conophorium* (Walnut)

Sub-group D_1: this group was treated with 10 ml/kg of distilled water

Sub-group D_2: this group received 14.14 mg/kg *Tetracarpidium conophorium* (medium dose)

Sub-group D_3: this group received 21.21 mg/kg of *Tetracarpidium conophorium* (high dose)

Group E – Administered Cimetidine + Ascorbic acid

Sub-group E_1: this group was treated with 10 ml/kg of distilled water

Sub-group E_2: test group treated with medium dose of Cimetidine (475 mg/kg) + medium dose of Ascorbic acid (250 mg/kg)

Group F – Administered Lime Juice + Walnut

Sub-group F_1: this group was treated with 10 ml/kg of distilled water

Sub-group F_2: test group treated with medium dose of Lime Juice (1000 mg/kg) + medium dose of Walnut (14.14 mg/kg)

2.3 Sample Collection

After 21 days of oral administration of cimetidine, *ascorbic acid, Citrus aurantifolia* juice and *Tetracarpidium conophorium extract*, the rats in the different groups were weighed again and on 22nd day, they were anesthetized using chloroform. The chloroform was applied on a swap of cotton wool and placed inside a desiccator. Each of the animals was then placed in the desiccator for a period of 30-40 seconds till completely anaesthetized. The fore and hind limbs of the rats were pinned to the dissecting board at the skin. Skin incisions were made underneath the thoracic region using a sterile pair of scissors to expose the heart. A 5 ml syringe fitted with a needle was used to aspirate blood from the right ventricle of the heart of each of the rats a procedure called "cardiac puncture". Different sterile syringes and needles were used for the different rats. The blood gotten was emptied into properly labelled plain sample bottles.

2.4 Sample Analysis

The blood samples from the rats were kept in plain sample bottles and the samples were allowed to settle before being placed in a centrifuge machine and spun for 15 minutes at a speed of 4000revolutions per minute. After that, the samples were removed from the centrifuge and the blood serum was separated from the packed cells using a 2 ml syringe fitted with a needle and then placed into another well labelled sample bottle. The separated serum samples were stored in the refrigerator before the hormonal assay was carried out.

2.4.1 Serum FSH, LH, and prolactin measurement

The FSH, LH and prolactin were determined based on the principle of the sandwich method using an enzyme-linked immunoassay (ELISA) technique. The assay system utilizes a high affinity and specificity monoclonal antibody (enzyme conjugated and immobilized) directed against a distinct antigenic determinant on the impact FSH, LH and PROLACTIN molecule. The test sample is allowed to react simultaneously with the two antibodies, resulting in the FSH, LH and PROLACTIN molecule being sandwiched between the solid phase and enzyme-linked antibodies. After incubation, the wells are washed with a washing solution to remove unbound labelled antibodies.

Tetramethylbenzidine substrate is added and incubated, resulting in the development of a blue

colour. The colour development is stopped with the addition of stopping reagent changing the colour to yellow. The concentration of FSH, LH and prolactin is directly proportional to the colour intensity of the test sample.

2.4.2 Serum testosterone hormone measurement

Testosterone level was determined using competitive micro plate enzyme immunoassay. Plates are quoted with anti-testosterone antibodies. Calibrators and specimen are first added to micro-plate well. The enzyme-testosterone conjugate is added. Testosterone present in the sample competes with enzyme testosterone conjugate for binding with anti-testosterone coated micro-plate to form an antigen-antibody complex. Unbound conjugate is removed by washing. The enzyme activity in the antibody-bound fraction is inversely proportional to the native testosterone concentration. The enzyme activity is revealed by a colour change in tetramethyl-benzidine substrate solution.

2.5 Toxicity Study and Dosage Design

2.5.1 Toxicity test for lime juice

Nine adult male mice weighing between 15 – 21 g were randomly placed in 3 cages, containing an equal number of 3 mice per cage. The animals fasted for 24 hours before administration of the lime juice. The nine mice were treated in aqueous extract of *citrus aurantifolia* at dosages 3000 mg/kg, 4000 mg/kg and 5000 mg/kg depending on their body weight. They were observed for 24 hours for signs of toxicity. No mortality was recorded.

2.5.2 Dosage design

LD 50 values greater than 5000 mg/kg are of no practical interest [16]. The absence of lethality at such a higher value indicates that the substance is relatively non toxic. Therefore, using Miller and Tainter's method of 10% low dose, 20% medium dose and 30% high dose [17], the dosage for the experiment was designed (Appendix I).

2.5.3 Toxicity test for walnut

The medium lethal dose (LD50) of the plant extraction was also determined by using Lorke's method [16]. The LD_5o was done on 2 phase. The 1st phase was done using 21 mice and 3 mice in each group. They were treated in aqueous extract of *Tetracarpidium conophonum* at dosages 5000 mg/kg, 4500 mg/kg, 4000 mg/kg, 3500 mg/kg, 3000 mg/kg, 2000 mg/kg and 1000 mg/kg depending on their body weight and the administration was intraperitoneal. They were observed for 24 hours for signs of toxicity. After 24 hrs all the 21 mice were dead and others signs of toxicity were noticed 2-4 hours after extract administration. There was decreased locomotion, weighting and decreased in sensitivity touch. Also, there was decreased feed intake after 15 hours of extract administration. The second phase, dosages of 100 mg/kg, 200 mg/kg, 300 mg/kg, 400 mg/kg, and 500 mg/kg for a total of 10 mice, 2 per group. They were well labelled and administered intraperitoneal, allowed for 24 hrs for toxicity. All 10 mice survived and the median lethal dose was calculated to be 70.7 mg/kg body weight.

