# INDUCTION OF VITELLOGENIN GENE EXPRESSION AS A MARKER TO ASSESS THE REPRODUCTIVE TOXICITY EFFECTS OF DI(2-ETHYLHEXYL)PHTHALATE (DEHP) IN *BOMBYX MORI*

**PRATIMA KHANDAYATARAY**

**DEPARTMENT OF ZOOLOGY**

**MIZORAM UNIVERSITY**

# Induction of vitellogenin gene expression as a marker to assess the reproductive toxicity effects of di(2-ethylhexyl)phthalate (DEHP) in *Bombyxmori*

**BY**

**Pratima Khandayataray**

**DEPARTMENT OF ZOOLOGY**

**Submitted in partial fulfillment of the requirement of the Degree of Master of Philosophy in Zoology, Mizoram University, Aizawl**

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## DECLARATION

I, **Mrs. Pratima Khandayataray**, hereby declare that the subject matter of this dissertation entitled "**Induction of vitellogenin gene expression as a marker to assess the reproductive toxicity effects of di(2-ethylhexyl)phthalate (DEHP) in *Bombyx mori***" is the record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the dissertation has not been submitted by me for any research degree in any other university/Institute.

This is being submitted to the Mizoram University for the degree of Master of Philosophy in Zoology.

(PRATIMA KHANDAYATARAY)

(HEAD) (SUPERVISOR)

**Department of Zoology**
**School of Life Sciences**
**MIZORAM UNIVERSITY**
***(A Central University)***
**P.B. No. 190, Tanhril, Aizawl - 796 009, Mizoram, India**

**Dr. G.GURUSUBRAMANIAN Ph.D**
**Professor**

**Phone: 0389 - 2331021(O)**
**E-mail: gurus64@yahoo.com**
**Mobile: 9862399411**

## CERTIFICATE

I certify that the dissertation entitled "**Induction of vitellogenin gene expression as a marker to assess the reproductive toxicity effects of di(2-ethylhexyl)phthalate (DEHP) in *Bombyx mori***" submitted to Mizoram University for the award of the degree of Master of Philosophy in Zoology by **Pratima Khandayataray** a record of research work carried out during the period of 2019 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this university or any other university or institution of higher learning.

(PROF. G. GURUSUBRAMANIAN)
Department of Zoology
Mizoram University

**MIZORAM UNIVERSITY**
(A Central University Established by Act of Parliament)
(NAAC Accredited Grade "A" institution)
Aizawl-796004, Mizoram, **INDIA**

**PROFESSOR G.S. SOLANKI**,
Ph.D., FAZ, FZSI, FNC, FNIE, FASEA, Ph: 91-09436374596 (m)
Head, Department of Zoology e-mail : gssolanki02@yahoo.co.in
Ex-Dean, school of Life science drghanshyam.solanki@gmail.com

## CERTIFICATE OF HEAD OF DEPARTMENT

This is to certify that **Mrs. Pratima Khandayataray**, a M.Phil. Scholar, Registration No. **MZU/M.Phil./530 of 17.05.2019** has worked on the dissertation entitled "**Induction of vitellogenin gene expression as a marker to assess the reproductive toxicity effects of di(2-ethylhexyl)phthalate (DEHP) in *Bombyx mori***". She has fulfilled all the criteria prescribed by the UGC (Minimum Standard and Procedure governing Ph.D Regulation). She has fulfilled all the mandatories are required for M.Phil degree (enclosed). It is also certified that the scholar has been admitted in the department through an entrance test followed by an interview as per M.Phil - UGC Regulation, 2016.

(Prof. G. S. Solanki)
Head
Department of Zoology
Mizoram University

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PRATIMA KHANDAYATARAY

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# CHAPTER-1

# INTRODUCTION

## 1. Introduction

Di(2-ethylhexyl) phthalate (DEHP, CAS No. 117-81-7) is the PA and 2-ethyl-1-hexanol (2-EH) branched-chain diester (Figure 1). In lipophilic liquid, it is colorless, viscous, and soluble. DEHP is the plasticizer most commonly used, making plastic more flexible and more elastic. It is used internationally and its shipping volume of 103,499 t represents around 50% of the overall phthalates in Japan in 2018 (Japan Plasticizer Industry Association, 2019).

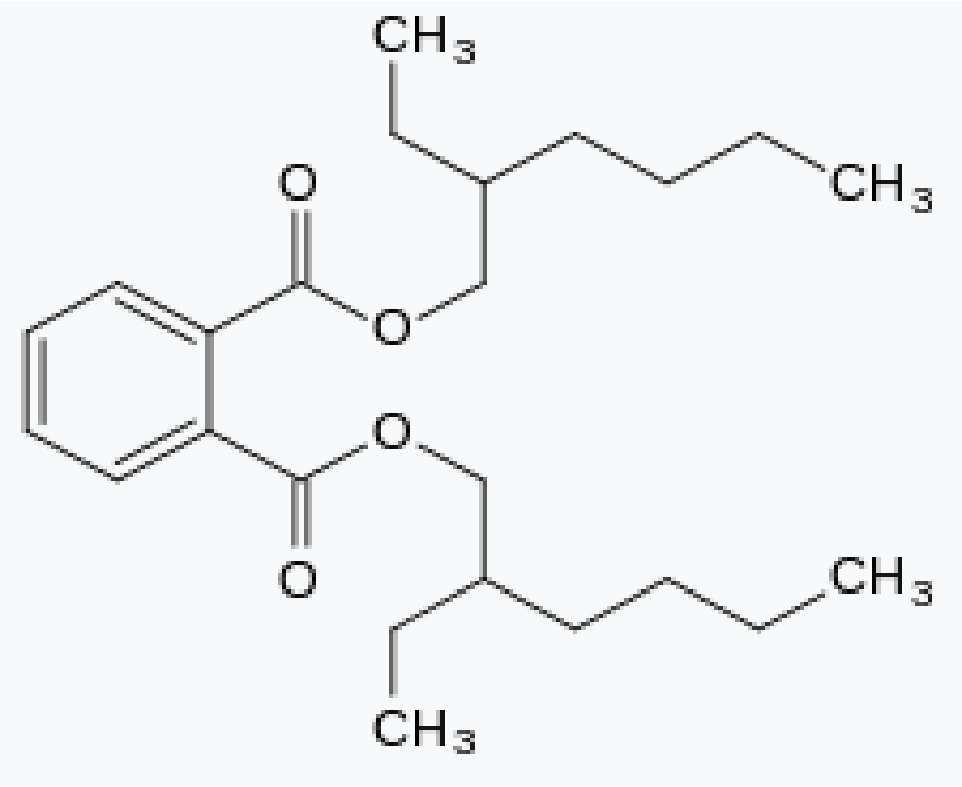


**Figure 1**. Structure of Di(2-ethylhexyl) phthalate (DEHP).

Di-2-ethylhexyl phthalate (DEHP) is ubiquitous in nature (soil, water, air, and lipid-rich food respectively) and causes serious health risks in infants, pregnant women, and elders also, due to non-covalent binding to the plastic matrix which leads to leach out to the outer environment. It is a good plasticizer, present in facial products (cosmetics), hair products ( shampoos), blood storage bags, nasogastric tubes, toys, and child products (Bourdeaux et al., 2004; Petersen and Breindahl, 2000; Serrano et a., 2014).

DEHP can cause numerous toxic effects on living organisms such as neurotoxicity (Du et al., 2017; Wu et al., 2019), hepatotoxicity (Zhang et al., 2017), immunotoxicity (Huang et al., 2015), and reproductive toxicity (Rowdhwal and Chen, 2018). Because of its omnipresence, the general population is likely to be exposed to DEHP. Increasing attention has recently been focused on DEHP's dangers, especially in the areas of carcinogenesis and child health. As DEHP is absorbed by the body, mono(2-ethylhexyl) phthalate (MEHP) and 2-EH are first metabolized (mainly by lipase). A element of MEHP is then conjugated by UDP-glucuronosyltransferase (UGT) with UDP-glucuronide and excreted in the urine. The remaining MEHP is excreted directly into the urine or is then oxidized to dicarboxylic acid or ketones by cytochrome P450 (CYP) 4A by alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH). 2-EH is metabolized primarily by the catalytic action of ADH and ALDH to2-ethylhexanoic acid (2-EHA) through 2-ethyl-1-hexanal (Albro, 1986).

Animal models are widely used for a deeper understanding of specific scientific issues in life sciences and other fields. In many cases, these studies use higher organisms such as mammals. The extensive use of mammals, however, will raise issues not only of biosafety but also of animal feelings, animal rights, and many other bioethical issues (Levy, 2012). Hence, in current life science research, selecting an acceptable model animal to replace mammals or through the use of mammals becomes a concern. Studies have shown that approximately 80% of pathogen infection experiments using mammals can be replaced with insects, and many experts have accepted and suggested using lower animals instead of higher (Renwick and Kavanagh, 2007).

A typical representative of lepidopteric insects, silkworm (*Bombyx mori*), is of great agricultural and economic importance. With a short generation, a clear genetic background, rich genetic resources and a considerable number of human homologous genes, silkworms have been widely used in various life science studies. In 2003, China and Japan initiated the silkworm genome project and completed the draft map, fine map, and multistrain genome resequencing of the silkworm (Xia et al., 2004; Xia et al., 2009), which greatly encouraged the advancement of sericulture science, and stimulated the use of silkworm as an ideal model organism for scientific

research. The application of the silkworm model has increasingly appeared in the field of life science ( Nwibo et al., 2015), such as antipathogenic drug screening, therapy evaluation, and environmental safety monitoring. This model organism has shown a very promising future.

Silkworm (*Bombyx mori* (L.); Lepidoptera: Bombycidae) has a large progeny size and a short time of generation with a larval stage of about 25-30 d (Figure 2). There are also numerous mutant strains and morphological mutations in silkworm. Its death does not include any bioethical problems, whether hereditary or forced (Chen et al., 2014). Furthermore, the silkworm has a moderate body size and can be easily dissected to obtain many tissues and organs like midgut, fat body, silk gland, and hemolymph. In addition, silkworms may be used to conduct an experiment as same as mammals (oral administration and intravenous injection). With the completion of the silkworm genome project and the development of the silkworm database and protein database, the silkworm is beginning to stand out in scientific research as a successful animal (Mita, 2008).

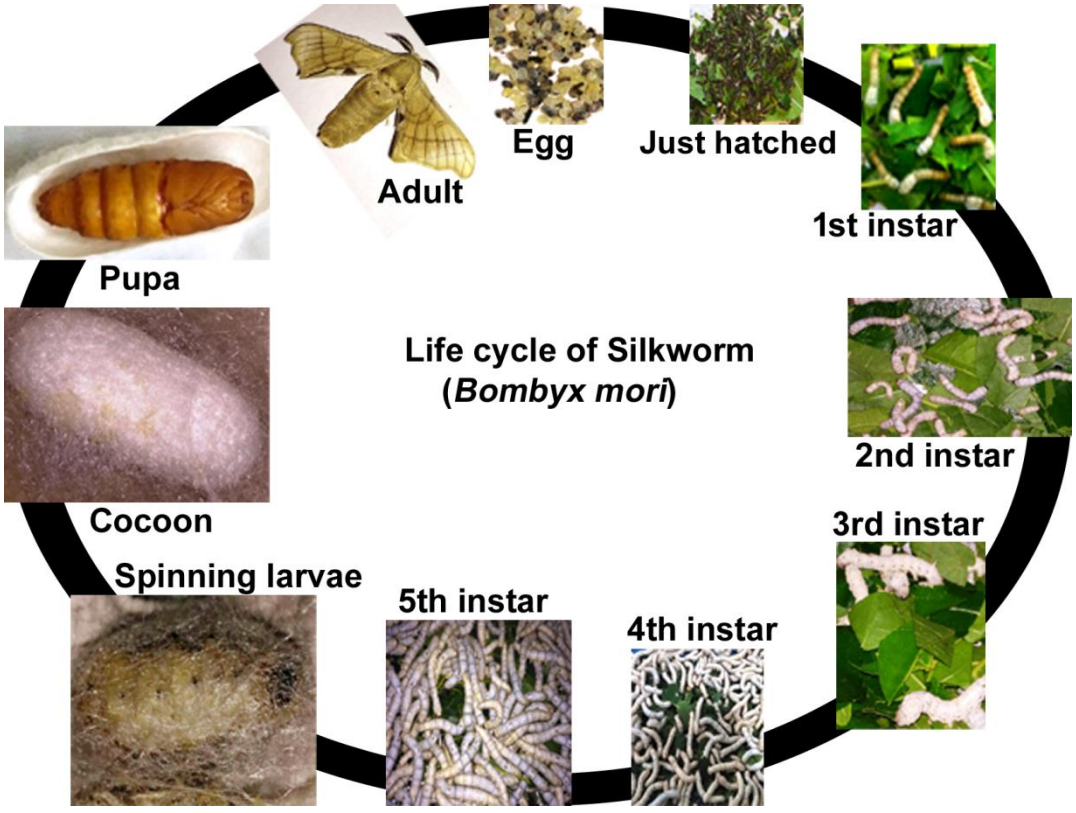


**Figure 2**. Life cycle pattern of silkworm (*Bombyx mori*).

Vitellogenin (Vg) is a egg yolk protein (phospholipoglycoprotein), present in liver in vertibrates whereas in insects it is present in fatbody equal to the liver (Matozzo et al., 2008). Generally, vitellogenin is absent in males but exposed to the chemical toxicants the vitellogenin may be induced in males aswellas in midges (Hahn et al., 2002; Jobling et al., 2003; Hannas et al., 2011). Vg expression is thus commonly used in the aquatic environment as a good biomarker for endocrine distrupting effects. Vg production in vertebrates is regulated by the pathway of the estrogen receptor (Flouriot et al., 1996). The hormones involved in the regulation of the Vg gene in insects can be independent of estrogen. Ecdysteroid and juvenile hormones in fact orchestrate Vg synthesis in insects with juveniles that usually induce vitellogenesis and ecdysteroids that have either a stimulating or suppressive effect, depending on the organism (Swevers and Iatrou, 2003; Handler and Postlethwait, 2005). Nevertheless, it remains unclear the DEHP toxicological mechanism with known endocrine disrupting effects.

