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EFFECTS OF SUPER7 - A POLYHERBAL ANTIMALARIAL DRUG – ON FEMALE WISTER RATS GONADOTROPIN HORMONES

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Acute toxicity, antimalarial, antioxidants, gonadotropin hormones, phytochemical analysis, polyherbal drug, Super7

ABSTRACT

Background: the increasing prevalence of infertility worldwide necessitated studies to elucidate its etiology and to profer solutions. This study evaluated the effects of Super7 – a polyherbal antimalarial drug - on female Wister rats' gonadotropin hormones. **Methods**: Phytochemical analysis and acute toxicity test of Super7 were done. The test animals were randomly allocated into six groups I-VI (n=7). Group I – III received 507.3 mg/kg, 1,014.6 mg/kg and 2,029.2 mg/kg body weight of Super7 respectively. Group IV, V and VI rats served as the general, positive and negative controls and received 5 ml/kg body weight of distilled water, 0.3 mg/kg body weight of Levonorgestrel, 50 mg/kg body weight of Clomiphene respectively. Treatments were administered daily for 30 days. Body weights, daily food and water intakes were measured. Both pre-treatment and post-treatment gondotropin hormonal assays were conducted. Anti oxidant properties were also tested. **Results**: The LD_{50} was > 5,000 mg/kg body weight with no signs of acute toxicity. Tannins, alkaloids, flavonoids, saponins, cardiac glycosides, steroids and terpenoids were present while proteins were absent; flavonoids being the most abundant in Super7. Groups I - V had increases in body weights which were statistically significant ($P < 0.05$). Group VI had none statistically significant increase in body weights ($P > 0.05$). The test drug showed positive gonadotropin properties and good antioxidant potentials by increasing post treatment LH levels and mean superoxide dismuthase enzyme units respectively. **Conclusion**: Super7 has a pro-fertility effect that is comparable to that of Clomiphene.

INTRODUCTION

Parenthood is regarded as a thing that is absolutely necessary for the attainment of full development. This is so in most African societies where passionate premium is placed on procreation and the family setting remains precarious till confirmed through child bearing. Children are sources of strength and economic fortune for the family. Infertility therefore entails a loss of something thought to be tangible and therefore impacts negatively. The inability of couples to achieve live births could be due to socio-demographic risk factors and a proportion of these couples will need therapeutic and/or surgical interventions to resolve the fertility problems [1]. Clinically, infertility is failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse [2]. Infertility is associated with psychological distress, depression, low self-esteem among other consequences [3]. Female factors and medicinal plants alteration of gonadotropin hormones are among the major causes of infertility.

As puberty approaches, there is increasing secretion of gonadotropins [4]. The pathophysiological pathways involving the gonadotropin hormones have been investigated and many conditions have been associated with these hormonal imbalances. Some of these conditions include polycystic ovarian syndrome (PCOS) and associated menstrual irregularities [5]. These hormonal imbalances can manifest as either excess or deficiency of the gonadotropins. In females, hypogonadotropins can result from failure of the hypothalamus to release gonadotropin releasing hormone (GnRH) [6]. GnRH pulses stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. Although produced in the same gonadotrope cell, concentrations of LH and FSH vary throughout the menstrual cycle. In the early follicular phase FSH is predominant over LH, whereas LH is dominant over FSH in the late follicular and luteal phases. Hypogonadotropic hypogonadism can also be a result of some physiological factors such as: amenorrhea, low calorie intake, physiological stress, excessive exercise [7] and Kallmann syndrome [8]. Elevated levels of FSH are associated with unresponsive gonads or hyperfunctioning pituitary adenomas [9], premature ovarian failure [10], Turner Syndrome [11] among other causes. LH deficiency can manifest in females or males as delayed puberty, hypogonadism at any age, or reproductive abnormalities that can be dramatic or subtle [12]. Careful analysis of the presenting problem, the patient's overall health, and

the hormonal profile is often necessary to determine the cause of gonadotropin hormonal imbalances and thus the most appropriate treatment.

A polyherbal formula will likely have a multisystem effect. Super7 is a multiherbal formula commonly sold and consumed in Nigeria. The constituents include: *Azadirachta indica*, *Anthocleista vogelli*, *Aloe vera*, *Antiaris toxicaria*, *Carica papaya*, *Moringa oleifera* and *Xylopia aethiopica*. Apart from malaria, other label indications include enteric fever, hepatitis, diabetes mellitus, anti-toxins, sexually transmitted diseases, immune and sperm booster, analgesic, antibacterial and anti-fungal. The effects of the various constituents on sex hormones have been reported with exception of *Antiaris toxicaria.* Neem *(Azadirachta indica)* oil when administered to female rats caused a reduction in the levels of LH, estradiol and progesterone but increased the level of FSH and decreased the number of mature ovarian follicles [13]. *Anthocleista vogelii* ethanol extract administered to female rats decrseased CD4+ and CD8+ lymphocyte counts and increased estradiol level [14]. CD4 and CD8 counts correlate negatively with T helper type 2 (Th2) cells level and enhanced Th2 level is required to establish pregnancy and implantation [15]. Optimum level of estradiol is required for stimulation of follicular growth and maturation and induction of estrous behaviour [16]. A study on *Aloe vera* reported that oral administration of 7 mg/kg body weight of aqueous extract of the plant to female rats' prolonged proestrus and estrus phases; increased the level of estradiol with no significant effect on the level of progesterone [17]**.** Oral administration of methanolic extract of unripe *Carica papaya* fruit to female rats prolonged metestrus and diestrus pattern in each cycle thereby lowering the frequency of the oestrus phase and thus ovulations [18]. The leaf extract of *Moringa oleifera* in female rats, increased the estradiol and progesterone level; the number of implantation sites was also increased. In animals treated with other plant parts, the level of estradiol and progesterone was decreased and no site of implantation was reported [19]. *Xylopia aethiopica* fruits extract on male sex hormones caused a fall in FSH, LH, testosterone, prolactin and estradiol levels; thus the potential of the extract to cause male fertility disorder [20]. From these documentations above, five of the constituents of Super7 have antifertility effects while only two have profertility effects on female Wister rats. This justifies the present study which sets out to investigate the effects of the solution of these seven components on gonadotropins.

