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Prolonged Exposure of Pyrethrin Insecticides Affects Reproductive Organs of Female Wistar Rats

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Abstract **Original Research Article**

Pyrethrins are pesticides found naturally in some Chrysanthemum flowers. They are commonly used to control mosquitoes, moths and many other pests. However, exposure to these Pyrethrins by direct or indirect inhalation by the population through various means could also pose potential risks to human health and the environment. Hence, this study aims to investigate the effect of chronic exposure of plant derived insecticides (pyrethrins) on female reproduction, using Wistar rats. Thirty (30) female wistar rats were randomly divided into three (3) groups (A, B and C). Group A was the control group, group B with mild exposure to the insecticide while group C with severe exposure to the insecticide for three weeks. Rats in each group were sacrificed after three (3) weeks of prolonged exposure to pyrethrin. Sera obtained from the rats were assayed for sex steroid hormones; Estradiol (E2) and progesterone, using Enzyme-linked Immunosorbent Assay (ELISA). Tissue samples of ovaries and gravid uteri were collected for histological processing. Vaginal smear samples were taken from each rat for the three (3) weeks of exposure. There was significant decrease in the body weights and relative uterine weight of the animals in groups exposed to pyrethrin, however, a significant increase was seen in ovarian weight of the test groups when compared to control. Serum hormonal levels of progesterone for the test groups were significantly increased while the serum estradiol level for the test groups was significantly reduced, when compared to the control group. There were pathological changes in the tissues of the ovaries and gravid uteri of the test groups compared to the control. There is significant difference in the progression of the estrous cycle of rats in the test groups when compared to control. More so, all rats presented a longer estrus phase and an irregular cycle by keeping same phase for long period. Hence, prolonged exposure to plant-derived insecticide (pyrethrin) affects female reproductive organs, steroid hormone synthesis and increase free radicals leading to oxidative stress. This may severely interfere with normal reproductive functions, and consequently lead to infertility.

Keywords: Female Reproductive System, Insecticides, Oestrus Cycle, Pyrethrins, Reproductive Hormones.

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INTRODUCTION

Mosquito-transmitted human diseases, such as malaria and dengue fever, represent significant burdens to global human health and cause considerable human suffering. One of the most effective measures to reduce disease transmission involves the use of insect repellents to prevent human contacts with mosquitoes. Pyrethrum extract from the dried and crushed flower heads of Chrysanthemum (Tanacetum cinerariifolium) began to be used as an insect repellent against biting arthropods thousands of years ago (Casida and Quistad 1995; Moore and Debboun 2007). Pyrethrins, the major insecticidal components, are used to control a wide range of pests in both agricultural and non-agricultural settings and may pose harmful effects on bees

when used outdoor. In addition, pyrethrum-producing Chrysanthemum spp. is recommended as "companion" plants to repel pest insects (Riotte, 1998). Pyrethrins and pyrethroids exert potent insecticidal activities by hyper-activating insect voltage-gated sodium channels, thereby causing rapid paralysis, known as knockdown, and eventual lethality (Bloomquist, 1996; Narahashi, 2000; Soderlund, 2005). Pyrethrins also have the advantage over other synthetic insecticides of being rapidly broken down upon exposure to light and air, are metabolized quickly, and can be used in the production of organic farm products, and they generally considered to be non-polluting (Sun *et al*., 2020).

There have been no studies to investigate the effects of chronic exposure of Pyrethrin on the reproductive organ of female Wistar rats. This report is probably the first of such studies on Wistar rats. Study of this nature can help us determine the effect of the chronic exposure of this Pyrethrin on humans too and the precautions measures to take when handling these insecticides.

MATERIALS AND METHODS

Thirty (30) Female Wistar rats were used. The Wistar rats were kept in cages in the LASUCOM Animal House and maintained at room temperature with approximately 12 hours dark and 12 hours light cycle. The rats were provided with standard rat chow as feed, and water ad labitum during this study. LASUCOM Animal Research Ethics Committee approved the study with ethics approval number (Ref. No: AREC/2022/03).