In this research, we used the silkworm to determine DEHP reproductive toxicity and focused on controlling and transporting vitellogenesis. Our data suggest that the Vg gene is a possible biomarker that can be used to determine the toxic effects of DEHP on silkworm reproductive growth.

# CHAPTER-2

# REVIEW OF LITERATURE

## 2. Review of literature

Previous studies have been reported on the adverse health effect of environmental agents like pesticides, plasticizers, and several industrial chemicals (Dalsenter et al., 2006). Endocrine disruptor substances (EDS) are a group of chemicals having detrimental effects on hormonal balance (Albert and Jegou, 2014). Phthalate is a synthetic diesters of phthalic acid which are first produced in 1920 (Kimber and Dearman 2010; North et al., 2014).

### 2.1. DEHP effect on pregnancy

Once DEHP has taken inside the human body, it is converted into MEHP, by enzymes that are found in the blood, gut, and lungs. So MEHP can go to fetal circulation by crossing the placenta (Whyatt et al., 2009). Prenatal exposure to DEHP can cause first-trimester pregnancy loss (Arbuckle et al., 2014). As the DEHP exposure can be influenced by the time duration at a specific dose to parent, which can further lead to damage or abnormal growth of developing fetus, particularly preterm (Latini et al., 2003b). The reduced anogenital distance in male infants can be considered as a potential marker of reproductive toxicity in humans (Arbuckle et al., 2014).

### 2.2. DEHP effect on reproductive system

At the developmental stage, the male reproductive system is more prone towards toxic effect of pthalte ester (Hannas et al., 2011). From many experimental studies, it is already proven that phthalates and their metabolites might alter the developmental process which affects to fetal health. DEHP develops adverse effect towards male reproductive system and also induces DNA damage in human lymphocytes (Matsumoto et al., 2008). Many experimental data exist on toxic effect of DEHP on animal with exposure during utero development. DEHP causes reproductive toxicity during exposure to developmental stages as well as adult life (Kavlock et al., 2002a). Other studies also have done on animals, which revealed that long term oral exposure to DEHP also causes reproductive toxicity (Kavlock et al.,

2006). From many animal studies it is clearly seen that DEHP causes adverse effects on experimental animal. Low-dose utero exposure to DEHP (10mg/kg) increases T levels in testis, whereas higher dose (750mg/kg) reduces T level and AGD in rats (Lin et al., 2008). Developmental toxicity can be caused with higher dose exposure of DEHP (500 mg/kg/day) in rats (Dalsenter et al., 2006). After oral treatment of DEHP in rats, DEHP gone through rapid hydrolysis done by lipases and got converted into MEHP in the gut (Kavlock et al., 2002a). Therefore timing, duration and route of exposure plays a major role in abnormal steroid biosynthesis (Ma et al., 2006). DEHP may induce inauspicious folliculogenesis and steroidogenesis which increases risk of infertility and nonreproductive disorder (Hannon and Flaws, 2015; Patel et al., 2015). Exposure to DEHP at $7^{th}$ and $8^{th}$ days of gestational period leads to malformation and even death (Ventrice et al., 2013).

### 2.3. DEHP effect on neurodevelopment

Prenatal exposure of child to phthalates can cause abnormal neurobehavioral development (Serrano et al., 2014). Because of the effect of pthalates during development of the fetus increases the internalizing behaviors and also affect on motor development which leads to psychomotor development index, Particularly in girls(Whyatt et al., 2012). Likewise, it is also confirmed that the gender plays role in a adverse effect in neurodevelopment due to parental exposure to phthalates (Téllez-Rojo et al., 2013).

### 2.4. Carcinogen effect of DEHP

Phthalate esters may induce cancer in humans (Ventrice et al., 2013). Based on several animal studies it is found that DEHP induces liver cancer (Rusyn and Corton, 2012). DEHP causes a hepatic effect in animals, neural damage and malignant liver tumor. The effect of DEHP varies in animal species with repeated exposure required to cause a response.

The administration of certain phthalates to rodents, e.g. DEHP, resulted in ad verse liver effects including increased liver weights, histological changes, increased l iver enzyme levels and, in some cases, tumors. These effects have been associated

with peroxisomal proliferation, cholesterol and fatty acid metabolism process (Ward et al., 1998; McKee et al., 2002 ).

DEHP can affect the differentiation of granulosa cells and the development of follicles in postnatal female mice (Wang et al., 2016). DEHP inhibits testosterone and impaired in spermatogonial sperm cells which leads to reproductive organ damage (Moore et al., 2001; Doyle et al., 2013). Reactive oxygen species (hydroxyl radical, nitric oxide, superoxide anion, and singlet oxygen) often retain antioxidant enzymes and reduced GSH at normal levels, which can induce oxidative stress when the balance of minimal antioxidant defenses and excessive ROS formation is disrupted (Wen et al., 2013; Jenkins and Goldfarb, 1993). Increasing evidence has shown that chemicals can cause oxidative stress and plays a vital role in the toxicity of reproduction. Several studies have shown that in pancreatic β cells, human hepatoma (HepG2) cells, and leydig cells, DEHP may induce oxidative stress (Lehnert and Iyer, 2002; Abarikwu et al., 2015; Tiwari and Vanage, 2017; She et al., 2017).

Hence, there is a probability that DEHP can cause impairment to the reproductive system. Though, no proof displays that DEHP could cause the induction of vitellogenin in male and female silkworms.

# CHAPTER-3

# OBJECTIVES

## 3. Objectives

1. To investigate toxic effect of DEHP on growth, development, economic parameters and life cycle of *B. mori*.

2. To assess the oxidative insult-associated markers and cellular antioxidant capacity in DEHP-exposed *B. mori*.

3. To assess the association between reproductive toxicity of DEHP and induction of vitellogenin gene (Vg) expression in different developmental stages and in different tissues of *B.mori.*

# CHAPTER-4

# MATERIALS AND METHODS

## 4. Materials and methods

### 4.1. Silkworm eggs

Healthy and disease free Bivoltine (SK7 and SK6) strain/breed eggs were procured from Central Silk Board, Silkworm Seed Production Centre, Malavalli-571430 (Figure 3).

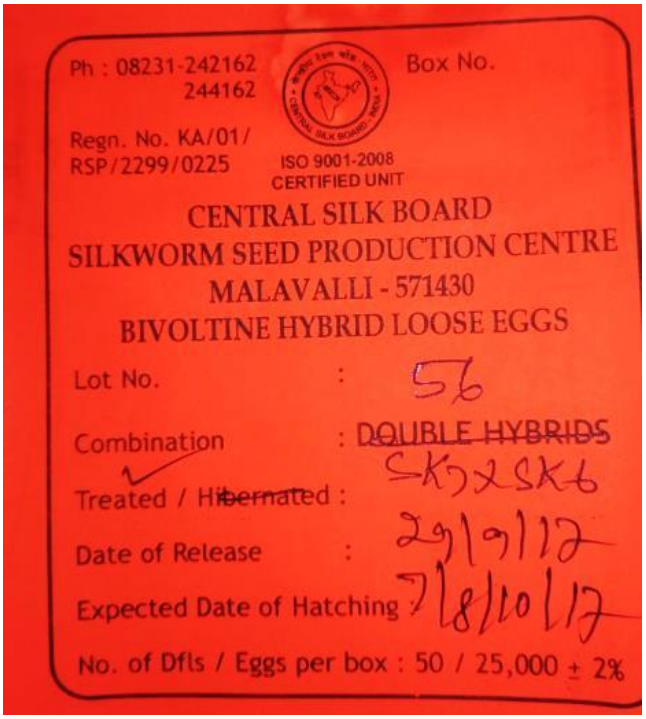


**Figure 3**. Eggs procured from Central Silk Board, Malavalli.

#### 4.1.1. Rearing and maintenance of silkworm eggs

The eggs of silkworm (*Bombyx mori*) were reared and maintained until hatching at standard laboratory conditions (25±1˚C and 75±5% relative humidity) in the darkroom. After hatching, the larvae were maintained at 16h light and 8h dark photoperiod conditions and fed with healthy and disease-free mulberry leaves collected from inside the Mizoram University campus (Jolly, 1987).

### 4.2. Di (2-ethylhexyl) phthalate (DEHP) stock preparation

The 1 mL of the 5% DEHP chemical (Sinopharm Chemical Reagent Co., Shanghai, China, purity > 99% (v/v)) stock solution was prepared by dissolving in

olive oil as a vehicle. (Luhua, Shanghai, China). For the preparation of the stock solution, we dissolved 5 µL of DEHP in 995 µL of olive oil.

### 4.3. Chemicals and reagents requirement

The analytical and molecular grade chemicals and reagents were used in this study. Alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) kits were purchased from Coral Clinical Systems, Tulip Diagnostics (P) Ltd. Mumbai. Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), 5, 5′ -dithiol-bis (2-nitro benzoic acid) (DTNB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethidium bromide (EtBr), hydrogen peroxide (H2O2), ethanol and all other fine chemicals were procured from Merck (Germany).

### 4.4. Trypan blue experiment

As we know silkworm larvae are a very soft and smooth insect model. To test the DEHP chemical, whether going or not inside the silkworm, we used a trypan blue experiment (Matsumoto and Sekimizu, 2019). Trypan blue was injected at midgut of the fourth instar larvae and observed the changes in silkworm after few hours.

### 4.5. Acute toxicity studies of DEHP

According to OECD, 2001; Zhang et al. (2008) the acute toxicity assay was performed using the semi-static procedure in a standard laboratory environment. The acute toxicity of DEHP was conducted using a fourth instar silkworm larvae. DEHP was injected at the midgut of the fourth instar silkworm larvae (Figure 4) using six concentrations (0.1, 0.2, 0.3, 0.4, 0.5, and 1g/kg) of DEHP, based on a gram per kilogram bodyweight of the silkworm larvae (g/kg b.wt.) and control group also maintained at standard laboratory conditions and fed with hundred gram of healthy and disease-free mulberry leaves (Table 1). The mortality rate and LD50 were measured after 24 hours. The LD50 means it could kill 50% of silkworms after 24 hours of exposure at different concentrations. The LD50 was estimated using the probit analysis (Finney, 1971). Based on acute toxicity studies, sub-lethal

doses/concentrations were selected for further studies. The acute toxicity studies were conducted five times (n=30/concentration).

### 4.6. Study design

Based on acute toxicity studies, three sublethal doses were selected for the rest of the studies and with control. Four groups were present in our study. **Group-1:** Control silkworm larvae ( no DEHP treatment), **Group-2:** 0.05 g/kg DEHP treated silkworm larvae, **Group-3:** 0.1 g/kg DEHP treated silkworm larvae, and **Group-4:** 0.2 g/kg DEHP treated silkworm larvae. 200 silkworm larvae were considered for each group (n=200/group) (Table 2).