2.6 Statistical Analysis

The results were analyzed for statistical significance by Student T-TEST method using the SPSS statistical program and Post Hoc Test (LSD) between groups using Microsoft Excel program, all data are expressed as mean SEM. P values < 0.05 are considered significant [18].

3. RESULTS

3.1.1 Comparative effect of Cimetidine, *Citrus aurantifolia*, *Tetracarpidium conophorium* and vitamin C on follicle stimulating hormone

In Fig. 1, Experimental Subgroup A treated with cimetidine, FSH level was significantly (P<0.05) higher in the medium dose (0.15±0.02 µmol/mL) when compared to control (0.10±0.00), though a marginal decrease was observed in the high dose (0.13±0.02).

Experimental subgroup B treated with ascorbic acid had a significant (P<0.05) difference in FSH level in high dose sub-group (0.37±0.11) compared to control (0.10±0.00) and the medium dose sub-group (0.10±0.00) as shown in Fig. 1.

FSH Levels in experimental subgroup C treated with *Citrus aurantifolia*, showed no significant difference in the control, medium dose and high dose subgroups as shown in Fig. 2.

In Fig. 2, Experimental sub – groups D treated with aqueous extract of *Tetracarpidium conophoruim*, FSH level was a significantly (P<0.05) increased in medium dose (0.50 ± 0.14) compared to control (0.10 ± 0.14), significantly

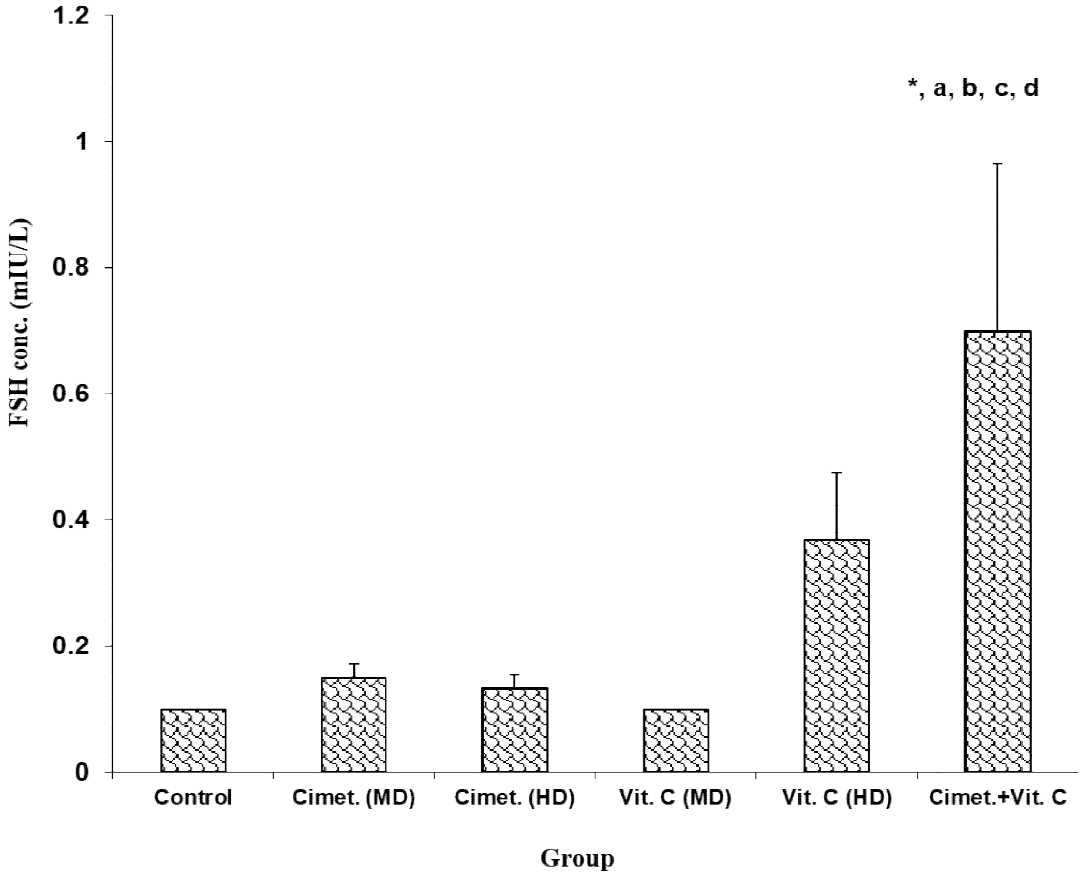

Fig. 1. Comparison of FSH conc. in groups treated with ascorbic acid and cimetidine

*Values are mean ± SEM, n = 6. *p<0.05 vs control; a = P<0.05 vs cimet. (MD); b = P<0.05 vs cimet. (HD); c = P<0.05 vs Vit. C (MD); d = P<0.05 vs Vit. C (HD)*

(*P*< 0.01) increased in high dose (0.17 ± 0.02) compared to control (0.10 ± 0.14), and significantly (*P*<0.05) decreased in high dose (0.17 ± 0.02) compared to medium dose (0.50 ± 0.14).

Experimental subgroup E treated with cimetidine and ascorbic acid in Fig. 1, showed there was a significant (*P*<0.05) increase in the FSH level of the combined group (0.70±0.27) compared to control (0.10±0.00), cimetidine medium (0.15±0.02) and high dose group (0.13±0.02), Vitamin C medium dose (0.10±0.00) and Vitamin C high dose group (0.37±0.11).

In Fig. 2, FSH Levels in experimental subgroups F treated with aqueous extract of *Citrus aurantifolia* and *Tetracarpidium conophorium* showed significant increases in *Tetracarpidium conophorium* medium dose group (*P*<0.05) compared to control (0.10±0.00) and *Citrus aurantifolia* medium dose (0.10±0.00), a significant (*P*<0.05) increase in the combined group (0.92±0.25) compared to control (0.10±0.00), *Citrus aurantifolia* medium dose (0.10±0.00) and *Citrus aurantifolia* high dose (0.25±0.10).