Experimental Design

Thirty (30) female Wistar rats were used and they were divided into three groups (A, B and C) with 10 rats in each group. Group A contains animals that served as the test group (control), group B are animals with mild exposure to the insecticide while group C are animals with the severe exposure to the insectide. The animals in group C (high dose) were exposed to three puffs of the insecticide in a closed space to prevent the insecticide from escaping for about 40-45 minutes and two times a day (morning and evening). While the animals in group B (low dose) were exposed to two puffs of the insecticide in a closed space to prevent the insecticide from escaping for about 40-45 minutes once a day (morning only). Group A (control group) were given water and feed daily. After three weeks exposure to allow the insecticide to take effect on the animals being exposed to, vaginal smear was gotten from the animals in groups A, B & C for another three weeks while the exposure continue. After six weeks, groups 1-3 were sacrificed. The rats were administered intraperitoneally with 0.2ml/100g (de Carvalho *et al*., 2011) of Ketamine and were monitored for indications of anaesthesia such as sleepiness and loss of consciousness before they were sacrificed when reflexes were lost. Blood sample of each rat was collected by retro-orbital method using heparinised tube and/or cardiac puncture using 5mls syringe. The samples were placed in plain bottles and allowed to clot, followed by centrifuging at 2500 rpm for 20 minutes using a desktop centrifuge (Surgifriend centrifuge, Model SMBO-2, England). This process separates the sera from the blood cells. The sera were aliquoted into Eppendorf tubes and stored at -20◦. The frozen blood samples were used for hormonal assay. Some samples of all the tissues removed were preserved in 10% buffered formalin which were later used for histological purposes

Hormonal Assay

Hormonal assay was carried out on the serum from groups A, B, & C as previously described Estrus cycle was determined as earlier described by Ajonuma *et al*. 2017a, Ajonuma *et al*. 2017b and Ajonuma *et al*. (2018). The reproductive hormones; Follicle Stimulating Hormones (FSH), Luteinizing Hormone (LH), Estradiol, and Progesterone concentrations were given quantitatively using enzyme linked immune-sorbent assay (ELISA) kits purchased from Monobind Inc., CA, USA. The procedure had previously been described (Ajonuma et al. 2017a, Ajonuma et al 2017b).The assays were done according to the instructions of the manufacturer. Assay kits were brought to room temperature. The following steps were taken: The needed number of micro strips were placed in the frame, with 12 wells for the calibrator samples and 12 wells for each serum sample .50µl of Calibrator sample were placed into the wells using pipette, 50µl of serum were also placed in the required wells and 50µl of hormonal Conjugate Reagent was dispensed into the wells and then incubated for 60 minutes at 37 degrees Celsius. The strips were rinsed five times with a prediluted washing solution after they had been incubated (containing surfactant in buffered saline) and a 20x dilution of the washing solution concentrate with distilled water was used to make the washing solution.50µl of 3,3,5,5'-Tetramethylbenzidine (TMB) substrate A solution was added into each of the wells followed by the addition 50µl of TMB Substrate B solution into all the well this was thoroughly mixed and incubated for 20 minutes at 26 degrees Celsius.100µl of stop solution was added into each of the wells using a pipette. Optical density (OD) was measured at 450nm/620nm on a STAT Fax 4700 ELISA micro plate reader.

Heamatoxylin and Eosin (H&E) Tissue Staining

This was carried out on the Ovaries, and gravid uterus that were previously fixed in 10% formalin as described (Ajonuma *et al*. 2005, Ajonuma *et al*. 2017a, Ajonuma *et al*. 2017b) with modifications. Fixed tissue is transferred to the mould containing paraffin wax and the wax was blown on the surface until a thin film of wax solidified. The mould containing the tissue was transferred to a container of cold water. It remained submerged until the wax hardens. The paraffin block was trimmed and placed on ice for 1 hour and the block was fixed in position on the microtome and sections were cut at 5pm. The thin section is floated with 20% alcohol in a warm bath at 40°C. The thin section was picked and dried on hot plate at 75°C. The section was taken into water and stained in Haematoxylin for 10 minutes. The stained section was rinsed in water and differentiated in 1% acid - alcohol. This was rinsed in water for 1 minute and counter stained with 1% Eosin for 1 minute. The Excess stain was washed, and the section was dehydrated in ascending order with 70%,90%, 100% alcohol

for 15 seconds each, the section was cleared in Xylene and mount in dihydroxy phthalate xylol (DPX). The stained section was evaluated using a light microscope (Olympus. China) and images of the section was captured using attached camera.