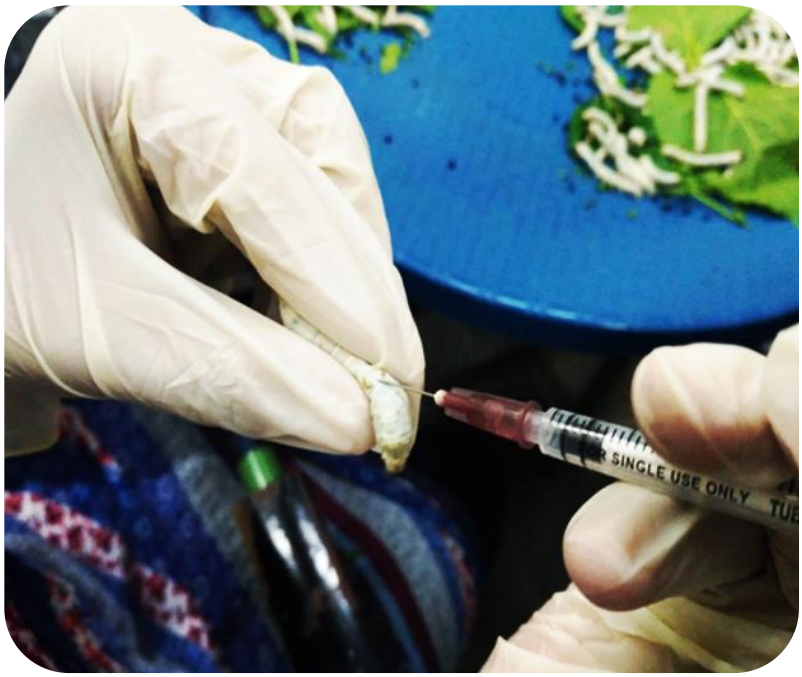


**Figure 4**. DEHP treatment to the midgut of the 4$^{th}$ instar silkworm larva.

**Table 1**. Acute toxicity study of DEHP on fourth instar silkworm larvae.

| Serial number | DEHP dose (g/kg b.wt.) | Number of silkworms |
|---|---|---|
| 1 | Control (0 g/kg) | 30 |
| 2 | 0.1 (g/kg) | 30 |
| 3 | 0.2 (g/kg) | 30 |
| 4 | 0.3 (g/kg) | 30 |
| 5 | 0.4 (g/kg) | 30 |
| 6 | 0.5 (g/kg) | 30 |
| 7 | 1 (g/kg) | 30 |

**Table 2**. Study design of the current study.

| Groups | DEHP treatment dose (g/kg b.wt.) | Number of silkworms |
|---|---|---|
| Group-1 | Control (0 g/kg) | 350 |
| Group-2 | 0.05 (g/kg) | 350 |
| Group-3 | 0.1 (g/kg) | 350 |
| Group-4 | 0.2 (g/kg) | 350 |

### 4.7. Morphological, biological and economic characteristics

#### 4.7.1. Morphological characteristics

Morphology and growth-related parameters like larvae ($4^{th}$ instar, $5^{th}$ instar, and spinning), cocoon and pupae weight, length, and width were studied respectively. We also studied the growth time of the life cycle (average time duration of each stage) after treatment with DEHP. Each group was allocated 50 larvae (n=50/group). The length and width of larvae ($4^{th}$ instar, $5^{th}$ instar, and spinning), cocoon, and pupae were measured using a scale whereas the weight of the larvae ($4^{th}$ instar, $5^{th}$ instar, and spinning stage), cocoon, and pupae were measured using the precise balance machine (Mettler Toledo, Sri Rounak Jewellery Equipment, N.S.C. Bose Road, Chennai, Tamil Nadu, India). This experiment was carried out five times.

#### 4.7.2. Behavioral and abnormal studies

Behavior (feeding and movement) and abnormal studies (malformed larvae, cocoon, pupae, and adults) were observed in DEHP treated groups and also in the control group. 50 larvae were kept per group (n=50/group). Further, the number of

abnormality larvae, pupae, cocoon, and adults were counted by vision (Kuribayashi, 1988). Behavioral and abnormal studies were carried out five times.

### 4.7.3. Biological and economic characteristics

Economical and biological parameters of silkworm such as Shell ratio (%), Filament length, Floss-Shell ratio, Denier (D), Cocooning yield (%), Growth index, Cocoon Shell Ratio (CSR%), Effective rearing rate (ERR) by number and weight, Moth emergence (%), Filament length (m), Ingesta, Digesta, Reference ratio (RR), Approximate digestability (A.D %), Consumption index (CI), Efficiency of conversion of ingested food (ECI %), and Efficiency of conversion of digested food (ECD %) were studied (Rahmathulla et al., 2007; Sabhat et al., 2011; Rajitha and Savithri, 2015). These parameters were calculated as follows. Each group contained 50 silkworm larvae (n=50/group) and repeated five times.

1. Pupation rate (%) = No. of healthy or good cocoons/kg/actual No. of cocoons per Kg×100
2. Shell ratio (%) = Cocoon shell weight/cocoon weight×100
3. Filament length = {Length of raw silk(m) x 1.125 (circumference)}/No of reeling
4. Floss- Shell ratio = {[Weight of the floss (25 cocoons)]/ [Weight of the shell (25 cocoons)]} x 100
5. Denier (D) = {Filament Weight in grams/Filament length in meters} x 9000
6. Cocooning percentage = {Total no. of cocoons/Total no of matured larvae} x 100
7. Growth index = Final weight of the larvae (g) - Initial weight of the larvae (g)/Initial weight of the larvae
8. Cocoon Shell Ratio (CSR) (%) = Shell Weight(g)/cocoon weight(g)×100
9. Moth emergence (%) = Number of moths emerged/number of cocoons kept for moth emergence×100
10. Efficiency of conversion of ingested food (ECI %)

10.1. ECI to larva (%) = Dry weight gained by larvae/dry weight of food ingested×100

10.2. ECI to cocoon (%) = Cocoon weight/ingesta×100

10.3. ECI to cocoon shell (%) = Shell weight/ingesta×100

11. Efficiency of conversion of digested food (ECD %)

11.1. ECD to larva (%) = Dry weight gained by larvae/dry weight of food digested×100

11.2. ECD to cocoon (%) = Cocoon weight/digesta×100

11.3. ECD to cocoon shell (%) = Shell weight/digesta×100

12. Effective rearing rate (ERR)

12.1. ERR by Number: Total no. of good cocoons harvested X 10,000

12.2. ERR by weight: Weight of good cocoons harvested in kg X 10,000

13. Filament length (m)= Number of rotations X Circumference of the wheel($\chi$)

14. Ingesta =Weight of leaf offered – the weight of leaf un-utilized.

15. Digesta = Ingesta - weight of excreta

16. Leaf: egg recovery ratio = Quantity of leaves consumed/Actual weight of eggs recovered

17. Reference ratio (RR) = Dry weight of food ingested/Dry weight of excreta

18. Approximate digestability (A.D) (%) = Digesta/Ingesta X 100

19. Consumption index (CI)=Fresh weight of food eaten (g)/Mean fresh wt. of larvae during x Larval duration of feeding

20. I/g. cocoon = ingesta/cocoon weight×100

21. D/g. cocoon = digesta/cocoon weight×100

22. I/g. shell = ingesta/shell weight×100

23. D/g. shell = digesta/shell weight×100.

### 4.8. Oxidative stress markers, antioxidants, and biochemical parameters

#### 4.8.1. Oxidative stress markers

Oxidative stress markers like hydrogen peroxide ($H_2O_2$), lipid peroxidation (LPX) and protein carbonyl content (PC) were studied in the hemolymph of the pupae. For the estimation of oxidative stress markers, 30 larvae were used per group (n=30/group) and repeated five times.

#### 4.8.1.1. Hydrogen peroxide ($H_2O_2$) estimation

Hydrogen peroxide ($H_2O_2$) concentration was measured spectroscopically at 610 nm wavelength in the hemolymph of the pupae using horseradish peroxidase (HRP) and $H_2O_2$ as standard. The basic principle of this method is the conversion of Hydrogen peroxide ($H_2O_2$) into water ($H_2O$) and oxygen molecules ($O_2$) in the presence of a catalyst (Pick and Keisari, 1981).

$$2\ H_2O_2 \xrightarrow{\text{Catalyst}} 2H_2O + O_2$$

#### 4.8.1.2. Lipid peroxidation (LPX) assay

This test is a calorimetric test that takes advantage of the ability of hydroperoxide to generate free radicals after reacting with transitional metals when a buffered chromogenic substance is added; a colored complex appeared. This colored complex was measured spectrophotometrically. Lipid peroxidation levels in the testis were measured using thiobarbituric acid reactive substances (TBARS). The testis was then homogenized in ice-cold 1X PBS (10%) and the concentration of TBARS was expressed as nmol of MDA per mg protein. The absorbance was read at 540 nm (Ohkawa et al., 1979). Homogenized tissue in 1X PBS (10%) if the weight of tissue is 50 mg, 500µL of PBS was added. (wt of tissue × 10). 500µL of homogenate tissue and PBS amount is taken µL of 10% TCA in one Eppendorf tube and centrifuged. The supernatant was taken out carefully. The amount of supernatant taken should be known. 0.8% TBA was added in 1 (protein) : 2 (TBA) ratios. If more pink is observed, it contains more malondialdehyde. 1 mL of supernatant and 2 mL of TBA (1:2) was taken. Boiled for 45 min and were observed for the formation of a colored complex. The slight pink color is expected to come. Absorbance at 540 nm was taken. The concentration of MDA (nmol MDA/mg of protein) was estimated using the molecular extinction coefficient ($1.56 \times 10^5$ M/cm).

#### 4.8.1.3. Protein carbonyl content (PC)

Protein carbonyl content was estimated in the hemolymph by detecting carbonyl groups to determine the content of oxidatively modified proteins (Levine et

al., 1990). The main principle of this assay was the formation of derivatives of hydrazine after the reaction between carbonyl groups and 2,4-dinitrophenyl hydrazine (DNPH). The PC content (nmol PC/mg protein) was measured spectroscopically at 366 nm wavelength and using the extinction coefficient (22,000M/cm).

#### 4.8.2. Enzymatic and non-enzymatic antioxidants

Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and non-enzymatic antioxidant reduced glutathione (GSH) were analyzed in the hemolymph of pupae. Every group contained 30 larvae (n=30/group). These assays were performed 5 times.

##### 4.8.2.1. Enzymatic antioxidants

###### 4.8.2.1.1. Superoxide dismutase (SOD)

SOD is an enzyme that catalyzes the dismutation of two superoxide anion into hydrogen peroxide and molecular oxygen. SOD is one of the most important enzymes in the front line of defense against oxidative stress. The reagents were required for the estimation of SOD as given below.

Phenazine methosulfate (PMS): 0.06 mg dissolved in 1 mL of distilled water, Nitrobluetetrzolium (NBT): 2.5 mg dissolved in 1 mL of distilled water, NADH: 0.6 mg dissolved in 1 mL of distilled water, Acetic acid, and n-Butanol. For each reaction, PMS (50µL), NBT (15µL), NADH (100µL) and sample (50µL) were required and followed by incubated for 90 sec at 30 $^{o}$C and the reaction was stopped by adding acetic acid (500µL). Subsequently, n-Butanol (2mL) was added to the reaction mixer. The absorbance was taken at 560 nm (Das et al., 2000). The calculation was done using the formula as follows:

% inhibition = {Blank-Test/Blank} x 100; 50% inhibition = 1 unit; 1% inhibition = 1/50

SOD unit = 1/50 × % inhibition.

#### 4.8.2.1.2. Catalase activity (CAT)

The assay was performed as described by Aebi et al. (1974). Catalase catalyzes the dismutation of $H_2O_2$ and thus competes with GSHPs for the common substrate. It is considered to be the primary scavenger of intracellular $H_2O_2$. In the UV range, $H_2O_2$ absorbs maximally at 240nm. Catalase rapidly breaks down $H_2O_2$ leading to a decrease in absorbance. A difference in the absorbance at 240 nm per unit time is measured of Catalase activity.

**Reagents**

1.50 nM phosphate buffers, pH 7 was made by mixing A and B in a 1:1.5 v/v ratio.
A) 6.8 g----$KH_2PO_4$

B) 8.9 g----$Na_2HPO_4$

2. 30 mM $H_2O_2$ made by diluting 340µL 30% $H_20_2$ to 100 mL with phosphate buffer pH-7.0.

3. The sample used: 10% (w/v) homogenate of hemolymph.

**Procedure**

| **Pipette successively into the cuvettes** | **Blank** | **Sample** | **Concentration in assay mixture 50 mmol/L** |
|---|---|---|---|
| Phosphate buffer | 1 mL | - | |
| Sample | 2 mL | 2 mL | |
| $H_2O_2$ | - | 1mL | 10 mmol/L |
| The reaction was started by adding $H_2O_2$. The absorbance was taken at 10-sec intervals up to 30 sec. | | | |

The calculation was done using the formula $0.23 \times \log A_1/A_2$. Where $A_1$ was the blank at 0 sec and $A_2$ is the test at 15 sec.

#### 4.8.2.2. Non-enzymatic antioxidant

##### 4.8.2.2.1. Reduced glutathione (GSH)

Glutathione is measured by its reaction with DTNB 5, 5 dithiols 2-nitro benzoic acid (Ellman's reaction) to give a compound that absorbs light at 412 nm. 10.2 M $Na_2HPO_4$: 28.392g was weighed and dissolved in 1L distilled water. 0.425mg in 15 mL. 2.10 mM DTNB: 39.63mg DTNB was dissolved in 10 mL of 0.2M $Na_2HPO_4$. Dithionitrobenzene 4mg in 1 mL. Blank was prepared using $Na_2HPO_4$ (900µL), DTNB (20µL), and Distilled water (80 µL). The test sample was prepared as same as the blank except sample (80 µL) instead of distilled water in a reaction mixture. Absorbance was measured at 412 nm after 5 min incubation (Ellman, 1959).

### 4.8.3. Biochemical studies

Alanine aminotransferase (ALT/SGPT), aspartate aminotransferase (AST/SGOT), and Alkaline phosphatase (ALP) were estimated in the hemolymph of pupae (n=30). Biochemical studies were performed 5 times.

