Evaluation of reprotoxic effects of orally administered endocrine disruptor Pyriproxyfen in adult female Sprague Dawley rats: A biochemical and histopathological study

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

By

Sajid Ali

DEPARTMENT OF ZOOLOGY

FACULTY OF BIOLOGICAL SCIENCES QUAID-I-AZAM UNIVERSITY ISLAMABAD, PAKISTAN

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"In the Name of ALLAH, the most Gracious, the most Merciful"

DECLARATION

I hereby declare that the material and information presented in this thesis in my original work. I have not previously presented any part of this work "**Evaluation of reprotoxic effects of orally administered endocrine disruptor Pyriproxyfen in adult female Sprague Dawley rats: A biochemical and histopathological study.**"

Sajid Ali

DEDICATION

This dissertation is dedicated to all the

DOWNTRODDEN

of our society.

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LIST OF ABBREVIATIONS

AF	Atretic Follicle
ANF	Antral Follicle
ANOVA	Analysis Of Variance
BM	Basal Membrane
BMD	Basal Membrane Disruption
BMI	Body Mass Index
BP	Binding Proteins
CEC	Cornified Epithelial Cells
CLD	Corpus Luteum Degeneration
DDT	Dichlorodiphenyltrichloroethane
DGC	Degenerating Granulosa Cells
EC	Emulsifiable Concentrate
EDCs	Endocrine Disrupting Chemicals
FAO	Food And Agriculture Organization
GCD	Granulosa Cells Distortion
HDL	High Density Lipids
HR	Hormone Receptors
IGR	Insect Growth Regulator
IPCS	International Program On Chemical Safety
IUPAC	International Union Of Pure And Applied Chemistry
LKC	Leukocytes

NCL	Newly Formed Corpus Luteum
NEC	Nucleated Epithelial Cells
OB	Ovarian Bursa
OC	Organochlorine
OES	Optical Empty Spaces
OP	Organophosphate
PBS	Phosphate Buffered Saline
PCL	Secondary Follicle
PMF	Primordial Follicle
PYR	Pyriproxyfen
RBCs	Red Blood Cells
RF	Rupturing Follicles
SEM	Standard Error Of Mean
SF	Secondary Follicle
TC	Total Cholesterol
TGL	Triglycerides
WHO	World Health Organization

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First of all, I am grateful for all the blessings that **Allah Subhan Wata'ala** Has bestowed upon me. Secondly, I am thankful to **Professor Dr. Sarwat Jahan** for giving me the opportunity to pursue my MPhil and the support throughout my degree. I am also thankful to all my friends especially, **Hina Afaqi, Mohammad Shoaib, Mohib Ullah, Nadir Akhtar,** and **Mohammad Aijaz** for their unconditional love and support through every phase of my life. Furthermore, I would like to thank **Rahmat Ali, Kashif Rahman, Asfandyar Ali, Ashiq Hussain,** and **Samina Farheen** for their support and love.

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Sajid Ali

ABSTRACT

The phenomenon of endocrine disruption has been acknowledged for a long time and since the discovery of the first hormone in 1902. Endocrine disruptors can be pharmaceuticals, plasticizers, polychlorinated biphenyls, organochlorinated pesticides. With the growing population of the world there is a growing demand for food, thus, a growing demand for pesticides to increase crop production. Approximately 5.6 billion pounds of pesticides are being used annually in the world, and this usage is unexpectedly rising, of which 95% possess the ability to be widely dispersed in the environment and to affect non-target organisms. PYR is one of the most widely used pesticides in the world and is well known for its ability as an embryogenesis inhibitor in insects. It gets accumulated in the environment leading to detrimental effects in non-target organisms (plants, fish, amphibians, birds, and mammals etc.) through food web. The adverse effects due to continuous exposure of PYR include growth retardation, disruption of hormonal balance, impaired reproduction, and neurodevelopmental abnormalities. In order to check the reprotoxic effects of PYR in female Sprague Dawley rats, we designed an experiment consisting of four groups i.e., control, G1, G2, G3 and administered them with distilled water, 62, 124, and 186 mg/kg PYR, respectively. After 28 days the rats were dissected, different samples were collected, and analyzed for organ weights, BMI, blood glucose levels, total protein concentration, lipid profile, and ovarian histology. The results showed that PYR had non-significant ($p>0.05$) effects on body weight, and BMI but, caused a significant ($p<0.05$) reduction in the ovarian, uterine, kidney, heart, and liver weights. Also, it was found to be involved in significantly decreasing ($p<0.01$) the blood glucose levels and altering estrous cyclicity. In addition, non-significant decrease ($p>0.05$) was observed in total protein levels, while the lipid profiles were significantly ($p<0.05$) effected as a result of PYR administration. Moreover, PYR was found to have potential detrimental effects on the ovarian histoarchitecture. In conclusion, PYR was found to have reprotoxic effects and thus must be used with caution and only when necessary. Also, further research is needed to identify the possible mechanisms of PYR interaction with ovarian cells and other body organs.

INTRODUCTION

The phenomenon of endocrine disruption has been acknowledged for a long time (Figure 1) and since the discovery of the first hormone in 1902 (Bayliss & Starling, 1902). Since ancient times, the hormonal effects have been known, especially in the perspective of using castration to change male servants into eunuchs (Darbre, 2019). Nevertheless, appreciation of hormones, as detectable chemical messengers, initiated in 1902 once secretin's role was documented in digestion (Bayliss & Starling, 1902). Endocrine-disrupting action was registered earlier by US pig farmers (1920), who were troubled by lack of fertility in their swine herds fed on mouldy grains (McNutt *et al.,* 1928), followed up with reports from Western-Australia's sheep farmers (1940s) regarding infertility in their lamb after grazing on certain clover fields (Bennetts *et al.,* 1946). Both the above reports were found to be due to the intake of mycoestrogens contained in the mould (Bennett & Klich, 2003) and utilizing phytoestrogens present in the plant material respectively (Woods, 2003).

In recent years, scientific work has ever more centered on the involvement of EDCs in human pathophysiology (Lauretta *et al.,* 2019). An increasing amount of evidence, together with reports, reviews, and clinical trials feature new effects of EDCs (Lauretta *et al.,* 2019). Endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations (WHO/IPCS, 2002). In the definition above "adverse health effects" outlines any changes to an organism's bodily processes, morphology, and/or conduct that weakens its capacity to develop, grow, and/or reproduce (Bertram *et al.*, 2022). There are so many kinds of substances that are causing endocrine disruption (Gore, 2001).

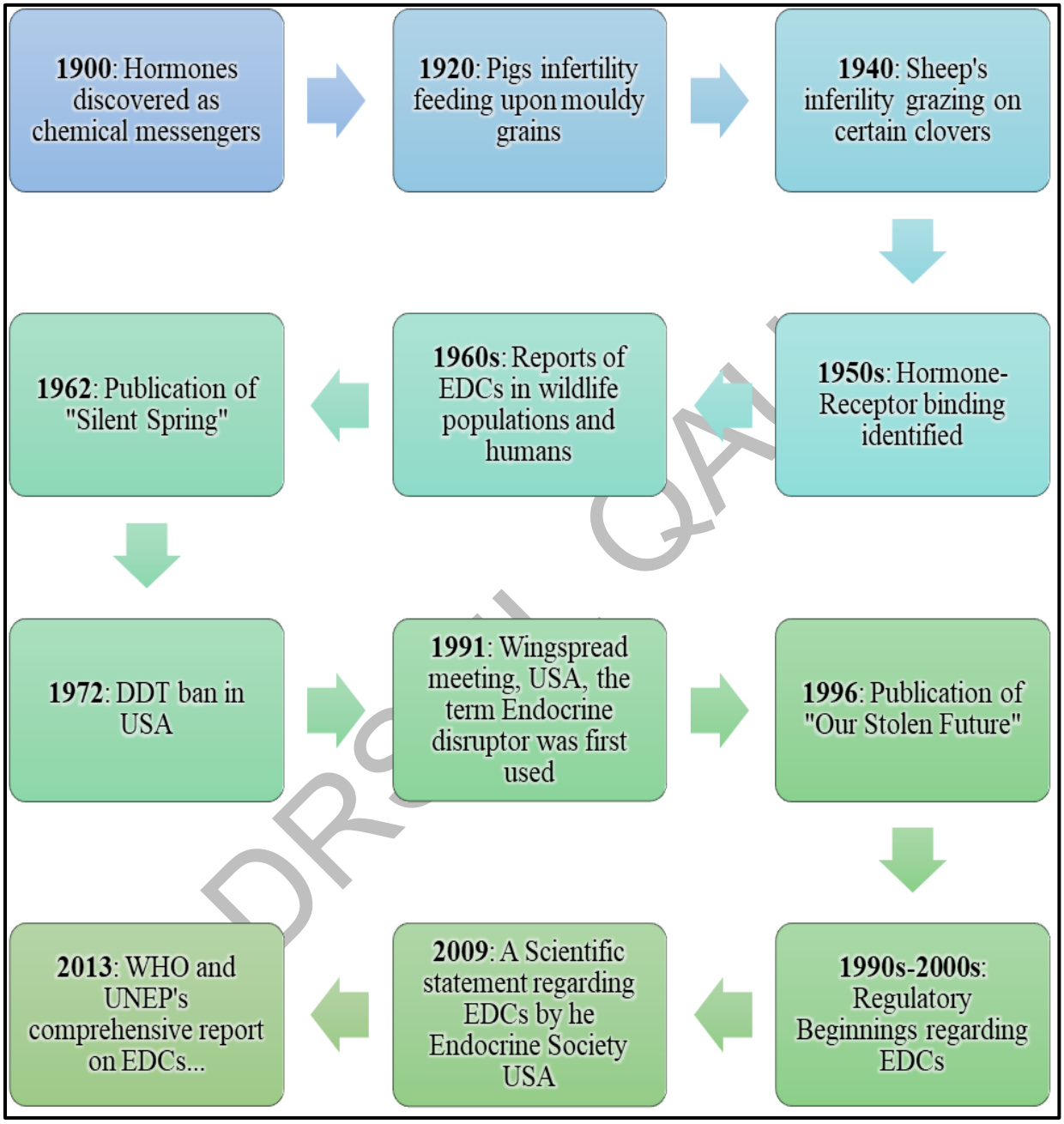

Figure 1. An outline showing key events in the recognition of EDCs and endocrine disruption.

On top of that, these substances can be both synthetic and natural, including dioxins and related compounds, pharmaceuticals, plasticizers, polychlorinated biphenyls, organochlorinated pesticides (Carpenter, 2013; Gore, 2001). Many everyday goods, including metal cans, plastic bottles, flame retardants, detergents, cosmetics, toys, food, and pesticides (Yilmaz *et al.*, 2020). The diverse mechanisms of actions that these EDCs can exhibit are: (1) binding to an HR and activating or inhibiting its signaling pathway; (2) interacting with receptor's downstream pathway components; (3) stimulating or inhibiting the biosynthesis of an endogenic hormone; (4) by binding to the hormone-binding proteins circulating in the body; (5) through promoting or preventing the synthesis or degradation of hormone-binding proteins; (6) promoting or inhibiting the expression of hormone receptor (Combarnous, 2017; Yilmaz *et al.*, 2020). These aforementioned mechanisms are summarized in Figure 2, so that the diverse kinds of EDCs, corresponding to their resemblances and differences compared to hormones, can be differentiated (Yilmaz *et al.*, 2020).

Food being most crucial item needed for existence, as it provides nutrients and energy for the body's maintenance, growth, and development. So, supplying enough food to feed the entire world's population has never been easy. Farmers all around the world deal with a variety of biotic and abiotic considerations regarding the process of production (Kumar & Kumar, 2019). Seeing that the world's human population is rising disproportionately, environmental sustainability and food security are the key concerns. Pests significantly decrease crop yields and raise the price of synthetic chemicals (Chattopadhyay *et al.*, 2017). The damage inflicted by far more than 10,000 insects and 30,000 weeds significantly decreases crop productivity (Dhaliwal *et al.*, 2010). The application of pesticides to manage pests and weeds and improve food production is one of the many demanding procedures involved in agriculture (de Souza *et al.*, 2020).

