International Journal of Phytopharmacology

www.onlineijp.com

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

Research article

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

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ABSTRACT

In the present study, the effect of AIT & AI 30on the reproduction system of sexually immature female rats have been investigated to elucidate the possible mode of action and the influence of AIT and AI 30 on the onset of sexual maturity and the ovarian steroidogenesis. The effects of the AIT & AI 30on the reproduction system of pre-pubertal rats were determined by studying the following parameters. i. Age of vaginal opening and first estrus, ii. Estimation of Cholesterol and Ascorbic Acid contents in treated immature rats ovaries, iii. Estimation of Glucose-6-phosphate dehydrogenase (G-6-PDH) and Δ^5-3β-Hydroxysteroid dehydrogenase (Δ^5-3β-HSD) Activities in treated immature rats ovaries. On the basis of the aforementioned results, it is inferred that the delay of the onset of puberty following treatment with AIT and AI 30could possibly be due to the depression in ovarian steroidogenesis.

Key words: Anti-steroidogenesis, Cholesterol, Ascorbic Acid, Glucose-6-phosphate dehydrogenase, Δ^5-3β-Hydroxysteroid dehydrogenase.

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INTRODUCTION

It is well established that the development of sexual organs in early life and the onset of puberty is closely related with ovarian steroidogenesis (Armstrong *et al*., 1964) under the influence of gonadotropins (Horikoshi H, Wiest WG, 1971). A small amount of estrogen has been shown to induce precocious puberty in immature female rats (Turner CD, Bonara JT, 1976; Short DJ, Wood DP, 1969). The estrogen directly acts on vagina to cause vaginal opening and cornification.

In the present study, the effect of AIT & AI 30on the reproduction system of sexually immature female rats have been investigated to elucidate the possible mode of action and the influence of AIT and AI 30 on the onset of sexual maturity and the ovarian steroidogenesis. The effects of the AIT & AI 30on the reproduction system of pre-pubertal rats were determined by studying the following parameters. i. Age of vaginal opening and first estrus, ii. Estimation of Cholesterol and Ascorbic Acid contents in treated immature rats ovaries, iii. Estimation of Glucose-6-phosphate dehydrogenase (G-6-PDH) and Δ^5-3β-Hydroxysteroid dehydrogenase (Δ^5-3β-HSD) Activities in treated immature rats ovaries.

Access this article online

Home page: http://onlineijp.com/

DOI: http://dx.doi.org/10.21276/ijp.2017.8.1.5

Quick Response code

Received:25.12.16 **Revised**:12.01.17 **Accepted**:15.01.17

MATERIALS AND METHODS

Animals

A total of forty albino wistar female rats procured from animal house of Vinayaga Mission College of Pharmacy, Salem, Tamil Nadu were reared from 35 to 70 days of age in four groups

(Group I, II, III and IV) consisting of ten rats in each group. Permission was obtained from Institutional Animal Ethics Committee with Approval No. P.col/64/2011/IAEC/VMCP, to conduct the experiment. Each rat was identified individually in a group with the markings of picric acid on the hairs at different sites of the body. They were maintained in polypropylene rat cages under standard laboratory hygienic conditions, providing balanced laboratory animal feed and water ad libitum. 35 to 70 days of age in four groups and selected for the present investigation.

Experimental design

This experiment was designed and carried out according to Gupta *et al*. The pre-pubertal rats were divided into four groups (n=10 in each group) and given the following treatment:

Group I (Vehicle control): 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 prepared as per HPI, respectively, suspended in Vehicle (Dosage – 0.2ml/kg.b.w., p.o)

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

The vehicles and test drugs were given orally from 35 days of age of rats on every day for 35 days. Initial body weight before treatment (i.e. at the age of 35 days) and final body weight during sacrifice (i.e. at the age of 70 days) were recorded. Five animals of each group were carefully noted to observe the onset of sexual maturity and left intact. Two measures of reproductive maturity were made: age at vaginal opening, age at first estrus. The rats were inspected between 7-9 a.m. for vaginal opening, a daily vaginal lavage was taken from each rat to determine the age at first estrus (cornified smear) (Whitten, 1965; Ojeda SR and Urbanski HF, 1994). The remaining five rats from each group were sacrificed by cervical dislocation 24 h after the last dose (i.e. at the age of 70 days) and 18 h fasting condition. Ovaries and uterus were dissected out, freed from fatty materials, weighed and kept on ice for further processing.