3.1.2 Comparative effect of cimetidine, vitamin C, lime and walnut on luteinizing hormone levels

In experimental subgroup A treated with Cimetidine**,** There was no significant difference in LH level between the experimental subgroups, though a marginal decrease was observed in the high dose group. Mean values of LH levels in control, medium dose and high dose were, 0.18±0.00, 0.18±0.02 and 0.13±0.02 respectively as shown in Fig. 3.

In Fig. 3, experimental subgroup B treated with ascorbic acid showed a significant (*P*<0.05) decrease in LH level of the medium dose sub-group (0.12±0.02) when compared to control (0.18±0.02).

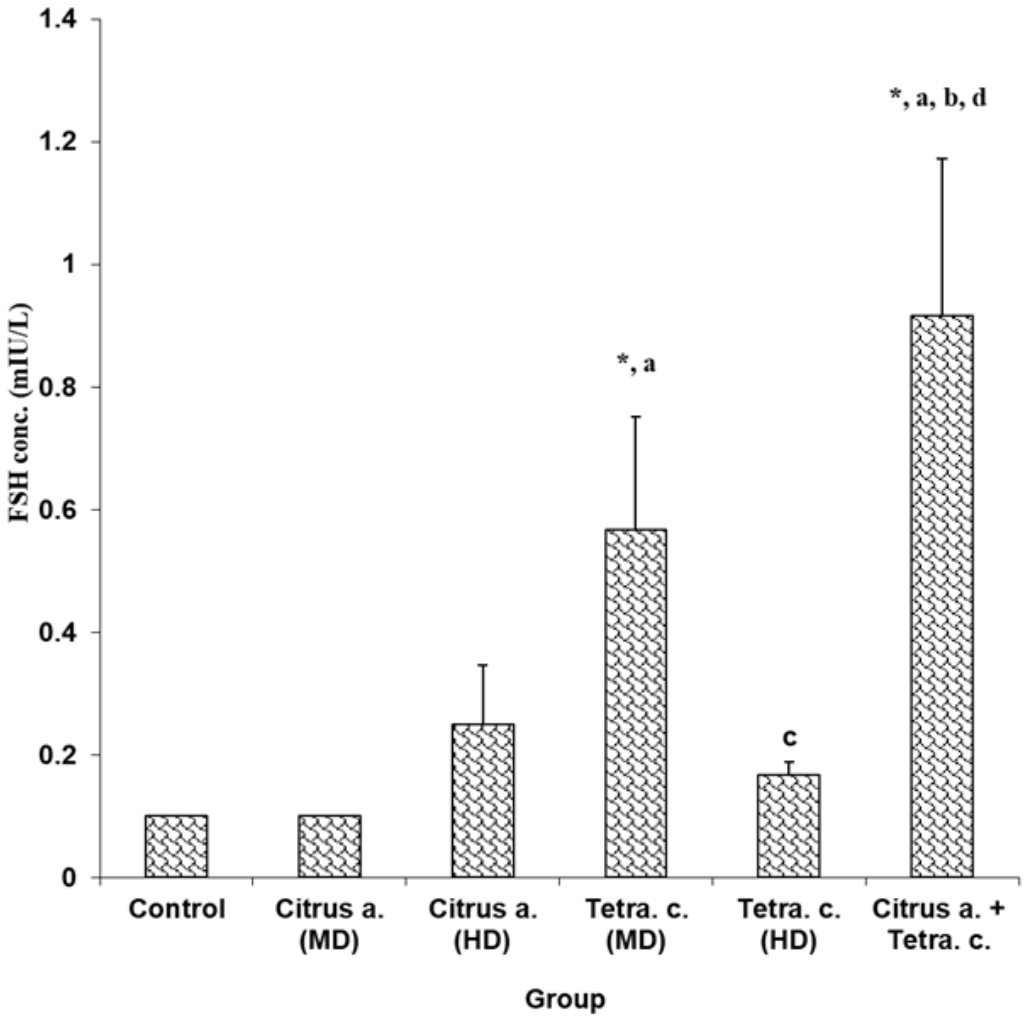

Fig. 2. Comparison of FSH conc. in groups treated with *Citrus aurantifolia* and *Tetracarpidium conophorium*

*Values are mean ± SEM, n = 6. *p<0.05 vs control; a = P<0.05 vs citrus a. (MD); b = P<0.05 vs citrus a. (HD); c = P<0.05 vs T. conophorium (MD); d = P<0.05 vs T. conophorium (HD).*

In experimental subgroup C treated with *Citrus aurantifolia,* LH level was significantly (*P*<0.05) decreased in the medium (0.12 ± 0.02) and high dose group (0.10 ± 0.02) when compared to control (0.18±0.02).

In Fig. 3, LH Level in experimental subgroup E treated with Cimetidine and ascorbic acid, showed significant (p<0.05) decrease in the combined group (0.10±0.00) compared to control (0.18±0.02) and cimetidine medium dose group (0.18±0.02), a significant (*P*<0.05) decrease in cimetidine high dose (0.13±0.02) compared to control (0.18±0.02) and cimetidine medium group (0.18±0.02), a significant (*P*<0.05) decrease in Vitamin C medium dose (0.12±0.02) compared to control (0.18±0.02) and cimetidine medium group (0.18±0.02).

3.3 Comparative Effect of Cimetidine, Vitamin C, Lime and Walnut on Prolactin Levels

In experimental subgroup e treated with cimetidine and ascorbic acid**,** there was a significant (*P*<0.05) increase in Vitamin C high dose group (1.72±0.91) compared to control (0.12±0.02), cimetidine medium (0.17±0.02) and high dose (0.55±0.32) as shown in Fig. 4.