Determining Stages of the Female Wistar Rats Oestrous Cycle

Estrus cycle was determined as earlier described by Ajonuma *et al*. (2018). Briefly, each stage of estrous cycle was determined following these steps of vaginal cytology involving the three groups of the rats. Frosted slides were labeled according to each rat and dated. Cotton bud dipped in normal saline was carefully inserted into the vagina of the restrained rat. The cotton bud swap was gently rolled and turned against the vagina wall before it was removed. The cells were transferred to the labeled frosted glass slide by rolling it across the slide. The slide was left for a little while to air dry before fixation inside 50% methanol. It was then allowed to dry before staining. The stains used were Field stain A and B. The slides were stained by dipping into Field stain A for 15 times and Field stain B for 8 times and allowed to dry. Each slide was viewed under a microscope to determine the estrous cycle stages of the rats.

Statistical Analysis

Data were expressed as Mean ± SEM (Standard error of mean) where applicable and statistical analysis was carried out using one-way Analysis of variance (ANOVA) followed by multiple comparisons using Tukey's post-hoc test. GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) version 8.0.2 statistical software was used for the analysis. P-value less than or equal to 0.05 was considered statistically significant**.**

RESULTS

Fig. 1: The effect of chronic exposure to Pyrethrins on the weights of the female Wistar rats across all groups after sixth weeks of exposure

There was statistical significant difference (P<0.05)) in the weights of rats in test groups compared to the control group.

