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ANTISTEROIDOGENIC EFFECT OF *AZADIRCHTA INDICA* TINCTURE (AIT) AND *AZADIRCHTA INDICA* 30 POTENCY (AI 30) IN MATURE FEMALE RATS

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ABSTRACT

The present work is to find the *Azadirchta indica* tincture (AIT) and *Azadirchta indica* 30 potency (AI 30) could be recommended for used as female contraceptives. The parameter chosen for the present investigation is ovarian activity in sexually mature female rats after treatment with the *Azadirchta indica* tincture (AIT) and *Azadirchta indica* 30 potency (AI 30). For this, arrest in the oestrus cycle, changes in the weighs of ovaries, biochemical estimation of ascorbic acid, total cholesterol, inhibition of the activity of enzymes, G-6-PD and Δ^5-3β-HSD that are responsible for the synthesis of steroidal hormones in the ovarian tissue, have been taken as the experimental parameters. From the overall results, it is evident that oral administration of the AIT & AI 30 causes inhibition of both steroidogenic enzymes and elevation of ovarian cholesterol and ascorbic acid content and induces functional sterility without toxic symptoms and they exert antisteroidogenic/antifertility activity in rats.

Key Words:- Antisteroidogenic, Antifertility, Steroidogenic Enzymes, *Azadirchta indica* Tincture, *Azadirchta indica* 30 Potency.

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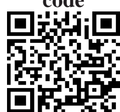
Home page: http://ijptjournal.com/ **DOI:** http://dx.doi.org/10.21276/ijpt.2017.8.1.5	**Quick Response code** 

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INTRODUCTION

The pattern of events in the estrous cycle may provide a useful indicator of the normality of reproductive neuroendocrine and ovarian function in the non-pregnant female. It also provides a means to interpret hormonal, histological, and morphological measurements relative to stage of the cycle, and can be useful to monitor the status of mated females. Estrous cycle normality can be monitored in the rat and mouse by observing the changes in the vaginal smear cytology (Long JA and Evans HM, 1922; Cooper *et al*., 1993). To be most useful with cycling females, vaginal smear cytology should be examined daily for at least three normal estrous cycles prior to treatment, after onset of treatment, and before necropsy (Kimmel *et al*., 1995). Daily vaginal smear data from rodents can provide useful information on (1) cycle length, (2) occurrence or persistence of estrus, (3) duration or persistence of diestrus, (4) incidence of spontaneous pseudo-pregnancy, (5) distinguishing pregnancy from pseudo- pregnancy (based on the number of days the smear remains leukocytic), and (6) indications of fetal death and resorption by the presence of blood in the smear after day 12 of gestation.

The technique also can detect onset of reproductive senescence in rodents (LeFevre J, 1988).

It is useful further to detect the presence of sperm in the vagina as an indication of mating. In non-pregnant females, repetitive occurrence of the four stages of the estrous cycle at regular, normal intervals suggests that neuroendocrine control of the cycle and ovarian responses to that control are normal. Even normal, control animals can show irregular cycles. However, a significant alteration compared with controls in the interval between occurrences of estrus for a treatment group is cause for concern. Generally, the cycle will be lengthened or the animals will become acyclic. Lengthening of the cycle may be a result of increased duration of either estrus or diestrus. Knowing the affected phase can provide direction for further investigation.

The persistence of regular vaginal cycles after treatment does not necessarily indicate that ovulation occurred, because luteal tissue may form in follicles that have not ruptured. This effect has been observed after treatment with anti-inflammatory agents (Walker *et al*., 1988). However, that effect should be reflected in reduced fertility. Conversely, subtle alterations of cyclicity can occur at doses below those that alter fertility (Gray *et al*., 1989). Irregular cycles may reflect impaired ovulation. Extended vaginal estrus usually indicates that the female cannot spontaneously achieve the ovulatory surge of LH (Huang HH and Meites J, 1975).

India within, few years of time span will be the leading country as far as the population growth is concerned. Since the population rising tremendously, this may affect drastically the economic growth of India. Family planning has been promoted through several methods of contraception, but due to side effect produced by the use steroidal contraceptive and use of abortifaciant drugs. There is a need of drug for contraception which is effective with lesser side effects.

