Research Paper

Ameliorative effect of *Withania somnifera* against Imidacloprid Induced Alterations in Oestrous Cycle of Female Albino Wistar Rats

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The present experiment was aimed to assess imidacloprid induced alterations in oestrous cycle and ameliorative effects of *Withania somnifera* in rats. 48 female albino Wistar rats were divided into four groups of 12 animals each. Group 1 is control, group 2 is imidacloprid (30 mg/kg body weight/d), group 3 is *Withania somnifera* (1 g/kg feed) and group 4 is imidacloprid+*Withania somnifera* (30 mg/kg body weight/d+1 g/kg feed). The test chemical was administered daily by oral gavage for 30 d. Seven consecutive estrous cycles were monitored in all the four groups by vaginal smear test. From each group, 6 rats were sacrificed on 16th d and remaining were sacrificed on 31st d. Blood samples were collected before sacrifice on 16th and 31st d of experiment for estimation of hormones in serum. A significant increase in length of oestrous cycle, follicle stimulating hormone levels and decrease in oestrogen levels were noticed in group 2. It is concluded that administration of *Withania somnifera* provided moderate protection against the imidacloprid induced toxicity by regularization of oestrous cycle in rats due to its antioxidant and gonadotropic properties.

Key words: Follicle stimulating hormone, imidacloprid, luteinizing hormone, oestrogen, oestrous cycle, *Withania somnifera*

Pesticides are widely used in agriculture and animal husbandry practices to increase the crop yield by controlling pests on plants and used as ectoparasiticides on animals. But widespread usage of insecticides leads to environmental pollution and persistence of their residues in the food chain resulting in accidental exposure and toxicity in human beings and other non-targeted organisms. Imidacloprid is a neonicotinoid, chloronicotinic insecticide, classified as category I due to its high leaching potential. It is extensively used to protect crops from piercing sucking pests, to control the household pests and parasites on animals[1-3]. Presently, neonicotinoids account for approximately one third of the world insecticide market[4]. It acts by inhibiting the nicotinic receptors of acetylcholinesterase and thus prevents acetylcholine from transmitting impulses between nerves, result in paralysis and death of insects[5]. It is highly and selectively toxic to insects due to its high affinity towards insect's nicotinic acetylcholine receptors compared to mammals[6,7]. It is known to induce reproductive toxicity and endocrine disruption in laboratory animals[8].

Withania somnifera (*W. somnifera*) is commonly known as Ashwagandha, Indian ginseng, poison gooseberry and winter cherry. Ashwagandha belongs to Solanaceae or nightshade family and commonly used as an Ayurveda medicine due to its anti-stress, antioxidant and free radical scavenging activities[9,10]. It is known to possess gonadotropic function which increases the gonadal weight by increasing the ovarian follicle size in females and by increasing the seminiferous tubular cell layers in male animals[11-14] and hence it was chosen as an ameliorating agent in current experiment to assess the protective role of it against the imidacloprid

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induced changes in oestrous cyclicity and reproductive hormone levels in female rats.

MATERIALS AND METHODS

Chemicals:

Imidacloprid was procured from Tropical Agrosystem India Pvt. Ltd., Chennai, Tamilnadu and *W. somnifera* was obtained from Herb leaf Organic, Haryana.

Experimental animals:

Forty-eight young female Wistar rats weighing 200-250 g were procured from Jeeva life sciences, Hyderabad. The rats were acclimatized to the experimental conditions for 10 d before commencement of the study. They were housed in solid bottom polypropylene cages at lab animal house in the Ruska labs, Hyderabad and were maintained in controlled environment (temperature 20°-22°) throughout the course of the experiment. Rice husk was used as a bedding material. All the rats were provided *ad libitum* with standard pellet diet (procured from Vyas Labs, Uppal, Hyderabad) and water throughout the experimental period. The experiment was conducted according to the guidelines and with prior approval of the Institutional Animal Ethics Committee (IAEC-No.7/22/C.V.Sc., Hyd./29.02.2020).