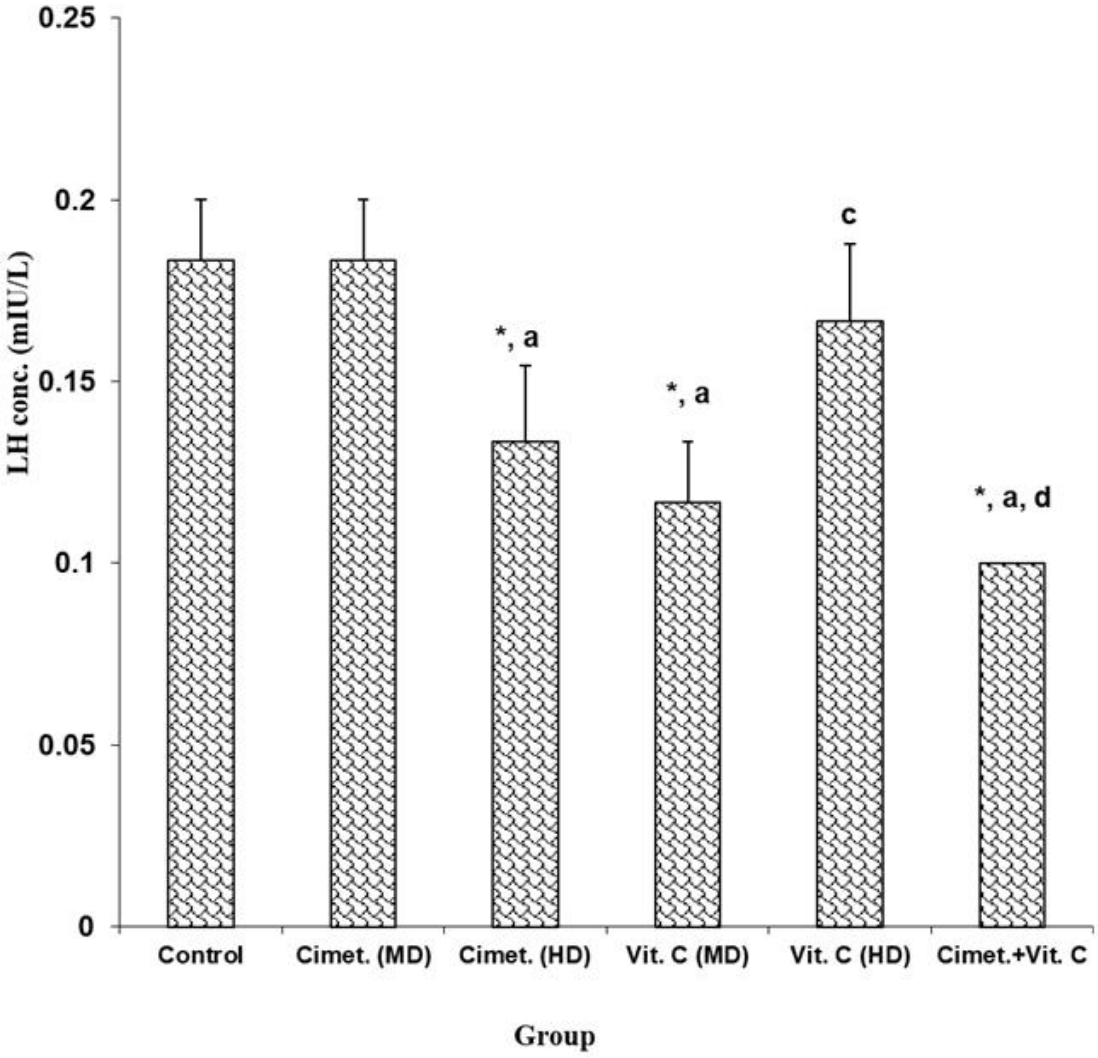

Fig. 3. Comparison of LH conc. in groups treated with ascorbic acid and Cimetidine
*Values are mean ± SEM, n = 6. *P<0.05 vs control; a = P<0.05 vs cimet. (MD); c = P<0.05 vs Vit C (MD); d = P<0.05 vs Vit C (HD)*

3.4 Comparative Effect of Cimetidine, Vitamin C, Lime and Walnut on Testosterone Levels

Testosterone Levels in experimental subgroup B treated with ascorbic acid, was significantly (P<0.05) increased in medium dose group (25.27±7.99) compared to control (6.45±1.10), also a significant (P<0.05) increase was seen in high dose group (30.93±8.12) compared to control sub-group (6.45±1.10) as shown in Fig. 5.

In experimental subgroup C treated with *Citrus aurantifolia,* there was a significant (P<0.05) increase in testosterone level of the medium dose group (28.63± 7.81) compared to control (8.12± 2.01), and a significant (P<0.05) increase in high dose group (22.58±5.21) compared to control (8.12± 2.01) as shown in Fig. 6.

In Fig. 5, testosterone Levels in experimental subgroup E treated with cimetidine and ascorbic Acid, showed a significant (P<0.05) increase in Vitamin C medium dose group (25.18±8.00) compared to control (8.12±2.01) and cimetidine medium dose group (9.77±1.59), a significant increase (P<0.05) in Vitamin C high dose (30.93±8.12) compared to control (8.12±2.01) and cimetidine medium dose (9.77±1.59) was also observed.