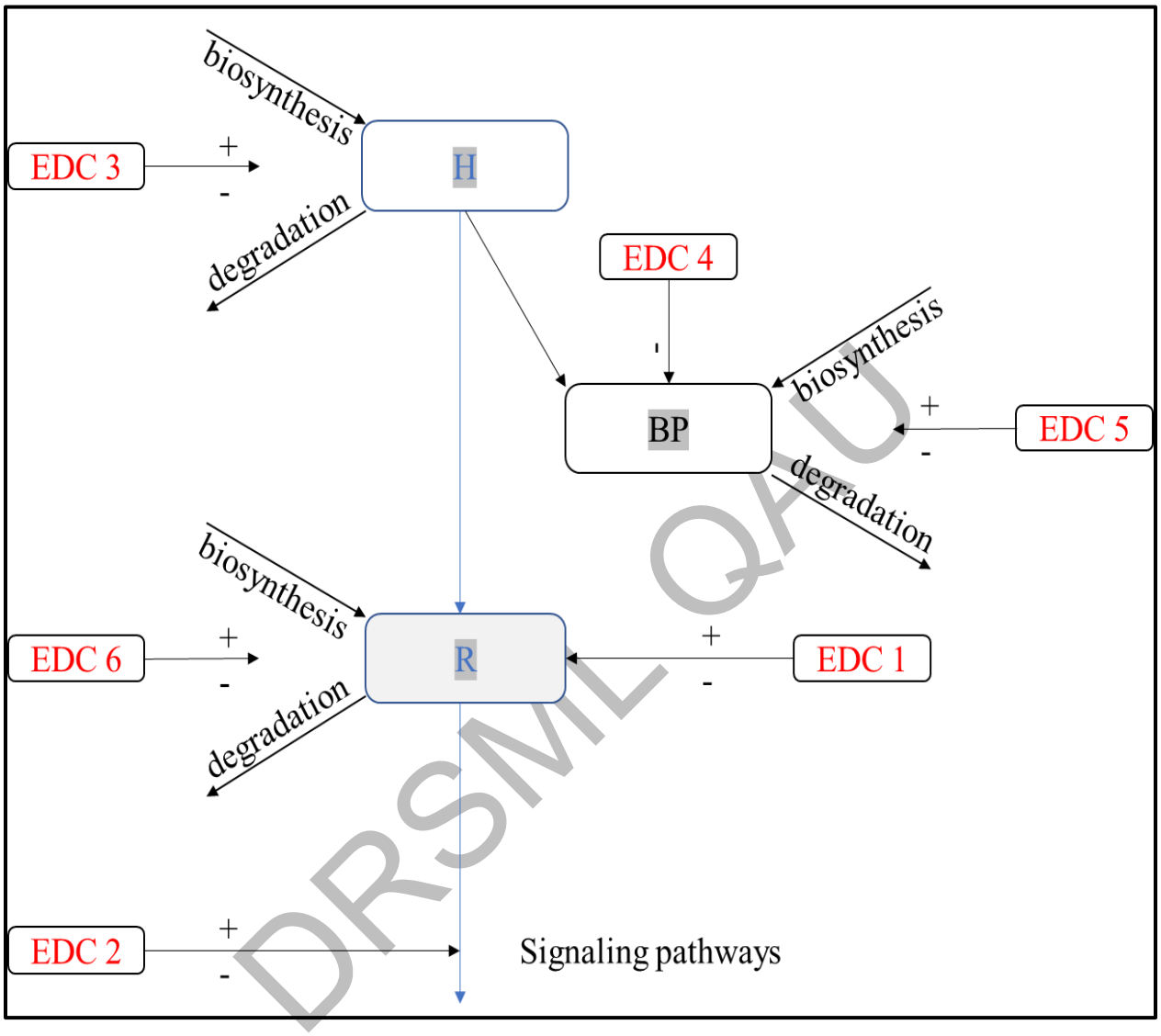

Figure 2. Graphical representation of EDCs' prospective mechanisms of action. The physiological-hormonal mechanism (in blue). The varied EDC mechanisms of action, (EDC 1 to EDC 9 in red), are presented directing to their place of action (black arrows, - inhibition; + stimulation).

According to the Food and Agriculture Organization (FAO), a pesticide is any substance intended to prevent, remove, or control any undesirable plant or animal species triggering destruction between or commonly prying with the generation, preparing shortage, transportation, or promotion of food, wood items, horticultural items, or animal foodstuff, or materials that may be directed to animals including vectors of human or creature ailment for the purpose of curing disease.

Pesticides are categorized according to their physical features, chemical composition, targeted individual, and means of action. Insecticides are used to kill insect pests of crops and flies, mosquitoes, and insect transmitters for human ailments. Herbicides are used to control undesired plants. Fungicides are used to kill fungus. Avicide is used to fight bird pests. Acaricides are used to kill ticks and mites. Scientists typically prefer to categorize pesticides based on their chemical structures since these structures affect their mode of action, toxicity, and other properties. Pesticides from the organochlorine (OC), organophosphate (OP), carbamate, and pyrethroid chemical families are among the most common (de Souza *et al.*, 2020).

Almost 5.6 billion pounds of pesticides are being used every year in the world, and this usage is unexpectedly rising (Alavanja, 2009). Pakistan is the second-highest consumer of pesticides among the South Asian nations, with the agricultural sector being its primary application (Khan *et al.*, 2020). Pesticides are being used extensively throughout the agricultural regions of Punjab, Pakistan. Pakistan utilizes more than 30 distinct varieties of fungicides, 5 distinct categories of acaricides, 39 diverse forms of weedicides, 6 distinct types of rodenticides, and 108 different types of insecticides (Mehmood *et al.*, 2017).

This has not always been the case, in ancient China followed by Middle Ages in Persia *Dalmatian pyrethrum* (containing 1.5% pyrethrin, an active insecticidal material) was used as an insecticide (Davies *et al.*, 2007). Ever since 1000 BC, natural chemicals are being used against pest insects (Popov *et al.*, 2003). WW2 opened the gates for *Modern Era of Chemical* control by introducing a new idea of controlling insects, synthetic organic insecticides, whose pioneer was DDT (Ware & Whitacre, 2004). As far as they are crucial for safeguarding the food safekeeping

and economic growth, improper and arbitrary use can be devastating both for the environment and human wellbeing (Christos, 2009). Due to elevated bioactivity, and, in some circumstances, long environmental persistence, the unfitting handling may consequence in severe acute poisonings; In addition, chronic, low-level exposures may also result in unpleasant health effects (Maroni *et al.,* 2006; Woodruff *et al.,* 1994).

Due to the prevalent dispersal of pesticides, a vast majority of people may be occupationally subjected to pesticides. People from various groups, with quite distinct patterns and extent of contact, are at jeopardy of negative effects. Workers from pesticide manufacturing industries and certain handlers in public health (e.g., destroyers of household pests) are occupationally exposed. While, In the farming sector, farmers and qualified appliers of pesticides get exposed to pesticides (Glass & Machera, 2009; Maroni *et al.,* 1999; Woodruff *et al.,* 1994; Ye *et al.,* 2013). As far as common people are concerned, persons may be exposed to pesticide remains in drinking water and food on a day-to-day base or to pesticide drift that occurs in housing areas that are in close vicinity to crop-dusting areas (Damalas & Eleftherohorinos, 2011).

More than 95% of the applied pesticides have the ability to effect non-target organisms and to become extensively spread in the environment (Simeonov *et al.,* 2014). The connection between cancer and pesticides has been stated by much research, including cohort and case control studies (Amr *et al.,* 2015; Koutros *et al.,* 2016; Provost *et al.,* 2007; Samanic *et al.,* 2008). Studies showing links between asthma and bronchial hyper-reactivity due to pesticides exposure are there (Amaral, 2014; Hernández *et al.,* 2011; Ndlovu *et al.,* 2014; Raanan *et al.,* 2015). Emerging scientific evidence indicates a positive relationship concerning diabetes and serum concentrations of several pollutants (Jaacks & Staimez, 2015; Sylvie Azandjeme *et al.,* 2013; Tang *et al.,* 2014). Epidemiological studies suggest the increased danger of Parkinson's disease as a result of pesticide exposure. Research have shown the involvement of pesticides in altering the thyroid gland function and reducing the circulating levels of thyroid hormone e.g., chlorophenoxy acids, chlorophenols, quinones, and organochlorines (Gray Jr & Kavlock, 1983; Van den Berg *et al.,* 1991). Also, one

of the most vital reasons of acute leukemia is pesticides exposure (Bailey *et al.,* 2015; Maryam *et al.,* 2015; Vinson *et al.,* 2011).

Majority of pesticides affect male reproductive system by reducing sperm activities, testis weights, inhibiting spermatogenesis, damaging its DNA, changing its morphology, inducing hypospadias (Mehrpour *et al.,* 2014; Michalakis *et al.,* 2014). Studies have also shown the effects of pesticides on female fertility ranging from effects on estrogen levels (Amita Rani & Krishnakumari, 1995; Eldridge *et al.,* 1994; Oduma *et al.,* 1995) e.g., DDT (Dees *et al.,* 1997; Tully *et al.,* 2000), pentachlorophenol (Danzo, 1997), endosulfan (Hodges *et al.,* 2000; Soto *et al.,* 1995). Pesticides also cause disturbances in the ovarian cycle, ovulation problems, impaired fertility, menstrual cycle disturbances, and infertility (Bretveld *et al.,* 2006).

Pyriproxyfen (PYR; IUPAC name, 4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy) propyl ether) is a pyridine-based broad-spectrum insect growth regulator (IGR) pesticide which works as an analog of juvenile hormone (Liu *et al.,* 2020; Maharajan *et al.,* 2018; Sullivan & Goh, 2008). PYR is an aromatic compound (Ginjupalli & Baldwin, 2013) (Figure 3), first manufactured in 1990 by Sumitomo Chemicals Co., Ltd. and marketed it under the trade name of "Lano® 10EC" (Dhadialla *et al.,* 1998; Payá *et al.,* 2013). Being a strong hormone agonist, PYR is categorized as an endocrine disruptor (Sullivan & Goh, 2008). Different means of identification for PYR and its physico-chemical properties are given in table 1 and table 2, respectively.

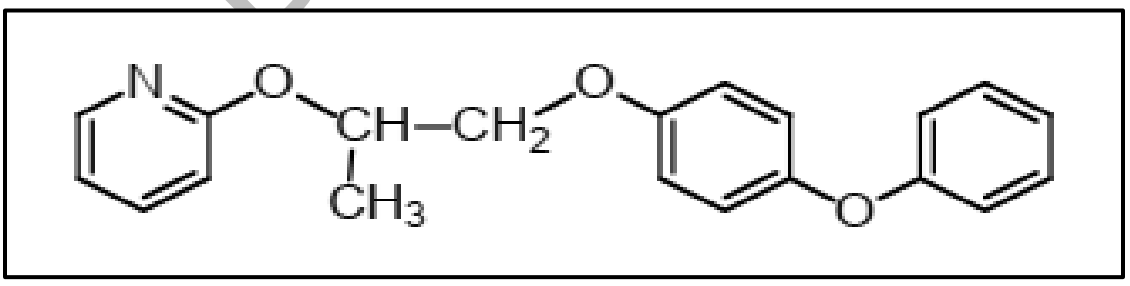

Figure 3. Structural formula of pyriproxyfen (Moermond, 2008).

Table 1. Identification of pyriproxyfen.

Parameter	Value	Unit	Source
Molecular weight	321.4	g/mol	(Netherlands, 2005)
Molecular formula	$C_{20}H_{19}NO_3$	-	(Tomlin, 2002)
Vapor pressure	1.33×10^{-5}	Pa	(Sullivan & Goh, 2008)
Water solubility	0.367	mg/l	(Netherlands, 2005)
Boiling point	318	°C	(Netherlands, 2005)
Melting point	48-50	°C	(Netherlands, 2005)
Color	Pale yellow	-	(Sullivan & Goh, 2008)
Physical state	Waxy solid	-	(Sullivan & Goh, 2008)

Table 2. Physico-chemical properties of pyriproxyfen.