Experimental design:

The rats were divided into four groups comprising of 12 in each. Group 1 served as control, group 2 was received with imidacloprid at the rate of 30 mg/kg body weight (b.wt)/d, group 3 was given with *W. somnifera* at the rate of 1 g/kg feed and group 4 was treated with both imidacloprid and *W. somnifera* (30 mg/kg b.wt/d+1 g/kg feed). The test compound was administered for a period of 30 d daily by oral gavage.

Evaluation of estrous cycle:

In the present study, seven consecutive oestrous cycles were monitored in all the four groups by vaginal smear test[15-17]. Daily vaginal smear was taken early in the morning by pipette smear technique. The method consists of flushing of cells from the vaginal lining by gently inserting the tip of a soft plastic pipette containing approximately 0.2 ml of 0.9 % normal saline into rat vaginal orifice to a depth of 2-5 mm and then the saline was flushed into the vagina and back out into the pipette 2-3 times by gently squeezing and releasing the bulb of the pipette. Then small amount of the cell suspension was expelled onto a labelled clean glass slide. Further slides were stained with Giemsa's stain and observed under light microscope[18]. In the vaginal smear, the stage of oestrous cycle was identified by the number and morphology of the cells in the smear.

The length of each oestrous cycle was calculated as per the method adopted by Mandl and Li[19,20]. The relative lengths of the various stages in oestrous cycle were determined by the method described by Astwood[21]. For each animal, the number of hours in each of the four stages in oestrous cycle were recorded and converted into number of days by dividing the number of hour with 24 h.

Hormone estimation:

From each group, 6 rats were sacrificed on 16th d and remaining rats were sacrificed on 31st d. Before sacrifice, on 16th d and 31st d of experiment, approximately 2 ml of blood was collected from each rat from retro orbital plexus through capillary tube into a clot promoting vacutainers and allowed to clot for 3 h to 4 h, later centrifuged at 20 k rpm for 10 min, serum was separated into Eppendorf tubes and stored at -20°. Later the serum samples were analyzed for Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and estrogen by using Enzyme-Linked Immunosorbent Assay (ELISA) kits.

Statistical analysis:

The data obtained from present study were subjected to statistical analysis by applying one way Analysis of Variance (ANOVA) using Statistical Package for Social Sciences (SPSS) version 16.0. Differences between means were tested using Duncan's multiple comparison test and significance was set at $p<0.05$[22].

RESULTS AND DISCUSSION

Vaginal smears in different group of rats revealed a large number of nucleated epithelial cells during proestrous, large numbers of cornified epithelial cells during oestrous, a combination of leukocytes with few cornified epithelial cells during metestrous and a combination of leukocytes with few nucleated epithelial cells during diestrous phase (fig. 1-fig. 4).

Group 2 rats showed a significant ($p<0.05$) increase in the length (d) of estrous cycle with a mean length of 6.10±0.11 as compared to group 1 which had a mean length of 4.25±0.03 whereas there was no significant difference in mean length of estrous cycle between groups 1, 3 (4.33±0.06) and 4 (4.28±0.08) respectively (fig. 5).