Organ weights

The remaining five rats from each group were sacrificed by cervical dislocation 24 h after the last dose (i.e. at the age of 70 days) and 18 h fasting condition. After sacrificing, they were dissected out and the left and right ovaries were collected in petridish containing 0.9% normal saline. The ovaries were trimmed and blotted with filter paper and weight of the ovaries was recorded with the help of electronic digital balance and kept on ice for biochemical estimation. Similarly, the weight of the uterus was also recorded (Narayana Swamy, 2004).

Number of Ovarian surface follicles

Total number of surface follicles on both the ovaries was counted with the help of stereozoom microscope to serve as the indicator of folliculogenesis (Ojeda SR and Urbanski HF, 1994).

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 $P<0.05$.

RESULTS

Effects of AIT and AI30 on the age at vaginal

opening and appearance of first estrus and body growth rate

The results showed in Table-1 indicated clearly that the oral administration of AIT and AI30 to the 70 days old female rats significantly ($P < 0.001$) retarded the onset reproductive maturity as indicated by the age at vaginal opening and appearance of first estrus when compared to vehicle control group. In the same condition a reduction in the rate of body growth (weight gain) accompanied the delay in sexual maturation. Whereas standard group animals showed significantly ($P < 0.001$) retarded the onset reproductive maturity and reduction in the rate of body weight (Table-1).

Effect of AIT and AI30 on the weight of reproductive organs

Marked reductions were observed in the weights of ovary and uterus, AIT, AI30, and standard -treated immature rats when compared with vehicle control. That is, among the three treatments, the standard drug showed a remarkably highest percent of reduction in the weights of ovary and uterus (24.70±0.3038& 81.20± 0.8602, respectively), followed by AI30 (26.39±0.4425 and 84.80 ±1.281, respectively), AIT (27.95±0.2396 and 88.6 ±1.208 respectively). The results are included in Table 2.

Ovarian surface follicles

The numbers of ovarian follicles were significant reduction of folliculogenesis process in Group II, Group III and Group IV, that received standard drug, AIT and AI30 respectively. standard drug, AIT and AI30 treatment groups was not enough to produce significant effect with respect to folliculogenesis. The results are included in Table 2.

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 3).

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 when compared with vehicle control (Table 4). 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. Mean age in days of immature female rats at two measures of sexual maturity and rate of body growth after treated with AIT and AI30

Treatment Design	Dose (mg/kg b.w)	Vaginal opening (mean age in days)	First estrus (mean age in days)	Body weight		
				Initial body weight (g)	Final body weight (g)	% Increase in b.w
Group-1: Control	10 ml	55.60 ± 0.600	75.60 ±0.6782	77.80±1.393	147.60 ±3.829	89.72
Group-2 : Standard	0.02mg	70.80±0.6633**	93.80±0.8602**	76.20± 1.744	131.20± 1.158**	72.18
Group-3: AIT	200mg (0.2ml/kg)	64.0±0.5477**	81.40±0.8124**	76.60±1.364	139.20±1.241*	81.72
Group-4: AI30	200 mg (0.2ml/kg)	66.20± 1.068**	87.60±0.8124**	77.40±1.568	135.80±0.8602**	75.45

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 2. Weights of ovary, Number of surface ovarian follicles and Weights of uterus of immature female rats after treated with AIT and AI30

Treatment Design	Dose (mg/kg b.w)	Weight of ovaries (mg)	Number of surface ovarian follicles	Weight of uterus (mg)
Group-1: Control	10 ml	31.09±0.6042	12.20±0.3742	99.80 ±1.175
Group-2: Standard	0.02mg	24.70±0.3038**	7.20±0.3742**	81.20± 0.8602**
Group-3: AIT	200mg(0.2ml/kg)	27.95±0.2396**	9.00±0.3162**	88.6 ±1.208**
Group-4: AI30	200 mg (0.2ml/kg)	26.39±0.4425**	8.40±0.2449**	84.80 ±1.281**

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 3. Effect of AIT and AI30on contents of ascorbic acid, cholesterol and protein in immature female rat ovaries

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	44.80± 1.068	70.00 ± 0.7071	9.60 ± 0.5099
Group-2: Standard	0.02mg	72.20 ± 0.800**	121.80 ± 1.281**	6.54 ± 0.0927**
Group-3: AIT	200mg (0.2ml/kg)	62.60±0.9274**	93.20 ± 1.241**	7.82 ± 0.2154**
Group-4: AI30	200 mg (0.2ml/kg)	66.80 ± 0.800**	106.40±01.7483**	6.98 ± 0.0663**

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.