Therefore objective of the present work is to find whether the *Azadirchta indica* tincture (AIT) and *Azadirchta indica* 30 potency (AI 30) could be recommended for use as female contraceptives or not. The parameter chosen for the present investigation is ovarian activity in sexually mature female rats after treatment with the *Azadirchta indica* tincture (AIT) and *Azadirchta indica* 30 potency (AI 30). For this, arrest in the oestrus cycle, changes in the weighs of ovaries, biochemical estimation of ascorbic acid, total cholesterol, inhibition of the activity of enzymes, G-6-PD and Δ^5-3β-HSD that are responsible for the synthesis of steroidal hormones in the ovarian tissue, have been taken as the experimental parameters.

MATERIALS AND METHODS

Animals used

Adult female albino rats of Swiss strain 150-180g were selected and housed in polypropylene cages and maintained under standard conditions (12 h light/12 h dark cycles; 26 ± 3°C; 45-60% relative humidity) in the laboratory for one week and given a standard pellet diet (Hindustan Lever) and water *ad libitum*. All the animal experiments were performed according to the guidelines of institutional animal ethics committee (Ref. No. P.col/64/2011/IAEC/ VMCP). Albino Female Wistar Rats showing four consecutive normal estrous cycles were then divided into four groups (n=6) and given the following treatment:

Group I: Treated as Vehicle control and received 1%tween 80 at the dose level of 10 ml/kg b.w, p.o

Group II: Standard group treat with Ethinyloestradiol and levonorgestrel (Ovral L) (Dosage – 0.02mg/kg.b.w., p.o)

Groups III: *Azadirchta indica* tincture (AIT) prepared as per HPI, respectively, suspended in Vehicle (Dosage – 0.2ml/kg.b.w., p.o)

Groups IV: *Azadirchta indica* 30 potency (AI 30) prepared as per HPI (Dosage – 0.2ml/kg.b.w., p.o)

The vehicles and plant drugs were given orally in their proestrus phase on every day over a period of 18 days. Body weight was noted and estrus cycle was observed every day. A drop of normal saline was pipetted in and out of the vagina and placed on a slide. The smear was stained with methylene blue and examined under the microscope (40 X) twice daily at an interval of 12 h. On the 19th day, 18 h fasting after the last dose, all rats of similar physiological state (vaginal smear showed diestrus) from each group were sacrificed by cervical dislocation. Ovaries were dissected out and kept in cold 0.2 M sucrose buffer at 4°C for further studies.

This experiment was designed and carried out according to Short and Woodnot, (1969); and Mazumder *et al*., (1997).

Biochemical estimations

Estimation of Cholesterol by the Method of Kingsley and Roscoe (1949)

The rats were sacrificed and ovaries were taken in glass mortar. The weighed ovaries were homogenized in a Potter Elvehjem homogenizer using chloroform-ethanol (2:1) mixture. The supernatant was transferred into centrifuge tube and aliquot was evaporated to dryness in hot water bath. Two drops of alcoholic potassium hydroxide was added and incubated at 60°C with occasional shaking. Then it was neutralized with 15% acetic acid after adding two drops of phenolphthalein. About 2 ml of alcohol-

acetone mixture (1:1) was added to the neutral solution and followed by the addition of the 1 ml of 0.5% digitonin solution in alcohol-acetone mixture. This mixture was then incubated overnight at 25°C and centrifuged at 3500 × g for 20 min. The supernatant was discarded, 3 ml of anhydrous ether was added to the precipitate was dried with hot air and 0.5 ml of glacial acetic acid was added to the precipitate. The tubes were stoppered and temperature of the mixture was brought to 35ºC, 1ml of color reagent (color reagent was prepared by adding 1 ml of concentrated sulphuric acid to 9 ml of acetic anhydride previously cooled in ice bath) was added to the mixture and mixed well. The tubes were removed after 0 min and kept into ice bath. A standard curve was prepared by dissolving cholesterol (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/ml) in chloroform, mixed with glacial acetic and color reagent accordingly. The optical density was determined in the spectrophotometer at 620 nm and total cholesterol content of the ovaries was determined from the standard curve.