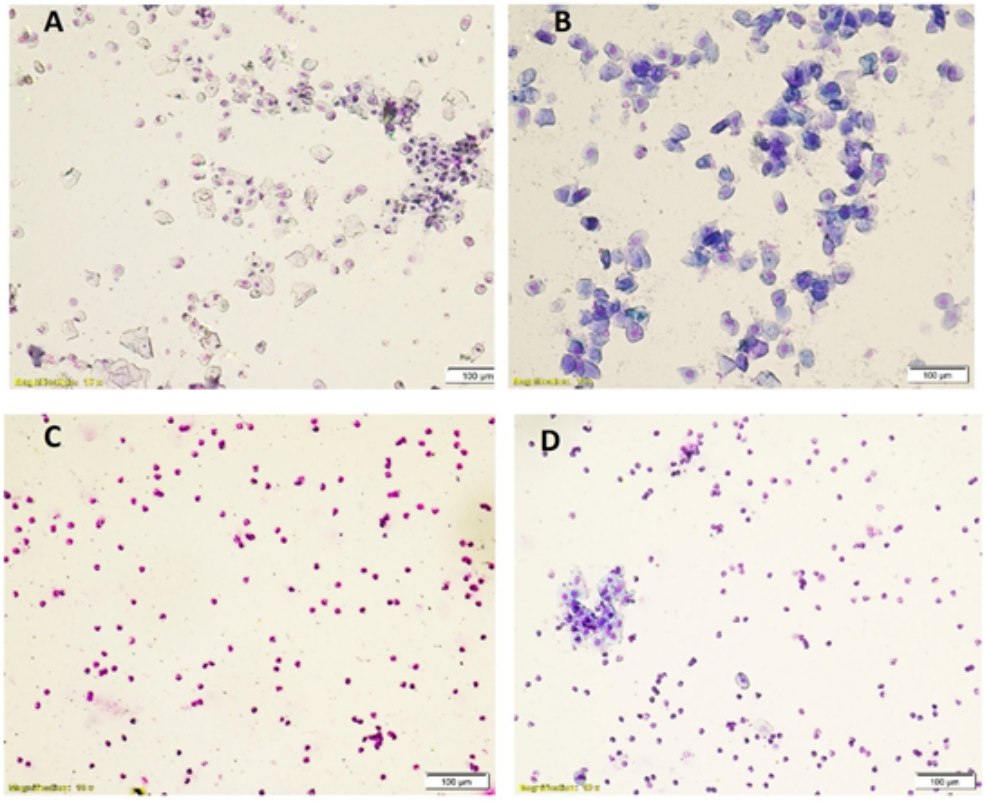

Fig. 1: Photomicrograph showing normal oestrous cycle, (A) Proestrous; (B) Oestrous; (C) Metestrous and (D) Diestrous, (Group 1): Giemsa 100×