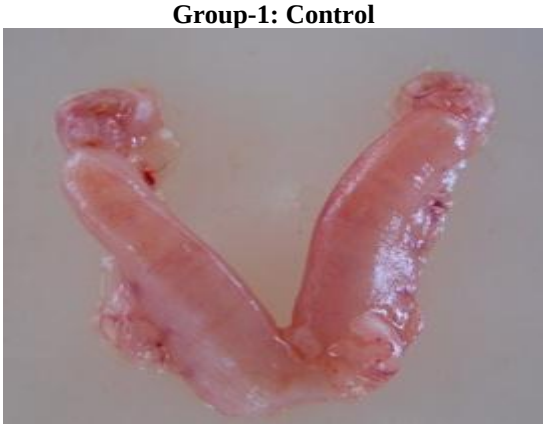
Table 4.Effect of AIT and AI30 on G-6-PDH and Δ^5-3β-HSD activities in immature female rat ovaries

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	3.16 ± 0.0509	1.04 ± 0.0139
Group-2: Standard	0.02mg	1.22 ± 0.0663**	0.70 ± 0.0196**
Group-3: AIT	200mg (0.2ml/kg)	1.96 ± 0.0504**	0.89 ± 0.0092**
Group-4: AI30	200 mg (0.2ml/kg)	1.62 ± 0.033**	0.81 ± 0.0044**

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.

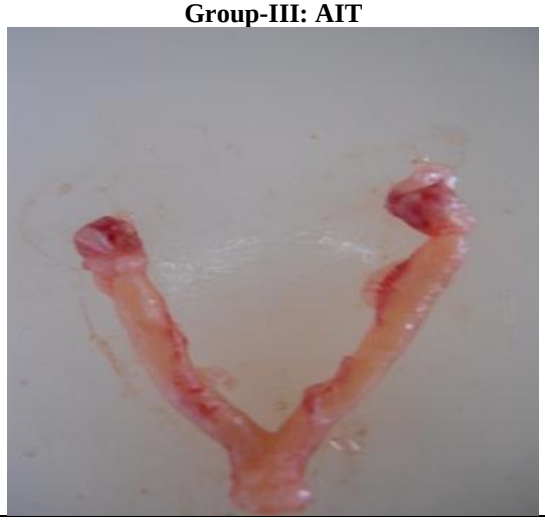
Figure 1. Photographs of Uterus with Ovarian Follicles in Immature Rats

Group-1: Control



Group-II: Standard

Group-III: AIT



Group-IV: AI30

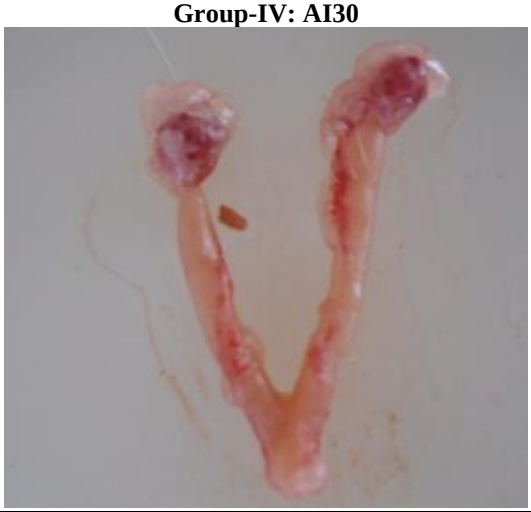
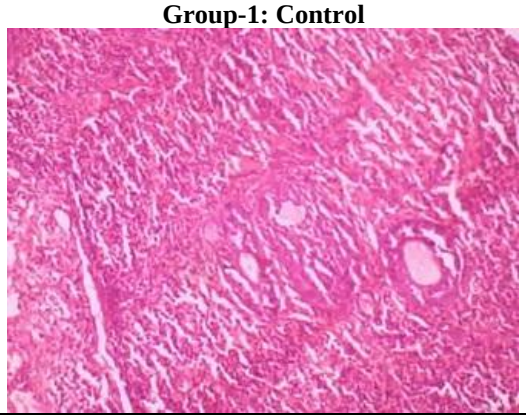
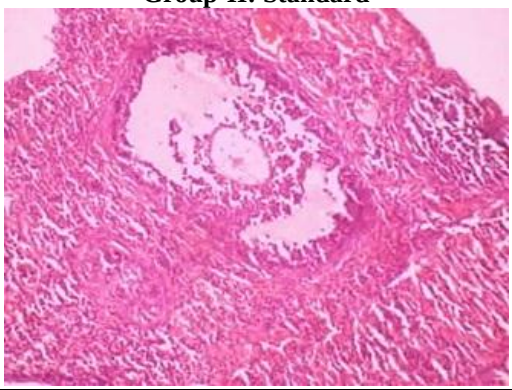