#### 4.8.3.1. Alanine aminotransferase (ALT/SGPT)

SGPT converts L-Alanine and α-Ketoglutarate to Pyruvate and Glutamate. The Pyruvate formed reacts with 2,4Dinitrophenyl hydrazine to produce a hydrazone derivative, which is an alkaline medium produces a brown colored complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGPT (ALT) is to read off this calibration curve (Reitman and Frankel, 1957).

| Addition sequence | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Enzyme Activity (U/L) | 0 | 28 | 57 | 97 | 150 |
| Substrate Reagent (L1) | 0.50 | 0.45 | 0.40 | 0.35 | 0.30 |
| Pyruvate Standard (S) | - | 0.05 | 0.10 | 0.15 | 0.20 |
| Distilled Water | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| DNPH Reagent (L2) | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Mixed well and allowed to stand at 25 $^0$C for 20 minutes. | | | | | |
| Working NaOH Reagent (L3) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |

Mixed well and allowed to stand at 25 $^0$C for 10 min. The absorbance of the tubes 2 – 5 against tube 1 (Blank) was measured at 505nm. Plot a graph of the absorbances of tubes 2 - 5 on the 'Y' axis versus the corresponding Enzyme activity on the 'X' axis.

#### 4.8.3.2. Aspartate aminotransferase (AST/SGOT)

SGOT converts L-Aspartate and α Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which is an alkaline medium produces a brown colored complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGOT (AST) is to read off this calibration curve (Reitman and Frankel, 1957).

| Addition sequence | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Enzyme Activity (U/L) | 0 | 24 | 61 | 114 | 190 |
| Substrate Reagent (L1) | 0.50 | 0.45 | 0.40 | 0.35 | 0.30 |
| Pyruvate Standard (S) | - | 0.05 | 0.10 | 0.15 | 0.20 |
| Distilled Water | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| DNPH Reagent (L 2) | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Mixed well and allowed to stand at 25 $^0$C for 20 minutes. | | | | | |
| Working NaOH Reagent (L3) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |

Mixed well and allowed to stand at 25 $^0$C for 10 min. The absorbance of the tubes 2-5 against tube 1 (Blank) was measured at 505nm. A graph of the absorbances of tubes 2-5 on the 'Y-axis versus the corresponding Enzyme activity on the 'X' axis was plotted.

#### 4.8.3.3. Alkaline phosphatase (ALP)

ALP at an alkaline pH hydrolyzes disodium Phenyl phosphate to form phenol. The phenol formed reacts with 4-Aminoantipyrine in the presence of Potassium Ferri-cyanide, as an oxidizing agent to form a red-colored complex. The intensity of the color formed is directly proportional to the activity of ALP present in the sample (Kind and King, 1954).

| **Addition Sequence** | **B (mL)** | **S (mL)** | **C (mL)** | **T (mL)** |
|---|---|---|---|---|
| Distilled water | 525µL | 500 µL | 500 µL | 500 µL |
| Buffer Reagent (L1) | 500µL | 500µL | 500 µL | 500 µL |
| Substrate Reagent (L2) | 50µL | 50 µL | 50 µL | 50 µL |
| Mixed well and allowed to stand at 37$^0$C for 3 minutes | | | | |
| Sample | - | - | 25 µL | 25 µL |
| Phenol Standard (S) | - | 25 µL | - | |
| Mixed well and allowed to stand at 37$^0$C for 15 minutes and add. | | | | |
| Colour Reagent (L3) | 500µL | 500µL | 500µL | 500µL |
| Sample | - | - | 0.025 | - |

It was mixed well after each addition. The absorbance of the Blank (Abs. B), Control (Abs. C) and Test (Abs. T) was measured against distilled water at 510nm. Total ALP activity (U/L) = Absorbance of T- Absorbance C/Absorbance of S – Absorbance of B×10.

### 4.9. Histopathology

Histology has been an important tool for assessing any kind of tissue damages microscopically. We observed the foregut, midgut and hindgut portions of the silkworm after treatment with DEHP. Each treatment group's gut portions were fixed in Bouin's fixative solution for 24 h. After fixation, dehydration was done

using 50%, 70% &, 90% and absolute ethanol for 1h. Later, tissues were subjected to xylene treatment and fixed in the wax. 5 μm sections of studied tissues were prepared using a microtome (Leica, model RM2125 RTS). The thin sections were placed on albumin coated glass slides for hematoxylin and eosin staining studies followed by hydration using 100%, 80%, 70%, and 50% of ethanol for a minute. Finally, slides were cleaned in distilled water to remove excess alcohol and any other debris material followed by staining with hematoxylin for 8 min. Later, slides were dipped in eosin stain (counterstain) for 16 min. After completion of staining, the slides were washed properly with xylene to remove excess stain fro the slides and mounted using DPX. Coverslips were used to cover the slides gently to evade air bubbles (Gurr, 1959; Bancroft and Gamble, 2008). The sections were observed for any histological changes in the light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany). Histological sections were performed five times and each group contains 10 silkworm larvae (n=10/group).

### 4.10. Chronic reproduction assay

Reproductive toxicity of DEHP treated groups was evaluated using fecundity and hatching rate parameters. For this, we mated 10 pairs of adult moths including control female and control male (1 pair), control female with treated males (3 pairs), control male with treated female (3 pairs), and treated male with treated female (3 pairs). Fecundity means the mean total number of eggs laid per female and hatching rate means the percentage of hatched larvae from eggs. Male and female were easily identified at the larval stage using length, abdominal and cresent mark characteristics. Generally, a crescent-shaped spot bordered with black line marks were more in male compared to female, the body size of a female is larger than the male, and the female has longer abdomen than the male (Figure 5).

For hatching rate, treated female and male adults were kept for pairing for 6 h in a plastic container and later mated females were separated and kept at dark conditions using standard laboratory protocols (Yuan et al., 2013). For fecundity

and hatching rate, 80 larvae were kept for each group (n=80/group) and repeated 5 times.


**Figure 5**. Sex determination of silkworm at the larval stage.

**4.11. RNA isolation, cDNA synthesis and Real-time quantitative PCR (qRT-PCR)**

Real-time quantitative PCR (qRT-PCR) of vitellogenin gene (Vg) expression was done from different life stages (egg, spinning larvae, pupae, and

adult) and different tissues (silk gland, foregut, midgut, and hindgut) of silkworm by using housekeeping gene actin-3 (A3) as a control gene. Forward and reverse primer sequences and their product size of vitellogenin (Vg), ecdysone receptor (EcR), and actin-3 (A3) genes were shown in Table 3.

**Table 3.** Genes with their primer sequences and their product sizes in basepair (bp) used in qRT-PCR.

| Gene name | Primers | Product size (bp) |
|---|---|---|
| Vitellogenin (Vg) gene | F-5'-GGGTCGCTTATCACTTC-3'<br>R-5'-GGTCCCTCTTTGCCTGTT-3' | 125bp |
| Ecdysone receptor gene (EcR) | F-5'-CCACGATGCCTTTACCAATG-3'<br>R 5'-GTCGAGGTGCAGGACCTTTC-3' | 179bp |
| Actin3 gene | F-5'-CTGCGTCTGGACTTGGC-3'<br>R-5' -CGAGGGAGCTGCTGGAT-3' | 184bp |

### 4.11.1. RNA isolation

RNA was isolated using 500 µL RNA extraction buffer (1X) from 30mg of tissue and made tissue homogenization using bead beater (BeadBug™ Microtube Homogenizer, 115V, Thomas scientific, USA). Subsequently, pipetting 250 µL of chloroform: isoamyl alcohol (4:1) mixer into the sample and centrifuged at 4 $^{0}$C, 8000 rpm for 10 min. The supernatant was transferred to the fresh sterilized centrifugal tubes and added 400 µL of Isopropanol. The samples were centrifuged at 4 $^{0}$C, 8000 rpm for 2 min followed by adding of 400 µL RNA wash buffer to the sampling tubes and again centrifuged at 8000 rpm for 1 min. The supernatant was collected into a fresh tube and added elution buffer (30 µL). After RNA isolation, the quality and quantity were measured using the agarose gel electrophoresis and UV spectrophotometer at 260nm wavelength (Chromous biotech).

### 4.11.2. cDNA synthesis

cDNA was synthesized using a commercial kit (RevertAid First Strand cDNA Synthesis Kit, Catalog number: K1621, Thermo Scientific, USA). Briefly, for cDNA synthesis master mix was prepared as follows.

2 µg of RNA, 150 ng of Random Hexamer, 3 µL of reverse transcriptase buffer (10X), 0.25 µl of RNase, 2 µl of dNTP mix, 5 µl of DTT, 2 µl of reverse transcriptase enzyme. The final volume (50 µL/reaction) was made using nuclease-free water.

### 4.11.3. qRT PCR

cDNA's of various tissues and different life stages of silkworm were used as templates and quantified using the Bio-rad q RT PCR system (CFX, Bio-rad, USA). Actin-3 was kept as a reference gene. Gene expression studies were done using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

qRT-PCR reaction mix (50 µL) prepared using 2 µL cDNA, 2 µL forward and reverse primer each, 25 µL SYBR Green master mix (2X) (SsoAdvanced Universal SYBR Green supermix, Catalog # 172-5270, Bio-rad, USA), and 20 µL of nuclease-free water. The qRT-PCR condition was set at reaction 94˚C for 5 m and followed by 40 cycles of 94˚C for 5 s, 52/54˚C for 10 s, and 72˚C for 10 s. The samples were loaded with qPCR plates. Each gene was amplified five times in triplicate (n=20/group).

## 4.12. Statistical analysis

Probit analysis was done using SPSS version 18.0. LD50 assay was performed five times (n=30/concentration). Morphological, biological and economic parameters were assayed five times (n=50/group) respectively. Oxidative stress markers, antioxidants, and biochemical parameters were performed five times (n=30/group) respectively. Histology analysis (n=10/group), chronic reproductive assay (n=80/group), and real-time quantitative PCR (n=20/group) were carried out

five times respectively. ANOVA was performed and Data were expressed as mean ± standard deviation using SPSS version 18.0. The p-value < 0.05 was showed significant variation in studied groups. Posthoc Tukey's test was done to compare the means of the respective groups.

# CHAPTER-5

# RESULTS

## 5. Results

### 5.1. Trypan blue experiment

After a few hours of trypan blue injection to the silkworm midgut, the entire silkworm body became blue in color. Based on this experiment, we confirmed that our chemical interest DEHP should be entered into the silkworm body (Figure 6).

**Figure 6**. Trypan blue injection to the 4$^{th}$ instar silkworm midgut.

### 5.2. Acute toxicity studies

Based on the acute toxicity studies, we analyzed that the mortality rate (%) was increased dose-dependently (Table 4). The percentage of the mortality rate was converted into probit mortality and doses were changed into $\log_{10}$ doses respectively. The graph was made between $\log_{10}$ doses and probit mortality. The LD50 was computed using probit analysis statistical method at various doses of DEHP after 24h exposure time. The LD50 of DEHP was 0.38 g/kg b.wt. of the silkworm. It was equal to 1.68 $\log_{10}$ doses (Figure 7). Based on LD50 studies of 24h, we selected three sub-lethal doses for further studies such as 0.05 g/kg, 0.1 g/kg, and 0.2 g/kg b.wt. of the silkworm. The slope (3.23 ± 0.07), was observed at, 95% confidential limit (1.25 ~ 4.95), and the intercept (0.06) was observed based on the regression equation (Table 5). The $R^2$-value was also observed at 0.95. Acute toxicity study was conducted five times (n=30) and showed great significant at $p < 0.009$ and F-value was 21.77.

**Table 4**. Probit analysis of percentage mortality of silkworm larvae opposite to DEHP (g/kg b.wt.)

| DEHP Dose (g/kg) b. wt. | Log dose | No. of larvae exposed | No. of larvae dead | % mortality | Probit mortality |
|---|---|---|---|---|---|
| 0.1 | 1 | 30 | 3 | 10 | 3.72 |
| 0.2 | 1.20 | 30 | 5 | 16 | 4.01 |
| 0.3 | 1.36 | 30 | 7 | 23 | 4.26 |
| 0.4 | 1.72 | 30 | 16 | 53 | 5.08 |
| 0.5 | 1.86 | 30 | 22 | 73 | 5.61 |
| 1 | 2 | 30 | 30 | 100 | 7.33 |

Acute toxicity experiment was done five times (n=30)

**Table 5**. Acute toxicity studies of silkworm against DEHP at 24h exposure.

| **Slope ± SE** | **95% CL** | **Chi square** | **p-value** | **F-value** |
|---|---|---|---|---|
| 3.23 ± 0.07 | 1.25 ~ 4.95 | 0.482 | 0.009 | 21.77 |

Acute toxicity assay was performed five times (n=30).