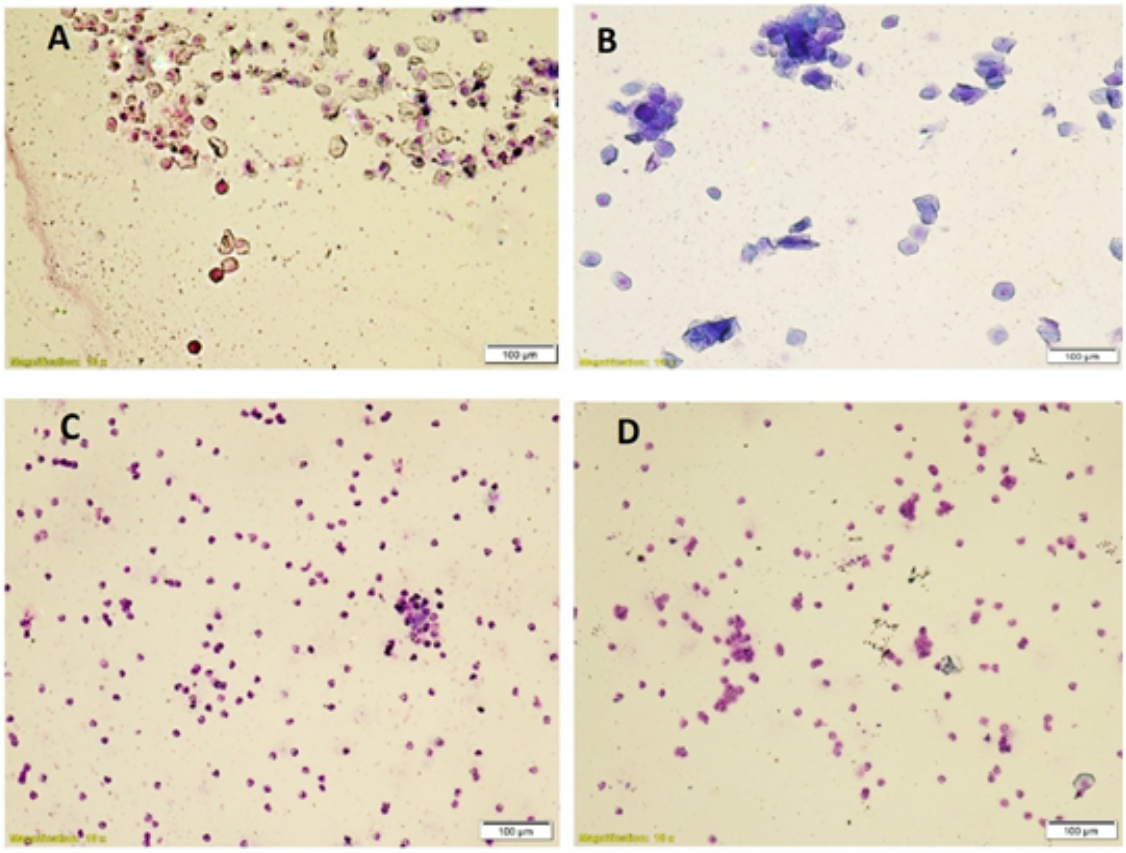

Fig. 2: Photomicrograph showing prolonged oestrous cycle with increased duration of diestrous, (A) Proestrous; (B) Oestrous; (C) Metestrous and (D) Diestrous, (Group 2): Giemsa 100×

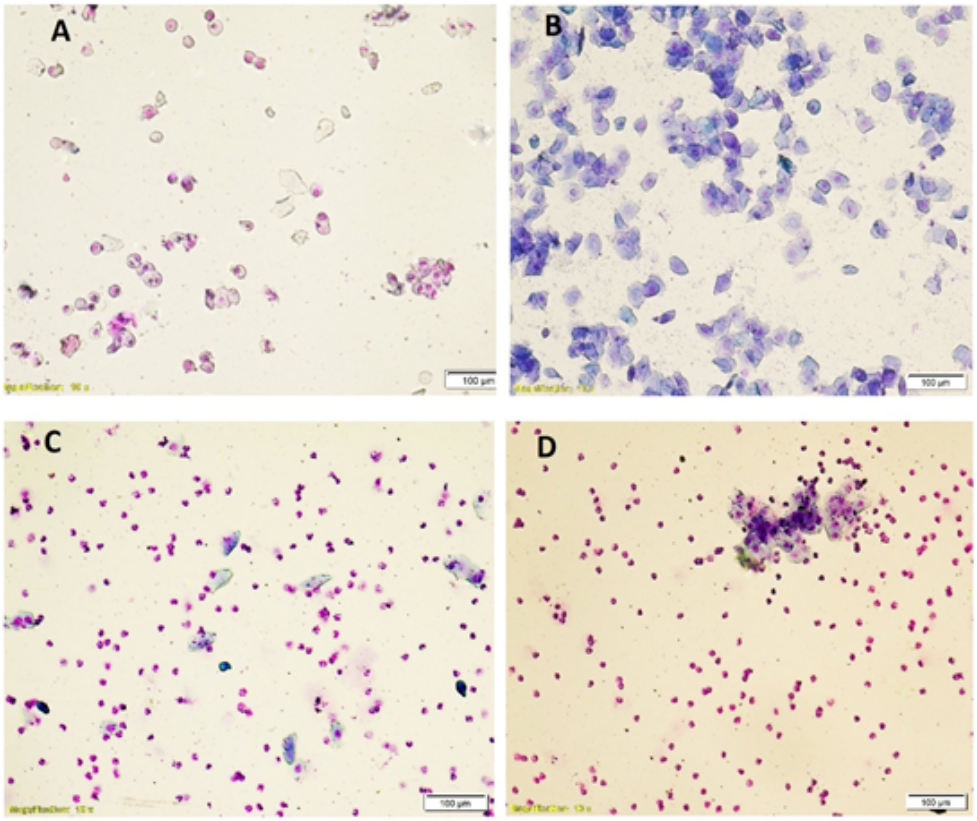

Fig. 3: Photomicrograph showing normal oestrous cycle, (A) Proestrous; (B) Oestrous; (C) Metestrous and (D) Diestrous) (Group 3): Giemsa 100×