Figure 2. Histopathological Studies of Uterus in Immature Rats

Group-1: Control



Group-II: Standard



Group-III: AIT

Group-IV: AI30

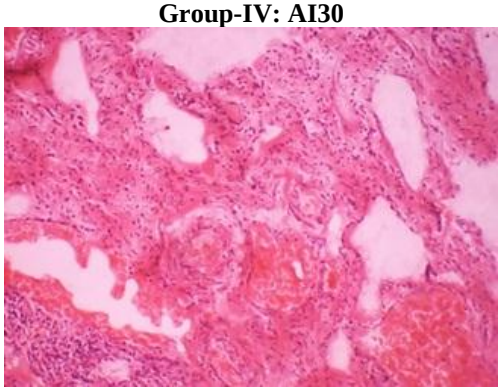
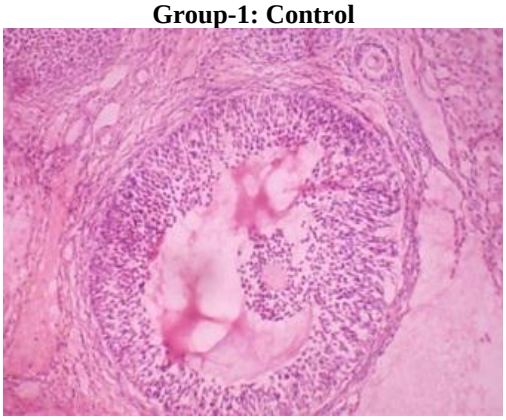
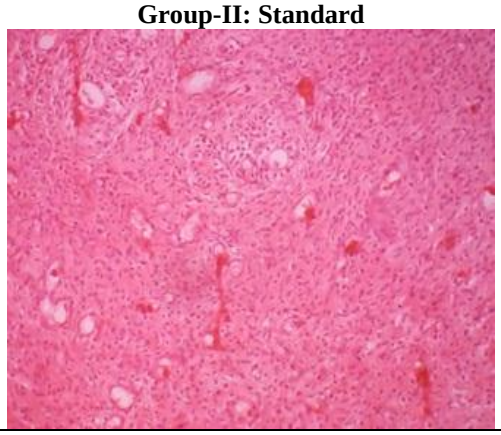