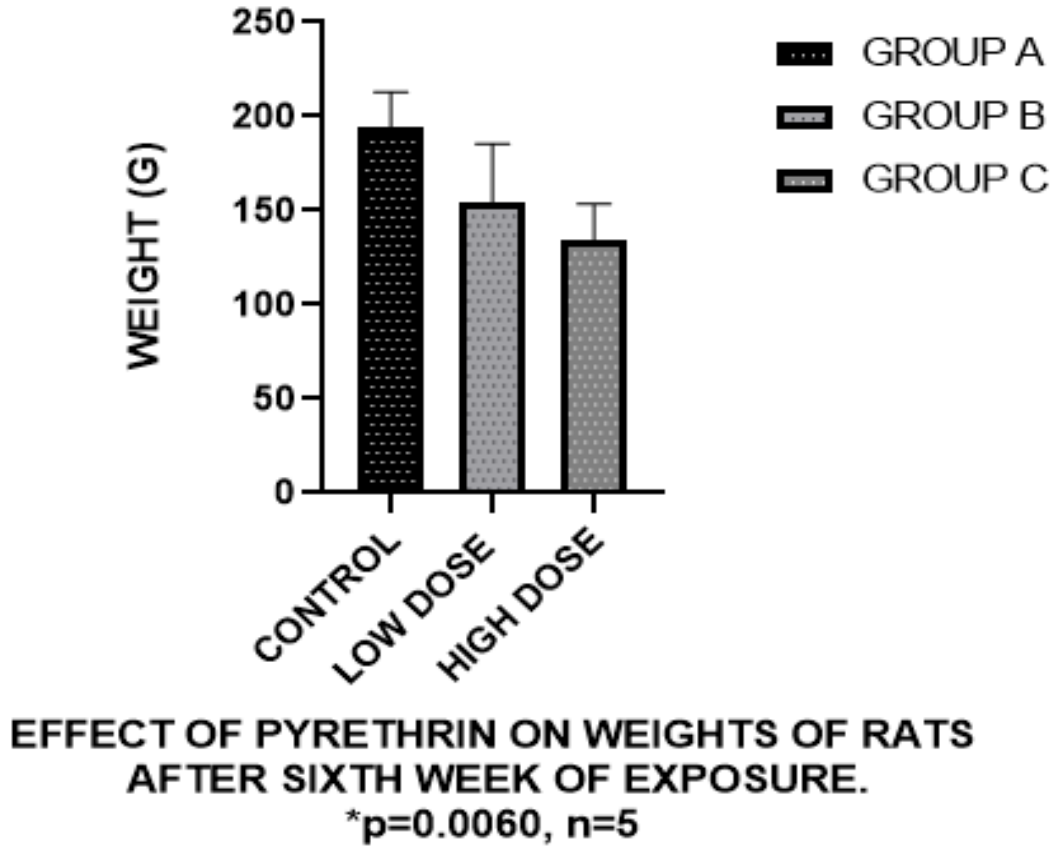
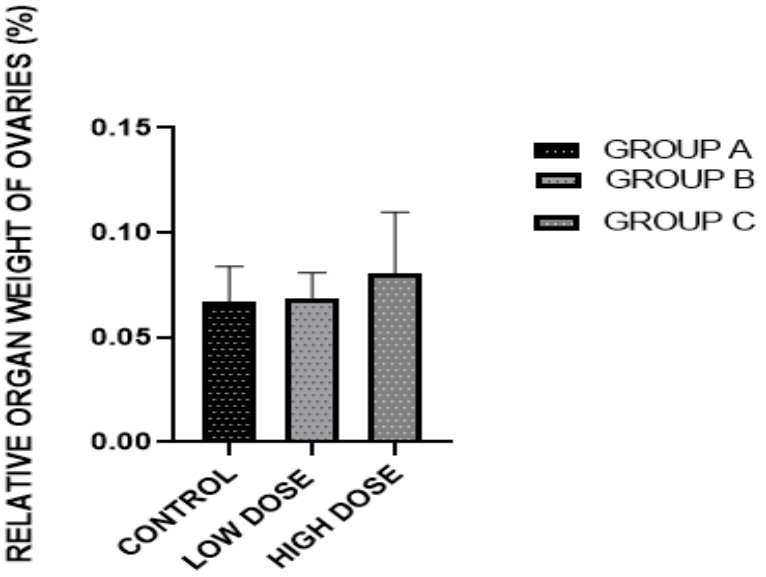
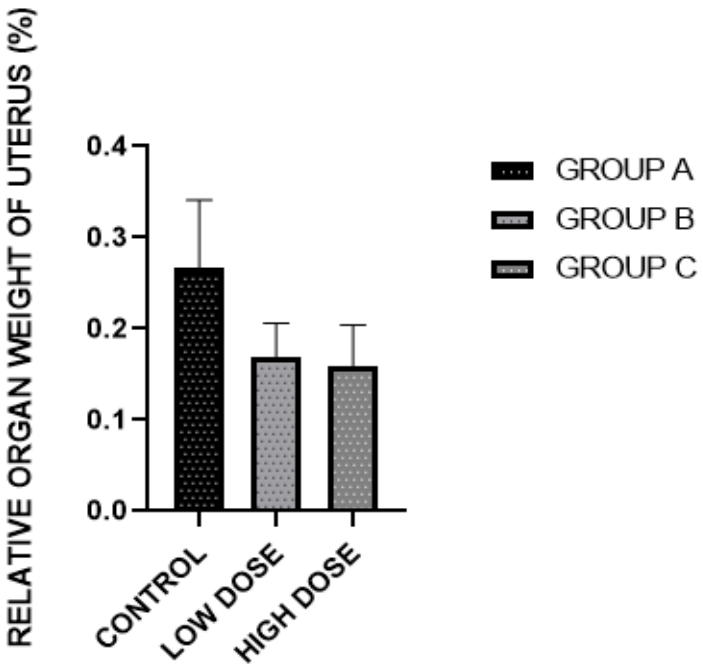


Fig. 2: The Effect of Plant Derived Insecticides (Pyrethrins) on Relative Weight of Reproductive Organs of the Female Wistar Rats.