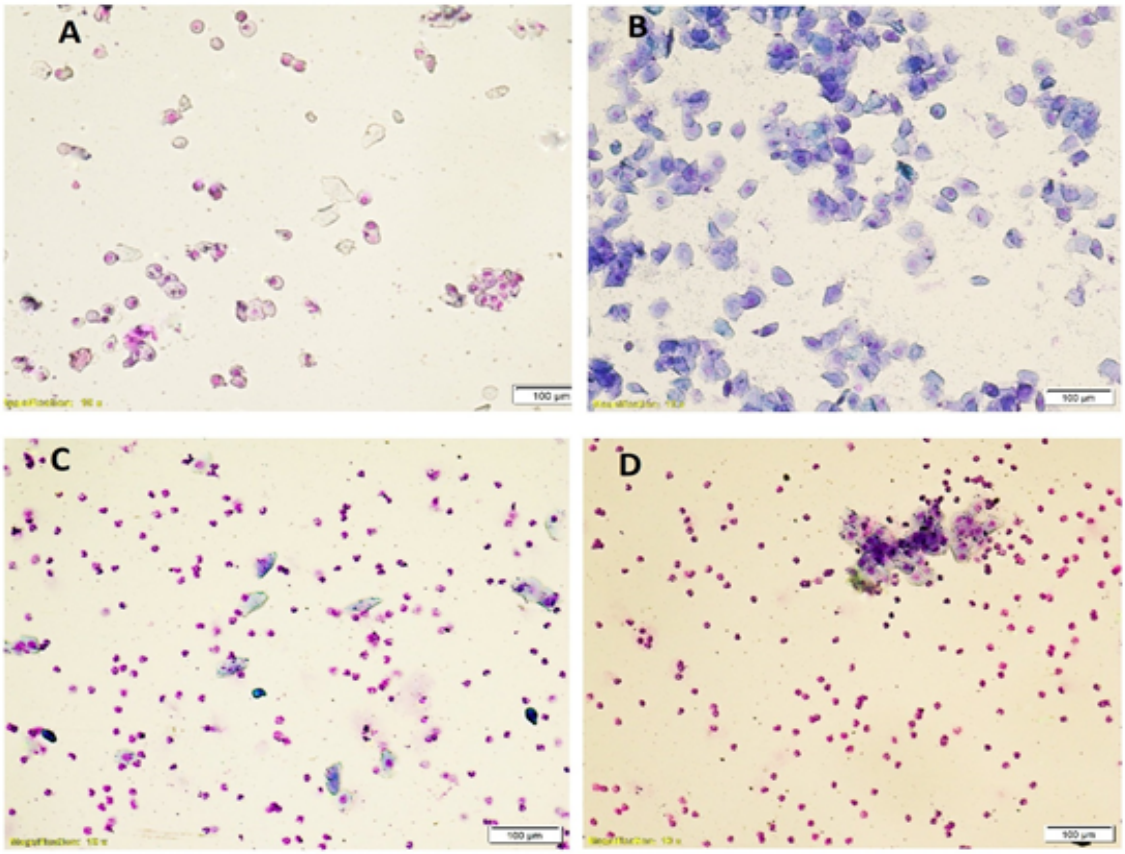

Fig. 4: Photomicrograph showing normal oestrous cycle, (A) Proestrous; (B) Oestrous; (C) Metestrous and (D) Diestrous, (Group 4): Giemsa 100×

No significant difference was observed in average length (d) of proestrous phase between groups 1 (0.47±0.05), 2 (0.42±0.03), 3 (0.45±0.04) and 4 (0.43±0.03) respectively.

The average length (d) of oestrous phase in group 2 (1.13±0.06), 3 (1.18±0.07) and 4 (1.23±0.05) did not differ significantly from control (1.08±0.04).

There was no significant difference in average length (d) of metestrous phase between groups 1 (0.37±0.04), 2 (0.38±0.04), 3 (0.32±0.03) and 4 (0.35±0.04) respectively.

The average length (d) of diestrous phase in group 1 is 2.33±0.06 whereas it was significantly ($p<0.05$) increased to 4.16±0.06 in group 2 and there was no significant difference in average length of diestrous phase between groups 1, 3 (2.38±0.05) and 4 (2.27±0.07) respectively (fig. 6).