Parameter	Identification	Reference
Common name (ISO)	Pyriproxyfen	(Netherlands, 2005)
IUPAC name	4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy) propyl ether	(Netherlands, 2005)
EC number	429-800-1	(Netherlands, 2005)
CAS number	95737-68-1	(Netherlands, 2005)
Chemical class	Insecticide	(Tomlin, 2002)
Acts as	Juvenile hormone mimic	(Tomlin, 2002)

Being well known for its ability as an embryogenesis inhibitor in insects and no recorded field resistance (Invest & Lucas, 2008). PYR is used in household, horticulture, and agriculture to control various insect species (Ginjupalli & Baldwin, 2013; Maharajan *et al.,* 2018). It is used as an insecticide against whitefly, thrips, aphids, scales, jassids, mealworms, cutworms, and bollworms (Aribi *et al.,* 2006; Dzieciolowska *et al.,* 2017; Ishaaya & Horowitz, 1992; Oouchi & Langley, 2005; Shahid *et al.,* 2019). In Pakistan and India PYR is used for controlling certain vectors, including *Culex quinquefasciatus* and *Anopheles stephensi* (Jambulingam *et al.,* 2008). It has also proven to be effective against insect pests concerning public health such as houseflies, mosquitos, cat fleas, and cockroaches (Sullivan & Goh, 2008).

Due to its extensive use and high stability in certain ecosystems, PYR gets accumulated in the environment leading to detrimental effects in non-target organisms (plants, fish, amphibians, birds, and mammals etc.) through food web (Mehrnoush *et al.,* 2013). The exposure can be dermal,

inhalation, or oral leading to toxicity in the respective individual (Cross, 2015). The adverse effects due to continuous exposure of PYR include growth retardation, disruption of hormonal balance, impaired reproduction, and neurodevelopmental toxicity (Maharajan *et al.,* 2018; Sartori *et al.,* 2020). PYR was found to have detrimental effects on root and shoot growth in pea, greengram, chickpea and lentil (Ahemad, 2014). PYR is shown to cause decrease in body and organ weight, lower the RBCs level, disturb structural integrity of kidney, liver, heart, and brain (Naseem *et al.,* 2022). PYR is also found to be altering the antioxidant levels and also causes DNA damage in visceral organs of *Labeo rohita* fish (Li *et al.,* 2022).

In addition, there are some reported evidence of PYR being involved in causing reproductive toxicity in some non-target organisms. A study performed on mice showed that repeated exposure of PYR lead to decreased weight gain in pregnant treated groups, reduced litter size, and increased stillbirths (Shahid & Saher, 2020). Another study performed on male mice showed its involvement in damaging testicular architecture, a potential evidence for its interference with spermatogenesis (Shahid *et al.,* 2019). In yet another study performed on zebrafish, PYR was found to be responsible for the decrease in testosterone levels in male zebrafish and also decrease in estrogen hormone levels in female zebrafish (Maharajan *et al.,* 2020). The study also showed histopathological alterations in adult zebrafish ovaries caused due to the exposure of PYR. Although some data shows the involvement of PYR in damaging the reproductive health of non-target organisms, still the data in not enough to be sure that PYR is a potential reprotoxic pesticide.

Hence, the purpose of our research was to find the reprotoxic effects that PYR has when given orally to adult female Sprague-Dawley rats. In order to accomplish that, we had set the following objectives:

- Examine the changes in estrous cycle due to PYR administration.
- Determine the outcomes of PYR exposure on complete protein profile.
- Investigate the effects of PYR on lipid profile of the rats.
- Examine the effects of PYR on the ovarian histology.

MATERIALS AND METHODS

The present study was conducted in the Reproductive Physiology Laboratory of Zoology Department, Quaid-i-Azam University, Islamabad, Pakistan. Number of animals used for the experiment, their handling, and scarification was approved by the departmental ethical committee. All the processes used in the study were performed keeping in view the recommendations for using research lab animals appropriately.

Animals

Twenty healthy adult female Sprague-Dawley rats (*Rattus norvegicus*) were collected from primates facility of Zoology Department, Quaid-i-Azam University, Islamabad. The average weight of selected rats was kept 160±15g. These rats were then casually sorted into four groups (5/group), each placed in a separate stainless-steel cage. The cages had sawdust as bedding and were placed in a separate well-ventilated room. The temperature of the room was maintained between 20-27˚C and the rats were subjected to 12-hour light and 12-hour dark cycles. All the rats were given food chaw and tap water during the experiment.

Chemicals

Pyriproxyfen (10.8%EC PYR), manufactured by Nantong Chemical Co., Ltd (China) and imported by Suncrop Pesticides Multan, was purchased from Anqa Agro Multan. It was diluted with distilled water to get the desired concentration of PYR for each group.

Experimental Design

Twenty healthy adult female rats (n=20), average weight 160±10g, were randomly sorted into four groups each group consisting of 5 rats (n=5). The doses of PYR used in our experiment were according to the previous studies performed by researchers (Sartori *et al.*, 2020; Shahid & Saher, 2020; Shahid *et al.*, 2019). All the groups were given doses orally for 28 consecutive days.

Control (C). It was served as control and given 3 ml distilled water.

Group 1 (G1). It was provided with 62 mg/kg of PYR.

Group 2 (G2). It was exposed to 124 mg/kg of PYR.

Group 3 (G3). It was given 186 mg/kg of PYR.

Sample Collection

The current study lasted for 28-days, and the respected dose of each group was administered routinely via oral gavage. At 29th day of our experiment, rats were weighed, lengths were measured and noted down; blood was collected through heart puncture in heparinized syringe, for plasma, the blood was centrifuged at 3000 rpm for 15 minutes, after that the plasma was stored at -20˚C up until further biochemical examination. The rats were then decapitated. After decapitation, ovaries, uterus, kidneys, liver, and heart were collected, washed in normal saline, and weighed. ovaries were then immediately fixed in 10% formalin solution for histological analysis.

Body Weight Determination

The body weight of rats was determined and noted down on 1st, 14th, and 28th day of the experiment. Top loading Sartorius Digital Balance (Germany) was used for weighing purposes.

Organ Weight Determination

After successfully dissecting the rats organs (ovaries, uterus, kidneys, liver, and heart) were collected and washed in normal saline. After washing, their weights were measured using Sartorius Digital Balance (Germany).

Body Mass Index (BMI) Determination

For calculating rat's BMI (g/cm^2), their body weight and body length were measured (Novelli *et al.*, 2007). The length was measured using measuring tape, and the subsequent formula was used to determine the BMI. The normal BMI for adult female rat ranges between 0.4504–0.5044 g/cm^2 (Engelbregt *et al.*, 2001).

$$BMI = \frac{Body\ weight(g)}{Body\ length(cm^2)}$$

Blood Glucose Determination

The rat's blood glucose levels were measured using a EasyGluco Auto-coding^{TM} (INFOPIA Co., Ltd. Korea) glucometer. Glucose levels were measured empty stomach early in the morning on 1st, 14th, and 28th day by pricking the tail tip with a sterile needle and then placing the blood drop on the edge of the glucometer's strip.

Determination of Estrous Cyclicity

The short length (4-5 days) of estrous cycle in rats make them an ideal for reproductive cycle studies. The estrous cycle of rats is distributed into 4 phases (proestrus, estrus, metestrus, and diestrus). For this purpose, vaginal cytology was performed on 1st, 7th, 14th, 21st, and 28th day; the specific stage of estrous cycle for each rat, on the respective day, was noted down.

While doing the assessment, rats were restrained, and their tails were raised to visualize the vagina. Next, the vaginal cells were rinsed gently by introducing a slight volume (10-20 µl) of normal saline (0.9%) through a pipette (repeat 4 to 5 times). Confirm that the pipette tip is sterile and is positioned at vaginal canal's opening. After that, the liquid was put on a glass slide, dried up in air, and then stained employing H&E staining. The slide then draped with a coverslip and observed under a light microscope at 10X and 40X magnification (Auta & Hassan, 2016).

Three types of cells make up the vaginal secretion. They are, cornified epithelial cells, nucleated epithelial cells, and leucocytes. The phase of estrous cycle is estimated based on the proportion of these cells (Auta & Hassan, 2016) in vaginal secretion (Figure 4).

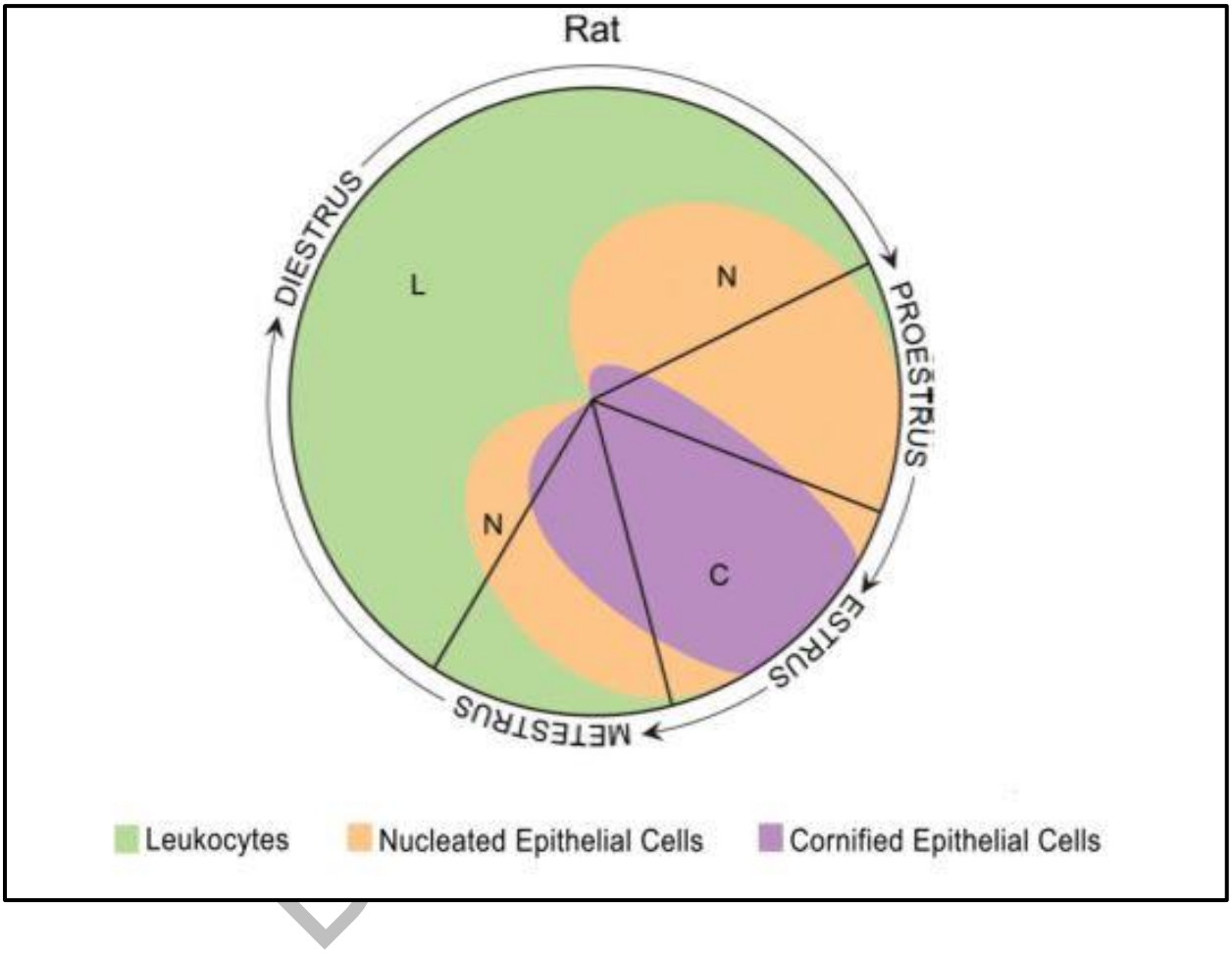

Figure 4. Estrous cycle wheel showing cell types and their proportion in each phase of the cycle (Ajayi & Akhigbe, 2020).

Total Protein Estimation

In order to quantitatively determine the total protein in serum/plasma, total protein estimation kit by AMP diagnostics (AMEDA Labordiagnostik GmbH, Austria) was used. It works on the principal of biuret reaction; in which a chelate (violet colored complex) is formed by the protein's peptide bonds and Cu^{2+} ions. The more intense the color, the more concentrated the protein in the sample.