Pyrethrin caused no significant difference (P>0.05) in the relative weight of the ovaries among the groups (Fig 2a)
Pyrethrin caused a statistical significant difference (P<0.05) in the relative weight of the uterus among the test groups (A and B) when compared to the control group (Fig 2b)



EFFECT OF PYRETHRIN ON RELATIVE WEIGHT OF OVARY.
p=0.4018, n=5



EFFECT OF PYRETHRIN ON RELATIVE WEIGHT OF UTERUS.
***p=0.0159, n=5**

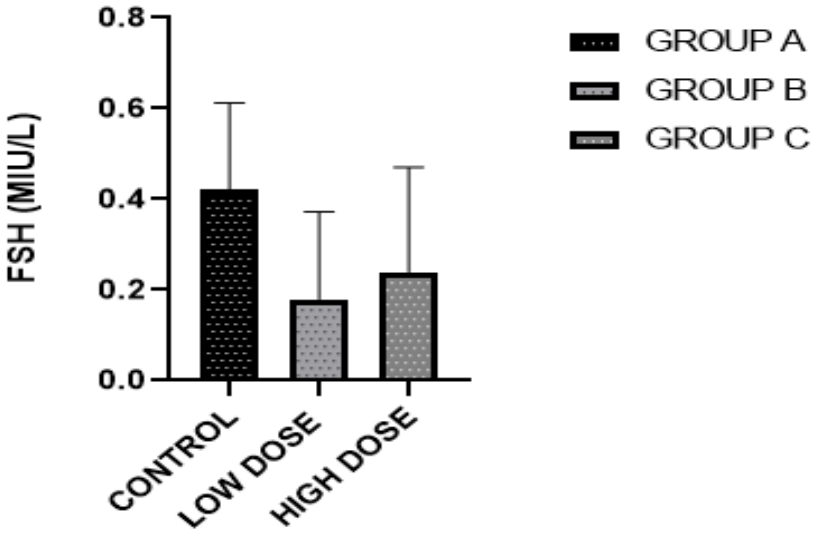
Fig. 3: The Effect of Exposure to Pyrethrin on Reproductive Hormones of Female Rats.

There was no statistical significant difference (P>0.05) in the serum follicle stimulating hormone level of the test groups that was exposed to pyrethrin (Fig 3a)

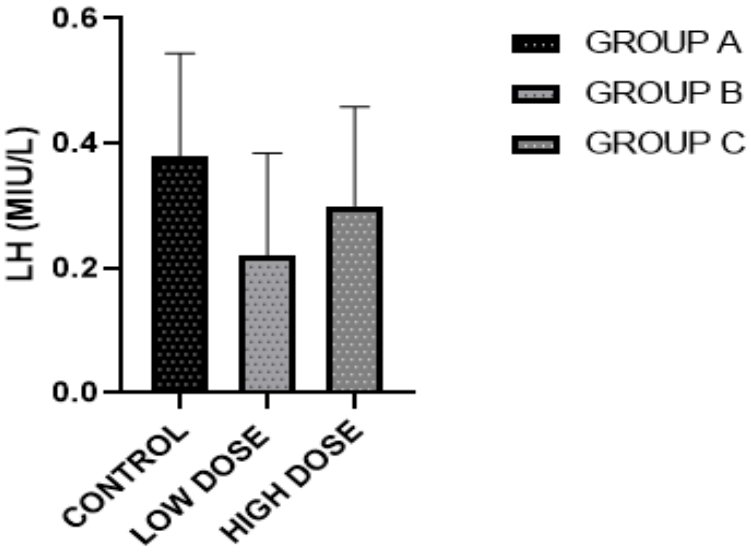
There was no statistical significant difference (P>0.05) in the serum luteinizing hormone level of the test groups that was exposed to pyrethrins (Fig 3b)