Figure 3. Histopathological Studies of Ovary in Immature Rats

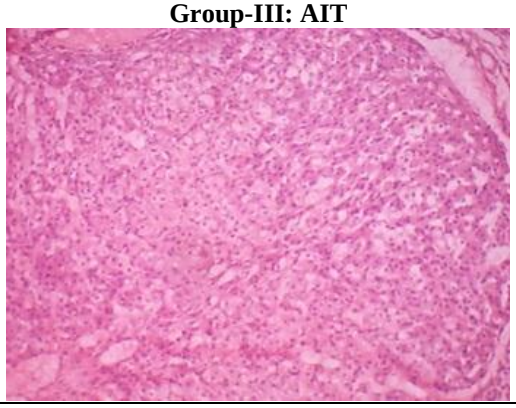
Group-1: Control



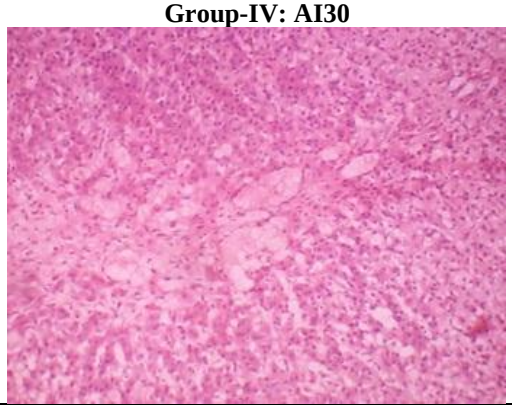
Group-II: Standard



Group-III: AIT



Group-IV: AI30



DISCUSSION AND CONCLUSION

At present it is supported by a large number of workers that onset of puberty is closely related with ovarian steroidogenesis (Armstrong *et al*., 1964; Horikoshi H, Wiest WG, 1971). The present study confirmed that the oral administration of AIT and AI 30 remarkably delayed the onset of sexual maturity as evidenced by the age at vaginal opening (Elbetieha *et al*., 1998) and appearance of first estrus. These disturbances in the reproductive cycle and the decrease in the weight of the ovary and uterus in the present investigation may be related with the diminution of ovarian steroidogenesis (Rindi *et al*., 1963). This was associated with an elevation in the level of cholesterol which serves as a precursor for the synthesis of steroid hormone in ovaries (Marcus R, Coulston AM, 1996; Rang *et al*., 1999; Wilks *et al*., 1970) and also precursor for the steroidogenesis of ovarian endocrine tissue (Strauss *et al*., 1981) suggesting thereby that cholesterol was not utilized. The depressed ovarian steroidogenic activity and hypo-functioning of the gland was evident by increase in ascorbic acid level after treatment with AIT and AI 30 (Deane, 1952). However, this accumulation of cholesterol and ascorbic acid was found to be more in ovaries of immature rats after treated with AIT and AI 30.

There was delayed in the age of attainment of puberty as evidenced by opening of vagina during canalization that was indicated by perforation in the treatment groups administered with AIT and AI 30. However, there was non-significant ($P>0.05$) delayed in the age of attainment of puberty in Group III & IV, which were administered with AIT and AI 30.

The significant ($P<0.05$) delayed in the age of attainment of puberty in rats, in the present study, could be attributed to AIT and AI 30that inhibited positive estrogenic feedback effects on the central nervous system that induced puberty which may be due to inhibition of steroidogenic activity of ovary under the hypothalamic effect. However, decreased weight of ovary was noticed in the rats administered with AIT and AI 30.The stereo zoom microscopic examination of ovaries revealed that the population of surface follicles on both the ovaries decreased significantly in Group II, III and IV. This inhibition of follicle population may be attributed to phytoconstituents present in AIT and AI 30that potentially decreased growth and development of follicles. The AIT and AI 30exhibited antifertility activity.

To substantiate these facts, the estimation of G-6-PDH and Δ^5-3β-HSD, the two key enzymes involved in steroidogenesis was performed (Armstrong, 1982; Suzuki *et al*., 1984). The importance of G-6-PDH of pentose phosphate pathway in the synthesis of estrogen in the sexually immature animals has been reported earlier (Dey*et al*., 1972). It is also well established that Δ^5-3β-HSD is associated with steroid biogenesis (Knorr *et al*., 1870). In the present study, the AIT and AI 30 significantly inhibited the activity of two key steroidogenic enzymes in a dose-dependent manner. Moreover, this diminution of ovarian G-6-PDH and Δ^5-3β-HSD activities were found to be more in immature rats after treated with AIT and AI 30. Thus, a fall of G-6-PDH and Δ^5-3β-HSD after treatment with AIT and AI 30 suggests a diminution of ovarian steroidogenesis (Mazumder *et al*., 1997; Dhanapal *et al*., 2005; Gupta *et al*., 2003).

Overall, the AIT and AI 30 delayed the onset of puberty and suppressed the ovarian steroidogenesis, which confirms the effect observed with AIT and AI 30. On the basis of the aforementioned results, it is inferred that the delay of the onset of puberty following treatment with AIT and AI 30could possibly be due to the depression in ovarian steroidogenesis.

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Cite this article:
Nirmal G and Arun Bhasme. Antisteroidogenic activity of *Azadirchta indica* tincture (AIT) and *Azadirchta indica* 30 potency (AI 30) in immature female rats. *International Journal of Phytopharmacology*, 2017;7(1):23-30.
DOI: **http://dx.doi.org/10.21276/ijp.2017.8.1.5**