MATERIALS AND METHODS

Materials

Animals

Pubertal female Wistar rats (42) weighing 70 – 100 g were procured from the Animal house of the faculty of Pharmaceutical sciences Agulu, Nnamdi Azikiwe University Awka. The animals were acclamatized for seven days under standard conditions of temperature and illumination (12 hours dark: 12 hours light) cycle. The rats were fed with commercially available rats' pellets (Amobyng feeds, Port Harcourt) and given access to drinking water ad libitum. All animal experiments were conducted in compliance with NIH guide for care and use of laboratory animals (Pub No: 85-23 Revised 1985) and approved by the Nnamdi Azikiwe University's Ethical Committee for the use of Laboratory Animals for Research Purposes.

Chemicals and Reagents

Mouse Anti-FSH Antibody-Horseradish Peroxidase (HRP) Conjugate (St John's laboratory UK), FSH Calibrators (Roche diagnostics USA), FSH (control) (Lee Biosolutions USA), Wash Buffer Concentrate (Sigma Aldrich Germany), Assay Buffer (Alpco USA), TMB Substrate (Cayman Chemical USA), Stop Solution (Cayman Chemical USA), Mouse Anti-hLH Antibody-Horseradish Peroxidase (HRP) Conjugate Concentrate (St John's laboratory UK), LH Calibrators (Monobind USA), LH control (Lee Biosolutions USA).

Chemicals for phytochemical analysis

Hydrochloric acid (Prime laboratories, India); Dragendoff reagent (Sigma Aldrich, United States of America); Ammonia (Shackti Industrial Gases, India), sodium hydroxide (Treveni Chemical Pvt., India); Ferric chloride (AkashPurochem. Pvt., India); Fehling's solution (Lab care Diagnostics, India); Million reagent (Interlab Chemical Pvt., India): Ethanol (TAJ

Pharmaceutical Ltd., India); Acetic anhydride (Ashok Organics Industries, India); Concentrated sulfuric acid (Navin Chemical Pvt., India), Acetic acid (Kayla Africa Suppliers, South Africa); Molisch reagent (Interlab Chemical Pvt., India); alcoholic alpha naphatol (Prat Industry Corcopation, India).

Drugs

Super7 (Tozok Natural, Obosi, Anambra State, Nigeria), Levonorgestrel (Gedeon Richter PLC, Hungary) and Clomiphene (Jianxi Xier Khanghai Pharmaceutical Industry Ltd, China)

Equipment

Precision pipettes (25, 50, 100 and 300 µl, 1,000 µL) (Labcompare USA); Disposable pipette tips (Labcompare USA); Distilled or deionized water (SnowPure Water Technologies USA); Plate shaker (Biocompare USA); Microwell plate reader (BioTek India); Centrifuge (Sharplex Filters Pvt., India); MouseAnti-FSH Antibody Coated Microwell Plate (Biocompare USA); Mouse Anti-hLH Antibody Coated Microwell Plate (Novus Biologicals USA); Vortex mixer (Bionics Scientific Technologies (P) LTD, India); Microplate mixer (United Technology Trade Corp. USA); Graduated cylinder for 500 ml (Boenmed Healthcare Co. Ltd, Hong Kong); Stop watch (Avi Scientific India); EDTA containers (Sure Care Corporation), heparinized capillary tube (Thomas Scientific, USA), disposable hand gloves (Supermax Malaysia), toilet tissue.

Methods

Qualitative phytochemical tests

Phytochemical tests were conducted according to the methods described by Biswal [21]

Alkaloids, flavonoids, saponins, tannins, terpenoids, steroids, proteins and cardiac glycosides were tested using standard testing methods which include: dragendorffs reagent, ammonium tests, froyhing test, ferric chloride test, acetic anhydride test (for both terpenoid and steroids), million reagents test and fehling solution tests respectively.

Quantitative phytochemical tests

Quantitative phytochemical tests were carried out using gas chromatography.

Acute toxicity studies

The acute toxicity and lethality (LD_{50}) of the test drug was conducted according to the method described by Lorke [22]. The tests were done in two phases; in the first phase three groups of rats (n=3) received oral administration of 10 mg/kg body

weight, 100 mg/kg body weight and 1,000 mg/kg body weight of test drug. The animals were observed for 24 hrs for number of deaths and for any sign of acute toxicity. In the second stage, new set of four groups of rats (n=1) were orally administered 2,000 mg/kg body weight, 3,000 mg/kg body weight, 4,000 mg/kg body weight and 5,000 mg/kg body weight of test drug and were observed for 24 hours for signs of toxicity and deaths.