Evaluation of the oestrous cycle in laboratory rodents is a useful measure of integrity of the hypothalamic-pituitary-ovarian reproductive axis and it can contribute an important information regarding the nature of a toxicant insult to the reproductive system[15]. In general,

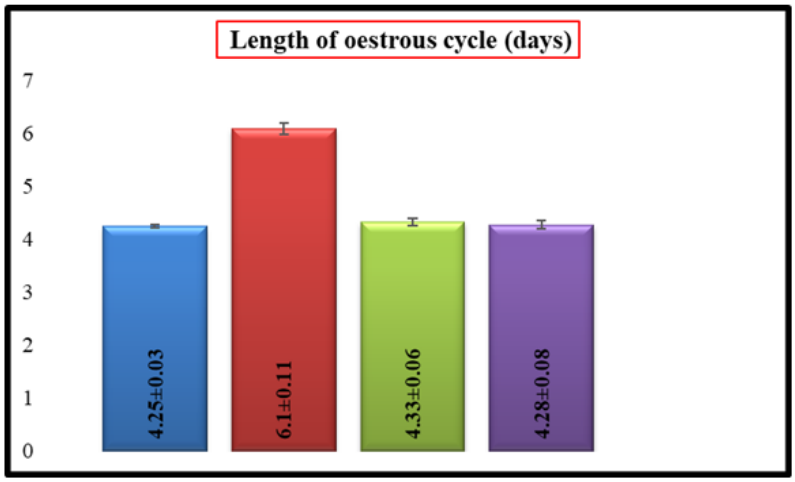

Fig. 5: Length of oestrous cycle (days) in different groups, values are expressed as mean±Standard Error (SE) (n=6) and one way ANOVA, (■) Group 1; (■) Group 2; (■) Group 3 and (■) Group 4

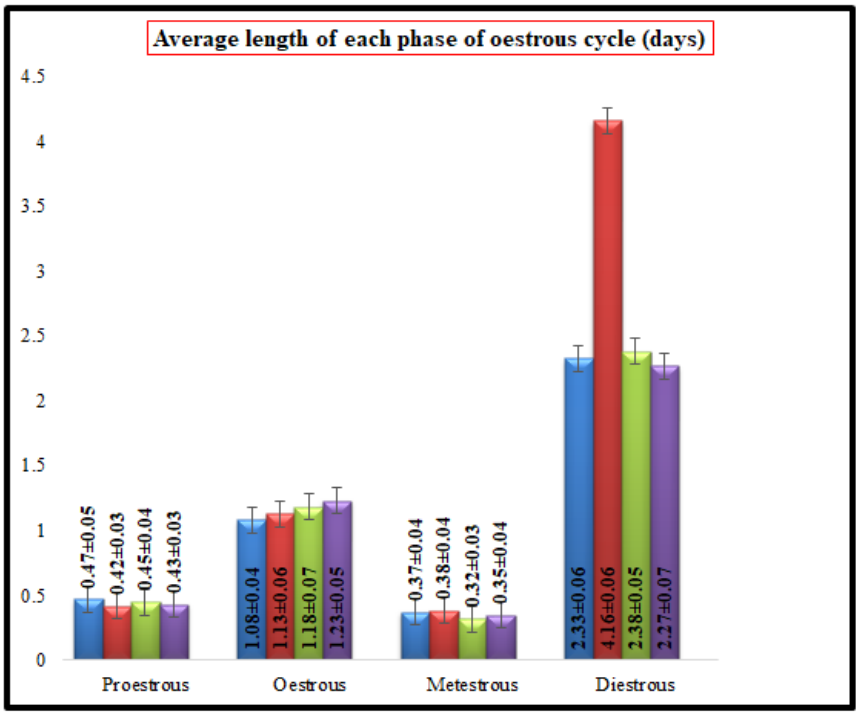

Fig. 6: Average length of each phase of oestrous cycle (days) in different groups, values are expressed as mean±Standard Error (SE) (n=6) and one way ANOVA, (■) Group 1; (■) Group 2; (■) Group 3 and (■) Group 4

the xenobiotic which induce the ovarian toxicity will alter the oestrous cycle in laboratory animals. In present study, increased duration of oestrous cycle is due to the prolonged diestrous phase in group 2 rats which might be resulted from disruption of the ovarian follicles and decreased levels of estrogen produced by imidacloprid induced oxidative stress. These results were agreed with previous authors[23,24].

However, simultaneous treatment of *W. somnifera* along with imidacloprid regularized the oestrous cycle in group 4 rats. It may be due to regeneration of ovarian follicles due to gonadotropic function of *W. somnifera*[11-14] that resulted in increased production of oestrogen by ovaries and led to regularization of oestrous cycle. Earlier, the role of *W. somnifera* in normalizing the oestrous cycle in letrazole induced polycystic ovarian syndrome in rats was reported by Saiyed *et al.*[25].

