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Research Paper

Effects of Ethanol Extract of *Chrysophyllum albidum* Fruit Pulp and juice on Female Reproductive Hormones.

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Abstract
Imbalance in the hormonal level of the female reproductive system may lead to hormonal disorder thereby causing infertility with numerous implications including psychological, physical, mental, spiritual, and medical effects to the patient. Drugs used to treat fertility problems caused by ovarian disorder has varying level of toxicities and therefore the need for alternative botanical source that is therapeutically effective with little or no toxicity. Therefore, this present research evaluated the phytochemical, acute toxicity and the effects of ethanolic fruit pulp extract and juice of C. albidum on the reproductive hormone levels of female rats. The result of the findings showed that the LD_{50} is above 500mg/kg. The findings also showed that the fruit pulp contained alkaloids, flavonoids, saponins, tannins, steroids, terpenoids while phenols were absent. The result of the study showed further that when C. albidum extract and juice was administered to female albino rats there was significant ($p<0.05$) reduction in the blood concentration of the luteinizing hormone and a significant increase in the blood concentration of the follicle stimulating hormone among the pregnant female albino rats except the group that was administered with 1000mg/kg of the juice that had no significant increase in the blood follicle stimulating hormone level and this implied that C. albidum extract and juice has a potentiating effect on the follicular development which may aid in the development and release the matured oocyte. However, there was no significant effect was noticed when the extract and juice of C. albidum was administered to non-pregnant female albino rats, although slight fluctuations were recorded. The findings suggest C. albidum extract may have triggered low frequency pulsation of gonadotropin-releasing hormone (GnRH) which stimulate the secretion and development of the follicle among the pregnant albino rats and this could have enhanced fertility effect among the pregnant subjects and this therefore, supports its usage by pregnant women of the south East Nigeria.

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I. Background

Female Reproductive systems are regulated by cascades of hormones. Imbalances in these hormonal levels may lead to hormonal disorder thereby causing infertility. Infertility has huge public health concern because it can cause psychological, physical, mental, spiritual, and other medical problems to a patient [1], hence, affect both the patient and the patient's partner and sometimes lead to divorce. Among causes of infertility, ovulatory disorder marked by suppression in follicular development caused by reduction in secretion of follicle stimulating hormone and luteinizing hormone account for about 25% of fertility problems in women, which implies that it is the leading cause of most infertility in women [1]. Ovulatory disorder also called oligo-ovulation or anovulation results in infertility due to non-secretion of the monthly oocyte and absence of an

oocyte leads to infertility. Hypothalamic amenorrhea caused by ovarian disorder results in a decrease in hypothalamic GnRH secretion [2]. This results in a decrease in the release of gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) from the anterior pituitary gland thereby causing abnormal follicle growth, anovulation, and low estrogen levels [3]. Drugs used to treat fertility problems caused by ovarian disorder have varying levels of toxicity. There are reports that the use of clomifene therapy causes nausea, vomiting, vasomotor flushes, visual blurring, spots or flashes, scotomata, ovarian enlargement with pelvic or abdominal pain [4]. Therefore, there is need to search for alternative source of fertility drugs that are botanical based that has strong therapeutic potency against infertility with little or no side effect. Numerous plants have been implicated and tagged effective for treatment of infertility in women. Plants such as *Lepidium meyenii* (maca), *Trifolium pretense* (red clover), *Dioscorea villosa* and *F. platyphylla* (wild yam) have all been folklorically and scientifically implicated in treatment of fertility in Nigeria [5] particularly the northern part of Nigeria.

The fruit of African star apple also called *Chrysophyllum albidum* is widely consumed by pregnant women in South Eastern part of Nigeria among other tribes. *Chrysophyllum albidum* is a forest fruit tree usually common to the tropical Africa. Folklorically, there is a general understanding/believe that this fruit generously consumed by pregnant women aid pregnancy development. Therefore, there is urgent need to determine the relationship between consumption of the fruit pulp of *C. albidum* and female reproductive hormones. Various parts of *C. albidum* have been shown to have different therapeutic potential for different public health disease conditions. Also, different researchers have investigated and reported different activities using different parts of the plants. Instances include [6] reported that the root back of ethanol extract of *C. albidum* lowered serum testosterone and Gonadotrophins concentration and therefore may lead to infertility in Men. The ethanol leaf extract of *C. albidum* was reported to have decreased hormonal profile [6]. Similarly, reduction in testosterone and gonadotrophins concentration using the ethanol extract root bark of C. albidum was reported by [7]. Furthermore, the antimalarial potential of the juice and seed of *C. albidum* among pregnant subjects was reported by researchers who suggested that the fruit pulp can be used as intermittent preventive therapy against malaria for pregnant subjects [8]. In the light of the above discussion, this present research investigated the effects of ethanolic fruit pulp extract and juice of *C. albidum* on the hormonal levels of female rats.