The LD_{50} was determined using the formula:

$LD_{50} = (HDM \times LDM)^{1/2}$

HDM = Highest dose that resulted to no mortality

LDM = Lowest dose that resulted to mortality

Reproductive function test

Experimental design

In this study, the test animals were randomly allocated into six groups I - VI (n = 7). Group I – III were the treatment groups and received 507.3 mg/kg body weight, 1,014.6 mg/kg body weight and 2,029.2 mg/kg body weight of the test drug. Group IV rats served as the control and received 5 ml/kg body weight of distilled water orally. Group V rats served as the positive control and received 0.3 mg/kg body weight of Levonorgestrel – a progesterone based contraceptive that has antigonadotropic effects thus preventing folliculogenesis and ovulation [23]. Group VI rats served as a negative control and received 50 mg/kg body weight of Clomiphene – a pro-fertility drug that enhances the serum level of FSH and LH with a resultant effect of improving folliculogenesis and ovulation [24].

Treatments were administered daily for 30 days. During the experimental periods, the effects of the treatment on body weight and daily food and water intakes were measured. Food intakes were quantified daily by placing a measured quantity of food in restrainer containers at zero hour. The animals were allowed to feed from the container. 24 hours later, the container was weighed and the difference in weight taken as the quantity of food taken by the rats. Food wastages were minimized by using pelleted feed instead of powdered feed. The fraction of the feed which mixed with urine in the floor of the cage was dried in open air before adding it to the remnant of the previous feed. Water intakes were quantified by placing measured volumes of water into calibrated animal water dispensers at zero hour. The animals were allowed to drink from the dispensers. At the end of 24 hours period, the volumes of water remaining in the containers were subtracted from the volumes at the zero hour to obtain the volume of water consumed by the animals. The water dispensers used have valve under the containers. The containers were placed on top of the cages and the rats leaked the water from the valves. This

prevented water spillage and reduced water wastages to the minimum. The rats were weighed weekly to determine the effects of the treatments on the body weights.

On day one, blood samples were withdrawn from rats 1 - 5 of each group via retro orbital plexus for FSH and LH analysis in order to get the basal concentrations of the two hormones. On day 31, post-treatment blood samples were withdrawn from the same five rats (1-5) of each group via retro orbital plexus. The blood samples were placed in serum separator tube and allowed to clot overnight at 4 ^{o}C; then centrifuged for 20 min at 1,000 x g. The serum was stored at –8 ^{0}C in a deep freezer until ready for determination of FSH and LH level. On the same day 31, three rats from each group were sacrificed by cervical dislocation. The livers from the three rats were harvested and homogenized for antioxidant assays marked by malondialdehyde concentrations and superoxide dismuthase activities.

Assay of the FSH

Assay of FSH was done according to the method described by Akyol [25]

About 50 µL each of six reference standard (calibrator), test samples (15 in numbers) and controls (15 in numbers) were added into the appropriate wells in duplicates. Then 50 µL of Detection Reagent A was added to each of the wells immediately. The microplate shaker was used to shake the plate vigorously. The plate was covered with a plate sealer and incubated for 60 min at 37 ^{0}C. The Detection Reagent A turned the solution milky. The set up was warmed to room temperature and mixed vigorously to make the milky color uniform.

The solution in the wells was aspirated and washed with 350 µL of Wash Solution using a multi-channel pipette. The set-up was allowed to stand for 120 s. The remaining liquid in the wells were completely removed by snapping the plate onto absorbent paper thrice. After the third wash, the remaining Wash Buffer was removed by aspiration. The plate was then inverted and blotted against absorbent paper. About 100 µL of Detection Reagent B (working solution) was added to each well and sealed with a plate sealer. The set-up was then incubated for 30 min at 37 °C. After incubation, 90 µL of TMB Substrate Solution was added to each well. Care was taken to protect the solution from light. The TMB Substrate Solution turned the solution blue. The set-up was then sealed with a new plate sealer and incubated for 10 - 20 minutes at 37 °C. Finally 50 µL of Stop Solution was added to each well and mixed vigorously. The Stop Solution turned the solution yellow. Care was taken to ensure that no drop of water was at the bottom of the plate. Measurement of absorbancies was conducted at 450 nm using the microplate reader.

Calculation of results

The average of the duplicate readings for each calibrator, controls, and samples were taken. A calibration curve was constructed on a graph paper with the mean absorbance obtained from each reference standard on the y-axis and FSH concentration on the x-axis. The best fit straight line was determined. Using the mean absorbance values for samples and controls, the corresponding concentrations of FSH in mIU/ml were determined from the standard curve. The mean of the FSH for each group was calculated.

Assay of LH

Assay of LH was done according to the method described by Steyn [26]

About 50 µL each of six reference standard (calibrator), test samples (15 in numbers) and controls (15 in numbers) were added into the appropriate wells in duplicates. 50 µL of Detection Reagent A was added to each of the wells. The microplate shaker was used to vigorously shake the plate. The plate was sealed with a plate sealer and incubated for 60 min at 37 ^{0}C. Reagent A turned the solution milky. The set up was warmed to room temperature and mixed vigorously. The warming and mixing made the milky color uniform. Using a multi-channel pipette the solution was aspirated into each well and washed with 350 µL of 1X Wash Solution. The set-up was allowed to stand for 120 s. The liquid in the well was removed by snapping the plate unto an absorbent paper. This was done at least thrice. After the last wash, any remaining Wash Buffer was removed by aspiration or decantation. The plate was inverted and blotted against absorbent paper. A 100 µL of Detection Reagent B working solution was added to each well sealed with a plate sealer and incubated for 30 minutes at 37 °C. Substrate Solution (90 µL) was added to each well and sealed with a new plate sealer. The set-up was then incubated for 30 min at 37 °C. Care was taken to protect the set-up from light. Addition of Substrate Solution turned the solution blue. Finally, 50 µL of Stop Solution was added to each well. The Stop Solution turned the solution yellow. Caution was taken to ensure that there was no water or finger print at the bottom of the plate, measurement was then conducted at 450 nm using the microplate reader.