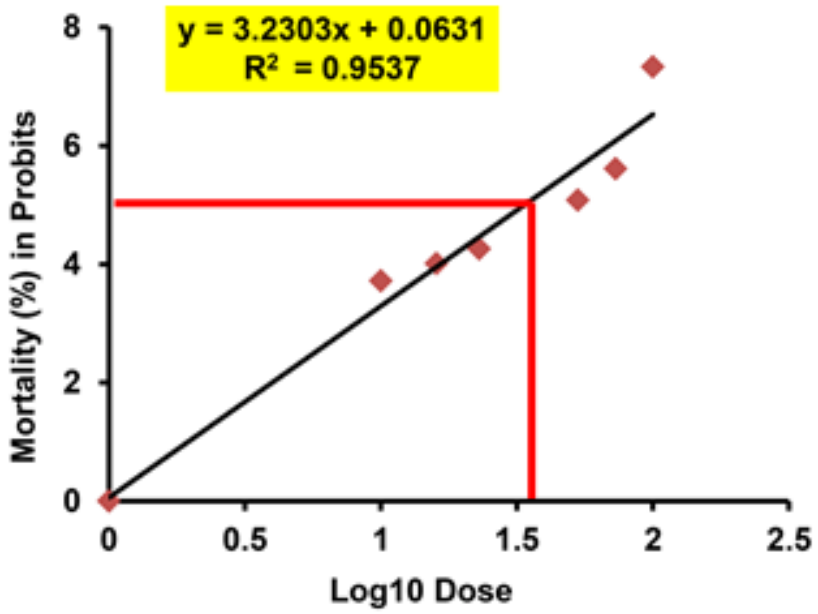


**Figure 7**. Probit mortality analysis graph of silkworm treated with DEHP (g/kg b.wt.)

### 5.3. Morphological, biological and economic characteristics

### 5.3.1. Morphological studies

Morphology and growth-related parameters like larvae (4th instar, 5th instar, and spinning), cocoon and pupae weight, length, and width were significantly decreased in DEHP treated groups ($p < 0.0001$).

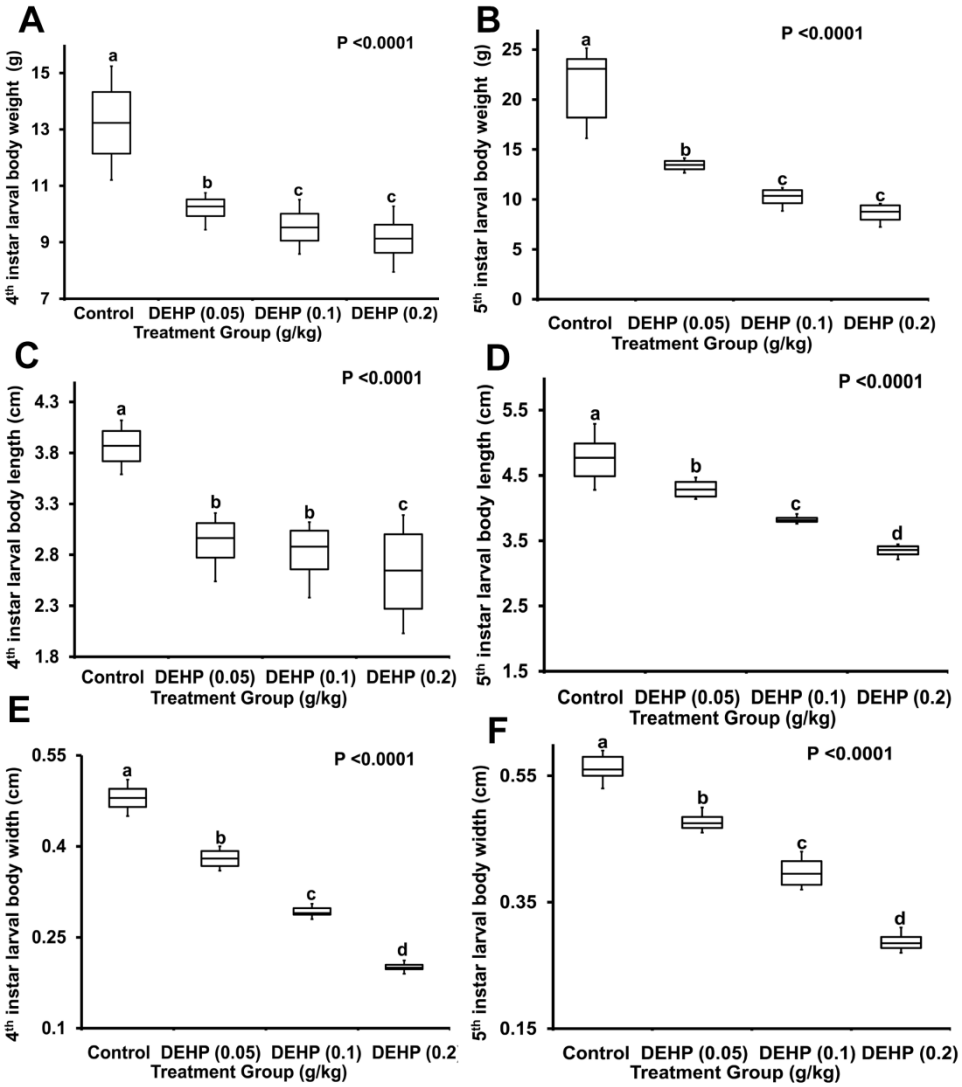


**Figure 8**. Morphological and growth parameters of 4th and 5th instar silkworm larvae respectively. A, B; weight (g) of 4th and 5th instar silkworm larvae, C, D; length (cm) of 4th and 5th instar silkworm larvae, and E, F; width (cm) of 4th and 5th instar silkworm larvae respectively. Bar with different alphabets showed significance ($p <$

0.0001) between treated groups. Data were expressed as medial ± standard deviation (S.D.). This was performed five times (n=50/group).

The weight (Figure 8A and 8B) length (Figure 8C and 8D), and width (Figure 8E and 8F) of $4^{th}$ instar and $5^{th}$ instar larvae were reduced significantly in DEHP treated groups respectively compared to the control group ($p < 0.0001$). The weight (8.62 ± 0.5 and 7.96 ± 0.8), length (2.27 ± 0.35 and 3.29 ± 0.09), and width (0.19 ± 0.002 and 0.27 ± 0.007) of $4^{th}$ and $5^{th}$ instar larvae were observed in 0.2 g/kg DEHP treated silkworms whereas in control group the weight (12.14 ± 1.09 and 18.2 ± 4.88), length (3.71 ± 0.15 and 4.49 ± 0.28), and width (0.46 ± 0.01 and 0.55 ± 0.01) of $4^{th}$ and $5^{th}$ instar larvae were observed (Figure 8). Larvae parameters were decreased while increasing the dose of DEHP. The experiment was carried out 5 times (n=50/group).

The weight of spinning larvae, cocoon, and pupae was significantly ($p < 0.0001$) reduced in DEHP treated groups whereas time duration of the life cycle like $5^{th}$ instar to cocoon, cocoon to adult, and adult life span was decreased significantly ($p < 0.0001$) in DEHP treated groups compared with control (Figure 9). The weight of spinning larvae was 1.09 ± 0.08 in a higher dose (0.2 g/kg) of DEHP whereas in control 2.98 ± 0.37 was observed in Figure 9A. The weight of the cocoon (Figure 9C) and pupae (Figure 9E) were also showed the same significant trend in studied groups. The time duration of $5^{th}$ instar to a cocoon (Figure 9B), cocoon to adult (Figure 9D), and life span of adult (Figure 9F) were reduced in DEHP treated groups. The reduction of time duration in the life cycle of silkworm showed greater significance ($p < 0.0001$) when compared to the control group. The time duration of $5^{th}$ instar to a cacoon, cocoon to adult, and adult lifespan in control (7 ± 0.12, 18 ± 1.25, and 7 ± 0.05) was observed in control group whereas in 0.2 g/kg DEHP treated group showed significant reduction in time duration of $5^{th}$ instar to a cacoon, cocoon to adult, and adult lifespan (4 ± 0.01, 6 ± 0.06, and 2 ± 0.02) respectively (Figure 9). The length of spinning larvae (cm), cocoon length (cm), cocoon width (cm), weight of the cocoon filament (g), size of the cocoon filament (d), length of the cocoon filament (m), weight of the floss (g), weight of the shell with floss (g), weight of the shell without floss (g), pupae length (cm), and pupae width (cm) were

significantly ($p < 0.0001$) reduced in DEHP treated groups while compared with control group (Table 6). The above showed parameters were significantly reduced while increasing the dose of DEHP. The study was performed five times (n=50/group).

### 5.3.2. Behavioral and abnormal studies

Based on behavioral studies, we were found that feeding behavior was significantly reduced in DEHP treated groups while compared with the control group whereas the abnormalities like the blackish spot on the skin, bending upward, agony type movement, oozing out hemolymph through the anus, malformed cocoons, pupae-adult intermediates, lack of adult wings, and even death of adults and pupae (Figure 10) were increased dose-dependently in DEHP treated groups. The food intake was reduced significantly ($p < 0.0001$) from 120 g (control) to 41.32 g (0.2 g/kg) in DEHP treated group. The data were not shown in the thesis. The abnormalities of larvae, cocoon, and pupae were significantly ($p < 0.0001$) increased in a dose-dependent manner. The data was not shown in the thesis. The abnormal experiment was done five times (n=50/group).

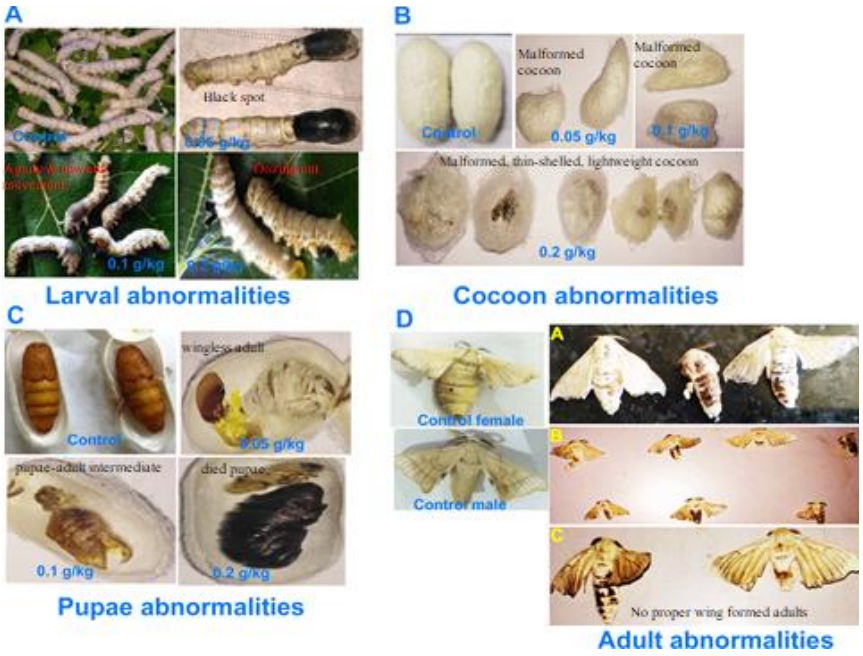

**Figure 10**. List of larvae, cocoon, pupae, and adult abnormalities. A, larval abnormalities (blackish spot on the skin, bending upward, agony type movement, oozing out hemolymph through the anus); B, Cocoon abnormalities (malformed, thin-shelled, and lightweight cocoons); C, Pupae abnormalities (pupae-adult intermediate, died pupae, no wing formed adults inside the cocoon); and D, adult abnormalities (no proper wing formed adults) respectively.

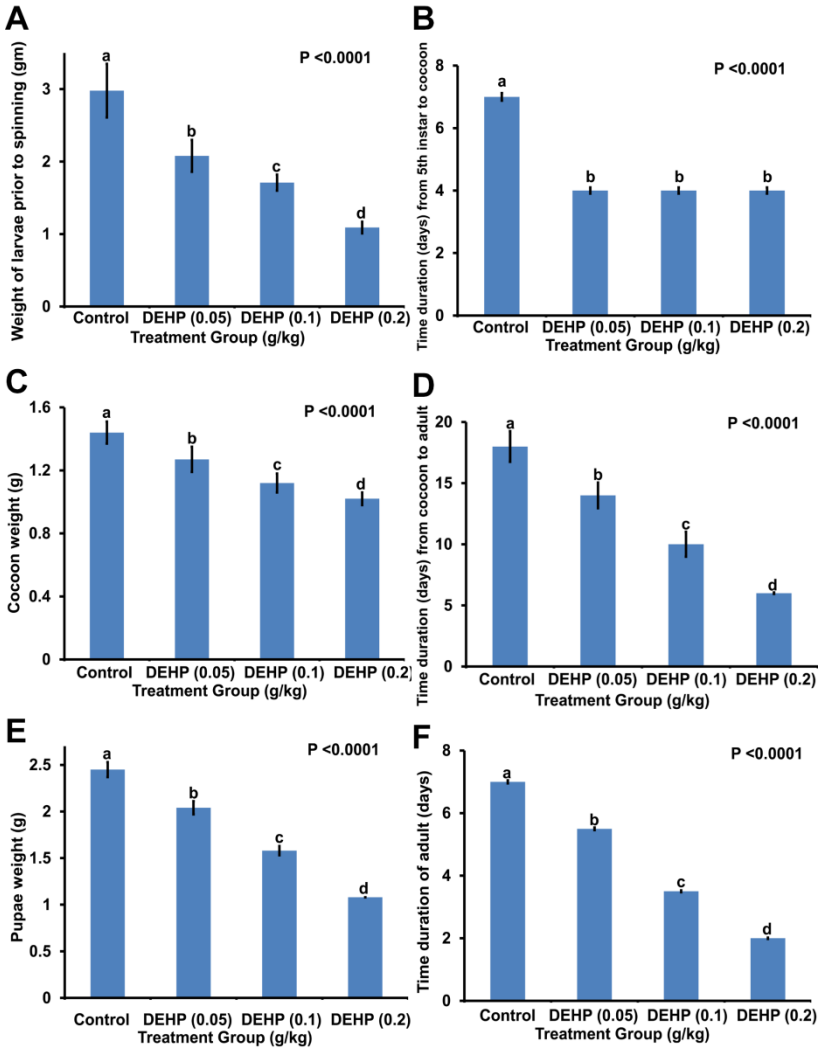


**Figure 9**. Growth parameters of spinning larvae, cocoon, pupae and adults of silkworm worm moth. A, C, and E; the weight of spinning larvae (g), cocoon (g), and adults (g) after DEHP treatment with different doses along with control. B, D, and F; time duration of the life cycle in days (B, 5$^{th}$ instar to cocoon; D, Cocoon to adult; and F, the life span of the adult) of the silkworm moth after DEHP treatment

compared with control. Different alphabet letters were showed significance ($p < 0.0001$) in studied groups. One way ANOVA was performed among the studied groups. Data were expressed as mean ± S.D. The experiment was performed five times (n=50/group).