Estimation of Ascorbic Acid by the Method of Omaye *et al* (1979)

The weighed ovaries were homogenized in a Potter Elvehjem homogenizer using 5% (w/v) metaphosphoric acid and 10% acetic acid solution (5 g of Metaphosphoric acid dissolved in 80 ml of distilled water and 10 ml of glacial acetic acid was added and the volume was made up to 100 ml with distilled water). The mixture was centrifuged at 3500 × g for 20 min. The supernatant was taken into hard glass test tube; a drop of bromine solution was added to it, mixed and kept for 10 min for complete oxidation of ascorbic acid into dehydroascorbic acid form. The excess bromine was then completely removed by bubbling air through the solution. About 2 ml of this solution was transferred to another test tube and 2 ml of Thiourea reagent (2 g of 2, 4-dinitrophenyl hydrazine dissolved in 100 ml of 10 (N) sulphuric acid, 4 g of Thiourea was dissolved in this solution and mixed well by shaking) was added. The mixture was incubated for 4 h at 37°C.The test tubes were kept in ice bath and 205 ml of 85% of sulphuric acid was added drop wise to the solution and mixed thoroughly. The solution was kept at room temperature for about 80 min for the development of color. Standard ascorbic acid solutions were prepared (10 mg of ascorbic acid accurately weighed and taken into 100 ml volumetric flask containing 10 ml of 5% (w/v) metaphosphoric acid, volume made up to the mark with distilled water) using different aliquots (0.1, 0.2, 0.3, 0.4, and 0.5 ml) of the solution and prepared in the similar manner as followed for test sample. A blank was prepared simultaneously using 2 ml of water in place of bromine treated tissue extract. After development of color the optical density was measured at 540 nm against the blank. The amount of ascorbic acid present in ovaries was determined from the standard curve.

Estimation of Glucose -6-Phosphate Dehydrogenase (G-6-PDH) Activity By the method of Lohr and Waller (1974)

The weighed ovaries were homogenized in a Potter Elvehjem homogenizer with the help of purified sand in cold. This mixture was centrifuged at 1000 × g for 5 min at 0°C and the supernatant was kept on ice for the assay of glucose-6-phosphate dehydrogenase. In a 0.5 ml cuvette, 0.2 ml of Tris HCI buffer (pH 8.3, 0.5 M), 0.01 ml of 20 mM NADP, 0.025 ml tissue extract and 0.025 ml of distilled water were added and mixed well. This mixture was kept in spectrophotometer at 340 nm and set to zero. Then 0.01 ml of 100 mM glucose-6-phospate (substrate) was added to the mixture and mixed well and extinction was observed for 10 min. The protein content of tissue extract was determined and the specify activity was expressed as Unit per mg of protein.

Estimation Δ^5-3β-Hydroxysteroid Dehydrogenase (Δ^5-3β-HSD) Activity by the Method of Rabin *et al* (1961)

Weighed ovaries were taken in a glass mortar and kept on ice, 1 ml of normal saline, 1 ml of 0.1 M phosphate buffer (pH 7.4) were added and then homogenized in a Potter Elvehjem homogenizer with the help of purified sand. This tissue mixture was centrifuged at 0°C and 1000 × g for 10 min. Then the supernatant was taken in a conical flask (50 ml) and 0.2 ml of NAD (6 mg dissolved in 2 ml of 0.1 M phosphate buffer, pH 7.4), 0.1 ml of DHEA (0.5 mg of dehydroepiandrosterone dissolved in 0.1 ml of propylene glycol) were added and mixed well. This solution was kept in shaking incubator at 37°C for 90 min, acidified with 0.1 ml of 3 M acetate buffer (pH 5.0). This solution was then extracted with 10 ml of ethyl acetate and evaporated to dryness. This residue was dissolved in 2.0 ml of ethanol and optical density was read by spectrophotometer at 240 nm ethanol as the blank. The protein content of tissue was determined and specific activity was expressed as Unit per mg protein.