II. Materials And Methods

Materials

The materials used in this research were of analytical grade and they includes: Plant sample, ethanol, rotary evaporator, No.1 whatman filter paper, refrigerator, healthy female albino rats, surgical blade, clomiphene (Clomid, USA), levonorgestrel (Postinor 2, Hungary), centrifuge machine, ELISA kit (Accubind), test tubes, beakers and measuring cylinders.

Methods

Plant extraction

Fresh pulp of *Chrysophylum albidum* was collected by carefully removing the epicarp and seed. The pulp was meshed using mortar and pestle. **Four hundred grams** of the meshed sample was macerated in 2 Litres of ethanol for 72hrs. The mixture was sieved with muslin cloth, and filtered with No.1 whatman filter paper. The extract was concentrated using rotary evaporator at reduced temperature of 20 ^{o}C and 150 pressure. It was further dried using water bath at 40^{0}C. The extract was stored in the fridge until required for use.

Phytochemical screening of the plant

The phytochemical screenings were done according to [9-12]. The extract was dissolved in ethanol and used for the phytochemical test as follows:

Test for saponin :

About 5ml of the extract solution was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth.

Test for steroids :

This was done using the method of Liebermann-Burchard (1946). Three mils (3 ml) of the extract was mixed with 3 ml of chloroform. Thereafter, 1–2 ml of acetic anhydride and 2 drops of concentrated H_2SO_4 from the side of test tube were added. The presence of steroid was indicated by colour changes to red, followed by blue, and finally green.

Test for flavonoids

Alkaline reagent test was used. Two mils (2 ml) of the test solution was treated with 2 ml of sodium hydroxide solution, which showed intense yellow colour that became colorless on addition of few drops of dilute hydrochloric acid . That showed the presence of flavonoids

T**est for terpenoids (Salkowski test):**
Salkowski test was used to detect terpenoids. Extract (5 ml) was mixed with chloroform (2 ml), and concentrated sulphuric acid (3 ml) was carefully added to form a layer. A reddish-brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Test for cardiac glycosides (Keller-Killani test):
Using Keller-Killani test, 3 ml of the extract was treated with 1ml of glacial acetic acid and one drop of 5 % ferric chloride solution ($FeCl_3$). This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicated deoxy sugar, a characteristic of cardenolides. A violet ring appeared below the brown ring, while in the acetic acid layer, a greenish ring formed just gradually throughout thin layer.

Reducing Sugar Test
Fehling's test was used. In a test tube 1 ml of Fehling's A and 1 ml of Fehling's B solutions were added and mixed together. The mixture was boiled for 1 min. Thereafter, equal volume (2 ml) of the test solution was added and the entire test tube contents was heated in boiling water bath for 5 min and observed for yellow and later brick red precipitate that indicated the presence of reducing sugar.

Acute toxicity test
The LD_{50} was carried out according to the method of Dietrich Lorke [13] as reported in [14]. A total of 13 mice was used. The study was done in two phases. Phase I: Here, total of nine mice were used and the crude extract were administered orally. They were grouped into three groups of three mice each. Group 1 was given 10 mg/kg extract of *Chrysophylum albidum* Group 2 received 100 mg/kg extract of *Chrysophylum albidum* and group 3 received 1000 mg/kg of the extract. The animals were constantly monitored for the next 1 hour, intermittently for the next 3 hours and finally, 24 hours post treatment for behavioral changes and mortality and no death was recorded at 1000 mg/kg. In this phase, total of four mice were used. They were grouped into four of one each. Mouse in group 1, 2, 3 and 4 were given 1200, 1600, 2900 and 5000 mg/kg body weight, respectively of the extract
combined with honey.

Hormonal level determination for non-pregnant rats

Forty (40) adult non-pregnant female rats were used in this study according to the method of Obi C.C et al., 2018 with slight modifications. The rats were grouped into eight (8) groups of five rats each. Group one served as the negative control and were given 1ml/kg distilled water. Group two and three served as positive control and were given clomiphene and levonorgestrel respectively. Group four were given 50mg/kg of the extract while animals in groups 5, 6, 7 and 8 received 150mg/kg, 300mg/kg, 500mg/kg and 1000mg/kg of the extract respectively. The animals were treated for 28 days. Their blood samples were collected through ocular puncture in a plain tube. The blood serum was centrifuged for 10 minutes at 4000rpm and analysed for FSH and LH levels using ELISA kit (Accubind).