There was an increase (P<0.05) in the serum progesterone level seen in the test group (B&C) that was exposed to pyrethrin compared to the control group (Fig 3c)

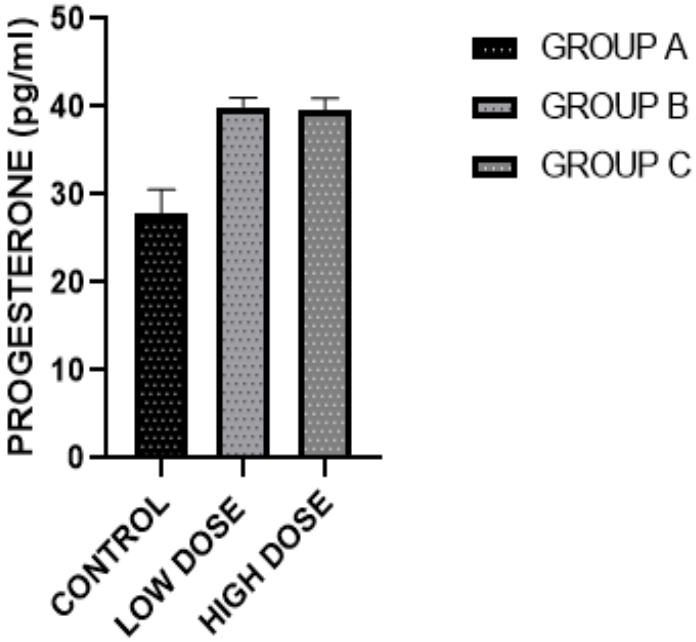
There was a decrease (P<0.05) in serum estradiol level in the test group (A&B) that were expose to pyrethrin compared to the control group (Fig 3d)



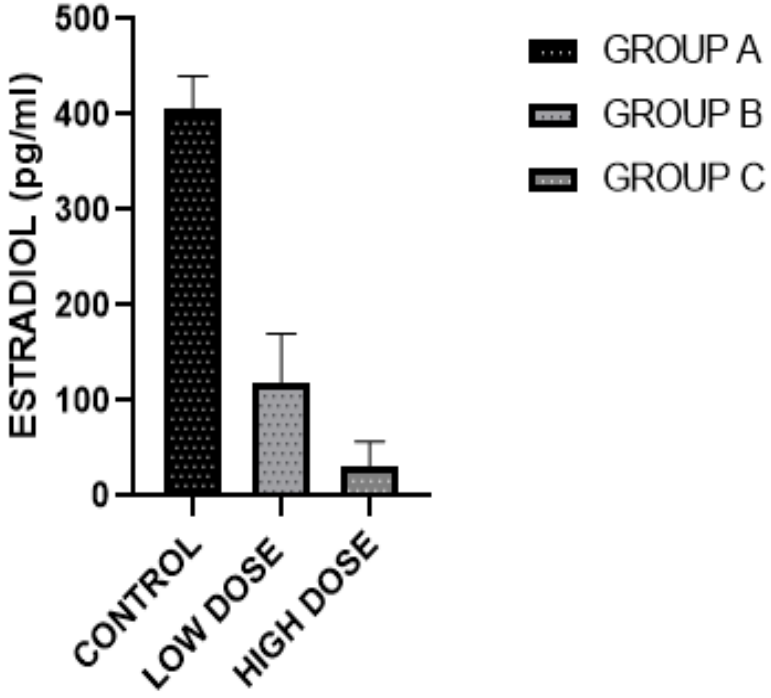
EFFECT OF PYRETHRIN ON SERUM FSH LEVEL.
p=0.2005, n=5