Estimation of protein Content by the method of Lowery *et al* (1951)

Reagents

Reagent A: 2% sodium carbonate in 0.1 N NaOH.

Reagent B: 0.5% $CuSo_4$, $5H_2O$, in 1 % sodium potassium tartarate
Reagent C: Alkaline copper solution (50 ml of Reagent-A mixed with 1 ml of Reagent B- freshly prepared.
Reagent D: Folin and Ciocalteu's reagent (diluted 2:1 with distilled water).
Standard Protein: Bovine serum albumin (BSA) (1 mg/ml).

Procedure

To the 0.1 ml of the tissue homogenate, 0.5 ml of reagent C was added and kept for 10 minutes at room temperature. To 0.5 ml of this solution, 0.5 ml distilled water and 5.0 ml reagent C were added. Then 0.5 ml reagent D was to this solution and kept for 30 min at room temperature and optical density was recorded at 660 nm against reagent blank. Standard curve was prepared by dissolving BSA (0.1 0.2, 0.3, 0.4, and 0.5 mg/ml) in distilled water and prepared in the similar manner as followed for test samples.

Statistical analysis

All the results are expressed as mean ± standard error of the mean (SEM). Statistical comparison was performed by using GraphPad Prism 3.0 (GraphPad Software Inc, San Diego, CA). Results were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test. Values were considered significant at P<0.05 or less.

RESULTS

Effect of AIT and AI30 on estrus cycle, total body weight and the ovaries

The oral administration of AIT and AI30 under study significantly (P<0.05) arrested the normal estrus cycle at diestrus phase during 18 days of treatment (Table 1). That is, the AIT, AI30, and standard drug arrested the normal estrus cycle at diestrus phase after the 9th & 5th dose, and 3rd dose, respectively when compared with vehicle control.
There was no significant change in body weight of rats treated with AIT, AI30, and standard drug as compared to vehicle control group (Table 2).

On the other hand, significant (P<0.05) reduction in the wet weight of ovaries was noted in rats after treated with AIT, AI30, and standard drug (Table 3). AIT, AI30, and standard drug produced reduction in the wet weight of ovaries when compared with vehicle control.

Effect of AIT and AI30 on ovarian cholesterol, ascorbic acid and protein content

When compared to vehicle control group, AIT, AI30, and standard drug significantly (P<0.05) elevated the ovarian cholesterol and ascorbic acid content, whereas reversibly protein content was reduced significantly (P<0.05) (Table 4).

Effect of AIT and AI30 on ovarian Δ^5-3β-HSD and G-6-PDH activities

The oral administration of AIT, AI30, and standard drug under study significantly (P<0.05) inhibited the ovarian G-6-PDH and Δ^5-3β-HSD activities in a dose-dependent manner when compared with vehicle control (Table 5). AIT, AI30, and standard drug produced a remarkably more reduction in ovarian G-6-PDH activity when compared with vehicle control. Whereas, the AIT and AI30 produced a moderate reduction in ovarian Δ^5-3β-HSD activity when compared with standard drug treated group animals.

Table 1. Effect of the AIT and AI30 on oestrus cycle in rats during 18 days of treatment

Treatment Design	Dose (mg/kg b.w)	Different phases of oestrus cycle in consequent 18 days																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Group-1: Control	10 ml	O M	D D	P P	O M	D D	D P	O O	M D	D P	O M	D D	P P	O M	D D	D P	O O	M D	D P
Group-2: Std	0.02mg/kg	O O	M D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D
Group-3: AIT	200mg (0.2ml/kg)	D D	P P	O P	D D	D P	O O	M D	D P	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D
Group-4: AIT30	200 mg (0.2ml/kg)	P P	O M	D D	D P	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D	D D

[**P**=Proestrus phase; **M**=Metestrus phase; **D**=Diestrus phase; **O**= Oestrus phase]

Table 2. Body weights of female rats after treated with AIT and AI30 for 18 days

Treatment Design	Dose (mg/kg b.w)	Body weight (g)		
		Initial	Final	% increase
Group-1:Normal Control	10 ml	163.83 ± 2.242	184.67 ± 2.459	12.72
Group-2: Standard	0.02mg	165.67 ± 3.712	175 ± 3.864	5.63
Group-3: AIT	200mg (0.2ml/kg)	170.17 ± 2.242	186.83 ± 2.315	9.79
Group-4: AI30	200 mg (0.2ml/kg)	169 ± 2.671	184.5 ± 3.631	9.17

Data are represented as mean ± S.E.M. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control (n = 6 in each group).