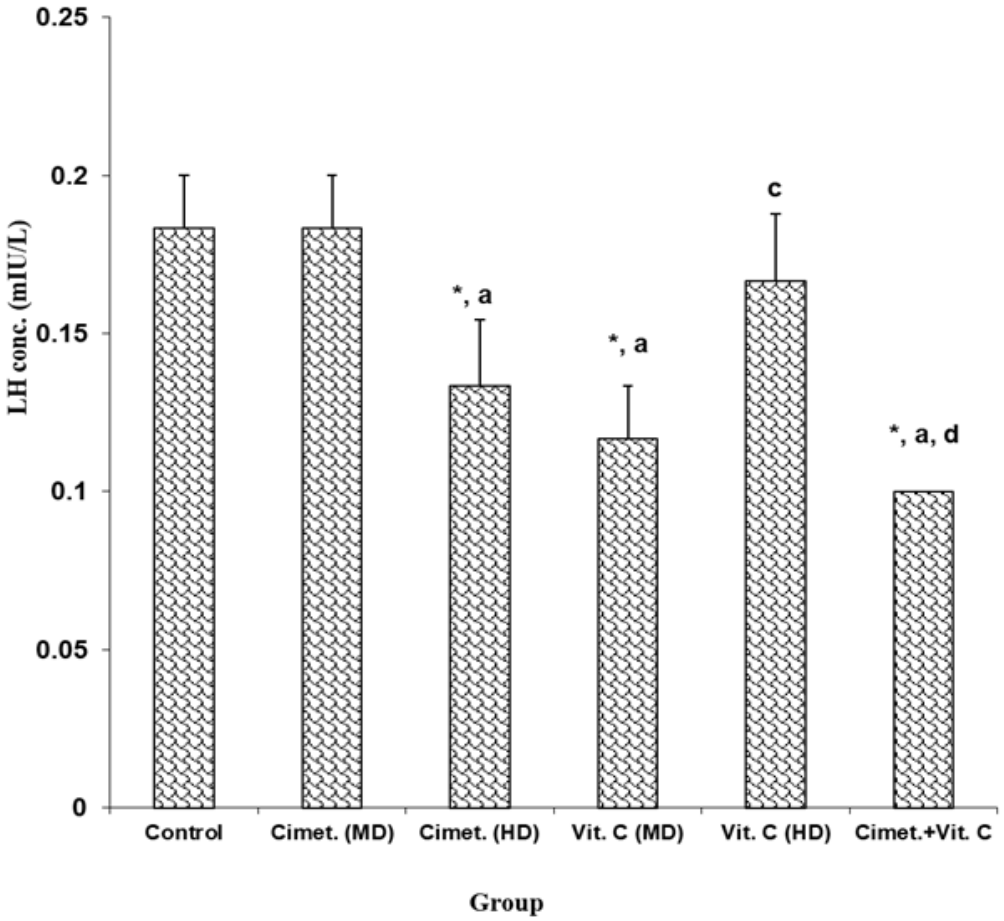

Fig. 4. Comparison of Prolaction conc. in groups treated with ascorbic acid and cimetidine.
*Values are mean ± SEM, n = 6. *p<0.05 vs control; a = P<0.05 vs cimet. (MD); b = P<0.05 vs cimet. (HD); c = P<0.05 vs Vit. C (MD); d = P<0.05 vs Vit. C (HD).*

In experimental group F treated with aqueous extract of *Citrus aurantifolia* and *Tetracarpidium conophorium*, a significant (*P*<0.05) increase in testosterone level was observed in the *Citrus aurantifolia* medium dose group (26.97±8.53) compared to control (8.12±2.01), a dose dependent marginal decrease (*P*<0.05) in *Citrus aurantifolia* high dose group (22.58±5.21), a significant (*P*<0.05) increase in the combined group (31.68±7.76) compared to control (8.12±2.01), *Tetracarpidium conophorium* medium dose group (13.18±5.61) and *Tetracarpidium conophorium* high dose group (13.58±3.05) as shown in Fig. 6.

4. DISCUSSION

Follicle Stimulating Hormone is known to regulate development, growth, pubertal maturation, and reproductive processes of the body. It stimulates primary spermatocytes to undergo the first division of meiosis, to form secondary spermatocytes. It enhances the production of androgen-binding protein by the Sertoli cells of the testes by binding to FSH receptors on their basolateral membranes [19], and it is critical for the initiation of spermatogenesis. When the seminiferous tubules fail to produce sperm, secretion of FSH by the anterior pituitary increases. Conversely, when spermatogenesis proceeds too rapidly, pituitary secretion of FSH diminishes, this occurs as a result of negative feedback on the anterior pituitary by inhibin [20]. In the cimetidine treated group, (experimental sub – groups A) there was significantly elevated FSH levels in the medium dose group. Similar results have been reported by Wang et al. [21] using single blood samples, and others have found no significant change in basal FSH and LH during cimetidine therapy