When performing the procedure, sterile Eppendorf tubes and micropipette tips were used. First of all, 1ml reagent was taken into an Eppendorf tube to be treated as blank. Then, 1 ml reagent and 20 µl standard were taken in another tube, treated as standard. Next, 20 µl serum from each sample was added into Eppendorf tubes followed by the addition of 1ml reagent into each. After that, all the samples were gently mixed and incubated for 10 minutes at 37˚C. Finally, the absorbance of samples and standard was read against reagent blank at a wavelength of 540nm using Piccos 05 Chemistry Analyzer (AMP Diagnostics, GmbH, Austria). The final concentration of Total Protein was calculated using the formula:

$$Total\ Protein(\frac{g}{dl}) = \frac{A(sample)}{A(standard)} * C(standard)$$

Total Cholesterol Estimation

Cholesterol is a significant element of cell membranes, precursor for steroid hormones, and bile acids, thus, plays a significant role in normal functioning of an organism. Blood cholesterol concentration tells us about the cardiovascular diseases, and also about the gallbladder and liver function. For the estimation of total cholesterol (TC), AMP diagnostic kits were used, manufactured by AMEDA Labordiagnostik GmbH (Graz/Austria). Standard protocol provided by the manufacturer was followed precisely, and all the samples were analyzed using Piccos 05 Chemistry Analyzer (AMP Diagnostics, GmbH, Austria).

Before starting the experiment, the samples and reagents were brought to room temperature. Next, they were put into Eppendorf tubes by using sterile micropipette tips to prevent contamination. After that incubation for 5-10 minutes at 37˚C. Finally, the absorbance was noted down at a wavelength of 500 nm. Final concentration of cholesterol was determined using formula:

$$Cholesterol\ Conc. = \frac{Absorbance(sample)}{Absorbance(standard)} * Standard\ Conc.$$

Triglycerides Estimation

Serum/plasma concentration of triglycerides was estimated using the kit provided by AMP diagnostics (Graz/Austria). It works on the principal of enzymatic hydrolysis of triglycerides (TGL), through series of reactions, yielding dihydroxyacetone phosphate and hydrogen peroxide as final products. In the end, peroxidase catalyzed coupling of phenol and 4-aminoantipyrine with hydrogen peroxide produces a red chromogen related to the concentration of TGL in the test.

While doing the procedure, contamination was avoided as much as possible, by using clean pipettes, Eppendorf tubes, and vials. Prior to the experiment, samples and reagent were brought to room temperature. Next, specific volumes of reagent, samples, and standard were taken through pipetting. Followed by gentle mixing and then, incubation for 5 minutes at 37˚C. Finally, absorbance of blank, sample, and standard were read and noted down at a wavelength of 500nm. Final concentration of TGL was determined using formula:

$$Triglycerides\ Conc.(mg/dl) = \frac{Absorbance(sample)}{Absorbance(standard)} * Standard\ Conc.$$

HDL-Cholesterol Estimation

The key role of HDL in the metabolism of lipids is the uptake and transportation of cholesterol to the liver from peripheral tissues. Thus, Low levels of HDL-C in an organism means a heightened danger of coronary artery disease. In order to measure the HDL-C levels in our samples we used kits provided by Bio-active Diagnostic Systems (Voehl, Germany). While performing the procedure, all the reagents and samples were taken and then mixed. After that, the mixture was incubated for 5 minutes at 37˚C after that the addition of reagent to into blank, sample, and calibrator. It was incubated again for 5 minutes at 37˚C. In the end, readings of calibrator and samples were taken against the reagent blank at 620 nm, using Piccos 05 Chemistry Analyzer (AMP Diagnostics, GmbH, Austria). Final concentration of HDL-C direct was calculated using given formula:

$$HDL\ direct\ Conc. = \frac{Absorbance(sample)}{Absorbance(calibrator)} * Calibrator\ Conc.$$

Ovarian Histology

Once the ovaries were successfully secured after dissection, they were fixed in 10% PBS formalin for 24-hours, it stabilizes and preserves the tissue for further processing. Since paraffin wax is hydrophobic hence, water from the sample was removed by dipping samples in a succession of ethanol solutions (increasing concentration) up until pure alcohol was reached i.e.,

- Ethanol (70%) for 60 min.
- Ethanol (80%) for 60 min.
- Ethanol (90%) for 60 min.
- Ethanol (100%) for 30 min.
- Ethanol (100%) for 45 min.
- Ethanol (100%) for 60 min.

At this stage, the tissue is water free but, it still cannot be infiltrated with wax as ethanol and wax are mostly non-miscible. So, we used a clearing agent that is mixable with both paraffin wax and ethanol. This process in performing histology is called “clearing.” For this process, the ovaries were placed in xylene for 30, 45, and 60 minutes. Next in the process of histology is the wax infiltration and embedding step. For this purpose, we dipped our samples into molten paraffin wax, removed air bubbles (if any), and then solidified. The wax blocks were then mounted onto wooden blocks for tissue sectioning.

Tissue sectioning (5 µm thick) was performed using microtome (Thermo, UK). The ribbon containing tissue section was fixed onto a formerly albumenized glass slides at 60˚C using slide warmer (Fischer). Followed by overnight incubation for removing any trapped air bubbles. Upon successful completion of fixation onto slides, the sections were then stained using H&E staining.

In order to achieve this, the wax was removed by placing in xylene overnight. Followed by the hydration of samples in the plunging grades of ethanol i.e.

- Ethanol (100%) for up to 5 min at 20-25˚C.
- Ethanol (80%) for up to 5 min at 20-25˚C.
- Ethanol (60%) for up to 5 min at 20-25˚C.
- Ethanol (50%) for up to 5 min at 20-25˚C.
- Ethanol (30%) for up to 5 min at 20-25˚C.

After successful hydration, slides were bathed with tap water and then plunged in hematoxylin (3-4 times). Again, slides were bathed using tap water, until the tissue sections were blue in color. The dehydration steps (mentioned earlier) were followed, and the slides were then dipped in Eosin, washed using tap water until satisfactory color appears. Slides were again dehydrated and placed in xylene for up to 10 minutes. After the staining process was done the slides were mounted with Canada balsam. After that, xylene dipped coverslips were used to cover the tissue sections on slides.

Microscopy and Microphotography

Slide containing different sections of ovaries were examined using Olympus light microscope (Tokyo, Japan) with attached Canon digital camera (Tokyo, Japan) for taking microphotographs. The sections were examined at 10X and 40X magnification. Microphotographs were taken at both the magnifications and evaluated.

Statistical Analysis

Statistical analysis of all the data was performed using IBM® SPSS® Statistics version 25 (IBM Corp.). The data was analyzed by applying one way Analysis Of Variance (ANOVA) followed by Tukey's test for comparison of different groups to each other. Finally, all the data was presented as Mean ± SEM by setting the significance level at $p<0.05$.

RESULTS

Effects on Body Weight

Data related to administration of different doses of PYR into adult female Sprague Dawley rats and its effects on their body weights is shown in table 3 as Mean ± SEM. The average weight of each group (Control, G1, G2, and G3) on day 1 was kept as 160±10g. On day 14 and 28 of treatment a non-significant ($p>0.05$) decrease in the body weights of control was detected. In contrast, G1, G2, and G3 treated with 62, 124, and 186 mg/kg PYR respectively, followed a different trend in their body weights with a non-significant ($p>0.05$) rise on day 14 of treatment; Followed by a slight decrease in the body weights on day 28.

Table 3. Mean ± SEM body weights (g) of adult female Sprague Dawley rats on different days of treatment with different doses of PYR.

	Body Weight (g)			
Groups	**Day 1**	**Day14**	**Day 28**	**Sig. (p value)**
Control	155.3±7.6	152.3±6.2	150.9±4.7	0.882
G1 (62 mg/kg)	166.7±2.7	173.6±4.6	161.1±6.5	0.236
G2 (124 mg/kg)	161.3±5.8	168.9±4.4	157.3±4.3	0.270
G3 (186 mg/kg)	160.2±7.9	167.5±8.5	154.8±9.4	0.594

Effects on Organ Weight

Weights of different body organs of adult female rats treated with different doses of PYR are presented in table 4. The weight of ovaries of G1, G2, and G3 when compared to Control showed a highly significant decrease ($p<0.000$) in dose dependent manner. The lowest weight was witnessed in G3 (186 mg/kg PYR treated rats). But, when compared to each other the G1, G2, and G3 displayed non-significant difference ($p>0.05$) in ovary's weight. In case of uterine weight, the G1 and G2 showed an increased uterine weight, while a decreased weight in G3 compared to G2 was observed. The increase in uterine weight of G1 and G3 was non-significant ($p>0.05$) after compared to control but, G2 had a notably ($p<0.05$) increased uterine weight.

When compared to each other, the experimental groups (G1, G2, and G3) showed non-significant differences in kidney weight. But, compared to control all the experimental groups (G1, G2, and G3) showed significantly increased ($p<0.05$) kidney weight. All the experimental groups showed an increase in heart weight compared to control but, only G2 showed a significant increase ($p<0.05$). The difference in heart weight among experimental groups was non-significant ($p>0.05$) (Figure 5). Liver weight showed an increase in dose dependent manner (Figure 6). When compared to control, the increase in G1 liver weight was non-significant, while that of the G2 and G3 was highly significant ($p<0.05$ and $p<0.000$).

Table 4. Mean ± SEM organ weight (g) of adult female Sprague Dawley rats as a result of treatment with different doses of PYR.

Organs	Control	G1	G2	G3	Sig. (p value)
Ovary's Weight	0.12±0.01	0.09±0.01^{a***}	0.08±0.01^{a***}	0.07±0.01^{a***}	0.000
Uterine Weight	0.31±0.07	0.48±0.09	0.57±0.08^{a*}	0.43±0.09	0.209
Kidney's Weight	0.49±0.03	0.67±0.07^{a*}	0.70±0.06^{a*}	0.68±0.04^{a*}	0.059

Liver's Weight	5.79±0.30	6.27±0.26	7.14±0.43^{a*}	8.82±0.51^{a***b***}	0.000
Heart's Weight	0.66±0.04	0.76±0.04	0.80±0.04^{a*}	0.75±0.03	0.132

a (value compared to control), b (value compared to G1)

* ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

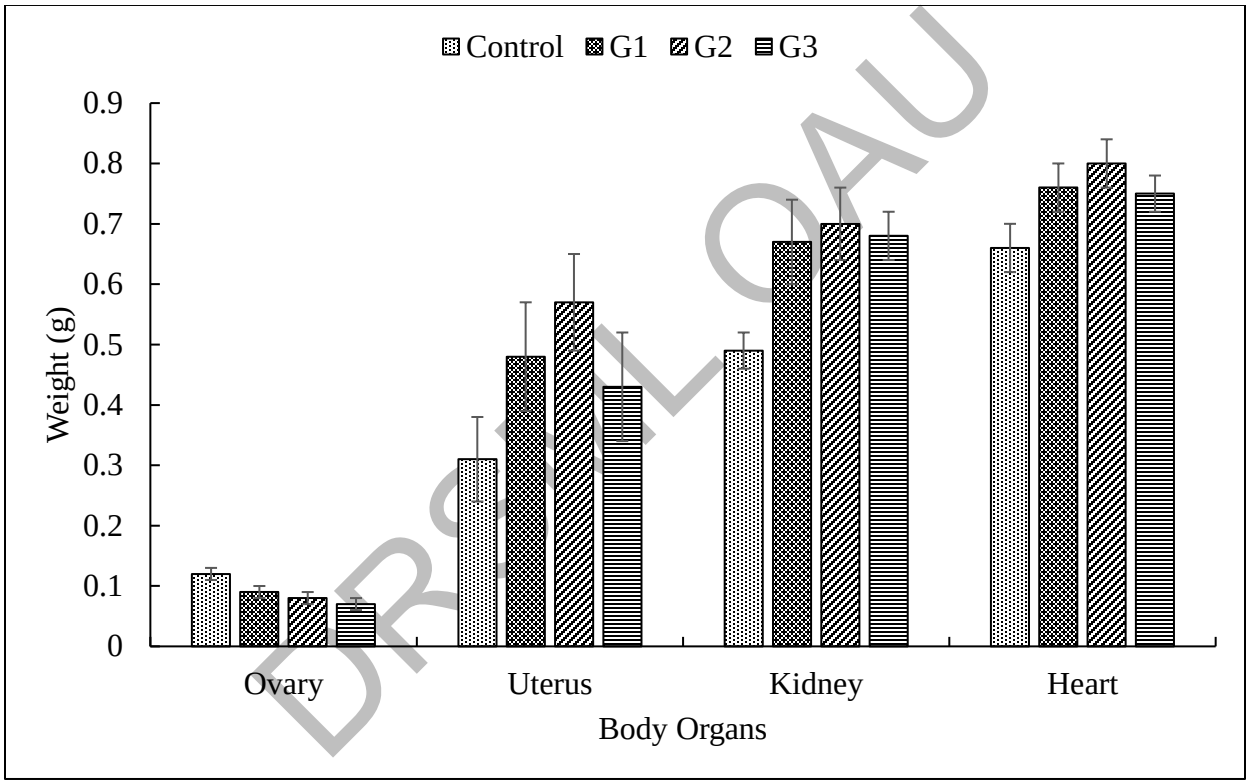

Figure 5. Graph showing the organ weights (g) of experimental groups compared to control as Mean ± SEM.