Hormonal level determination for pregnant rats

Sixty(60) adult albino rats were used in this study according to the modified method of Obi C.C et al., 2018. Pregnancy was induced in sixty adult female rats by bringing them to estrous after exposing them to male rats for three days at the ratio of three female to one male rat after which the animals were now allowed to mate. Blood samples were collected from the animals after the exposure to males, and tested for positive indication of pregnancy using pregnancy test strips. The pregnant rats were grouped into eight (8) of five rats per group. Group one served as negative control and were given 10ml/kg distilled water orally. Group two and three served as positive control and were given clomiphene and levonorgestrel respectively. Group four were given 50mg/kg of the extract orally, and groups 5, 6, 7 and 8 were given 150mg/kg, 300mg/kg, 500mg/kg and 1000mg/kg of the extract respectively. The animals were treated for 28 days. Their blood samples collected through ocular puncture in a plain tube. The blood serum was centrifuged for 10 mins at 4000rpm. The serum was an analysed for FSH and LH level using ELISA kit by bringing the serum and reference calibrators to room temperature after which the microplate wells were formatted for each serum reference calibration, control and test sample specimen to be assayed in duplicates. Then 50ul of the appropriate serum reference calibrator and sample specimen was pipetted into its assigned well and 100ul of hormone (FSH or LH) enzyme reagent solution was added to all the wells. The microplate was swirled gently for 30seconds for proper mix, covered and incubated for 60minutes at room temperature. The content of the microplate was discarded and blotted dry with absorbent paper, then 350ul of wash buffer was added and was decanted. This was repeated three times, then 100ul of working substrate solution was added to all the wells. They were incubated at room temperature for 15minutes, 50ul of stop solution was added to each well and gently mixed for 20seconds. The absorbance in each well was read at 450nm in a microplate reader.

Statistical analysis

Data obtained from the study was analyzed using Statistical ~~Packing~~ Package for Social Solution (SPSS-21). Results were expressed as mean ± SEM. Raw data were subjected to one-way analyses of variances (ANOVA) followed by Turkey's post hoc test and mean with P< 0.05 was considered statistically significant

III. RESULTS

Qualitative Phytochemical constituents of *Chrysophyllum albidum* PULP EXTRACT

The result of qualitative phytochemical constituents of *C. albidum* is presented in Table 1. The findings showed that *C. albidum* fruit pulp contained alkaloids, flavonoids, saponins, tannins, steroids, terpenoids while phenols were absent.

Table 1: Phytochemical Constituents of the fruit pulp of *C. albidum*

	Phytochemicals	Crude extract
1	Saponins	+ + +
2	Tannins	+++
3	Reducing Sugars	+
4	Flavonoids	+++
5	Alkaloids	+++
6	Cardiac Glycosides	+++
7	Terpenoids	++
8	Phenol	-

(-) => Not Present, (+) => Present in small concentration, (++) => Present in moderately high concentration, (+++) => Present in high concentration.

Acute toxicity (LD_{50}) profile of *C. Albidum*

The result of the LD_{50} ethanol extract of *C. albidum* pulp was above 5000mg/kg as no death was recorded at the highest dose after 24hours monitoring. There was no sign of weakness, anorexia, or restlessness observed within 24 hours monitoring which implied that *C. albidum* is relatively safe for consumption when consumed orally at acute level.

Effect of *C. albidum* fruit pulp on Luteinizing hormone of pregnant albino rats

The result of the effect of *C. albidum* fruit pulp on hormonal level of pregnant female albino rats are presented in Figure 1.0. The findings showed a significant decrease ($p<0.05$) in the level luteinizing hormone among the pregnant rats administered with ~~of~~ 0.33mg/kg of Levonorgesterol while administration of 55.18mg/kg of Clomifen to the pregnant rats showed a non-significant ($p>0.05$) decrease in the level of luteinizing hormone when compared to baseline study respectively. The findings further showed that there was no significant ($p<0.05$) change (decrease) in the level of the luteinizing hormone when the *C. albidum* juice and *C. albidum* ethanol extract were administered at 50mg/kg and 150mg/kg to the pregnant rats respectively when compared to baseline. However, there was a significant ($p<0.05$) decrease in the level luteinizing hormone among pregnant rats in the groups administered with the *C. albidum* juice and *C. albidum* ethanol extract administered at 300 mg/kg, 500 mg/kg, and 1000 mg/kg respectively when compared to baseline. The finding further showed that there was no significant effect in the level of luteinizing hormone of the pregnant rats in the control group that was administered distilled water only. These findings showed a dose dependent effect of the luteinizing hormone of female albino rats.