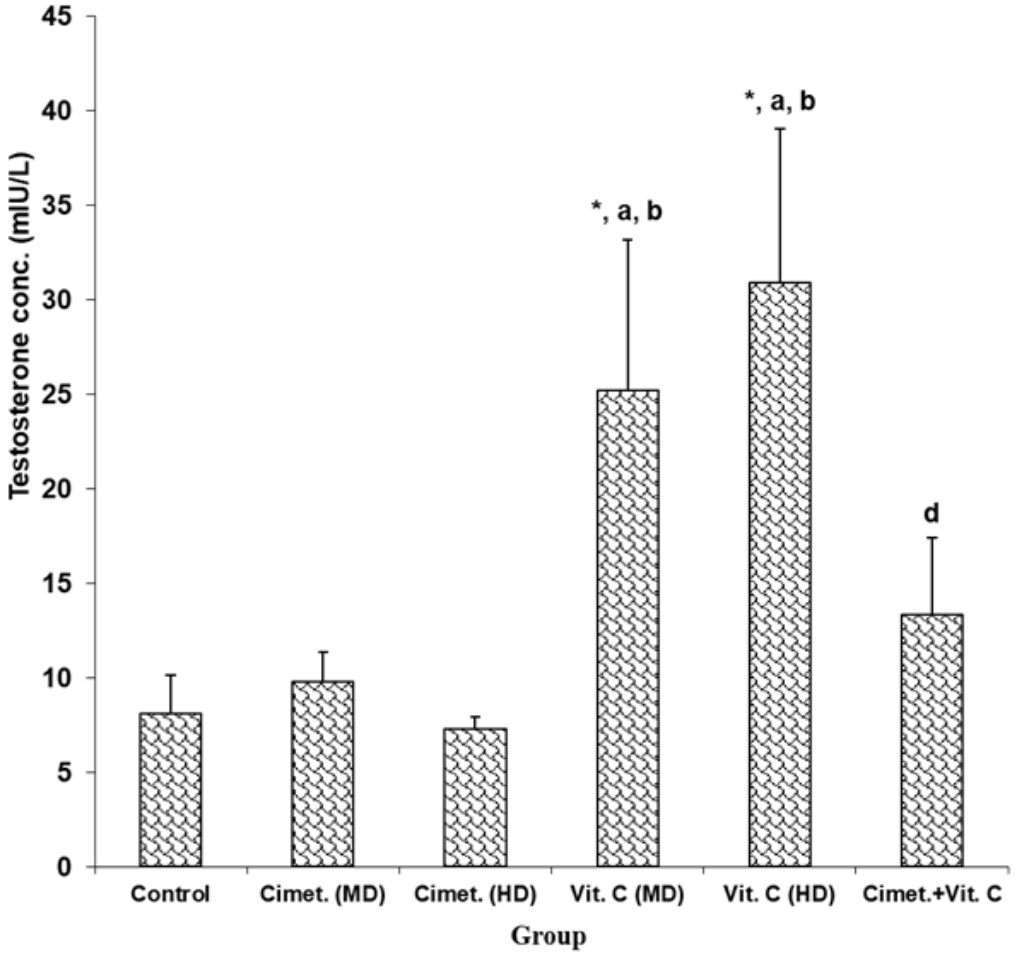

Fig. 5. Comparison of testosterone levels in groups treated with ascorbic acid and cimetidine
*Values are mean ± SEM, n = 6. *p<0.05 vs control; a = P<0.05 vs cimet. (MD); b = P<0.05 vs cimet. (HD); c = P<0.05 vs Vit. C (MD); d = P<0.05 vs Vit. C (HD).*

[22,23,24]. Increase in FSH has been reported to occur in patients with severely impaired spermatogenesis [25,26,27]. Therefore, cimetidine being a weak anti-androgen could be said to have completely prevented FSH from binding to its receptor and as a result, the action of Sertoli cells is inhibited which in turn adversely affects spermatogenesis and thus leading to an elevated serum FSH by negative feedback response.

Treatment with ascorbic acid (Vit.C) increased the FSH levels significantly in the animals as also reported by [28] and this could be as a result of the effect of ascorbic acid on the gonadotropes to improve fertility. Another possible cause for the increase in FSH at high dose could be as a result of reduced sperm concentration in the semen due to the failure of the seminiferous tubules to produce sperm. Vitamin C has long been associated with fertility and considered a major antioxidant in the testis. This vitamin is an essential water-soluble micronutrient required for an array of biological functions. It is unstable, easily oxidized acid and can be destroyed by oxygen, alkali and high temperature [29].

C. aurantifolia is said to have an anti-fertility effect. In experimental sub – Groups C, a marginal increase in the FSH level was observed in the lime treated group**,** which is in line with a report by Rosen and Weontral [25], Leonard et al [26] and De-krester et al. [27] where increase in FSH concentration occurs in patients with impaired spermatogenesis. However, this seems to contradict a report by Bakare et al. [30], where there was a significant decrease in FSH level in female rats, in turn reducing follicular