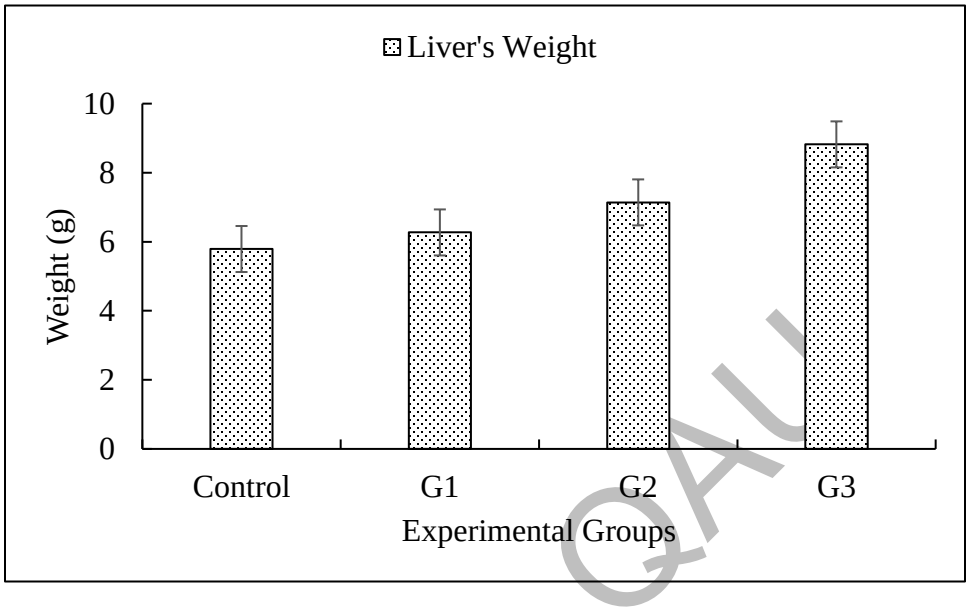

Figure 6. Graph showing liver weight (g) as Mean ± SEM of control and experimental groups treated with different doses of PYR.

Effects on BMI

All the treated groups (G1, G2, and G3) showed a decrease in BMI values (g/cm^2) when compared to the BMI values of control group (Figure 7). Nevertheless, the decrease shown by the treated groups was non-significant ($p>0.05$) when related to control and when compared to each other (table 5).

Table 5. Mean ± SEM values of BMI (g/cm^2) in control and treated adult female rats with PYR.

	Control	G1	G2	G3	Sig. (p value)
BMI(g/cm^2)	0.14±0.067	0.12±0.018	0.13±0.062	0.12±0.053	0.195

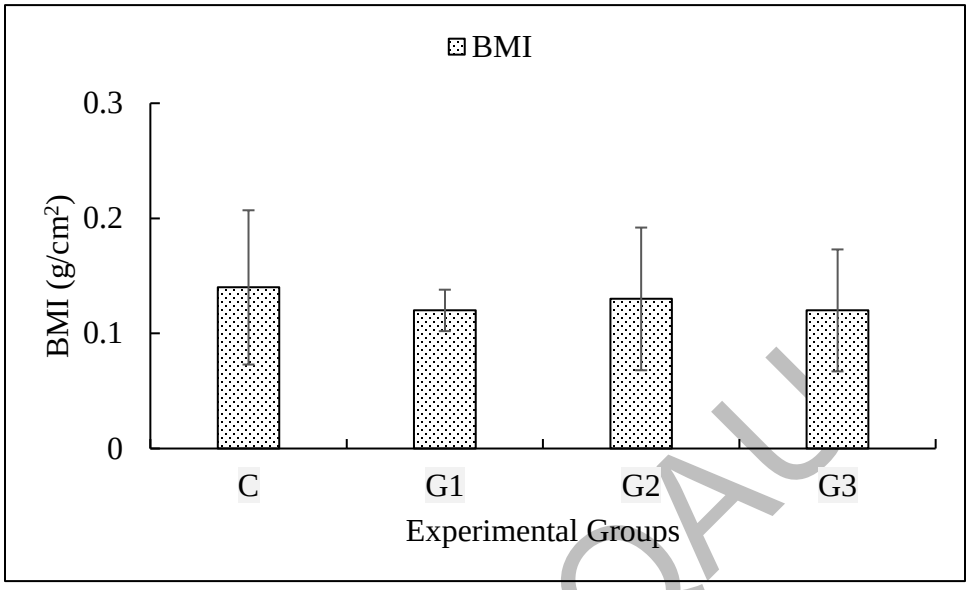

Figure 7. Graph presenting BMI (g/cm^2) values of control and treated groups as Mean ± SEM.

Effects on Blood Glucose Levels

Data related to the effects of PYR on blood glucose levels is shown in table 6 as Mean ± SEM. The blood glucose levels in control showed no significant differences on day 1st, 14th, and 28th day of the experiment. They showed very minute fluctuations through the experiment. However, the G1 showed a decrease in the blood glucose levels through experiment with highly significant ($p<0.000$) decrease on day 28th when compared to the glucose levels on day 1st and day 14th. The decrease on day 14th was not significant ($p>0.05$) compared to day 1st. In G2 the blood glucose levels slightly increased on day 14th followed by a slight decrease in levels on day 28th but, this rise and fall in glucose levels was not significant ($p>0.05$). In G3 treated with PYR the day 28th of experiment marked a highly significant decline ($p<0.001$) in the blood glucose levels as compared to day 1st and day 14th. Finally, the 28th day blood glucose levels of all treated groups (G1, G2, and G3) were significantly decreased ($p<0.001$) as compared to the 28th day blood glucose levels of control (Figure 8).

Table 6. Mean ± SEM blood glucose levels (mg/dl) of control and treated adult female rats with PYR.

Groups	Day1	Day14	Day28	Sig. (p value)
Control	107.4±2.1	111.8±1.5	108±0.9	0.139
G1	114.6±4.1	108.2±2.4	73.6±3.3^{a***b***}	0.000
G2	96±6.5	107.6±7.2	93.4±4.1^{a**}	0.251
G3	104±4.1	110±3.3	85.4±2.5^{a***b***}	0.001

a (value compared to control), b (value compared to day1).

* ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

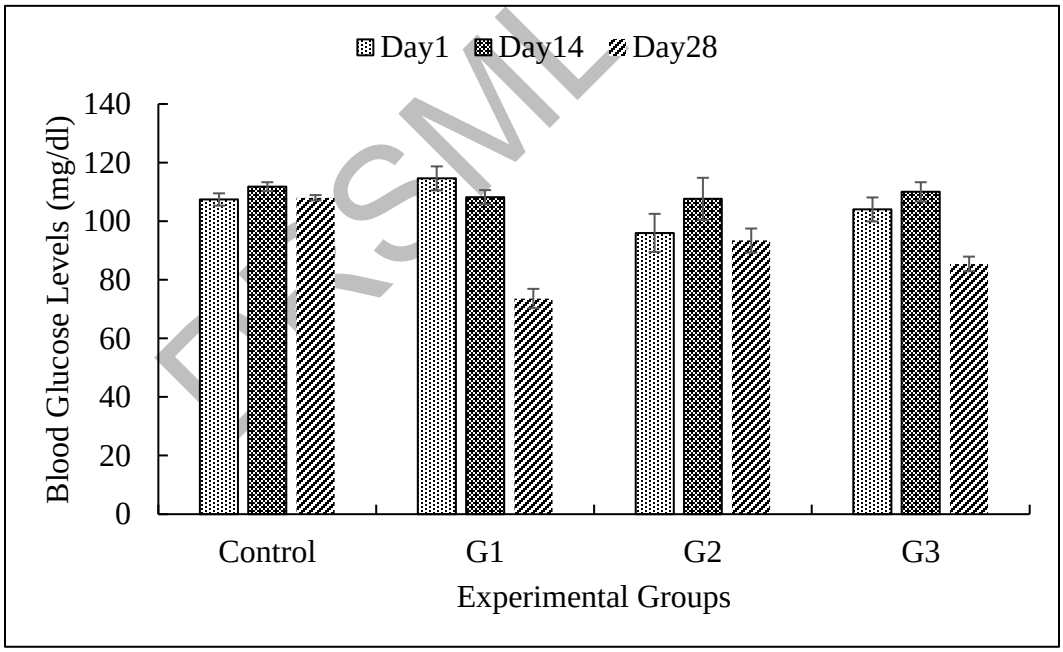

Figure 8. Graphical representation of effects of PYR on blood glucose levels (mg/dl) in adult female rats as Mean ± SEM.

Effects on Estrous Cyclicity

Vaginal smears of PYR treated groups were made and compared to those of control to see the effects on the estrous cycle. Low dose of PYR, in G1 rats, lead to a prolonged metestrus phase compared to control. However, at high doses, PYR treatment lead to shortened proestrus and prolonged metestrus in G2; and lengthened diestrus phase in G3 treated rats (table 7). The microphotographs of normal phases of estrous cycle in control group are shown in figure 9.

Table 7. Different stages of estrous cycle in control and PYR treated groups on different days of the experiment.

Groups	Day 1	Day 7	Day 14	Day 21	Day 28
Control	+	+++	++++	+	+++
G1	++	+++	+++	+++	+++
G2	+++	+	+++	+++	+
G3	++++	++	++++	+++	++++

proestrus (+), estrus (++), metestrus (+++), diestrus (++++).

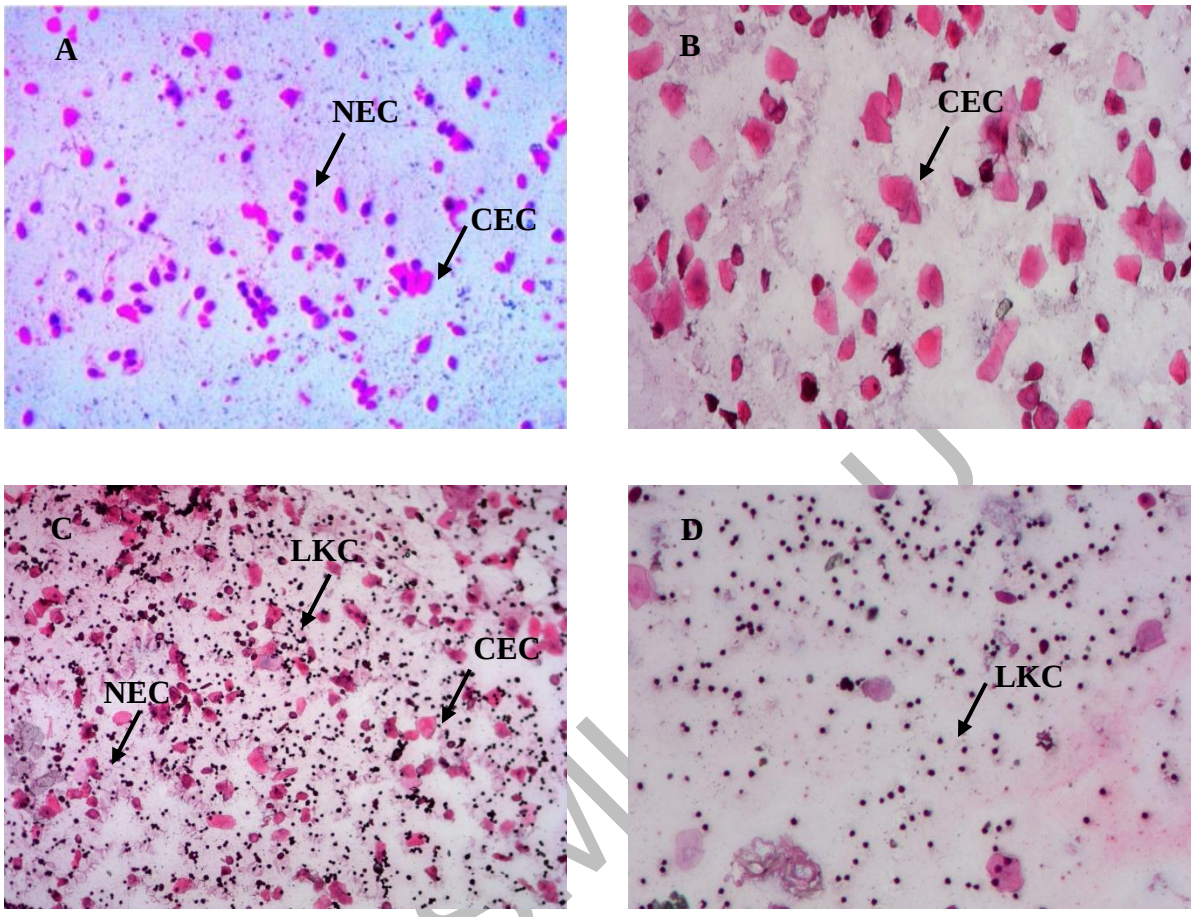

Figure 9. Photomicrographs (10X) of different stages of rat's normal estrous cycle. (**A**) the proestrus phase with an abundance of Nucleated Epithelial Cells (NEC) and a few Cornified Epithelial Cells (CEC). (**B**) estrus phase of the cycle with abundance of CEC. (**C**) metestrus phase with almost equal number of NEC, CEC, and Leukocytes (LKC). (**D**) the diestrus phase with prominent number of LKCs and a few NECs as well.