### 5.3.3. Biological and economic characteristics

Economical and biological parameters of silkworm such as Shell ratio (%), Filament length, Floss-Shell ratio, Denier (D), Cocooning yield (%), Growth index, Cocoon Shell Ratio (CSR%), Effective rearing rate (ERR) by number and weight, Moth emergence (%), Filament length (m), Ingesta, Digesta, Reference ratio (RR), Approximate digestability (A.D %), Consumption index (CI), Efficiency of conversion of ingested food (ECI %), and Efficiency of conversion of digested food (ECD %) were reduced significantly ($p < 0.0001$) in DEHP treated groups compared to control group (Table 7). This experiment was conducted five times (n=50/group).

## 5.4. Oxidative stress markers, antioxidants, and biochemical parameters

### 5.4.1. Oxidative stress markers

Oxidative stress markers like hydrogen peroxide ($H_2O_2$), lipid peroxidation (LPX), and protein carbonyl content (PC) were drastically changed in DEHP treated groups (Figure 11).

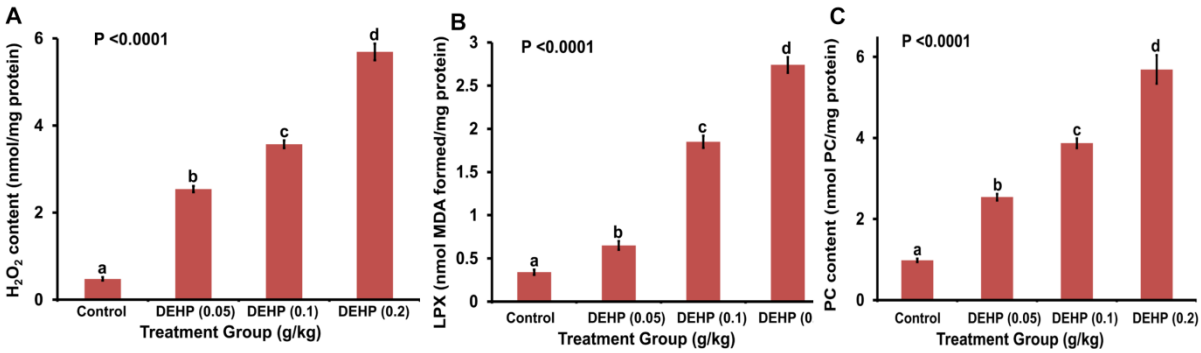


**Figure 11.** Oxidative stress markers in DEHP treated groups and also in the control group. A, hydrogen peroxide level ($H_2O_2$, nmol/mg protein); B, lipid peroxidation (LPX, nmol MDA/mg protein); and C, protein carbonyl content (PC, nmol PC/mg

protein). Data were expressed as mean ± S.D. Bar with different letters was showed significance between the groups at $p < 0.0001$. All oxidative marker assays were performed five times (n=30/group).

Hydrogen peroxide level ($H_2O_2$, nmol/mg protein) (Figure 11A), lipid peroxidation (LPX, nmol MDA/mg protein) (Figure 11B), and protein carbonyl content (PC, nmol PC/mg protein) (Figure 11C) were increased in DEHP treated groups as compared to the control. $H_2O_2$ levels in the control group were 0.48 ± 0.04 nmol/mg protein whereas in 0.2 g/kg DEHP treated group was showed a significant increase ($p < 0.0001$) in $H_2O_2$ levels were about 5.69 ± 0.19 nmol/mg protein. LPX and PC content levels were also shown as same as $H_2O_2$ levels. The levels of LPX and PC were 0.34 ± 0.03 nmol MDA/mg protein and 0.98 ± 0.04 nmol PC/mg protein in the control group respectively whereas in DEHP treated group (0.2 g/kg) 2.74 ± 0.09 nmol MDA/mg protein and 5.69 ± 0.35 nmol PC/mg protein was shown significant increase ($p < 0.0001$) in LPX and PC content levels (Figure 11). All oxidative marker assays were performed five times (n=30/group).

### 5.4.2. Enzymatic and non-enzymatic antioxidants

Enzymatic antioxidants (SOD and CAT)) and non-enzymatic antioxidants (GSH) were significantly decreased in DEHP treated groups as compared to the control group ($p < 0.0001$) (Figure 12). The superoxide (SOD, U/mg protein) levels were reduced (3.85 ± 0.12 U/mg protein) significantly ($p < 0.0001$) in a higher dose of DEHP (0.2 g/kg) whereas in control group the levels of SOD was (10.81 ± 0.65 U/mg protein) observed (Figure 12A). The catalase (CAT, pkat/mg protein) levels were also decreased significantly ($p < 0.0001$) in DEHP treated groups (Figure 12B). The levels of catalase were significantly decreased with increasing DEHP dose. The reduced glutathione (GSH, mMol/L) levels were significantly ($p < 0.0001$) decreased in 0.2 g/kg DEHP treated group (1.24 ± 0.01 mMol/L) as compared to the control group (5.28 ± 0.06 mMol/L) (Figure 12C). Enzymatic and non-enzymatic antioxidant assays were performed five times (n=30/group).

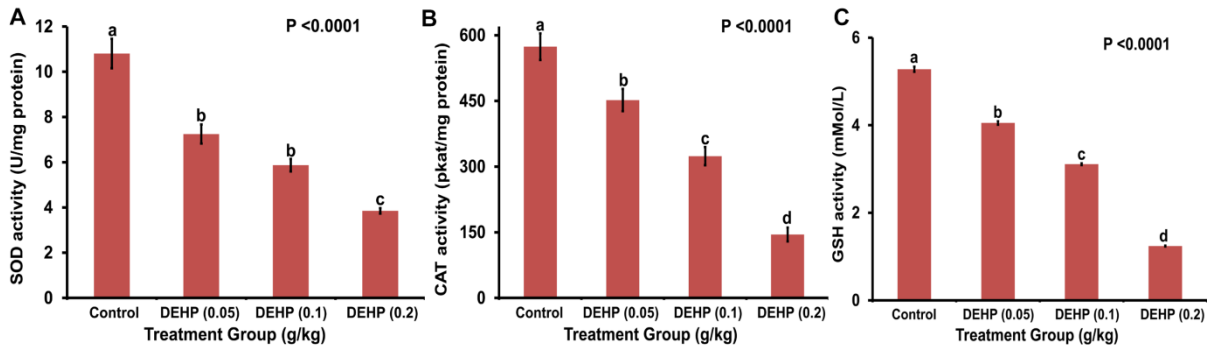


**Figure 12**. Enzymatic (SOD and CAT) and non-enzymatic (GSH) antioxidant enzymes level in DEHP treated groups and in the control group. A, superoxide dismutase (SOD, U/mg protein); B, catalase (CAT, pkat/mg protein); and C, reduced glutathione (GSH, mMol/L). Data were shown in mean ± S.D. Bar with different alphabetical letters showed the significance between studied groups. The p-value was < 0.0001. Enzymatic and non-enzymatic antioxidant assays were performed five times (n=30/group).

### 5.4.3. Studies on biochemical parameters (ALT, AST, and ALP)

Alanine aminotransferase (ALT, U/mL), aspartate aminotransferase (AST, U/mL), and alkaline phosphatase (ALP, U/mL) were significantly increased in DEHP treated groups compared to the control group (Figure 13). The levels of ALT (0.37 ± 0.02) (Figure 13A), AST (4.65 ± 0.08) (Figure 13B), and ALP (39.15 ± 3.05) (Figure 13C) was significantly reduced ($p < 0.0001$) in a higher dose of DEHP treated group whereas in control group, ALT (0.02 ± 0.001), AST (0.5 ± 0.05), and ALP (12.45 ± 1.25) were observed respectively. Biochemical parameters (ALT, AST, and ALP) were performed five times (n=30/group).

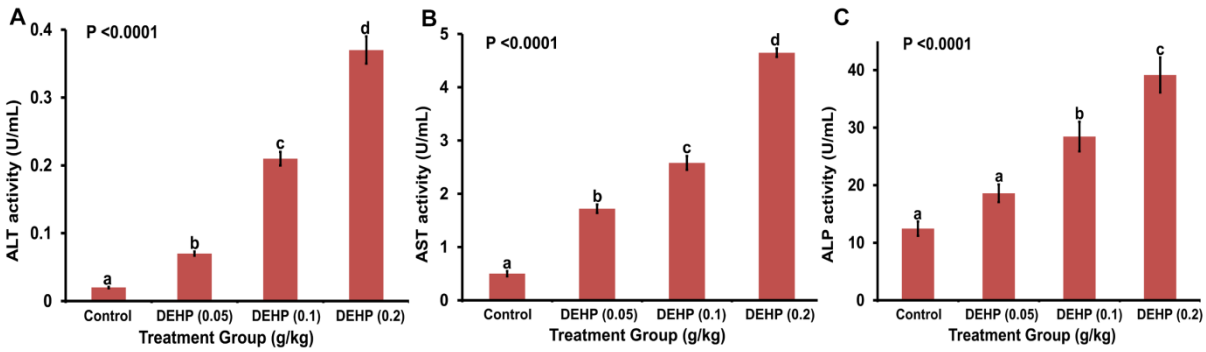


**Figure 13**. Biochemical parameters (ALT, AST, and ALP) of hemolymph were studied in control as well as in DEHP treated groups. A, alanine aminotransferase (ALT, U/mL); B, aspartate aminotransferase (AST, U/mL); and C, alkaline phosphatase (ALP, U/mL) respectively. Oneway ANOVA was performed and data were expressed in mean ± S.D. Bars with different alphabets were shown significant variation in studied groups. The p-value was < 0.0001. Biochemical parameters (ALT, AST, and ALP) were performed five times (n=30/group).