EFFECT OF PYRETHRIN ON SERUM LH LEVEL.
p=0.3307, n=5



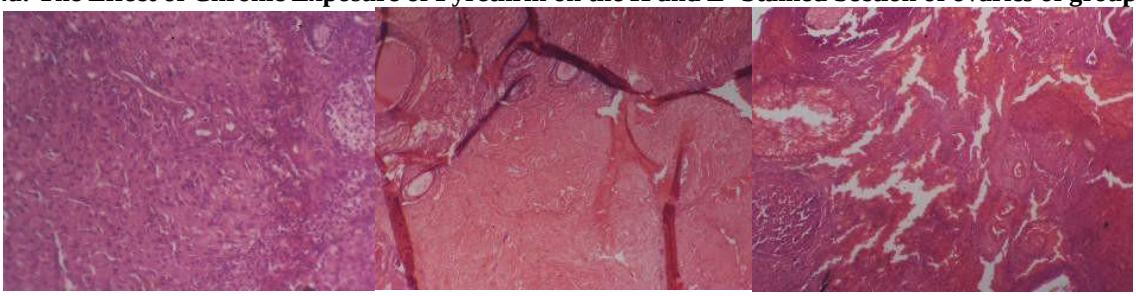
EFFECT OF PYRETHRIN ON SERUM PROGESTERONE LEVEL.
***p<0.0001, n=5**



EFFECT OF PYRETHRIN ON SERUM ESTRADIOL LEVEL.
***p<0.0001, n=5**

Fig. 4: The Effect of Chronic Exposure of Pyrethrin on the Heamatoxylin and Eosin (H & E) stained Section Ovaries and Uterus.

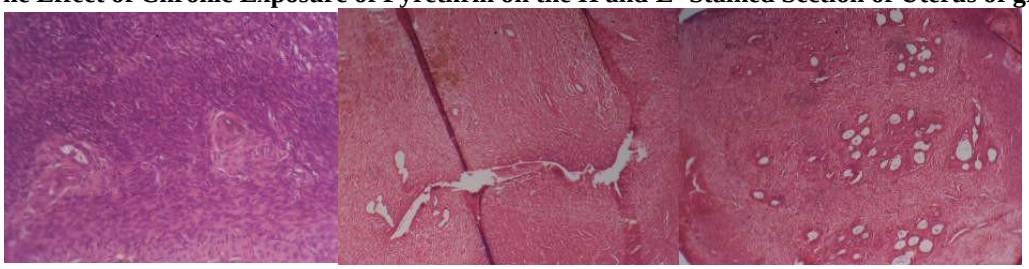
Fig. 4a: The Effect of Chronic Exposure of Pyrethrin on the H and E -Stained Section of ovaries of group A-C



A B C

Photomicrograph of the ovaries in group A, B and C. Group A showing a normal histological features. Pyrethrin caused significant effect on the ovary in group B which included; Degeneration of the ovarian follicle and stroma, there is also spaces within the stroma and fibrosis, follicular cystic expansion can also be noticed. For group C, chronic exposure to Pyrethrin caused degeneration of the ovarian follicle and stroma, there are also spaces within the stroma and fibrosis, follicular cystic expansion can also be noticed, there was loss of vascular architecture. Magnification×10.

Fig. 4b: The Effect of Chronic Exposure of Pyrethrin on the H and E -Stained Section of Uterus of group A-C



A B C

Photomicrograph of the uterus in groups A, B and C. For group A showed normal histological features. For group B there was degeneration of grandular cells. For group C, there was also a degeneration of glandular cells. Magnification×10

EFFECT OF CHRONIC EXPOSURE OF PLANT DERIVED INSECTICIDES (PYRETHRINS) ON FEMALE WISTAR RATS.

Estrous Cycle of the Control Group (Group A)

TABLE 1: Estrous cycle of the control group

DAYS N=5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
A1	P	E	M	M	M	D	P	E	M	M	D	P	E	M	D	P	E	M	M	M	D
A2	P	E	M	M	D	P	E	M	M	D	D	P	E	E	M	M	D	P	E	E	M
A3	E	M	M	M	D	D	P	E	M	M	D	P	E	M	M	D	P	E	M	M	D
A4	P	E	M	M	M	D	D	P	E	M	M	D	D	P	E	M	M	D	P	E	M
A5	D	P	P	E	E	M	D	D	P	E	M	D	P	P	E	M	D	D	P	P	E

Where P is proestrus, E is estrus, M is metestrus and D is diestrus, N= no of rats.