Effects on Total Protein Levels

In table 8 total protein concentration (g/dl) of control and treated groups is shown as Mean ± SEM. The results showed an elevated levels of total protein concentration in dose dependent manner compared to control. Although the results showed increased levels in all treated groups (G1, G2, and G3), they were still non-significant ($p>0.05$) compared to control and to each other (Figure 10).

Table 8. Mean ± SEM total protein concentration (g/dl) of control and all PYR treated groups.

	Control	G1	G2	G3	Sig. (p value)
Protein Concentration	5.63±0.36	5.72±0.38	6.29±0.28	6.29±0.16	0.287

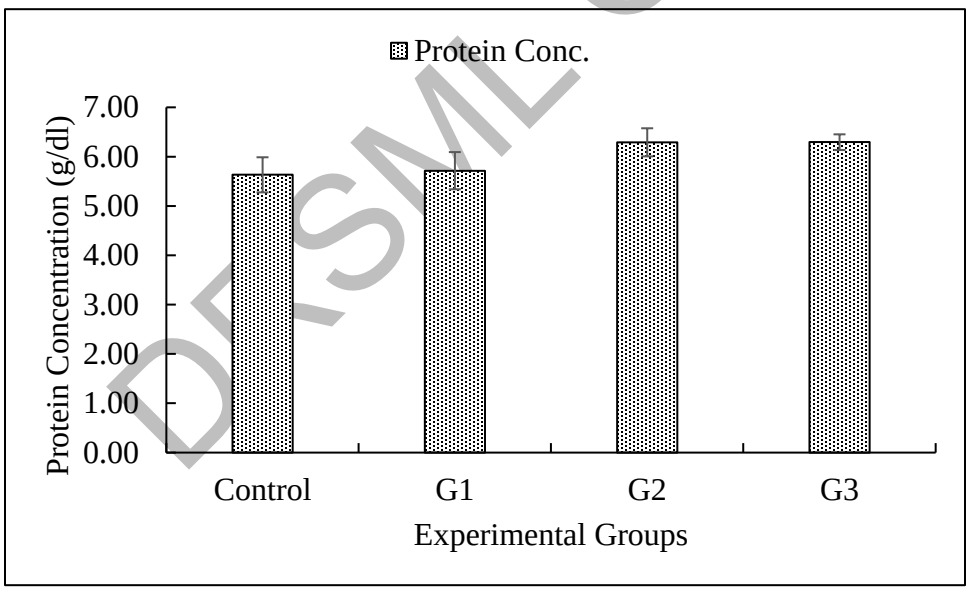

Figure 10. Graphical representation of total protein concentration (g/dl) in different groups of experiment. Data is presented as Mean ± SEM.

Effects on Total Cholesterol

To check the effects of PYR on total cholesterol concentration the assay results of treated groups and control were compared. It was observed that the treated groups had increased levels of cholesterol in their plasma as compared to control (Figure 11). However, only G2 displayed a significant increase ($p<0.05$) compared to control, increased levels of G1 and G2 were insignificant ($p>0.05$) in comparison to control (table 9). The treated groups showed no significant difference when compared with each other.

Table 13. Mean ± SEM plasma cholesterol levels (mg/dl) in control and PYR treated rats.

	Control	G1	G2	G3	Sig. (p value)
Total Cholesterol	53.07±4.25	62.83±4.77	67.09±2.91[a*]	59.53±4.62	0.157

a (value compared to control)

* ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

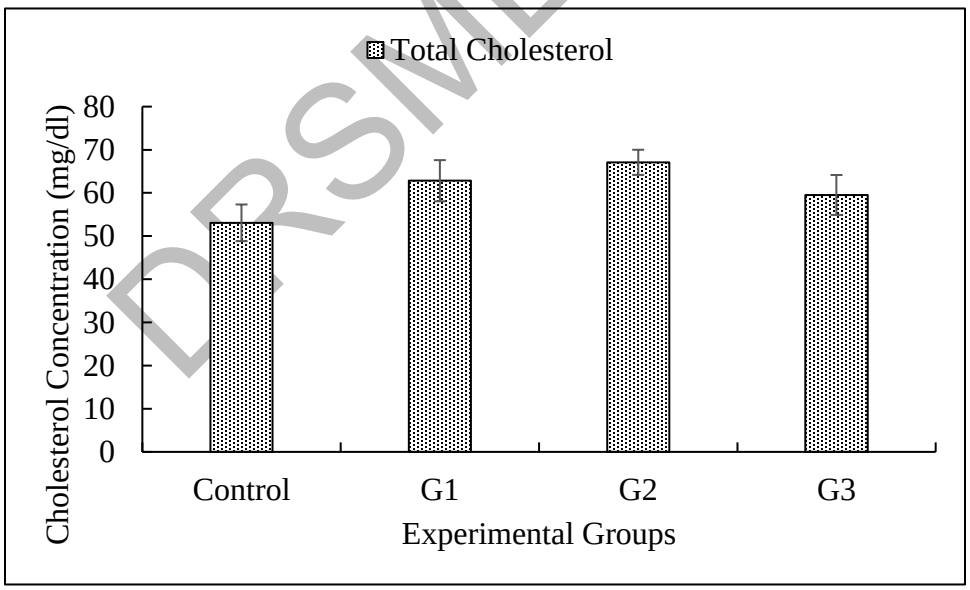

Figure 11. Plasma cholesterol concentration (mg/dl) of control and PYR treated rats, presented graphically as Mean ± SEM.

Effects on Plasma Triglyceride Levels

Results of plasma TGL levels are showed in table 10 as Mean ± SEM. After comparison of TGL levels of treated groups to the TGL levels of control, a noteworthy rise in G1 TGL levels was seen. Also, non-significant decrease ($p>0.05$) was detected in G2 and G3 rats TGL levels as compared to control. G1 rats also showed a significant ($p<0.05$) surge as compared to the G2 and G3 rats (Figure 12).

Table 10. Mean ± SEM plasma TGL levels (mg/dl) of control and PYR treated adult female Sprague Dawley rats.

	Control	G1	G2	G3	Sig. (p value)
TGL level	$78.91\pm4.25^{b*}$	$93.99\pm3.93^{a*}$	$74.1\pm4.24^{b**}$	$76.5\pm4.05^{b**}$	0.015

a (value compared to control), b (value compared to G1)

* ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

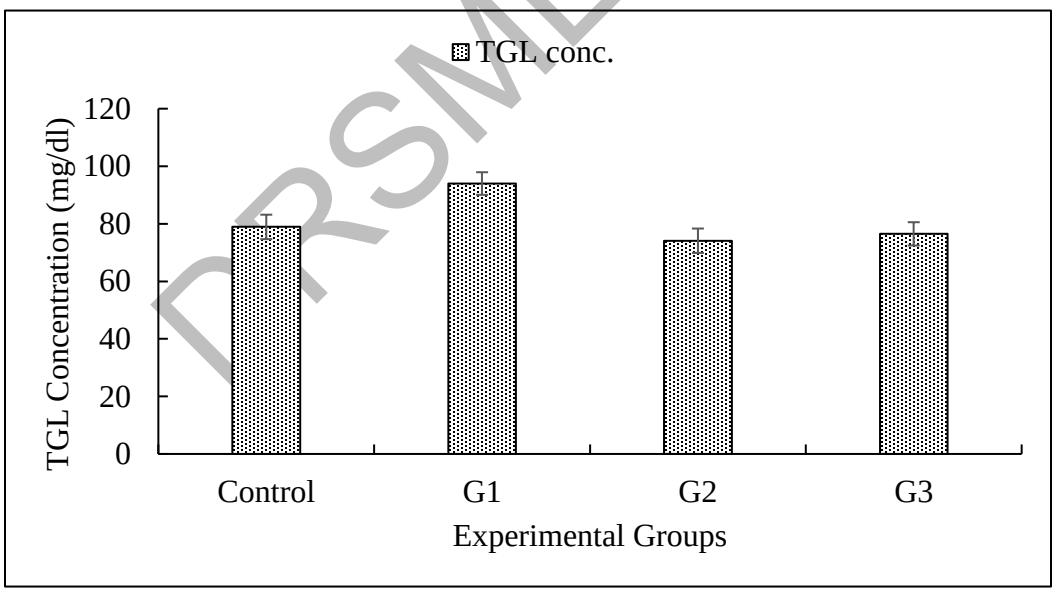

Figure 13. Plasma TGL concentration (mg/dl) of control and PYR treated adult female rats. Values are shown as Mean ± SEM.

Effects on Plasma HDL-C Levels

Our study revealed that PYR administration caused a decrease in plasma HDL concentration (table 11). The lowest HDL concentration was seen in G2 treated rats. In addition, the changes in G1 and G3 were non-significant ($p>0.05$) when compared to control. While G2 indicated a significant decrease ($p<0.01$) in contrast to control and to G1 and G3 treated groups (Figure 13).

Table 11. Mean ± SEM plasma HDL-C levels (mg/dl) of control and PYR treated adult female Sprague Dawley rats.

	Control	G1	G2	G3	Sig. (p value)
HDL-Cholesterol	65.53±2.55	63.66±1.13	43.72±3.9^{a***b***}	55.71±4.02^{a*}	0.001

a (value compared to control), b (value compared to G1)

* ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

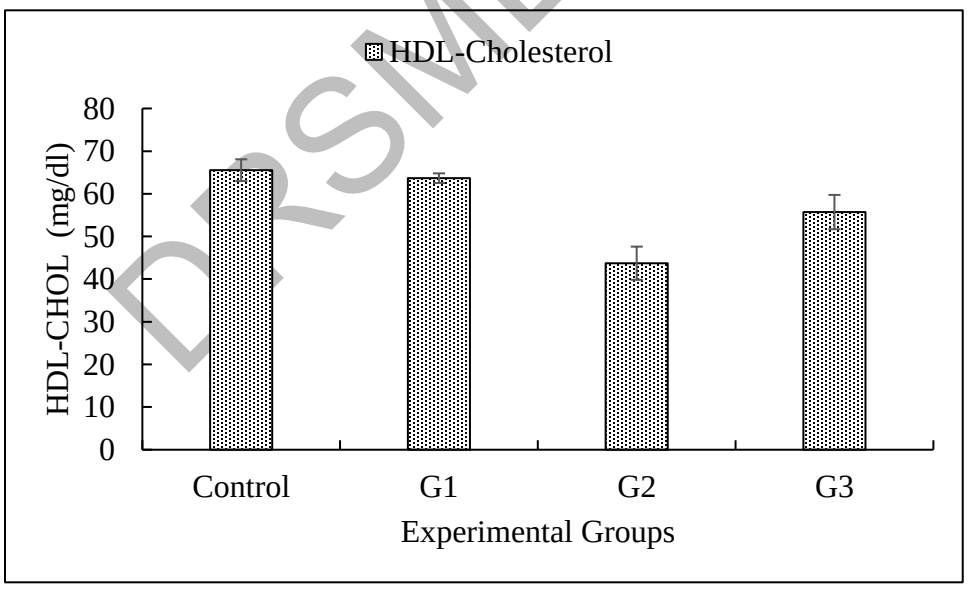

Figure 13. Plasma HDL-C concentration (mg/dl) of control and PYR treated adult female rats. Values are shown as Mean ± SEM.