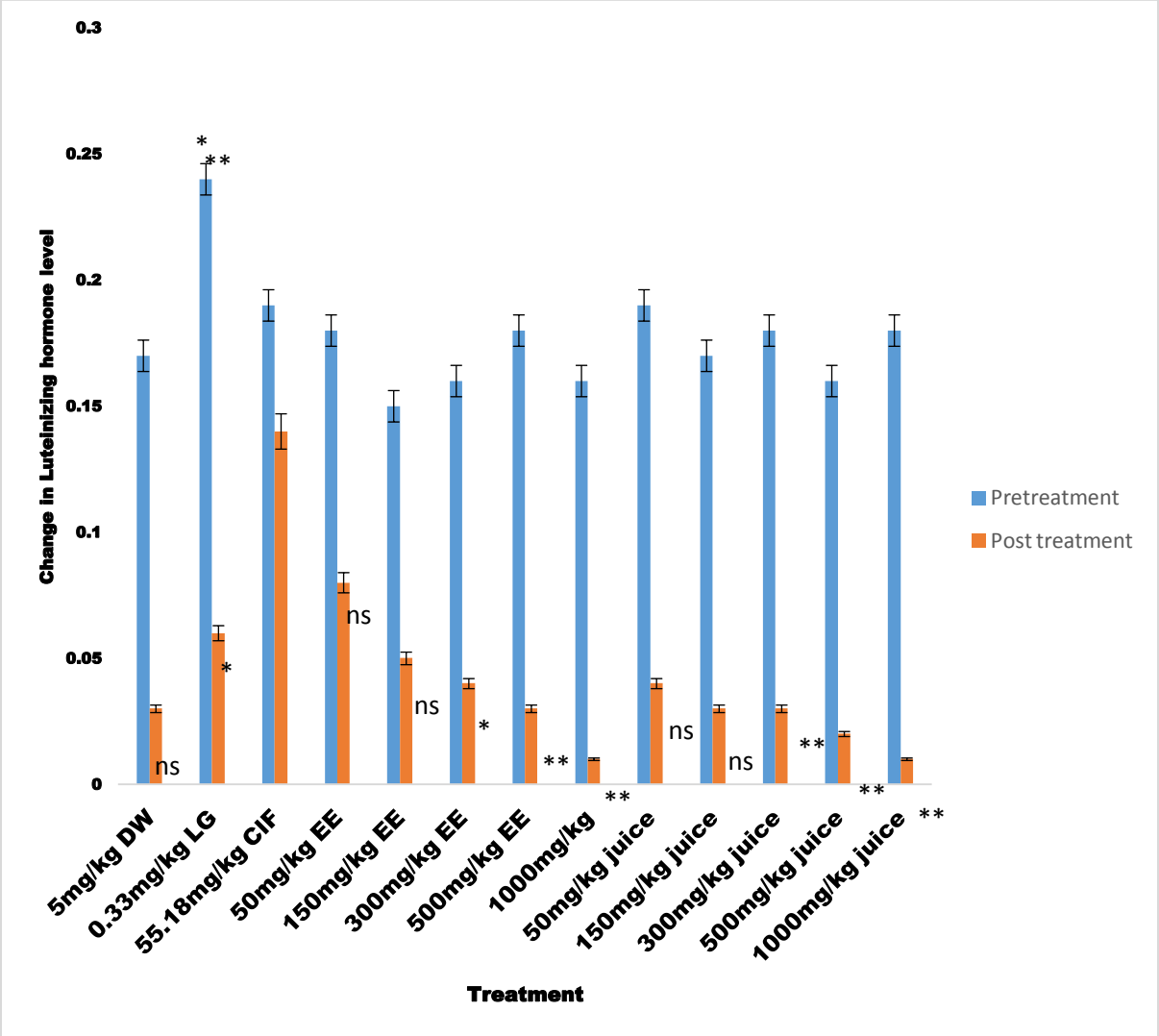

Fig 1: Effect of *C. albidum* luteinizing hormone level in pregnant rats (Note: DW = distilled water, LG= Levonorgesterol, EE = ethanol extract, CIF =Clomifene).

Effect of *C. albidum* fruit pulp on follicle stimulating hormone (FSH) of pregnant Albino rats

The result of the effect of *C. albidum* on follicle stimulating hormone of pregnant female albino rats is presented in Figure 2. The result showed a dose dependent change in the level of follicle stimulating hormones across the treatments except the pregnant rats treated the *C. albidum* juice at 1000mg/kg. The result findings further showed a significant decrease ($p<0.05$) in the level follicle stimulating hormone in the pregnant rats administered with ~~of~~ 0.33mg/kg of Levonorgesterol while administration of 55.18mg/kg of Clomifene showed a significant ($p>0.05$) increase in the level of follicle stimulating hormone when compared to baseline study respectively. Also, the findings further showed significant ($p<0.05$) increase in the level of follicle stimulating hormone of rats administered with both the C. *albidum* juice and *C. albidum* ethanol extract administered at 50, 150, 300, 500, and 1000 mg/kg respectively when compared to baseline except the groups administered with C. *albidum* juice at 1000mg/kg which showed increase that was not significant when compared with the baseline. The finding further showed that there was no significant effect in the level of follicle stimulating hormone of the pregnant rats in the control group that was administered distilled water only.