Calculation of results

The average of the duplicate readings for each calibrator, sample and control were taken. A calibration curve was constructed on a graph paper with the mean absorbance obtained from each reference standard on the y-axis and LH concentration on the x-axis. The best fit straight line was determined. Using the mean absorbance values for samples and controls, the corresponding concentrations of LH in mIU/ml were determined from the standard curve.

Antioxidant assay

Malondialdehyde assay - The assay was conducted according to the method described by Alam [27]

Preparation of sample for malondialdehyde (MDA)

On day 31 post-treatment, 18 female rats (three from each group) were decaptitated by cervical dislocation and the liver rapidly isolated and placed on ice and weighed. Each of the tissues was homogenized in cold saline (1/10 w/v) with about 10 up-and-down strokes at approximately 1,200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 mins at 3,000 x g. This process yielded a pellet and a low-speed supernatant (SI). The former was discarded and the latter kept for lipid peroxidation assay. The SI fraction was divided into two portions; one portion was used for MDA assay while the other portion was used to assay the SOD activity.

Assay of malondialdehyde activity

The lipid peroxidation assay was conducted with 18 clean test tubes labeled according to the six groups (three test tubes per group). 0.2 ml of each sample was added to the appropriate test tube. 1 ml of 1% thioberbituric acid (TBA) in 20% sodium hydroxide (NaOH) was added. Then 1 ml of glacial acetic acid was added. Each tube was mixed and incubated in boiling water for 15 mins. It was cooled and the optical density (OD) was read at 532 nm using spectrophotometrer. The MDA concentration in nmol/ml was calculated using the formular:

$$\text{MDA (nmol/ml)} = \frac{(OD)}{156{,}000} \times 1{,}000{,}000$$

(1.56×10^5 = molar extinction coefficient)

Determination of superoxide dismutase activity

The study will be conducted in accordance with the method described by Jing [28]**.** The procedure involves:

Preparation of the sample for superoxide dismutase (SOD) activity

The second portion of the SI fraction obtained on day 31 post-treatment was used to assay the SOD activity.

Assay of superioxide dismutase activity

18 clean tubes were labeled according to the six groups; three tubes per group. 0.4 ml of different samples was added to their respective tubes. 5 ml of 0.05 M carbonate buffer (PH 10.2) was added and mixed. It was allowed to equilibrate for 5 mins at 37^{o}c. Then 0.6 ml of 0.3 nM of freshly prepared epinephrine was added. The absorbances were read at 480 nm at 30 secs and at 150 secs. A blank sample was also tested; the blank contain distilled water in place of carbonate buffer. The absorbance of the blank was also read at 480 nm at 30 secs and 150 secs. The actual optical density reading for all the samples were calculated using the formular:

$$\text{Actual OD reading (R)} = (OD_{150} - OD_{30})/ 2$$

% Inhibition = $[(R_{blank} - R_{test}) / R_{blank}] \times 100$

50% inhibition is taken as 1 enzyme unit

Emzyme unit (μ/ml) = (% inhibition / 50) x Y

Where Y = common dilution factor (weight in mg of tissue in the sample used = 40 mg) [29].

Statistical analysis

Results were presented as mean ± Standard error of mean (S.E.M). Means were analyzed using one way analyses of variance (ANOVA) followed by post hoc Turkey's test for multiple comparisons. Mean FSH and LH values "before and after treatment" were compared using paired samples T-test. $P < 0.05$ was set to be statistically significant. Results analysis was conducted using Statistical Package for Social Science, SPSS- version 20.

RESULTS AND DISCUSSION

Results

Results of the phytochemical tests

Table 1: Qualitative phytochemistry of super7 polyherbal drug formulation

S/n	Phytochemical constituents	Qualitative values
1	Tannins	+
2	Alkaloids	++
3	Flavonoids	+++
4	Proteins	-
5	Saponins	++
6	Cardiac glycosides	+
7	Steroids	++
8	Terpenoids	++

\- *Absent*

\+ *Low concentrations*

++ *High concentrtions*

+++ *Very high concentrations*

The result showed that flavonoids were the most abundant phytochemical constituent in Super7 drug while protein is lacking.

Table 2: Quantitative phytochemistry of super7 polyherbal drug formulation

S/n	Components	Conc (µg/ml)	Classes	Percentages (%)
1	Spartein	0.0018	Alkaloid	7.70
2	Lunamarine	0.7638	Alkaloid	
3	Ribalinidine	0.1641	Alkaloid	
4	Rutin	1.6048	Flavonoid	60.83
5	Anthocyanin	0. 0112	Flavonoid	
6	Kaempferol	0.0423	Flavonoid	
7	Catechin	5.6841	Flavonoid	
8	Sapogenin	1.4603	Saponins	12.10
9	Phytate	0.1971	Phosphorus (P)	1.63
10	Oxalate	0.3185	Oxidative product of carbohydrate	2.64
11	Phenol	1.8231	Steroids, flavonoids, etc.	15.10
12	**TOTAL**	**12.0711**		**100%**

Flavonoids were mostly abundant with 60. 83% occurrence while tannins, cardiac glycosides and terpenoids were lacking quantitatively.

According to the results of acute toxicity studies, no death was recorded and there wrer no signs of acute toxicities at the tested doses of Super7. LD_{50} was > 5,000 mg/kg body weight.