Effects on Ovarian Histology

PYR effects on the morphology of ovaries was inspected through histology. The stitched photomicrographs of whole ovarian cross sections from control, G1, G2, and G3 were examined and compared with each other (Figure 14). The photomicrographs were examined for any changes made to the tissue integrity, follicular structure, and phases of follicular development. The ovarian histoarchitecture was seen to be normal in H&E sections of control with ovarian follicles at different stages of development; also, there were very fewer empty spaces and no follicular cell dispersion. The ovarian surface epithelium (called Basal membrane) was well intact (Figure 15). In the ovarian cross sections of G1 rats, increased optical spaces were seen, accompanied by the distortion of basal membrane, thus, causing follicular cells dispersion and damage to the tissue integrity of ovaries. In G1 most of the follicles were in primary and secondary phase of folliculogenesis; in addition, previously formed corpora lutea were degenerating (rupturing). Mostly, there were secondary follicles, a few newly formed corpora lutea, and a few atrial follicles (Figure 16).

The H&E sections of G2 ovaries showed tormented basal membranes, leading to increased empty spaces and disturbed tissue compaction. The photomicrographs showed large numbers of previously and newly formed corpora lutea. Many of the seen follicles were degenerating and a few were found to be in the secondary stage of folliculogenesis (Figure 17). While examining the cross sections of ovaries of G3 the ovarian surface epithelium was seen to be disassembled, with lots of optical empty spaces in the ovary. The granulosa cells and theca folliculi were detached from each other. Also, there was follicular degeneration in ovaries of G3 rats. There were few secondary follicles and majority of corpora lutea (Figure 18).

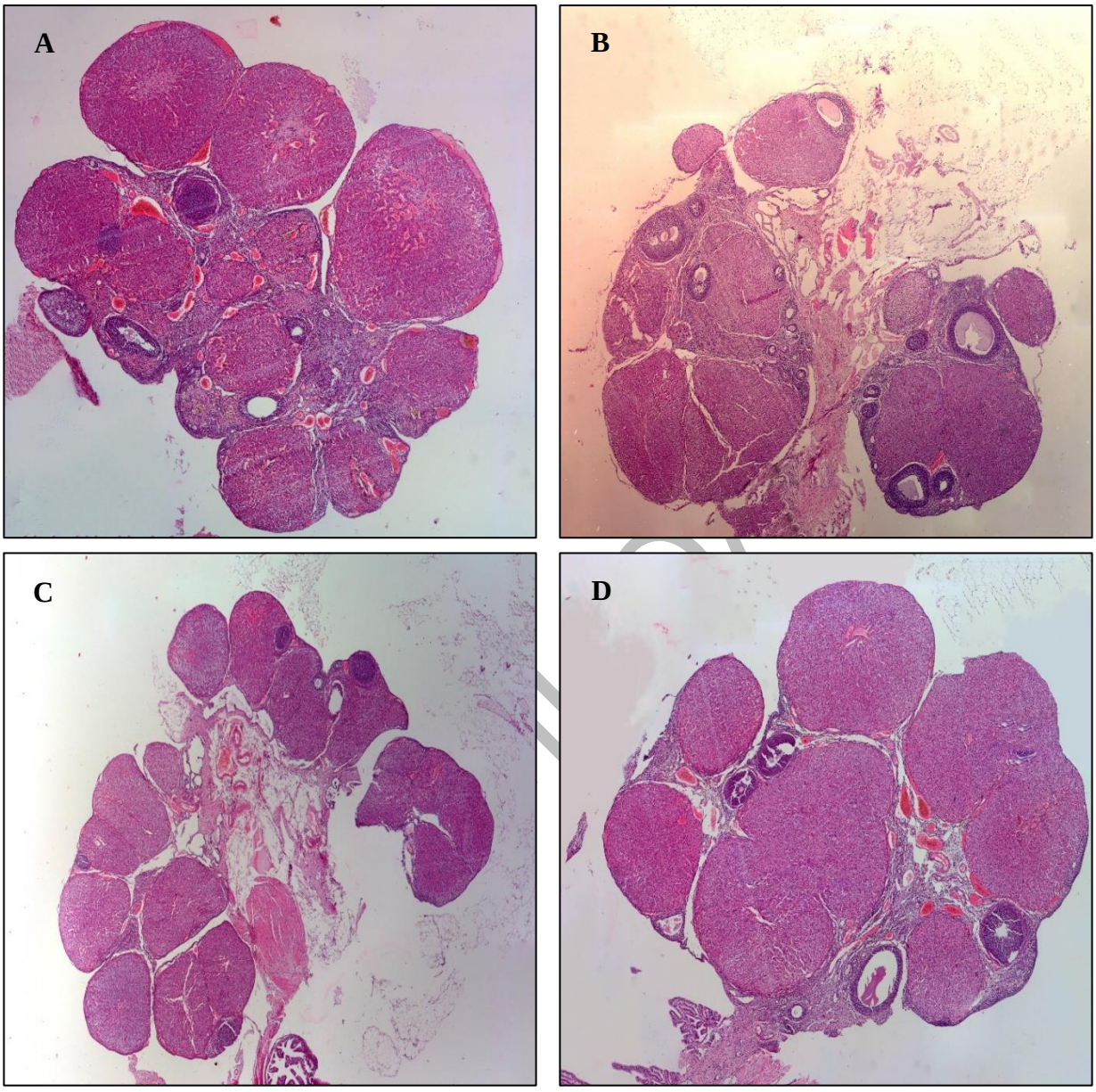

Figure 14. Stitched photomicrographs of rats' ovaries (10X). (A) H&E cross section of ovary from control group animal, (B) H&E cross sectional view of ovary from G1 (62 mg/kg PYR) rat, (C) H&E cross section of ovary from G2 (124 mg/kg PYR) rat, and (D) H&E cross section of an ovary from G3 (186 mg/kg PYR) rat.

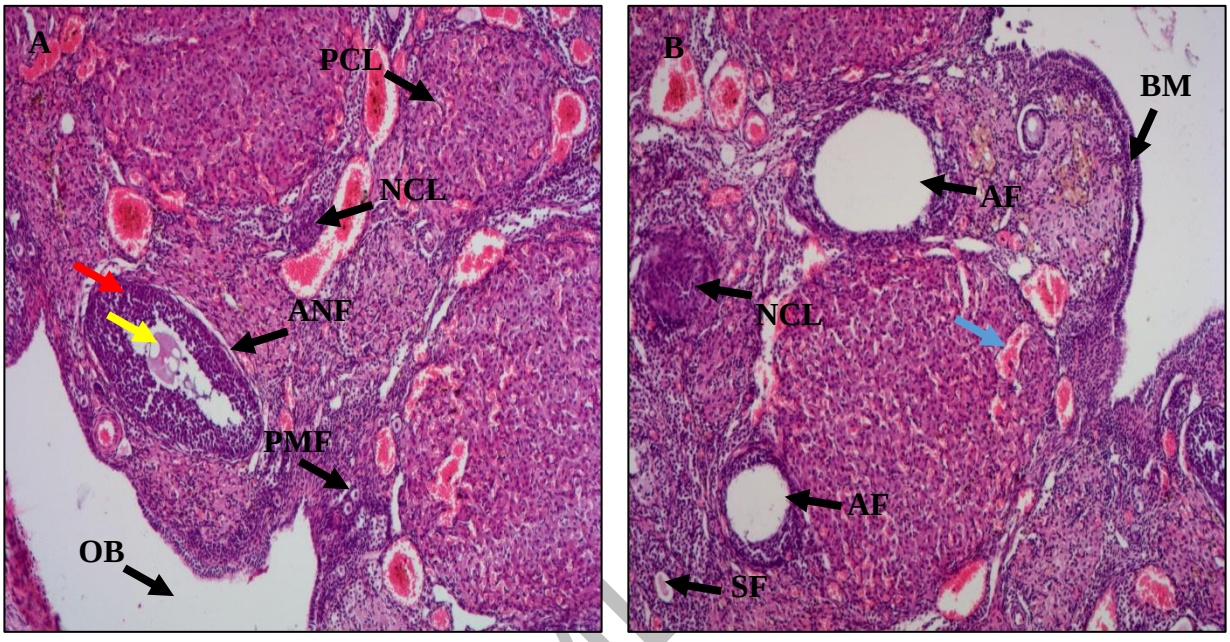

Figure 15. Photomicrograph (10X) of rat ovary of control group. (A and B) The H&E cross section shows the previously formed corpus luteum (PCL), newly formed corpus luteum (NCL), antral follicle (Tertiary follicle, ANF), primordial follicle (PMF), ovarian bursa (OB), well intact basal membrane (BM), atretic follicle (AF), secondary follicle (SF), oocyte (yellow arrow), undistorted granulosa cells (red arrow), ovarian blood vessels (blue arrow).

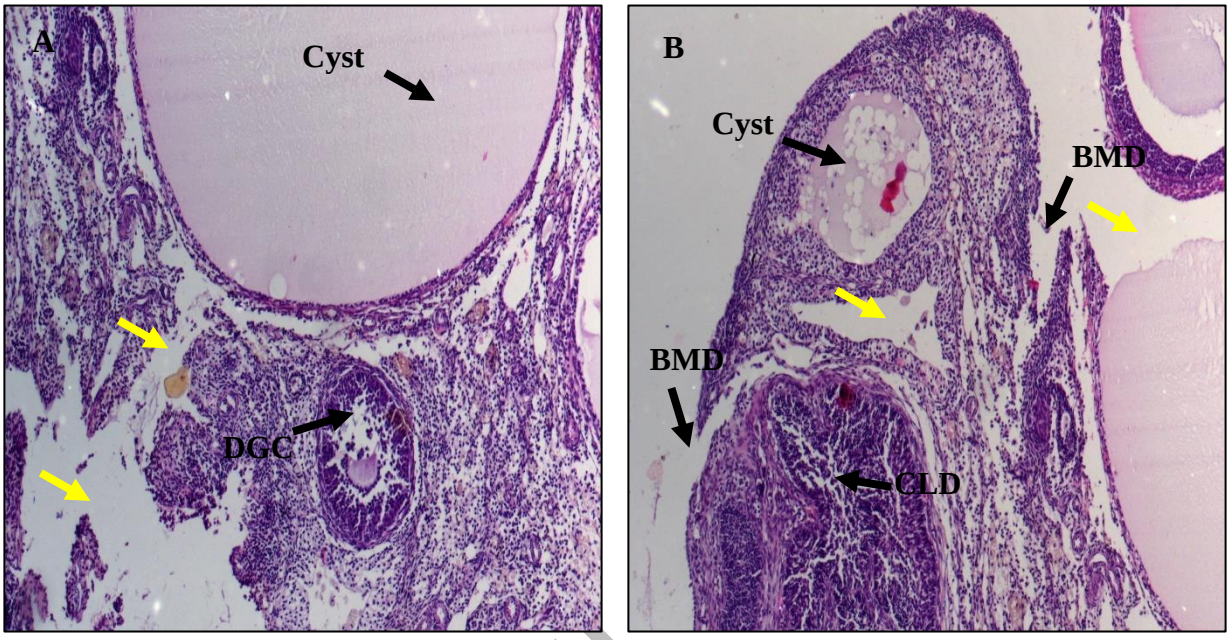

Figure 16. Photomicrographs of G1 ovary, treated with 62 mg/kg PYR. (A and B) shows the distortions in granulosa cells of follicles (GCD), large water filled cysts (Cyst), lots of disruption in basal membrane (BMD), also the degeneration of previously formed corpus luteum (CLD), and many optical empty spaces (yellow arrows).