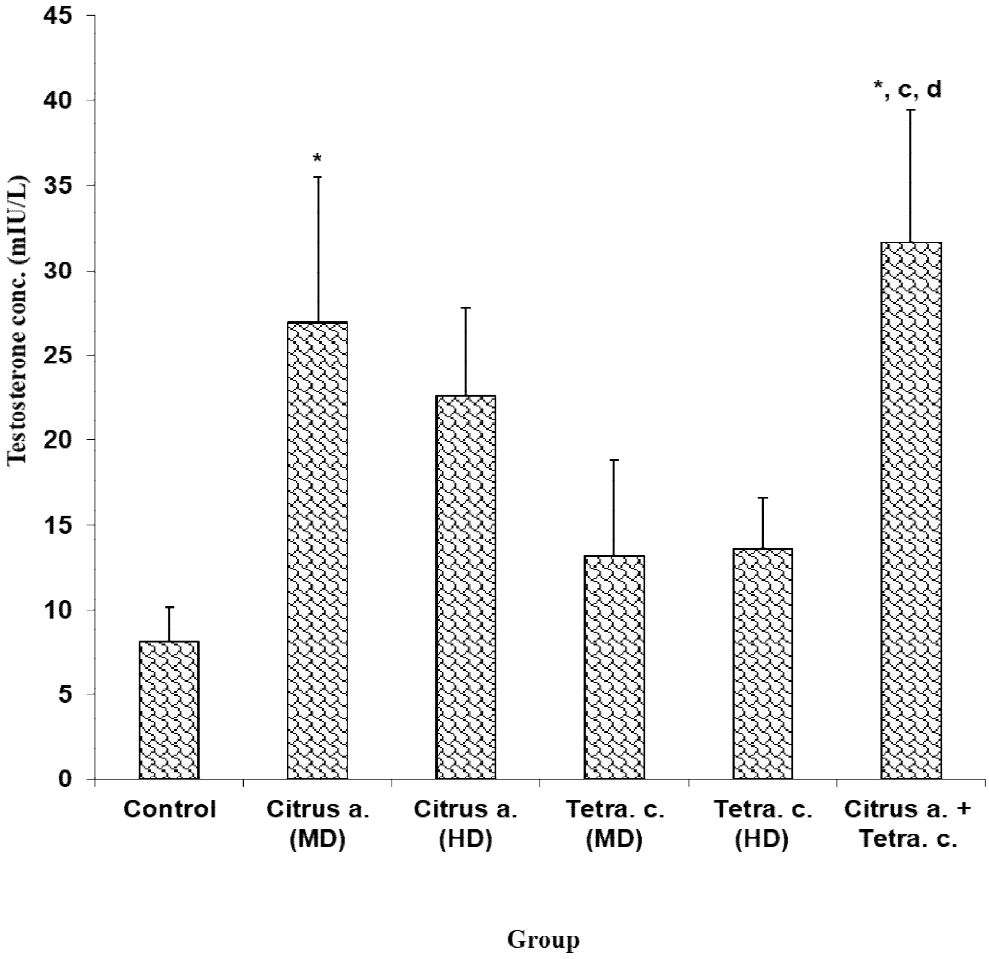

Fig. 6. Comparison of testosterone conc. in groups treated with *Citrus aurantifolia* and *Tetracapidium conophorium*

*Values are mean ± SEM, n = 6. *p<0.05 vs control; c = P<0.05 vs tetra c (MD); d = P<0.05 vs tetra c (HD)*

development and ovulation, on the application of lime juice. This could indicate that lime juice effect on FSH level could be sex dependent.

In the *T. conophorium* treated group (experimental subgroup D), a significant increase in FSH level was observed. It is interesting to mention the fact that the seeds of walnut contain important bioactive component [31] whose effect can synergistically or singly enhance the biosynthetic processes underlying hormonal production. This presupposes that the said bioactive components in the seeds might have probably enhanced FSH in the rats.

Also, the elevated FSH level observed may be due to a disruption in the spermatogenic process thus leading to decrease in sperm production [32], an imbalance in gonadal-pituitary feedback mechanism or may represent a hormone of reduced biological activity [33].

The combined group of Cimetidine and Vitamin C showed a significant increase in FSH level. The increase was compared to cimetidine medium dose and high dose group and Vitamin C medium dose group in Fig. 1. It can be deduced that both drug acted synergistically to increase FSH. The marginal increase of FSH observed in the Vitamin C high dose group compared to the other single dose groups suggests that the FSH increment had more to do with the Vitamin C high dose group, as such, exerts its effect on the gonadotropes as shown in Fig. 1.

The elevated FSH level observed in the combined group of lime and walnut compared to lime medium and high dose group also shows

that the elevation in FSH level had more to do with the walnut as seen by a significant increase in walnut medium dose in Fig. 2. This increase in FSH could be as a result of impaired spermatogenesis or an imbalance in the gonadal-pituitary feedback mechanism.

The primary role of Luteinizing Hormone in the male is to stimulate the production of testosterone by the Leydig cells. From this study, Cimetidine group showed no significant difference. It has been reported that using either integrated LH or a mean LH determined from multiple samples generally would provide a more accurate reflection of the secretion than single LH measurements [34]. An alternative explanation for the rise of FSH without an increase in LH could be related to the slight inhibition of spermatogenesis [35].

Studies have shown that the concentration of ascorbic acid in seminal plasma directly reflects dietary intake, and lower levels of vitamin C may lead to infertility and increased damage to the sperm's genetic material [36]. Fraga et al. [37] demonstrated this by reducing ascorbic acid intake in healthy men from 250 mg to 5 mg per day. Seminal plasma levels of vitamin C decreased by 50 %, with a concomitant 91% increase in sperm with DNA damage. Treatment with Vitamin C and Lime showed a significant decrease in LH level in experimental subgroups 2 and 3. LH significant decrease is a reverse of what happened in FSH level, in which both are gonadotropins released from the gonadotrophs of the anterior pituitary when stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. The decrease in LH could be as a result of a negative feedback on the anterior pituitary caused by increase in testosterone has observed in this study [20].

For *T. conophorium* treated group, LH increased insignificantly in both medium and high dose group. Isolated elevation of LH level may suggest the presence of a cross reacting substance such as hCG [38]. Increase LH level in medium and high dose groups compared to the control group may be due to the presence of hCG or a mutation of the LH receptor gene as reported by Sulthan et al [39]. In addition to that, LH has a prolonged half-life, increase serum level of LH may be due to its prolonged half-life. Also, the hyperactivity of the LH axis leading to the LH induced infertility could be due to mutation of the LH receptor gene [39].

The significantly decreased LH level observed in the combined group of cimetidine and Vitamin C. compared to the marginal increase observed in cimetidine medium dose group might have resulted from the inhibitory effect of testosterone on the pituitary as shown in Fig. 3. This can be a confirmation of a synergic action between cimetidine and Vitamin C.

A marginal increase was observed in prolactin levels of both medium and high dose cimetidine group compared to control, however, there was no significant difference observed as shown in Fig. 4. With inference on the duration of cimetidine oriented therapy, it is generally observed that in most cases treatments last for more than a month, but this research work only lasted for 21 days. It is, therefore, possible that with a long duration of treatment, prolactin level will have attained a significant peak. Unusually highly elevated levels are suspected to be responsible for decreased level of testosterone, impotence and loss of libido in men while it decreases the levels of estrogen in women [40]. The non-uniformity of response after oral cimetidine can account for the discrepancies in prolactin levels reported in previous studies [41, 42, 43, 44]. The mechanism of action of cimetidine on Prolactin secretion is unknown, although it probably acts through mechanisms other than pituitary dopamine receptors [41]. Alternatively, it has also been found that compared to un-mated males, fathers and expectant fathers have increased prolactin concentrations [45].