Table 3: Effects of super7 polyherbal drug formulation on body weight

Groups	Mean basal body weight (g) ± SEM	Mean final body weight (g) ± SEM	Mean weight gain (%) ± SEM	p-value
I	79.20 ± 1.36	147.62 ± 3.24	46.35 ± 1.51	0.026
II	80.91 ± 3.61	148.20 ± 6.28	45.41 ± 1.07	0.026
III	76.54 ± 1.78	133.17 ± 4.10	42.53 ± 1.99	0.045
IV	89.24 ± 2.72	159.31 ± 2.73	43.98 ± 1.35	Control
V	74.41 ± 2.37	135.69 ± 4.45	45.16 ± 1.48	0.040
VI	77.80 ± 1.42	122.63 ± 3.54	36.56 ± 1.04	0.068

Values were expressed as mean ± standard error of mean (S.E.M), n = 7.

From Table 3, there were significant differences ($p < 0.05$) in body weight gains of animals in all the groups except group VI when compared with controls in group IV.

Table 4: Effects of super 7 polyherbal drug formulation on feed intake

Groups	Week 1 Mean feed intakes (g) ± S.E.M	Week 2 Mean feed intakes (g) ± S.E.M	Week 3 Mean feed intakes (g) ± S.E.M	Week 4 Mean feed intakes (g) ± S.E.M	Mean Mean ± S.E.M	P - value
I	115.79 ± 17.04	98.29 ± 7.22	126.84 ± 10.84	128.49 ± 2.78	117.35 ± 9.47	0.02
II	118.23 ± 8.81	109.73 ± 6.06	122.54 ± 8.62	137.33 ± 5.96	121.96 ± 7.36	0.02
III	128.43 ± 15.61	100.63 ± 14.29	138.01 ± 15.00	135.17 ± 4.19	125.56 ± 12.27	0.02
IV	144.67 ± 17.01	176.93 ± 8.29	145.24 ± 11.84	115.74 ± 14.51	145.65 ± 12.91	-
V	103.77 ± 8.40	97.84 ± 5.92	138.40 ± 11.47	122.40 ± 13.78	115.60 ± 9.89	0.03
VI	137.40 ± 10.54	97.73 ± 9.26	143.51 ± 5.37	96.80 ± 15.99	118.86 ± 10.29	0.02

Values were expressed as mean ± standard error of mean (S.E.M), n = 7.

The means of the weekly mean feed intakes had statistically significant decreases (P < 0.05) in all the groups when compared with the control group IV.

Table 5: Effects of super 7 polyherbal drug formulation on water intake

Groups	Week 1 Mean water intakes (ml) ± S.E.M	Week 2 Mean water intakes (ml) ± S.E.M	Week 3 Mean water intakes (ml) ± S.E.M	Week 4 Mean water intakes (ml) ± S.E.M	Mean Mean ± S.E.M	P-value
I	151.10 ± 15.64	151.67 ± 12.78	153.01 ± 11.28	223.24 ± 9.61	169.76 ± 12.33	0.01
II	173.56 ± 15.21	162.14 ± 12.09	175.76 ± 9.71	228.57 ± 14.21	185.01 ± 12.81	0.01
III	127.27 ± 14.36	151.13 ± 7.61	188.97 ± 20.80	232.86 ± 10.85	175.06 ±13.41	0.01
IV	158.84 ± 9.30	184.26 ± 9.96	197.87 ± 10.47	245.71 ± 16.74	196.67 ±11.62	-
V	132.94 ± 7.33	148.51 ± 8.91	181.23 ± 12.37	221.43 ± 12.04	171.03 ±10.16	0.02
VI	147.59 ± 18.21	174.10 ± 19.30	165.41 ± 17.46	222.86 ± 8.65	177.49 ±15.91	0.01

Values were expressed as mean ± standard error of mean (S.E.M), n = 7.

From Table 5, all the groups had statistically significant decreases (P < 0.05) in mean of weekly mean water intakes when compared with the control group IV.

Table 6: Effects of super 7 polyherbal drug formulation on FSH level

Groups	Mean FSH levels (mIU /ml)			
	Baseline (B) ± S.E.M	**After treatment (AT) ± S.E.M**	**(AT - B) ± S.E.M**	**P-values**
I	2.13 ± 2.13	0.00 ± 0.00	- 2.13 ± 2.13 ≡ 0.00 ± 0.00	0.01
II	4.40 ± 2.75	0.00 ± 0.00	- 4.40 ± 2.75 ≡ 0.00 ± 0.00	0.03
III	0.02 ± 0.02	0.27 ± 0.27	0.25 ± 0.27	0.04
IV	5.20 ± 1.79	0.05 ± 0.05	- 5.15 ± 1.79 ≡ 0.00 ± 0.00	Control
V	6.96 ± 6.31	0.13 ± 0.13	- 6.83 ± 6.31 ≡ 0.00 ± 0.13	0.003
VI	5.20 ± 4.01	0.27 ± 0.16	- 4.93 ± 4.01 ≡ 0.00 ± 0.00	0.001

Values were expressed as mean ± standard error of mean (S.E.M), n = 7. P < 0.05 implies statistically significantly difference from baseline value; only group 3 recorded increase in FSH level.

Negative values were considered to be equivalent to zero.