No significant difference was observed in mean LH (ng/ml) values between groups 1 (0.55±0.02 and 0.56±0.03), 2 (0.58±0.05 and 0.57±0.05), 3 (0.54±0.03 and 0.53±0.04) and 4 (0.52±0.05and 0.51±0.03) on 16th and 31st d of experiment respectively (fig. 7).

Significantly ($p<0.05$) increased mean values of FSH (mIU/ml) were recorded in group 2 (5.13±0.27 and 5.57±0.30) when compared with group 1 (2.58±0.22 and 2.75 ±0.16) on 16th d and 31st d of experiment respectively. There was no significant difference in mean values of FSH between groups 1, 3 (2.48±0.31 and 2.85±0.24) and 4 (2.63±0.17 and 2.93±0.23) on 16th d and 31st d of experiment respectively (fig. 8).

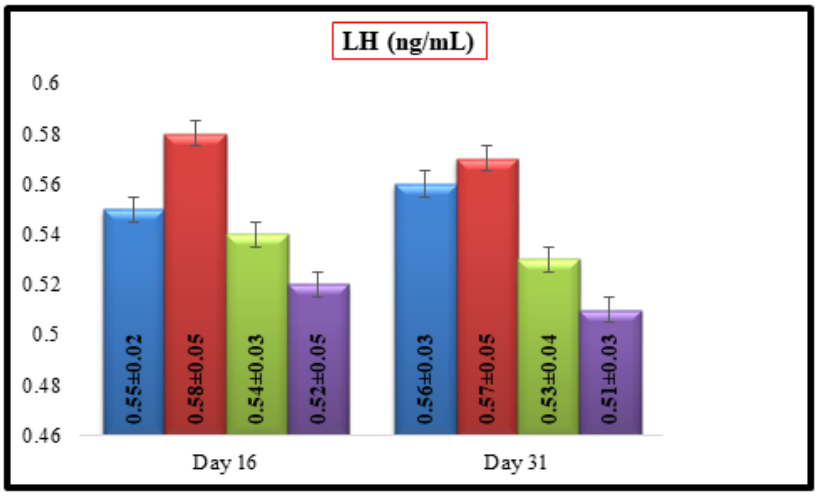

Fig. 7: LH (ng/ml) in different groups, values are expressed as mean±Standard Error (SE) (n=6) and one way ANOVA, (■) Group 1; (■) Group 2; (■) Group 3 and (■) Group 4

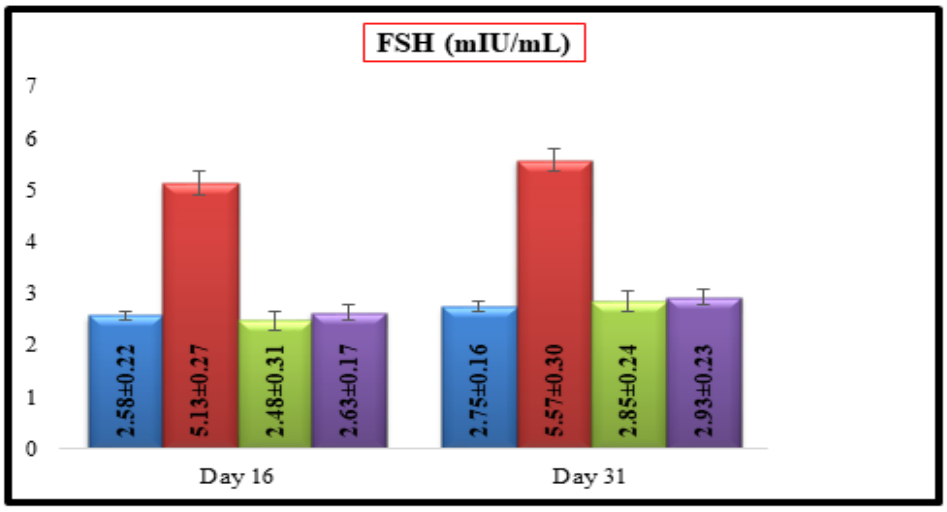

Fig. 8: FSH (mIU/ml) in different groups, values are expressed as mean±Standard Error (SE) (n=6) and one way ANOVA, (■) Group 1; (■) Group 2; (■) Group 3 and (■) Group 4