There is little or no significant changes in the progression of the estrous cycle stages. Estrous cycle synchronization occurred among rats in Group A (control). However some rats presented longer cycles.

TABLE 2: The effect of chronic exposure of plant derived insecticides (pyrethrins) on estrous cycle of Group B (LOW DOSE).

DAYS N=5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
B1	M	M	M	D	D	P	E	E	E	E	M	M	D	D	P	P	E	E	E	D	D
B2	P	E	E	E	M	D	P	E	E	M	D	P	E	E	D	D	P	E	E	M	D
B3	E	E	M	D	P	E	E	E	E	M	D	D	P	P	E	E	M	D	E	E	E
B4	M	M	D	P	E	E	E	M	M	D	D	P	E	E	E	M	D	P	E	E	E
B5	D	P	E	E	M	M	D	P	P	E	E	E	E	M	D	P	E	E	E	M	D

Where P is proestrus, E is estrus, M is metestrus and D is diestrus, N= no of rats..

TABLE 3: The effect of chronic exposure of plant derived insecticides (pyrethrins) on estrous cycle of Group C (HIGH DOSE).

DAYS N=5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
C1	D	P	E	E	E	E	D	P	E	E	E	M	D	P	E	E	M	D	D	P	P
C2	E	E	M	D	P	P	E	E	M	D	P	E	E	E	E	M	D	P	E	E	M
C3	P	E	E	D	P	E	E	E	M	D	P	E	E	M	D	D	P	P	E	E	E
C4	M	D	P	E	E	E	E	D	P	E	E	M	D	D	P	E	E	E	M	D	D
C5	P	E	E	E	E	M	D	E	E	E	M	D	P	E	E	M	D	D	P	E	E

Where P is proestrus, E is estrus, M is metestrus and D is diestrus , N= no of rats.

There is significant difference in the progression of the estrous cycle in Group C when compared to Group A which is control. Estrous cycle synchronization occurred among rats. However, all rats presented a longer estrus phase and an irregular cycle by keeping same phase for long period.

DISCUSSION

This study evaluated the effect of prolonged exposure to pyrethrin on the reproductive system of female Wistar rats. This study revealed that there was a reduction in the body weights of the female Wistar rats after six weeks of administering pyrethrin. This may be due to decreased appetite caused by the long term exposure to pyrethrin. There was also a significant reduction in the relative weights of the uteri of rats that were exposed to pyrethrin. The long term exposure to pyrtherin might have caused the uterus to reduce which can lead fertility issues. More so, it was noted that there was reduction in the estradiol level in rats exposed to pyrethrins, when compared to control. This may prevent sexual development and also lower sexual desires in females. However, there was a significant increase in serum level of progesterone in the test groups (B and C) when compared to the control group A, this could be because the pyrethrin act as an endocrine disruptor which have been reported to possess estrogenicity action by McCarthy et al., (2006).

There was also a significant effect of pyrethrin on the Hematoxylin and Eosin (H&E) Stained section of uterus and ovaries in both test groups. Histology revealed degeneration of the ovarian follicle and stroma, follicular cystic expansion, degeneration of glandular cells. All these abnormalities in ovary may lead to poor follicular growth which can affect reproduction.

There was also synchronization in the estrous cycle of rats administered with doses of pyrethrins. At every week of the cycle, there were varying difference between the test groups (B and C) and the control group A which indicate interference in estrous cycle activity and which may lead to poor follicular development (Raji and Hart, 2012). Prolonged estrus stage and metesrus stage in the test groups when compared to the control

group might be because of the increase in progesterone level in tests groups compared to the controls since the progesterone level peaks at estrus followed by metestrus. (Zenclussen *et al*., 2014).

In summary, prolonged exposure to pyrethrin (plant-derived insecticide) affects female reproductive organs, gonadotropins, steroid hormone synthesis and estrus cycle. This may severely interfere with normal reproductive functions, and consequently lead to infertility.

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