Table 7: Effects of Super 7 polyherbal drug formulation on LH level

Groups	Mean LH levels (mIU /ml)			
	Baseline (B) ± S.E.M	**After treatment (AT) ± S.E.M**	**(AT - B) ± S.E.M**	**P-values**
I	34.20 ± 12.71	43.58 ± 9.54	9.38 ± 11.67	0.01
II	14.40 ± 10.01	51.88 ± 18.31	37.48 ± 19.17	0.02
III	63.30 ± 38.60	18.64 ± 5.91	0.00 ± 2.50	0.01
IV	15.00 ± 7.65	12.64 ± 6.74	0.00 ± 1.24	Control
V	16.50 ± 10.21	10.98 ± 4.84	0.00 ± 2.35	0.003
VI	33.90 ± 16.16	0.04 ± 0.04	0.00 ± 0.00	0.02

Values were expressed as mean ± standard error of mean (S.E.M), n = 7. P < 0.05 implies significantly different from group IV. There were significant increase (P < 0.05) in LH levels of animals in group I and II after treatment when compared to their baseline levels.

Negative values were considered to be equivalent to zero.

Table 8: Effects of super7 on mlondialdehyde (MDA) and Super oxide dismutase (SOD)

Groups	Mean MDA concs (nm/ml) ± S.E.M	MDA P-values	Mean SOD concs (µ/ml) ± S.E.M	SOD P-values
I	2.85 ± 0.49	0.02	3.64 ± 2.78	0.01
II	3.36 ± 0.23	0.01	28.48 ± 20.31	0.04
III	4.26 ± 0.31	0.06	35.15 ± 6.15	0.06
IV	3.24 ± 0.34	Control	6.06 ± 6.06	Control
V	3.37 ± 0.23	0.01	22.43 ± 12.66	0.04
VI	4.18 ± 0.21	0.06	21.21 ± 21.21	0.002

Values were expressed as mean ± standard error of mean (S.E.M), n = 3 for both MDA and SOD.

Effects on MDA level:

There were significant alterations ($p < 0.05$) in mean MDA level of animals in groups I, II and V when compared with controls group IV. Only group I recorded less MDA concentration than the control group IV.

Effects on SOD level:

There were significant differences ($p < 0.05$) in mean SOD levels of animals in all the groups except group III which recorded non-significant increase ($P > 0.05$) when compared with control group IV. Super7 had dose dependent increases in SOD enzyme.

Discussion

In accordance with the results of the phytochemical analysis, the positive gonadotropin effects of Super7 might be mainly due to the presence of flavonoids, saponins and tannins. This is due to the following reasons: flavonoids and tannins had been reported to improve the serum levels of gonadotropins (FSH and LH) significantly [30]. Flavonoids and saponins have positive effects on androgen bioavailability [31]. On the other hand, alkaloids, cardiac glycosides and anthraquinones reduced significantly the serum level of FSH, LH, progesterone and estradiol [32]. Steroids including anabolic steroids and corticosteroids (cortisone, prednisone) could have a serious effect on fertility [33]. Some components of terpenoids (essential oils) have a high ovicidal activity [34].

The results of the acute toxicity tests implied that the drug have high safety profile. However, higher doses should be used with caution. A previous study on *Carica papaya*, a component of the test drug (Super7) had an LD_{50} above 5,000 mg/kg body weight with no signs of autonomic or other symptoms of toxicity [35]. But in the sub-acute study, the levels of alkaline phosphatase, gamma glutamyl transferase and bilirubin increased in a dose-dependent fashion, suggesting a possible damage to the hepato-billiary system [35]. The increased body weights was attributed to anabolic effects which caused increased metabolism, tissue generation and muscle building which resulted in general increases in body mass index [36]. Many factors might have been responsible for the decrement in feed intakes. Some of these factors include: possibility of side effects such as anorexia. The feed might contain some thermogenic ingredients which might have caused early satiety in the rats [37]. There might have also been drug – feed interaction which might have resulted in slowing down the gastrointestinal tract motility. Increased LH implied successful ovulation and formation of corpus luteum which will in turn produce estrogen and progesterone. These two hormones might have altered the threshold for osmotically induced arginine vasopressin (AVP), the primary hormone involved in the regulation of renal free water release and thirst onset [38]. This might have contributed to the decrement in water intakes observed in all the groups.

This reduction of FSH observed in group IV was not expected because group IV received distilled water. However, the blood samples might have been collected when the rats were in their luteal phases of menstral cycles. In the luteal phase, LH is dominant over FSH. In addition to this, the dominant follicle during its development secretes increasing levels of estradiol and inhibin B which act to reduce FSH secretion through a negative feedback mechanism [39]. At high dosage, the test drug could enhance FSH level thus having pro-fertility effect. The reduction observed in group V was as expected because group V received levonorgestrel, a standard anti-fertility drug [40]. The reduction in group VI might be due to the prolonged administration of the drug clomiphene. FSH might have risen to a level that triggered negative response that resulted to the decline in FSH level. Estradiol also exerts negative feedback on FSH secretion from the pituitary gland [41]. The test drug (Super7) had dose dependent increases in LH concentrations. Therefore, group III should have recorded an increase more than that obtained in groups I and II. This was not as expected and the reason could be due to the high dose coupled with prolonged administration. The LH might have been raised to a level that triggered negative response that resulted in the decline in LH level. LH release is stimulated by gonadotropin-releasing hormone (GnRH) and inhibited by estrogen in females and testosterone in males [42]. This was evident in group VI which received clomiphene – a standard pro-gonadotropin drug. LH is critical to luteinization of the ovarian follicles and post-ovulatory follicular function [43].

The test drug had dose dependent increases in MDA concentrations. This implied aggravation of lipid peroxidation – an oxidative process that modifies lipid and fatty acids in both sperm and oocyte membranes thereby increasing the likelihood of infertility [44]. Since the low dose of Super7 recorded a less MDA concentration when compared to the control group IV, it implied that appropriate down modification of the dosage and duration of treatment will unfold the lipid peroxidation lowering potential of the test drug [45]. The test drug had a good antioxidant property by increasing the concentration of super oxide dismuthase in a dose dependent manner. SODs form the front line of defense against reactive oxygen species (ROS) mediated injury [46].