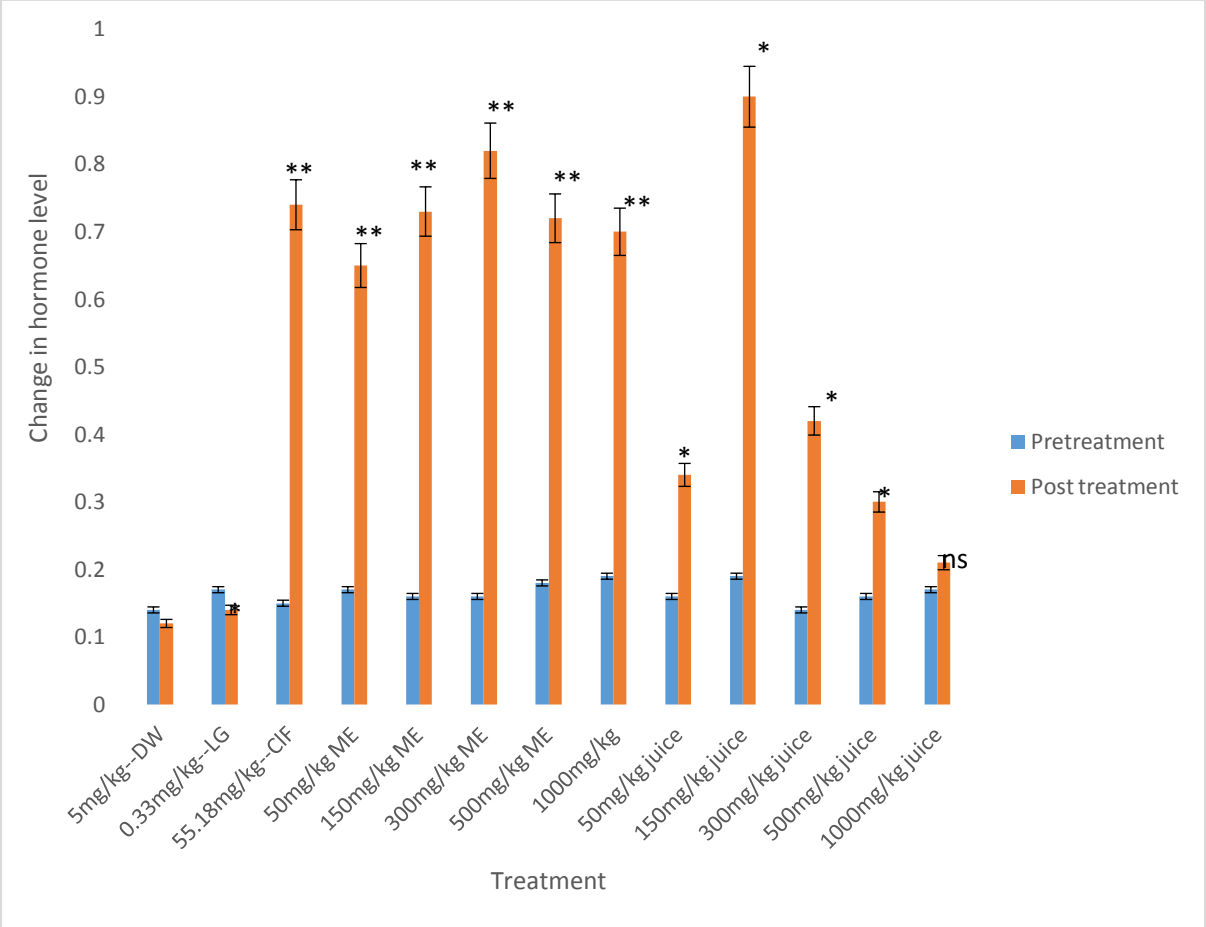

Fig 2: Effect of *C. albidum* on follicle stimulating hormone in pregnant rats (FSH). (Note: DW = distilled water, LG= Levonorgesterol, EE = ethanol extract, CIF =Clomifene).