### 5.5. Histology of foregut, midgut, and hindgut

Histopathological studies of foregut, midgut, and hindgut of 4$^{th}$ instar larvae, 5$^{th}$ instar larvae, and spinning larvae were observed respectively (Figure 14-22). The control's foregut, midgut, and hindgut of 4$^{th}$ instar larvae, 5$^{th}$ instar larvae, and spinning larvae showed no damaged symptoms/any morphological changes (Figure 14-22A). We observed epithelial and goblet cell uniformity and basement membrane and muscle integration in control tissues followed by DEHP treatment groups gut tissue damage levels were varied in4$^{th}$ instar larvae, 5$^{th}$ instar larvae, and spinning larvae (Figure 14-22 B, C, and D). In 0.2 g/kg b.wt. DEHP treatment, vacuolization increment, sloughed epithelial cells, displacement of the gut lining into the lumen, and membrane blistering/blebbing were observed (Figure 14-22D)

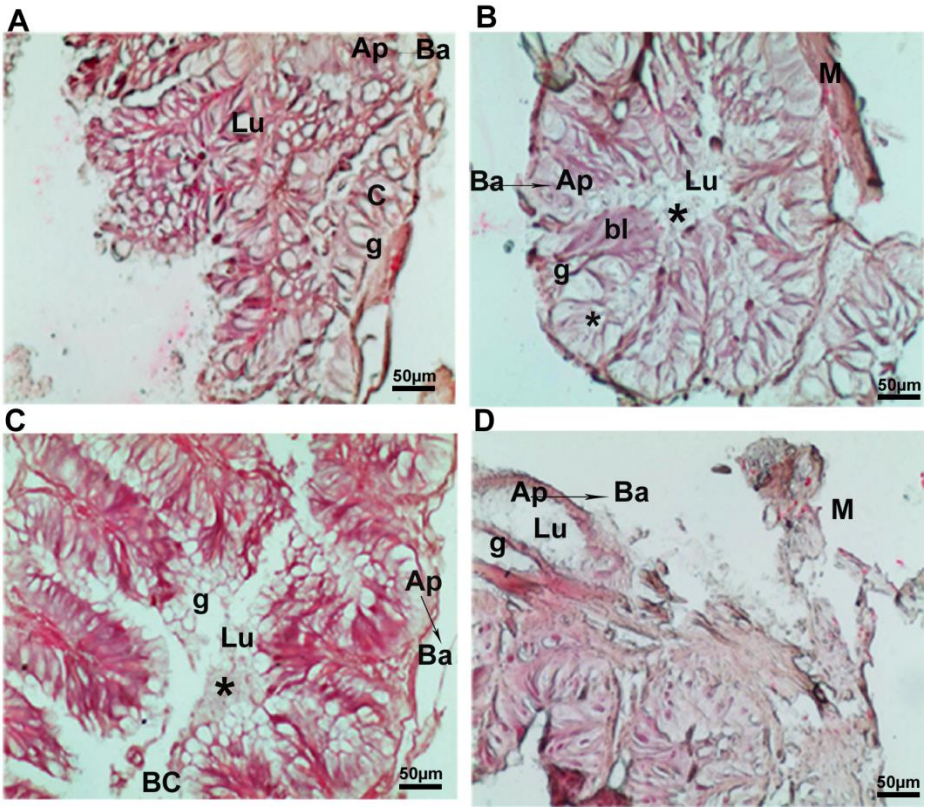


**Figure 14**. Histopathology of the foregut in the fourth-instar larvae of silkworm under DEHP treatment. A, Control showing normal structures of Lu-Lumen, Ap-Apical, Ba-Basolateral, g-goblet cells, C-columnar epithelial cells; B, Treated foregut tissue with DEHP (0.05g/Kg) showing damaged structures of Lu-Lumen, Ap-Apical, Ba-Basolateral, g-goblet cells, bl-blebbing/blistering of the cells, An asterisk (*) denotes cellular damage and displacement into the lumen, M-muscle; C, Treated foregut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, Ap-Apical, Ba-Basolateral, g-goblet cells, BC-body cavity, An asterisk (*) denotes cellular damage and displacement into the lumen, M-muscle; and D, Treated foregut tissue with DEHP (0.2g/Kg) showing damaged structures of Lu-Lumen, Ap-Apical, Ba-Basolateral, g-goblet cells, M-muscle respectively. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

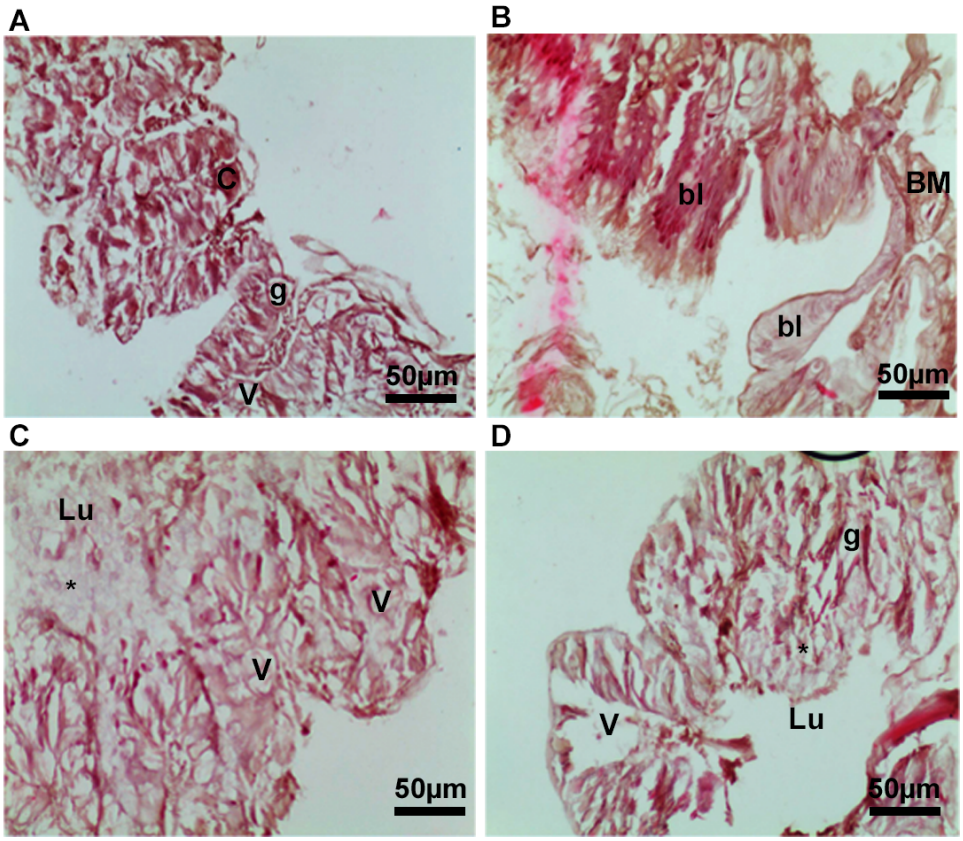


**Figure 15**. Histopathology of the foregut in the fifth instar larvae of silkworm under DEHP treatment. A, Control showing normal structures of v-vacuole, g-goblet cells, C-columnar epithelial cells; B, Treated foregut tissue with DEHP (0.05g/Kg) showing damaged structures of bl-blebbing/blistering of the cells, BM-basement membrane; C, Treated foregut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, An asterisk (*) denotes cellular damage and displacement into the lumen, v-vacuole; and D, Treated foregut tissue with DEHP (0.2g/Kg) showing damaged structures of Lu-Lumen, v-vacuole, An asterisk (*) denotes cellular damage and displacement into the lumen, g-goblet cells respectively. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

**Table 6**. Spinning larvae, Cocoon, and pupae morphological and growth parameters (length, weight, and width) in the control group as well as in DEHP treated groups respectively.

| Parameters | Control | DEHP (0.05g/Kg) | DEHP (0.1g/Kg) | DEHP (0.2g/Kg) | *p* value |
|---|---|---|---|---|---|
| **Length of larvae prior to spinning (cm)** | 5.84 ± 1.24a | 4.75 ± 1.08b | 3.72 ± 1.04c | 1.98 ± 0.28d | 0.0001 |
| **Cocoon length (cm)** | 3.62 ± 0.74a | 2.97 ± 0.54b | 2.07 ± 0.32c | 1.39 ± 0.04d | |
| **Cocoon width (cm)** | 2.39 ± 0.07a | 1.72 ± 0.04b | 1.08 ± 0.03c | 0.58 ± 0.02d | |
| **Weight of the cocoon filament (g)** | 0.32 ± 0.07a | 0.25 ± 0.05b | 0.14 ± 0.03c | 0.08 ± 0.01d | |
| **Size of the cocoon filament (d)** | 2.85 ± 0.08a | 2.26 ± 0.06b | 1.71 ± 0.05c | 0.98 ± 0.01d | |
| **Length of the cocoon filament (m)** | 1140.00 ± 25.12a | 1024.00 ± 24.75b | 945.00 ± 19.74c | 875.00 ± 19.05d | |
| **Weight of the floss (g)** | 0.12 ± 0.03a | 0.07 ± 0.01b | 0.04 ± 0.001c | 0.01 ± 0.002d | |
| **Weight of the shell with floss (g)** | 0.43 ± 0.07a | 0.29 ± 0.05b | 0.17 ± 0.04c | 0.06 ± 0.01d | |
| **Weight of the shell without floss (g)** | 0.31 ± 0.06a | 0.22 ± 0.05b | 0.13 ± 0.03c | 0.05 ± 0.01d | |
| **Pupae length (cm)** | 2.87 ± 0.09a | 1.87 ± 0.07b | 1.07 ± 0.04c | 0.08 ± 0. 02d | |
| **Pupae width (cm)** | 1.28 ± 0.05a | 1.05 ± 0.03b | 0.95 ± 0.02c | 0.58 ± 0. 01d | |

Data were expressed as mean ± S.D. One way ANOVA was done among the studied groups and different alphabetical letters were showed significance between studied groups. The p-value is < 0.0001.

**Table 7**. Economically important and growth parameters of the silkworm in DEHP treated groups and in a control group.

| Parameters | Control | DEHP (0.05g/Kg) | DEHP (0.1g/Kg) | DEHP (0.2g/Kg) | *p* value |
|---|---|---|---|---|---|
| **Moth emergence (%)** | 100.00 ± 10.45a | 88.23 ± 9.71b | 84.61 ± 8.25c | 66.66 ± 7. 35d | 0.0001 |
| **Cocoon yield (%)** | 100.00 ± 5.75a | 77.77 ± 5.04b | 66.66 ± 4.84c | 54.54 ± 3.74d | |
| **Floss-shell ratio (%)** | 38.71 ± 2.45a | 31.82 ± 2.12b | 30.77 ± 1.94c | 20.00 ± 1.46d | |
| **Cocoon-shell ratio (%)** | 29.83 ± 2.85a | 22.79 ± 2.72b | 15.09 ± 1.84c | 5.88 ± 1.05d | |
| **Shell ratio (%)** | 29.86 ± 4.87a | 26.77 ± 4.45b | 20.53 ± 3.75c | 17.64 ± 2.49d | |
| **Raw silk of centage of cocoon (%)** | 21.05 ± 2.47a | 16.47 ± 2.15b | 9.74 ± 1.57c | 4.27 ± 0.98d | |
| **Reelability (%)** | 93.00 ± 7.85a | 81.00 ± 7.08b | 71.00 ± 6.75c | 51.27 ± 4. 85d | |
| **Denier (g)** | 2.53 ± 0.14a | 2.20 ± 0.12b | 1.33 ± 0.06c | 0.82 ± 0. 04d | |
| **Reeling breaks** | 2.57 ± 0.38a | 1.97 ± 0.18b | 1.09 ± 0.06c | 0.52 ± 0. 01d | |
| **Pupation rate (%)** | 95.83 ± 10.89a | 86.36 ±10.18b | 75.00 ± 8.75c | 67.81 ± 7. 85d | |
| **Growth index** | 1.24 ± 0.05a | -0.28 ± 0.01b | -0.44 ± 0.03c | -0.49 ± 0.04d | |
| **ERR by Number** | 10000.00 ± 25.87a | 7700.77 ± 22.85b | 6600.66 ± 20.84c | 5400.54 ± 18.75d | |
| **ERR by Weight** | 706.94 ± 15.87a | 720.70 ± 16.24b | 804.93 ± 17.28c | 927.73 ± 18.65d | |
| **Ingesta** | 28.25 ± 2.14a | 26.50 ± 2.08b | 25.00 ± 2.01c | 23.00 ± 1.97d | |
| **Digesta** | 15.25 ± 2.06a | 21.50 ± 2.18b | 22.00 ± 2.19c | 21.00 ± 2.09d | |
| **Reference ratio (RR)** | 2.17 ± 0.04a | 5.30 ± 0.06b | 8.33 ± 0.09c | 11.50 ± 1.02d | |
| **Approximate digestibility (A.D) (%)** | 53.98 ± 2.45a | 81.13 ± 4.58b | 88.00 ± 5.86c | 91.30 ± 6.14d | |
| **Consumption index (CI)** | 12.36 ± 2.47a | 27.71 ± 3.58b | 34.25 ± 3.74c | 35.11 ± 3.81d | |
| **ECI to larva (%)** | 49.38 ± 4.57a | -11.70 ± 1.98b | -18.61 ± 1.74c | -21.91 ± 1.61d | |
| **ECD to larva (%)** | 91.48 ± 8.75a | -14.42 ± 1.45b | -21.23 ± 1.75c | -24.00 ± 2.05d | |
| **ECI to cocoon (%)** | 5.10 ± 1.09a | 4.80 ± 1.04a | 4.51 ± 0.98a | 4.44 ± 0.84a | |
| **ECD to cocoon (%)** | 9.45 ± 1.42a | 5.92 ± 1.16b | 5.12 ± 1.07c | 4.86 ± 1.04d | |
| **ECI to cocoon shell (%)** | 1.24 ± 0.08a | 0.91 ± 0.07b | 0.72 ± 0.05c | 0.39 ± 0.02d | |
| **ECD to cocoon shell (%)** | 2.30 ± 0.09a | 1.12 ± 0.07b | 0.82 ± 0.04c | 0.43 ± 0.02d | |
| **I/g. cocoon** | 1959.90 ± 35.28a | 2082.51 ± 32.58b | 2218.48 ± 33.24c | 2253.80 ± 31.84d | |
| **D/g. cocoon** | 1058.00 ± 100.28a | 1689.59 ± 121.25b | 1952.26 ± 127.84c | 2057.81 ± 130.25d | |
| **I/g. shell** | 8071.43 ± 150.28a | 11041.67 ± 135.64b | 13888.89 ± 140.58c | 25555.56 ± 214.98d | |
| **D/g. shell** | 4357.14 ± 157.84a | 8958.33 ± 187.25b | 12222.22 ± 241.25c | 23333.33 ± 284.98d | |
| **Leaf : egg recovery ratio** | 2.88 ± 0.85a | 3.85 ± 0.94b | 5.58 ± 1.02c | 8.28 ± 1.05d | |

Data were expressed as mean ± S.D. One way ANOVA was done among the studied groups and different alphabetical letters were showed significance between studied groups. The p-value is < 0.0001.