In the combined group treated with cimetidine and Vitamin C, a significant increase in Prolactin level was observed in Vitamin C high dose group compared to cimetidine group as shown in Fig. 4. The result shows that the increased prolactin had more to do with the action of Vitamin C on the gonadotropes. Melmed et al. [46] reported that Prolactin levels peak during REM sleep and in the early morning, and can rise after exercise, meals, sexual intercourse, or minor surgical procedures. This could be another reason for the increased prolactin level observed.

Prolactin is mostly found in nursing mothers in large quantities because of its role in lactation. Its action or contributions in spermatogenesis or testosterone secretion have not yet been fully understood. In men, high serum Prolactin concentrations decrease gonadotropin secretion, thus decrease testicular function resulting in low serum testosterone concentrations. The major

symptoms are loss of sexual desire, erectile dysfunction, muscle weakness, and infertility. Highly elevated levels of prolactin decrease the levels of sex hormones — estrogen in women and testosterone in men [40]. Prolactin acts in a cytokine-like manner and acts as an important regulator of the immune system. Prolactin has important cell cycle related functions as a growth-, differentiating- and anti-apoptotic factor [47]. As a growth factor binding to cytokine like receptors, it has also profound influence on haematopoiesis, angiogenesis and is involved in the regulation of blood clotting through several pathways [48].

In this study, cimetidine group showed a marginal increase in testosterone level in the medium dose and high dose group compared to control as shown in Fig. 5, which confirms similar results reported by Van-Thiel et al. [22] and Wang et al. [21]. Cimetidine being a weak anti-androgen [49] agent causes an increase in gonadotropin levels presumably by antagonizing the negative feedback control of gonadotropin secretion by androgens subsequent to increase in gonadotropin levels, elevations in testosterone concentration may occur [50]. A second possible mechanism of cimetidine may be the result of chronic antagonism of H2 receptors, vascular smooth muscle cells of the body are generally known to be H2 responsive [51]. And we believe those of the testis is likewise responsive although a report describing that peritubular cells possess H2 receptors have not been confirmed. Apoptotic cell activities likely took place at the vascular smooth muscles of the testis on the administration of cimetidine at the high dose as seen in the marginal decrease in testosterone level in the high dose group.

In Fig. 5 the ascorbic acid group, testosterone levels increased significantly in the experimental sub-groups, probably because of the effect of LH on the Leydig cells of the testes which caused an increase in the rate of conversion of cholesterol to pregnenolone and subsequently testosterone.

In the *C. aurantifolia* treated group, testosterone level showed a significant increase as presented in Fig. 6 In this study, LH significantly decreased and since LH stimulates the Leydig cells to release testosterone, it could, therefore, be said that the significant increase in testosterone had nothing to do with LH stimulation. The increase in testosterone level could be said to be attributed to the effect of alkaloid, a component of lime juice as previously reported by Ikpeme et al. [52] that alkaloids are known as the starting material in the manufacture of steroid hormones. This indicates that the increase in testosterone level might be triggered by alkaloids, thus could be a result of its effect on the synthetic pathway of testosterone.

In the combined group of cimetidine and ascorbic acid, the decreased testosterone level observed in the combined group could be as a result of the significantly reduced LH since it is the primary stimulus for the secretion of testosterone as shown in Fig. 5. Comparing this result to Vitamin C high dose group, it shows that the decrease had more to do with cimetidine rather than Vitamin C as shown in Fig. 5. The significant increase observed in testosterone of Vitamin C medium and high dose group compared to cimetidine medium dose group confirms that Vitamin C promotes fertility. A significantly increased testosterone level was observed in the combined group of *C. aurantifolia* and *T. conophorium* compared to lime medium and high dose group shows that the increment can be attributed to as confirmed by a significant increase in *C. aurantifolia* medium dose group which could be an effect of alkaloid present in *C. aurantifolia* as shown in Fig. 6.

5. CONCLUSION

The present study showed that Cimetidine significantly reduced FSH concentration. Ascorbic acid significantly increased FSH and testosterone concentration, significantly decreased LH level. *C. aurantifolia* significantly reduced LH levels and significantly increased testosterone levels. Walnut significantly increased FSH level. Combination treatment of Cimetidine and Vitamin C significantly increased FSH concentration, significantly decreased LH level. Combination treatment of lime and walnut significantly increased FSH and significantly increased testosterone level.

The following conclusions can be drawn from the observations made in the present study

I. Cimetidine causes adverse effects on male fertility through the inhibition of FSH from binding to the FSH receptor, which adversely affects the process of spermatogenesis.
II. Ascorbic acid promotes fertility.
III. *C. aurantifolia* has a dose dependent effect. At high doses, it severely affects fertility negatively having a mild effect at medium doses.

IV. *T. conophorium* boost fertility.
V. Combination of cimetidine and Ascorbic acid cannot completely remedy the effect of cimetidine on fertility.
VI. *T. conophorium* promotes fertility while *C. aurantifolia* does not, however combination of both *C. aurantifolia* and *T. conophorium* shows a synergic action thus boosting fertility.

CONSENT

It is not applicable.

ETHICAL APPROVAL

"All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX I

DOSAGE DESIGN

Medium dose:

$$\frac{20}{100} \times \frac{5000 \text{ mg/kg body weight}}{1} = 1000 \text{ mg/kg body weight}$$

High dose:

$$\frac{30}{100} \times \frac{5000 \text{ mg/kg body weight}}{1} = 1500 \text{ mg/kg body weight}$$

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