Effect of *C. albidum* fruit pulp on Luteinizing hormone (FSH) of non-pregnant Albino rats

The result of the effect of *C. albidum* on luteinizing hormone of non-pregnant female albino rats is presented in Figure 3. The result showed a dose dependent decrease in the level of luteinizing hormone across the treatments excepts the non-pregnant rats treated with the *C. albidum* juice and extract at 50mg/kg respectively. The result findings further showed a significant increase ($p<0.05$) in the luteinizing hormone among the non-pregnant rats administered with 55.18mg/kg of Clomifene while the non-pregnant rats administered with 0.33mg/kg of Levonorgesterol showed a significant ($p>0.05$) decrease in the level of luteinizing hormone when compared to baseline study respectively. However, at 50 mg/kg of the ethanol extract and juice of *C. albidum* respectively, there was a non-significant (*$p>0.05$*) increase in the post-treatment group compared to baseline study while at 100, and 150 mg/kg there was a non-significant ($p>0.05$) decrease in the level of luteinizing hormone when compared with the baseline before treatment. There was a significant reduction of luteinizing hormones among the rats treated with 300, 500 and 1000 mg/kg of the ethanol extract and juice of *C. albidum* respectively when compared with the baseline.

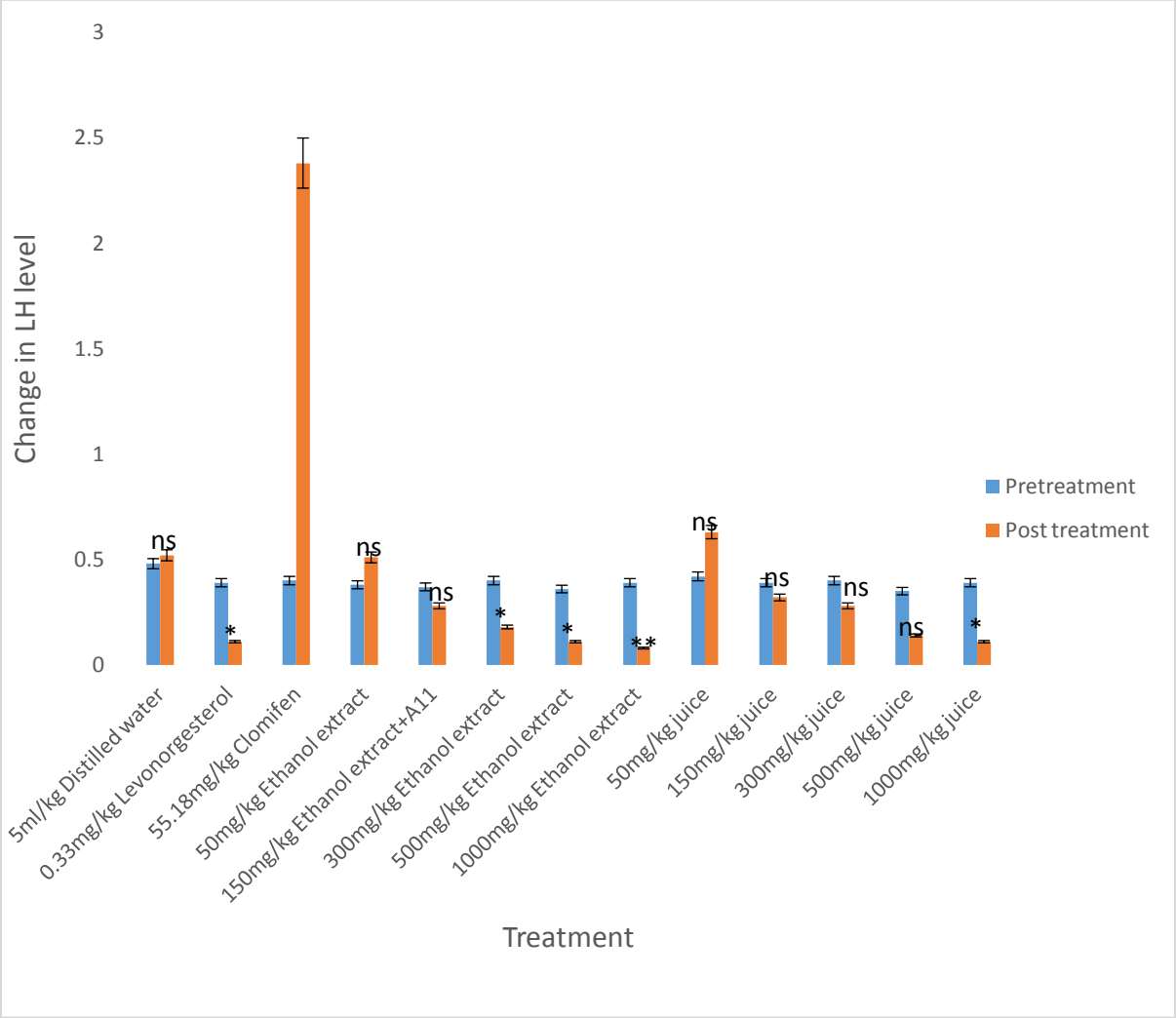

Fig 3: Effect of *C. albidum* pulp ethanol extract and juice on Luteinizing hormone of non-pregnant rats (LH) (Note: DW = distilled water, LG= Levonorgesterol, EE = ethanol extract, CIF =Clomifene).