Table 3. Weights of the ovaries of female rats after treated with AIT and AI30 for 18 days

Treatment Design	Dose (mg/kg b.w)	Wet weight of Ovary After treatment (mg/100gm of b.wt)
Group-1:Normal control	10 ml	40.09 ± 0.4862
Group-2: Standard	0.02mg	36.53 ± 0.3152*
Group-3: AIT	200mg (0.2ml/kg)	37.78 ± 0.4002*
Group-4: AI30	200 mg (0.2ml/kg)	37.61 ± 0.4298*

Data are represented as mean ± S.E.M. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control (n = 6 in each group).

Table 4. Effect of AIT and AI30 on contents of ascorbic acid, cholesterol and protein in rats ovaries after 18 days of treatment

Treatment Design	Dose (mg/kg b.w)	Cholesterol (µg/mg of ovary)	Ascorbic acid (µg/mg of ovary)	Protein (mg/100mg of ovary)
Group-1: control	10 ml	61.83 ± 0.7923	91.16 ± 0.8724	11.33 ± 0.4944
Group-2: Standard	0.02mg	99.67 ± 1.430**	143.33 ± 1.308**	5.72 ± 1.142**
Group-3: AIT	200mg (0.2ml/kg)	74.33 ± 1.174**	123.83 ± 1.078**	9.88 ± 0.2301**
Group-4: AI30	200 mg (0.2ml/kg)	82 ± 0.8563**	130.5 ± 0.8851**	8.35 ± 0.07638**

Data are represented as mean ± S.E.M. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control.

Table 5. Effect of AIT and AI30 on G-6-PDH and Δ^5-3β-HSD activities in rat ovaries after 18 days of treatment

Treatment Design	Dose (mg/kg b.w)	Specific activity (U/mg of protein)	
		G-6-PDH	Δ^5-3β-HSD
Group-1: Control	10 ml	4.13 ± 0.0494	1.23 ± 0.0204
Group-2: Standard	0.02mg	1.97 ± 0.0667**	0.75 ± 0.0236**
Group-3: AIT	200mg (0.2ml/kg)	2.91 ± 0.0696**	1.03 ± 0.032**
Group-4: AI30	200 mg (0.2ml/kg)	2.003 ± 0.0479**	0.9 ± 0.0134**

Data are represented as mean ± S.E.M. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control.

Figure 1. Photomicrographs of cells types in the vaginal smears of cycling female rats