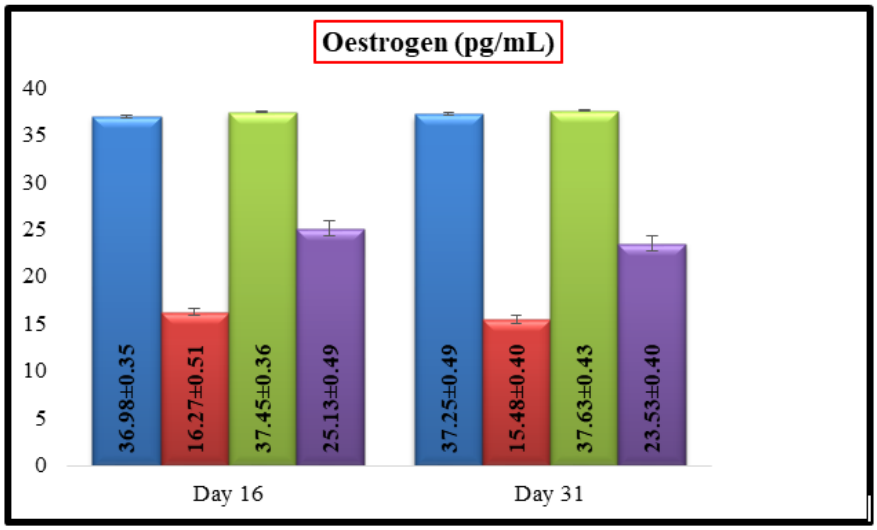

Fig. 9: Oestrogen (pg/ml) in different groups, values are expressed as mean±Standard Error (SE) (n=6) and one way ANOVA, (■) Group 1; (■) Group 2; (■) Group 3 and (■) Group 4

The mean values of oestrogen levels (pg/ml) in group 2 (16.27±0.51 and 15.48±0.40) and group 4 (25.13±0.49 and 23.53±0.40) were significantly ($p<0.05$) decreased when compared with group 1 (36.98±0.35 and 37.25±0.49) and group 3 (37.45±0.36 and 37.63±0.43) on 16th d and 31st d of experiment respectively. In group 4, a significant ($p<0.05$) increase in oestrogen levels were recorded in comparison to group 2 and no significant difference was observed in oestrogen levels between groups 1 and 3 (fig. 9).

The major source for production of estradiol is ovary and FSH is produced from anterior pituitary in response to gonadotropin-releasing hormone from hypothalamus in animals. In present case, a significant decrease in levels of estrogen were noted in imidacloprid treated rats which may be due to the oxidative damage to ovarian follicles resulted in decreased production of estradiol which might have further stimulated a negative feedback to the hypothalamus and resulted in increased secretion of FSH to stimulate the growth of ovarian follicles. But the matured ovarian follicles were again attacked by imidacloprid, this cycle was continued and lead to persistent reduction in oestrogen and elevation in FSH levels. These findings were in conformity with earlier researchers[26,27]. The hormonal imbalance in current experiment suggests that the imidacloprid would have induced toxic effects on hypothalamus-hypophysis-ovary axis.

However, simultaneous administration of *W. somnifera* along with imidacloprid to the rats demonstrated a significant ($p<0.05$) increase in oestrogen and decrease in FSH levels in group 4 rats in comparison with group 2 rats. This biological change might have triggered by gonadotropic function[11-14] of *W. somnifera*. It may also be due to prevention of oxidative damage to ovaries by its free radical scavenging activity and helped in production of oestrogen in appropriate quantities, which further regularized the production levels of FSH from anterior pituitary gland.

In conclusion, the study revealed that oral administration of imidacloprid at the rate of 30 mg/kg b.wt./d in female rats resulted in a significant alteration in oestrous cyclicity and serum hormone levels. However, supplementation of *W. somnifera* along with imidacloprid significantly ameliorated the toxic effects induced by imidacloprid.

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Conflict of interests:

The authors have no conflict of interest to report.

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