Effect of *C. albidum* fruit pulp extract and juice on follicle stimulating hormone (FSH) of non-pregnant Albino rats

The result of the effect of the C. *albidum* pulp extract and juice on follicle stimulating hormone of rats is presented in Table 4.0.

The result showed that the non-pregnant rats in the control group had a non-significant ($p>0.05$) increase in the level of follicle stimulating hormone when compared to base line while at 0.33mg/kg Levonorgesterol there was a non-significant ($p>0.05$) decrease in follicle stimulating hormone when compared to the baseline. A highly significant increase ($p<0.05$) in the follicle stimulating hormone level was seen in the post-treatment group administered with 55.18mg/kg Clomifene when compared with the control. However, when ethanol extract of *C. albidum* pulp was administered at 50mg/kg and 150mg/kg and *C. albidum* ~~of the~~ fruit juice was administered at 1000mg/kg ~~of there~~ ~~was~~ no significant ($p>0.05$) increase in the level of follicle stimulating hormone resulted when compared with ~~compared to~~ baseline study. Also, the findings further showed a non-significant ($p>0.05$) decrease in the level of follicle stimulating hormone at 300 and 500mg/kg of ethanol extract of *C. albidum* pulp while at 1000mg/kg ~~of~~ ethanol extract of *C. albidum* pulp showed a significant ($p<0.05$) decrease in the post treatment groups compared to baseline. Moreso, at 50, 150, 300, and 500 mg/kg of the fruit juice there was a non-significant reduction in the level of follicle stimulating hormone respectively when compared with the base line before the treatment.