Di-oestrus | **Pro-oestrus**

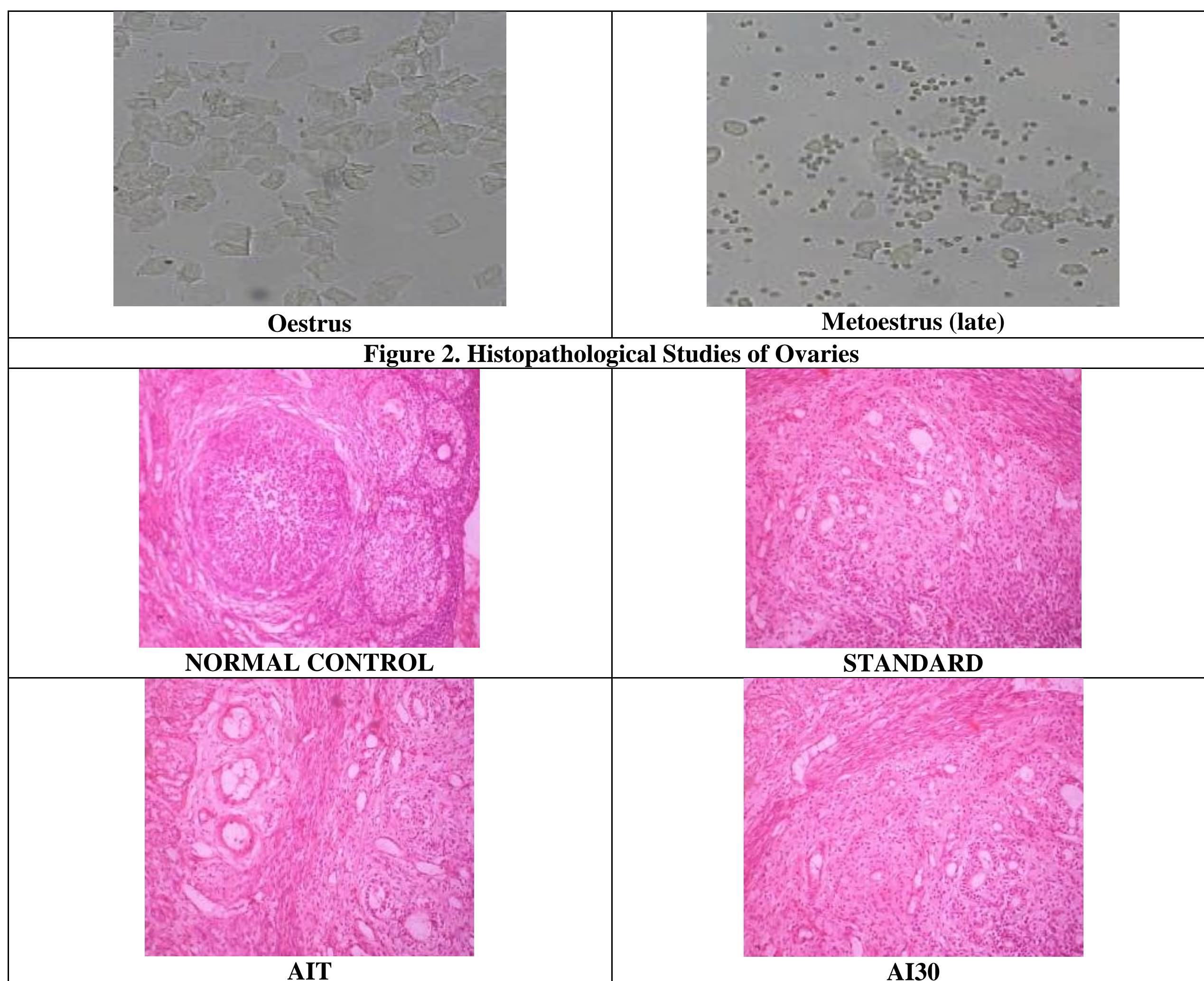

Figure 2. Histopathological Studies of Ovaries

DISCUSSION AND CONCLUSION

Ovary can be considered an aggregate of three endocrine tissues, the stroma, the follicle and the corpus luteum. The weights of these tissues constitute the net weight of the ovaries. During the estrous cycle the weight of the ovarian tissue increases under the influence of gonadotrophic and steroidal hormones (Wiest *et al*., 1963). In the present investigation, the decrease in the weight of ovaries of the mature rats after treated with AIT & AI 30 clearly indicates a decrease in the activity of the stroma, the follicle, and the corpus luteum in the ovary. Also, this decrease may be due to the non-availability of gonadotrophic or steroidal hormones or both.

In this study, AIT & AI 30 arrested the normal estrus cycle at diestrus stage where minimum activities of steroids were reported same like standard drug (Ramirez VD and McCann SM, 1964; Anderson RR and McShan WH, 1996; Tamooki B and Pincus G, 1961). This was associated with an elevation in the level of cholesterol, which is the precursor for the synthesis of steroid hormones in ovaries, suggesting thereby that cholesterol was not utilized (Guillemin R and Sakiz E, 1963). Ovarian cholesterol and ascorbic acid are responsible for synthesis of steroid hormones. The high accumulation of ovarian cholesterol & protein in the AIT & AI 30 treated rats may suggest the non-utilization of lipids towards hormonal biosynthesis in the ovaries.