Conclusions

This study showed that the test drug Super7 when used at the right dosage and for the right duration of treatment will have positive gonadotropin effects that will be comparable to those of a standard pro-fertility and positive gonadotropin drug Clomiphene. However, it has to be used during the luteal phase for a short duration and at a dose between 1,014.6 mg/kg body weight and 2,029.2 mg/kg body weight.

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References

1. Sarkar S and Pallavi G. Socio-Demographic Correlates of Women's Infertility and Treatment Seeking Behavior in India; *J Reprod Infertil*, 2016; 17(2): 123–132.
2. Vander B, Wyns C. *Fertility and infertility definition and epidemiology*. Clin Biochem, 2018: 62:2-10.
3. Sami N, Tazeen S A. Perceptions and experiences of women in Karachi, Parkistan regarding secondary infertility results from a community based qualitative study. *Hindawi.com/journals*, 2012; /ogi/108756/; pp.1-7.
4. Guglielmo B, Lucia G. Normal and abnormal puberty. Endotext last update, 2015. Available from: https://www.ncbi.nlm.nih.gov/books/NBK279024/
5. Zahra S., Arezoo K., Mohammad R., Jafarzadeh S., Amin T. Pathophysiological mechanisms of gonadotropins and steroid hormones related genes in etiology of polycystic ovary syndrome; *Iranian journal of basic medical sciences*, 2019; Article 2, Volume 22, Issue 1, Page 3-16.
6. Vaishali P. Gonadotropin-Releasing Hormone Deficiency in Adults; *Medscape: Drugs & Diseases > Endocrinology*; Updated 2017.
7. Stamatiades G. A, Kaiser U. B. Gonadotropin regulation by pulsatile GnRH: Signaling and gene expression. *Mol. Cell. Endocrinol*, 2018; 463:131-141.
8. Barbieri RL. The endocrinology of the menstrual cycle. *Methods Mol. Biol*. 2014; 1154:145-69.
9. Michelle O, Sarao M. S. Physiology, Follicle Stimulating Hormone. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing, 2018; Available from: https://www.ncbi.nlm.nih.gov/books/NBK535442/
10. Mahbod E and Firoozeh A. A. Pathogenesis and Causes of Premature Ovarian Failure*:* An Update *Int J Fertil Steril*, 2011; 5(2): 54–65.
11. Pouresmaeili F, Fazeli Z. Premature ovarian failure: a critical condition in the reproductive potential with various genetic causes. *Int J Fertil Steril*, 2014; 8(1):1-12.
12. Jennifer K. G. How Medications Can Affect Your Fertility; Explore parents, 2016; published online: https://www.parents.com/getting-pregnant/pre-pregnancy-health/how-medications-can-affect-your-fertility/

13. Parveen B., Bansal R., Verruchi G. Antifertility effects of *Azadirachta indica* (Neem) - ResearchGate publication/285274309. *Anals of biological research*, 2010; 1(2):108-133.
14. Oladimeji I J. O., Coleshowers C. L. S. O. Immunological biomarkers determined in female rats administered with pro-fertility extract of *Anthocleista vogelii* researchgate.net/publication, 2014; 304351875.
15. Oladimeji S. O, Lawal O. A and Steve A. 1. Some immune factors and hormones determined in female albino rats induced with infertility and administered with *Anthocliesta vogeli*; *Journal of Research and Review in Science*, 2017; 238-244; Volume 4.
16. Weinberg A., Enomoto L., Marcus R. and Canniff J. Effect of menstrual cycle variation in female sex hormones on cellular immunity and regulation. *J. Reprod. Immunol*, 2011; 89(1): 70 – 77.
17. Eman G. E. H, Rasha A. A. E, Hoda M.A. Studies on the Use of *Aloe vera* Extract as a Contraceptive in Female Rats. *The Egyptian Journal of Hospital Medicine*, 2015; Vol. 60, Page 271-281.
18. Odirichukwu E. O., Nneka V. S., Uchechukwu and David O. The Aqueous Methanolic Extract of Unripe *Carica Papaya* (Pawpaw) Fruit Distrupts Oestrous Cycle in Albino Rats. IOSR *Journal of Agriculture and Veterinary Science*, 2016; Volume 9, Issue 5Ver. II, PP 57-62.
19. Owolabi J. O., Ogunsola O. A., Fabiyi O. S., Nwobi N. L., Faluyi B, Akinbola A. S. Moringa Plant Parts Consumption Had Effects on Reproductive Functions in Male and Female Rat Models. OSR Journal of Dental and Medical Sciences, 2017; Volume 16, Issue 10 Ver. VII, PP 82-86.
20. Nnodim J. K., Nwanjo H. U., Okolie N. J., Opara A. U., Nwosu D. C., Okoroiwu I., Dike J., Okorie H., Nwadike C. N and Uduji H. I. Effects of *Xylopia Aethiopica* Fruits on reproductive hormonal level in rats. *Global Journal of Medicinal Plant Research*, 2013; 1(1): 29-31.
21. Biswal S. Phytochemical analysis and a study on the antiestrogenic antifertility effect of leaves of *Piper betel* in female albino rat. Ancient science of life, 2014; Volume : 34, Issue : 1 , Page : 16-22
22. Lorke D. A new approach to practical acute toxicity testing. *Arch Toxicol*, 1983; 54(4):275-87.
23. Stefano L and Andrea C. Ethynilestradiol 20 mcg plus Levonorgestrel 100 mcg: Clinical Pharmacology; *Int J Endocrinol*, 2014: 102184. Published online Doi: 10.1155/2014/102184.
24. Errol R. N, John O. S. Ovulation induction; Obstetrics and Gynecology at a Glance; Blackwell publishing, 2010; ISBN: 976-1-4051-8324-6.
25. Akyol S, Cınar S. A, Purisa S, Aydinli K. Relationship between lymphocytes, IL2 and the hormones E2, LH, PRG and FSH in menopausal and postmenopausal women. *Am J Reprod Immunol*. 2011; 66(4):304-9.
26. Steyn F. J, Wan Y, Clarkson J, Veldhuis J. D, Herbison A. E and Chen C. Development of a methodology for and assessment of pulsatile LH secretion in juvenile and adult male mice. Endocrinology, 2013; 154:4939-45.