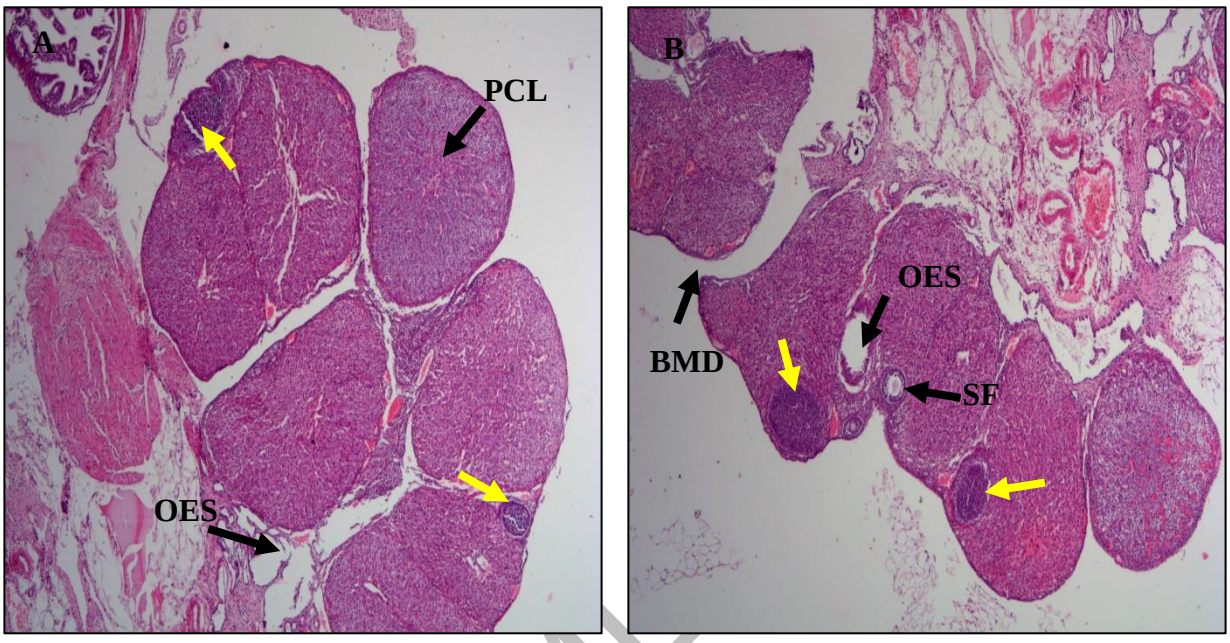

Figure 17. Photomicrographs of H&E sections of G2 rats' ovary treated with 124 mg/kg. (A and B) shows various optical empty space (OES) caused due to the degeneration of basal membrane (BMD), the abundance of previously formed corpus luteum (PCL) and newly formed corpus luteum (NCL), and secondary follicles (SF) with degenerating granulosa cells layer.

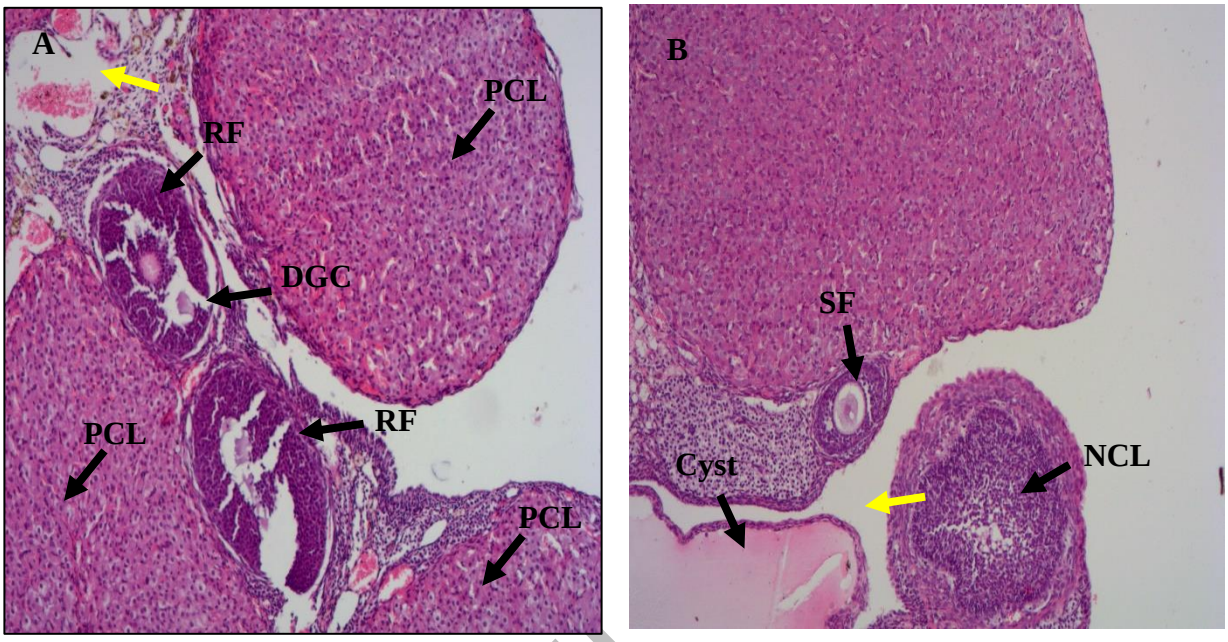

Figure 18. Photomicrographs of H&E cross sections of ovaries from G3 treated with 186 mg/kg PYR. (A and B) shows the rupturing follicles (RF) with degenerating granulosa cells (DGC), previously and newly formed corpus luteum (PCL, NCL), a cyst filled with water (Cyst), a secondary follicle (SF), and some empty spaces (yellow arrows).

DISCUSSION

The phenomenon of endocrine disruption has been acknowledged for a long time and since the discovery of the first hormone in 1902 (Bayliss & Starling, 1902). On top of that, these endocrine disruptors can be pharmaceuticals, plasticizers, polychlorinated biphenyls, organochlorinated pesticides (Carpenter, 2013; Gore, 2001). With the growing population of the world there is a growing demand for food, thus, a growing demand for pesticides to increase crop production. Approximately 5.6 billion pounds of pesticides are being used every year in the world, and this usage is unexpectedly rising (Alavanja, 2009). More than 95% of these employed pesticides have the ability to become widely distributed in the environment and to affect non-target organisms (Simeonov *et al.,* 2014). PYR is one of the most extensively used pesticides in the world and is well known for its ability as an embryogenesis inhibitor in insects (Invest & Lucas, 2008). It gets accumulated in the environment leading to detrimental effects in non-target organisms (plants, fish, amphibians, birds, and mammals etc.) through food web (Mehrnoush *et al.,* 2013). The adverse effects due to continuous exposure of PYR include growth retardation, disruption of hormonal balance, impaired reproduction, and neurodevelopmental toxicity (Maharajan *et al.,* 2018; Sartori *et al.,* 2020).

Our study investigates the reprotoxic effects of PYR in adult female Sprague Dawley rats. For this purpose, three different dosages of PYR, 62, 124, and 186 mg/kg were selected (Sartori *et al.,* 2020; Shahid & Saher, 2020; Shahid *et al.,* 2019). Oral administration of PYR was performed, because of its use in drinking water for mosquito control and on different crops for controlling insect pests, leading to its oral exposure through food and water.

At the end of our experiment, a non-significant reduction in the average weights of the treated groups was observed. The same effects of PYR on body weight was found in a study executed on *Labeo rohita* by (Naseem *et al.,* 2022) in which they administered 300, 600, and 900 µg/l of PYR into G1, G2, and G3 of fish, respectively. In yet another study performed on male Swiss albino mice the oral administration of PYR (1200, 600, 320, 200, 100, 40, 20, and 0 mg/kg)

caused significant drop in the body weights in relation to control group (Shahid *et al.,* 2019). In another study performed on pregnant female mice the oral administration of PYR (30, 100, 300, 1000 mg/kg) caused decrease in pup's body weights in dose dependent manner (Shahid & Saher, 2020).

Our study resulted in a decreased ovarian weight due to the PYR administration, in dose dependent manner. In another experiment where female rats were chronically administered with PYR (0, 80, 400, 2000, 10000 ppm) for six months, revealed a drop in ovarian weight as compared to control at higher doses (Koyama *et al.,* 1989). Also, a study on the reproductive toxicity of PYR in male mice showed a decline in testicular weight because of PYR administration (Shahid *et al.,* 2019). The existing study revealed that oral administration of PYR instigated a significant rise in absolute weights of uterus, kidney, liver, and heart as compared to control. These findings are supported by a research performed on male and female Sprague Dawley rats, in which oral administration of PYR for six months resulted in increased heart, liver, and kidney weights in relation to the control group (Koyama *et al.,* 1989). Yet another study, focusing on the toxic effects of PYR in *Labeo rohita* fish, showed that PYR administration lead to the elevation in weights of liver, kidney, brain, and gills (Naseem *et al.,* 2022). Our study revealed that the PYR had truly little (non-significant) effects on BMI of adult female rats. There are no previous studies relating PYR and BMI.

The present study revealed that PYR exposure lead to decrease in blood glucose levels with passing days. In PYR treated groups, rats showed significantly low blood glucose levels on the 28th day of experiment as compared to glucose levels on day 1st of experiment, while in control group no substantial decrease was observed in rats' blood glucose levels throughout the experiment. In previous studies, the blood biochemistry results of female rats after 26-weeks treatment with PYR displayed a significant decrease in the blood glucose levels (Koyama *et al.,* 1989). Yet another study performed on silkworm larvae showed that PYR had decreasing effects on the hemolymph Glucose levels after 24-hours of PYR administration (Etebari *et al.,* 2007).

In the current study the chronic oral administration of different doses of PYR were found to be directly affecting the reproductive cycle (estrous cycle) in adult female Sprague Dawley rats. It was found to be affecting the length of different phases of estrous cycle as compared to control group. Previous data regarding the effects of PYR on estrous cyclicity is lacking, but in studies performed on pregnant female mice it is found to be causing a reduction in litter size and the number of live births as compared to control (Shahid & Saher, 2020). In another study the fetal indices of Wistar rats treated with different doses of PYR (100, 300, and 500 mg/kg) showed a significant decrease in number of fetuses in the treated groups.

The present study showed increased concentrations of total plasma proteins in PYR treated groups as compared to control group. The same type of effects of PYR on total protein concentrations has also been reported in the past studies on different organisms. A group of researchers (Koyama *et al.,* 1989) reported that PYR exposure caused an increase in total protein concentration in female and male Sprague Dawley rats. In another research elevation in total protein in hemolymph of silkworm larvae was reported as a result of pyriproxyfen residue (Etebari *et al.,* 2007).

In the current study, it was found that the PYR administration lead to an increase in total cholesterol concentration and plasma triglycerides levels as compared to the control group. Also, there was a non-significant decrease in HDL concentrations in the PYR treated groups as compared to control. A study done on silkworm larvae showed that PYR had elevating effects of hemolymph cholesterol levels (Etebari *et al.,* 2007). In an another study performed on Sprague Dawley rats, PYR was also found to be responsible for the elevation in cholesterol and TGL concentrations in the plasma (Koyama *et al.,* 1989). Yet another study performed on *Labeo rohita,* showed that PYR exposure lead to increased concentration of Cholesterol and TGL (Naseem *et al.,* 2022). Additionally, there is no prior records related to PYR exposure and HDL concentration.

The current study showed deteriorating effects of PYR on the ovarian histoarchitecture of rats. The chronic ovarian administration had led to the basal membrane distortion, cysts formation, granulosa cells degeneration, increased optical empty spaces, increased corpus luteum formation,

and follicular dispersion. Although there are no previous studies related to the effects of PYR on ovarian histology, there are findings of its effects on the testicular histology in a study done on mice. In which the PYR administration (1200, 600, 320, 200, 100, 40, 20, 0 mg/kg) for 28 successive days caused shrinkage of seminiferous tubules, vacuolization in seminiferous tubules, and reduction in lumen diameter (Shahid *et al.,* 2019). Also, it was reported that chronic oral administration of PYR was responsible to cause a significant decrease in the ovarian weights of female Sprague Dawley rats (Koyama *et al.,* 1989). Another study performed on adult zebrafish reported the negative effects of PYR exposure on the ovary histology (Maharajan *et al.,* 2020). In another case a group of researchers performed experiments on Christmas Island red crab and reported that PYR had negative effects on the ovarian histology of red crabs (Linton *et al.,* 2009).

Conclusion

From the current findings of our study, we conclude that the alarming increase in the usage of PYR on crops and in drinking waters and because of its bioaccumulation in the environment, it is not safe for non-target organisms. In our present findings, and in previous studies performed on different organisms, it is found to be causing toxicity when administered. Therefore, a controlled and only necessary use of PYR is advised by so that its bioaccumulation in the environment and exposure to non-target organisms can be minimized. Furthermore, there is a need of molecular studies to find the mechanisms involved in the interaction of PYR with different cells of ovaries.

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