Ascorbic acid, an easily diffusable water-soluble reductant, is found abundantly in ovaries, where it plays an important role in ovarian steroidogenesis (Miller WL, 1988). Its deficiency leads to malfunction of ovaries along with the elevation of ascorbic acid levels. In the present study, AIT & AI 30 caused significant elevation of ascorbic acid levels due to the malfunction of ovaries.

Steroidogenic enzymes are responsible for the biosynthesis from cholesterol of various steroid hormones including glucocorticoids, mineral

ocorticoids, progestins, androgens, and estrogens. They consist of several specific cytochrome-P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases (Reincke *et al*., 1998). De novo synthesis of all steroid hormones starts with the conversion of cholesterol to Pregnenolone by CYP11A (cholesterol side-chain cleavage) (Leers-Sucheta *et al*., 1997). Pregnenolone is converted to progesterone by 3bhydroxysteroid dehydrogenase (3β-HSD), one of several non-CYP450 enzymes involved in steroidogenesis and which is found in both mitochondria and smooth endoplasmic reticulum. 3β -HSD is widely distributed in steroidogenic and nonsteroidogenic tissues and consists of two isoenzymes, which are regulated in a tissue-specific manner (Mason *et al*., 1997; Luu-The V, 2001).

The main role of the ovary is to produce eggs for fertilization and steroid hormones for sexual and reproductive function. The ovum inside the developing follicle is directly surrounded by layers of granulosa cells followed by thecal cells, which is where steroidogenesis predominantly takes place. The theca interna is highly vascularized and produces large amounts of progesterone and androgens, which act as precursor for estrogen synthesis in the granulosa cells. Androstenedione and testosterone diffuse into the neighboring poorly vascularized granulosa cells where they are converted to predominantly estradiol via the concerted action of aromatase and 17β -HSD types 1 and 7, which favor the conversion of estrone to estradiol (Fitzpatrick *et al*., 1997). In the preovulatory follicular stage, during which the follicle maturates, estrogen synthesis increases gradually due to upregulation of aromatase by LH and FSH. During this critical phase, estrogen appears to be responsible for the upregulation of LH receptors and the initiation of the positive feedback loop responsible for the LH and FSH surge which triggers ovulation (Baillie *et al*., 1960). Interference with the synthesis of estrogens during this critical window of time would prevent ovulation. After the LH surge, the follicle enters the luteal phase and becomes a corpus luteum which predominantly synthesizes progesterone. Decreased LH concentration and subsequently decreased aromatase expression result in declining estrogen production (Greenwald G and Roy S, 1994), while a concurrent increase in CYP11A and 3β-HSD activity promotes the synthesis of progesterone which via its receptor initiates the process of follicle rupture.

The steroidogenesis in ovaries is under the physiological control of two dehydrogenases namely Glucose-6-phosphate dehydrogenase (G-6-PDH) and Δ^5**-3β**-hydroxysteroid dehydrogenase Δ^5**-3β** −HSD). Both the enzymes that are directly involved in biosynthesis of steroidal hormones in ovaries have been reported (Armstrong DG, 1982). Any alteration in the activity of these two enzymes reflects on the hormonal production in ovaries. To substantiate this fact, the estimation of G-6-PDH and Δ^5-3β-HSD, the two key enzymes involved in steroidogenesis was estimated (Suzuki *et al*., 1984). The AIT & AI 30 were significantly inhibited the activity of two key steroidogenic enzymes.

It has been observed that blood and serum parameters are within the normal range indicating non-toxic action on general body metabolism. From the overall results, it is evident that oral administration of the AIT & AI 30 causes inhibition of both steroidogenic enzymes and elevation of ovarian cholesterol and ascorbic acid content and induces functional sterility without toxic symptoms and they exert antisteroidogenic/antifertility activity in rats.

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