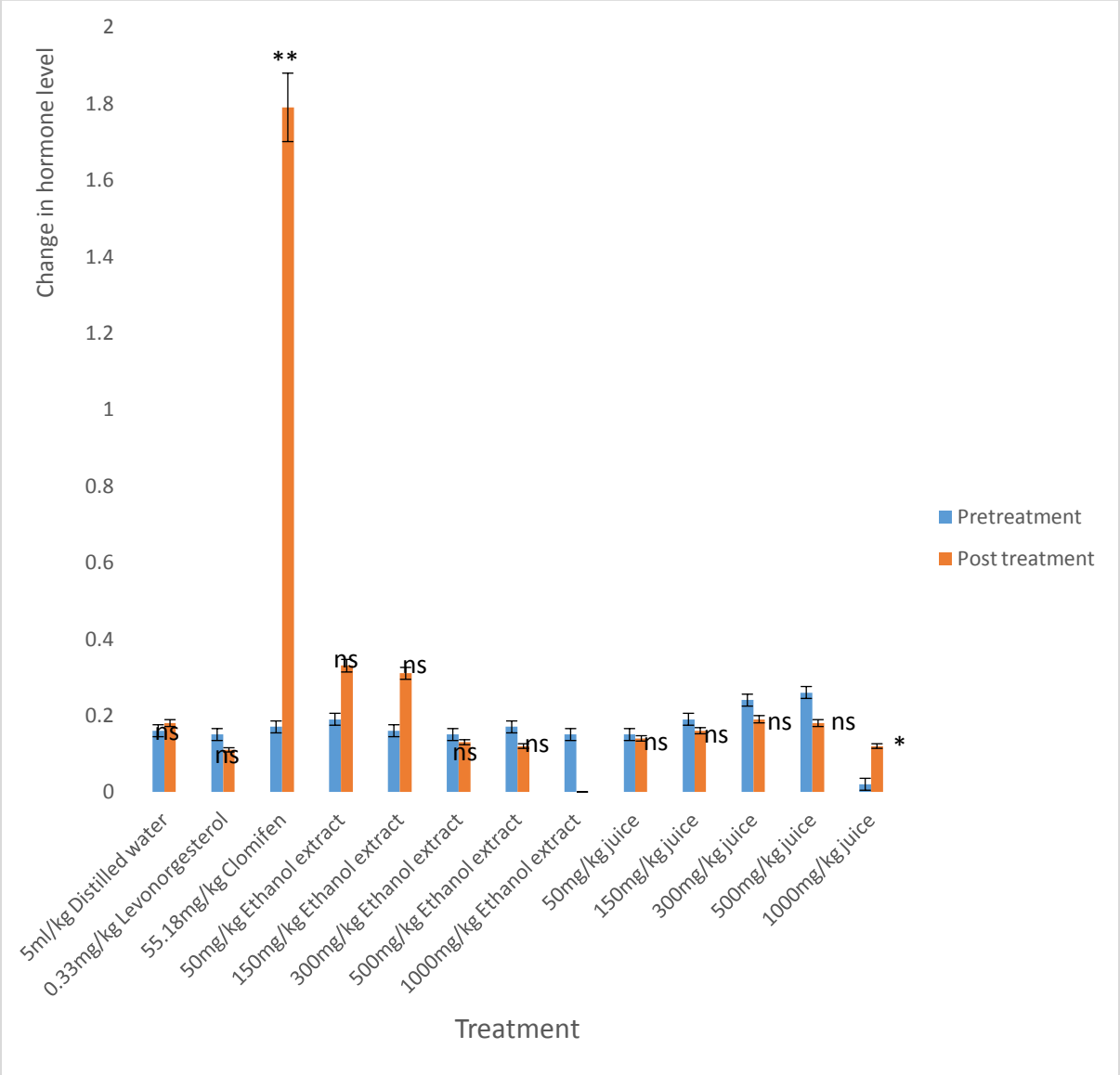

Fig 4. Effect of *C. albidum* on follicle stimulating hormone in non-pregnant rats (FSH)

IV. Discussion

The findings that the phytoconstituent screeing revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids and terpenoids is similar to the findings of [15] who reported the presence of carbohydrates, saponins, steroids, tannins, alkaloids and flavonoids in the methanol extract of the seed of the *C. albidum.* It is also in agreement with the findings of [16-18], who reported alkaloids, tannins, saponins, flavonoids, terpenoids, phlobatanins and cardiac glycosides in the fruit of *C. albidum.*

The results also showed that the fruit pulp and juice extract of *C. albidum*, LD_{50} was above 5000mg/kg which therefore implied that the fruit is safe for consumption. This is in agreement with the findings of [8, 19, 20] and this findings is aligned with the study findings revealing that the LD_{50} of the ethanol extract of *C. albidum* is greater than 5000mg/kg.

The findings showed decreased luteinizing hormone when C. *albidum* ethanolic extract and the fruit juice were administered to the female pregnant rats across all the dose ranges while C. *albidum* ethanolic extract and the fruit juice when administered to a non-pregnant rats had a non-significant increase in the luteinizing hormone at low doses of 50, and 150 mg/kg respectively. At increased dosage of 300, 500 and 1000mg/kg, the C. *albidum* ethanolic extract and the fruit juice showed decrease in luteinizing hormone level in pregnant rats. This finding showed that C. *albidum* ethanolic extract and the fruit juice may have the potential to suppress the secretion of the luteinizing hormone level in the blood and this depends on the concentration consumed. However, the findings of this research is in disagreement with the findings and report of [21] who reported significant increase in the level of LH and follicle stimulating hormone using ethanol extract of *C. albidum* leaves. The research findings also showed a non-significant reduction in luteinizing hormone level when

clomifene was administered and also a significant reduction in luteinizing hormone when levonorgesterol was administered among the pregnant rats. This finings is in line with the findings and report of [22-23] who reported a significant decrease in the LH activities following levonorgestrel administration respectively.

Follicle stimulating hormone is the central hormone of mammalian reproduction. It is essential for gonadal development, maturation at puberty and stimulation of gamete production during the fertile phase of life [24]. Both the follicle stimulating hormone and luteinizing hormone are stimulated by the gonadotropin-releasing hormone (GnRH) which is a tropic peptide hormone made and secreted by the hypothalamus through varied pulse frequency of the release hormone. Low frequency of the gonadotropin-releasing hormone pulse stimulates the secretion of follicle stimulating hormone while high frequency of the gonadotropin-releasing hormone is responsible for luteinizing hormone [25]

The resultant effect is that low levels of LH following the surge restarts the FSH production by the slow-pulsation frequency of GnRH release [26]. This was seen in this research which showed a significant increase in follicle stimulating hormone leading to enhanced fertilization and development of the fetus. The research further showed an increase in follicle stimulating hormone of the pregnant female albino rats when C. *albidum* ethanolic extract and the fruit juice were administered to the female pregnant rats and this implied that the extract and juice can stimulate the growth of the follicle in the ovary leading to follicular maturation, ovulation and pregnancy among female albino rats.

V. Conclusion

The study revealed that *C. albidum* pulp and juice are safe for human consumption because the LD50 is above 5000mg/kg, hence, its continued use as fruit by pregnant women of South eastern Nigeria. The findings showed that *C. albidum* pulp and juice impart stimulating effect on follicle stimulating hormone of the ovary and could be harnessed as a fertility booster among pregnant subjects when consumed at low dosage. This useful knowledge could be exploited during pregnancy for stabilizing and maintaining the developing foetus. In general, the findings of this study therefore encourages women (both pregnant and non-pregnant women) and support women of the south East Nigeria extraction to continue the consumption/usage of *C. albidum* fruits.

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