27. Alam Z and Fareed U. A Simple Spectrophotometric Method for the Determination of Thiobarbituric Acid Reactive Substances in Fried Fast Foods; *Journal of Analytical Methods in Chemistry*, 2016; Article ID 9412767.
28. Jing Z, Rui C, Zhen Y, and Lili X. Superoxide dismuthase (SOD); and catalase (CAT) activity. Bio-protocol, 2017; vol 7, ISS 16, Doi: 10.21769/Bioprotoc.2505.
29. Spreng S. N., Himadri S P., Nishith R. B and Bodhisattwa C. In vitro evaluation of antioxidant activity of Leucas plukenetii (Roth); *Asian Journal of Plant Science and Research*, 2012, 2 (3):254-262 254.
30. Chris K., Geisinger H. S and Rebecca P**.** Mechanism of action of levonorgestrel emergency contraception; Literature Review (PDF Available) *in* The Linacre quarterly, 2015; 82(1):18-33 ·
31. Abigail D., Jani R. J., and Dean M. How Laboratory Tests Contribute to Successful Infertility Treatments; AACC.org // ... // Clinical Laboratory News // All CLN Articles // Fertility Testing; Date: NOV.1.2012.
32. Daniel N, Singh G. Physiology, Luteinizing Hormone. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing, 2019; Available from: https://www.ncbi.nlm.nih.gov/books/NBK539692/
33. Pratap K and Sameer F. S. Luteinizing hormone and its dilemma in ovulation induction; *J Hum Reprod Sci*, 2011; 4(1): 2–7.
34. Chinyerum S. O and Ralf R. H**.** An Update on Oxidative Damage to Spermatozoa and Oocytes; Biomed Res Int: 2016; 9540142; Doi: 10.1155/2016/9540142.
35. Giuseppina B, Stefania P, Martina D, Chiara D, Alessia A, Giovanni P C, Giulio G, Marie A. C, Maria G and Fabrizio G. Lipid Peroxidation-Derived Aldehydes, 4-Hydroxynonenal and Malondialdehyde in Aging-Related Disorders. Open acess journal, 2018; *7*(8), 102.
36. Younus H. Therapeutic potentials of superoxide dismutase; *Int J Health Sci* (Qassim), 2018; 12(3): 88–93.
37. Akram A, Ali A O, Hamid H, Ghaedi E, and Mohammad R. R. N. Effects of Hydro-Alcoholic Extract of *Rhus coriaria* (Sumac) Seeds on Reproductive Complications of Nicotinamide-Streptozotocin Induced Type-2 Diabetes in Male Mice. *World J Mens Health*, 2014; 32(3): 151–158.
38. Akrayi H. F. S and Abdullrahman Z. F. A. Screening in vitro and in vivo the antibacterial activity of *Rhus coriaria* extract against *S. Aureus*. IJRRAS, 2013; 15:390–397.
39. Adebayo E. M. Effects of Oral Administration of a Decoction on Serum Levels of Leutinizing Hormone, Follicle Stimulating Hormone, Progesterone and Estradiol in Female Dutch-White Rabbits. *Research Journal of Medicinal*
Plants, 2015; Volume 9 (3): 141-145.
40. Jennifer L. E (2016). Luteinizing Hormone Deficiency; Mediscape; Updated: https://emedicine.medscape.com/article/255046.

41. José S. D., María P. Z, Jimena M. H, Romina P. P, Vanessa A. A and Julio A. Z. Terpenes: Natural Products for Controlling Insects of Importance to Human Health — a Structure-Activity Relationship Study. Psyche: *A Journal of Entomology*, 2016; Article ID 4595823, 17 pages http://dx.doi.org/10.1155/2016/4595823.
42. Charles A., Jemima A. A., Kwesi B. M., Priscilla K. M. Aqueous leaf extract of *Carica papaya* (caricaceae) linn. Causes liver injury and reduced fertility in rats; *Int J Pharm Sci*, 2015; Vol 8, Issue 2, 261-265.
43. Ndukui, J.G., Muwonge H., and Sembajwe L.F. Aphrodisiac potential and phytochemical profile of *Ekebergia capensis* (Cape ash) in male albino rats. Spatula DD, 2012; 2(4): 237-243.
44. Lucy C., Keri M., Martin R and Yeomans. Optimising foods for satiety; trends in Food Science & Technology, 2015; Volume 41 Issue 2, Pages 149-160.
45. Fintan H, Monty M, Hugh M. The sensitivity of the human thirst response to changes in plasma osmolality: a systematic review, Perioperative Medicine, 2018; 7:1.
46. Maria N and Chester W. B. Activins and Inhibins: Roles in Development, Physiology, and Disease Cold Spring Harb. Perspect. Biol, 2016; 8: a021881.