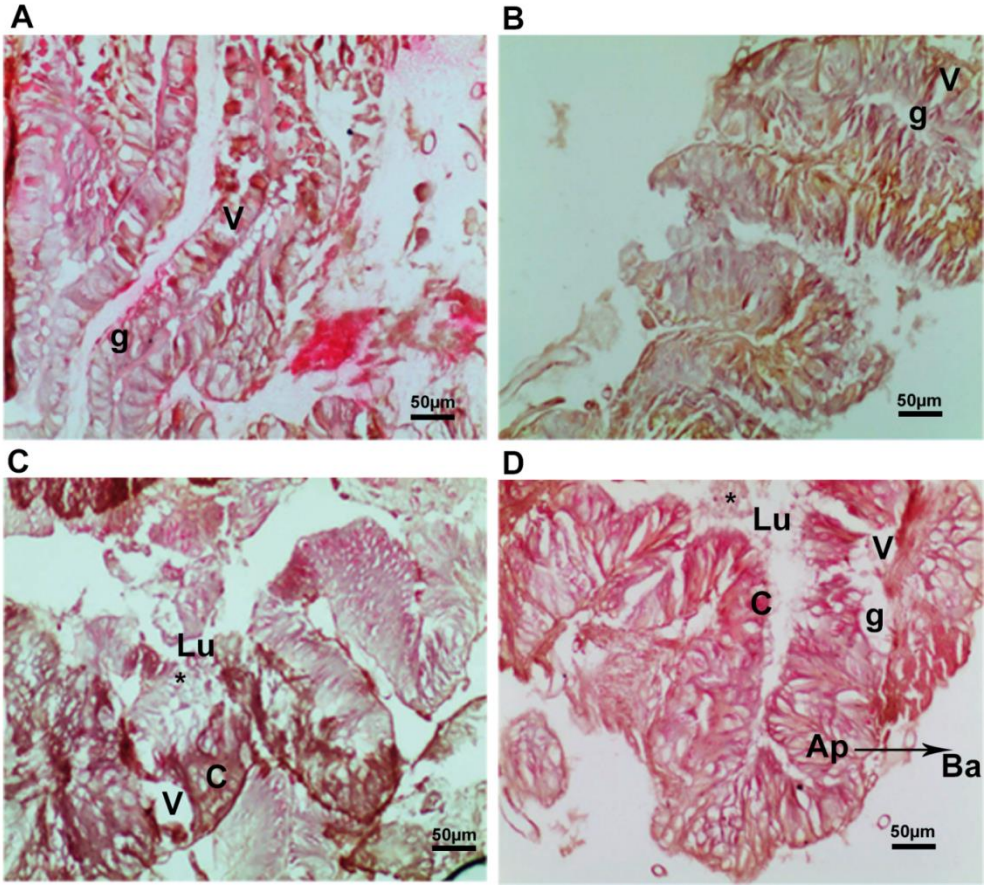


**Figure 16.** Histopathology of the foregut in the spinning larvae of silkworm under DEHP treatment. A, Control showing normal structures of v-vacuole, g-goblet cells; B, Treated foregut tissue with DEHP (0.05g/Kg) showing damaged structures of bl-blebbing/blistering of the cells, BM-basement membrane; C, Treated foregut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, An asterisk (*) denotes cellular damage and displacement into the lumen, v-vacuole, C-columnar epithelial cells; and D, Treated foregut tissue with DEHP (0.2g/Kg) showing damaged structures of Lu-Lumen, v-vacuole, An asterisk (*) denotes cellular damage and displacement into the lumen, g-goblet cells, C-columnar epithelial cells, Ap-Apical, Ba-Basolateral. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

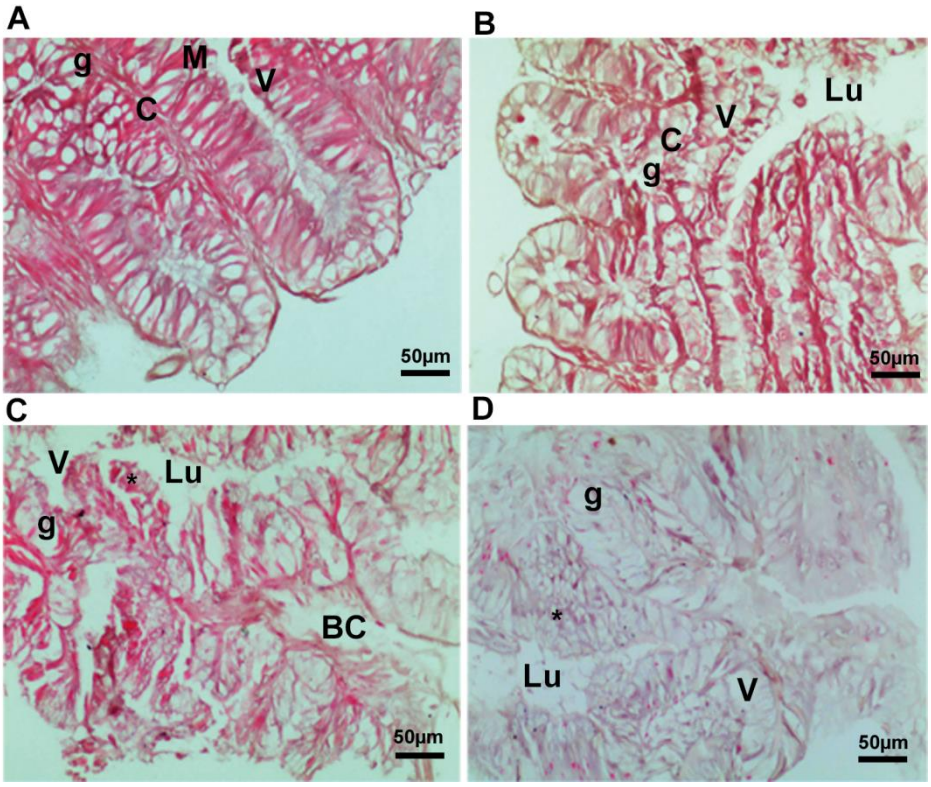


**Figure 17**. Histopathology of the midgut in the fourth-instar larvae of silkworm under DEHP treatment. A, Control showing normal structures of M-Muscle, v-vacuole, g-goblet cells, C-columnar epithelial cells; B, Treated midgut tissue with DEHP (0.05g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, M-muscle, v-vacuole; C, Treated midgut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, BC-body cavity, An asterisk (*) denotes cellular damage and displacement into the lumen, v-vacuole; and D, Treated midgut tissue with DEHP (0.2g/Kg) showing damaged structures of Lu-Lumen, An asterisk (*) denotes cellular damage and displacement into the lumen, g-goblet cells, v-vacuole. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

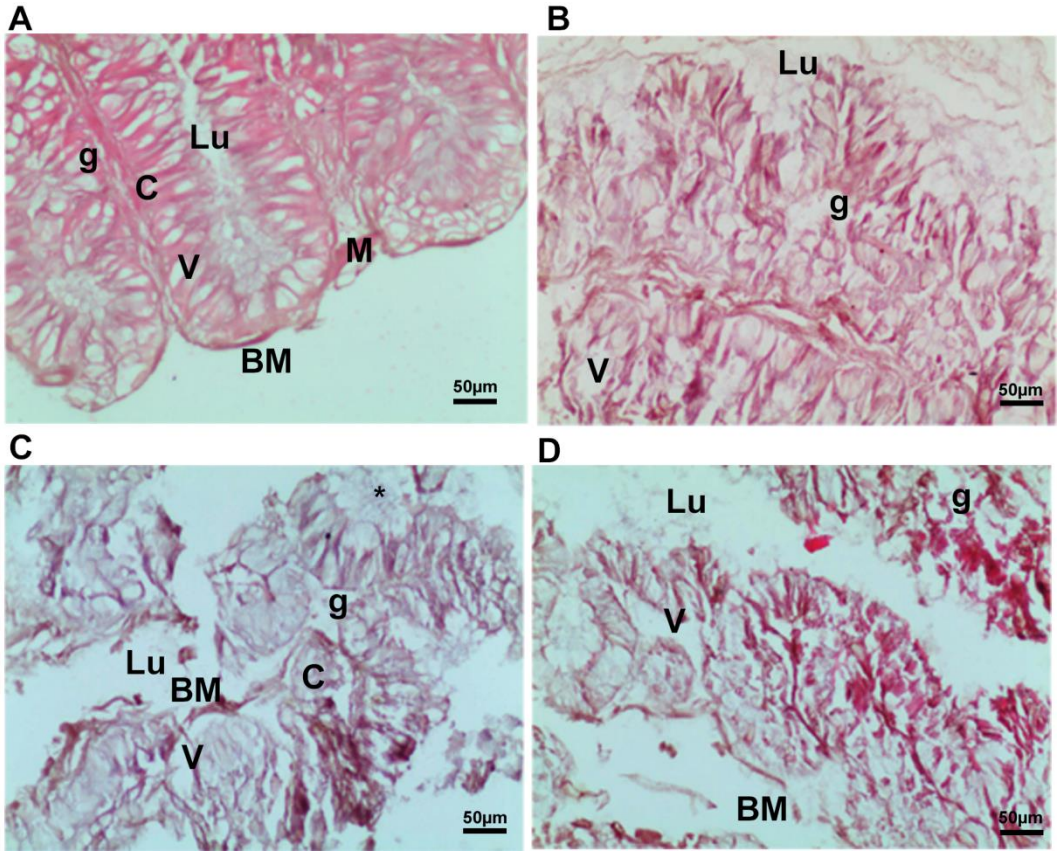


**Figure 18**. Histopathology of the midgut in the fifth instar larvae of silkworm under DEHP stress. A, Control showing normal structures of Lu-Lumen, BM-basement membrane, M-Muscle, v-vacuole, g-goblet cells, C-columnar epithelial cells; B, Treated midgut tissue with DEHP (0.05g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, v-vacuole; C, Treated midgut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, C-columnar epithelial cells, An asterisk (*) denotes cellular damage and displacement into the lumen, BM-basement membrane, v-vacuole; and D, Treated midgut tissue with DEHP (0.2g/Kg) showing damaged structures of Lu-Lumen, BM-basement membrane, g-goblet cells, v-vacuole. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

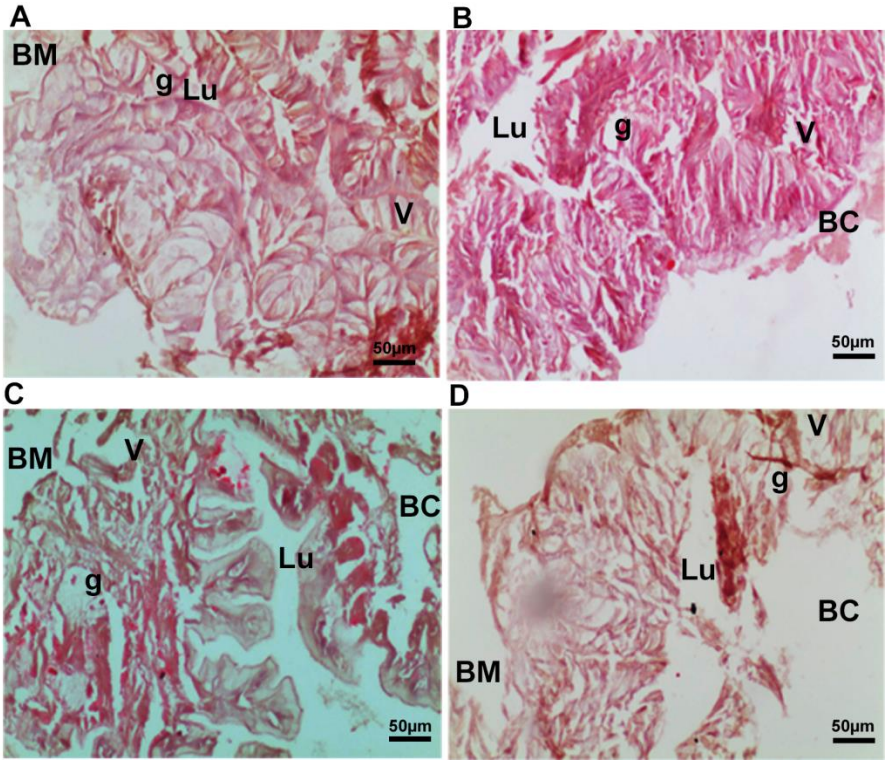


**Figure 19**. Histopathology of the midgut in the spinning larvae of silkworm under DEHP stress. A, Control showing normal structures of Lu-Lumen, BM-basement membrane, v-vacuole, g-goblet cells; B, Treated midgut tissue with DEHP (0.05g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, v-vacuole, BM-basement membrane; C, Treated midgut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, BC-Basal cavity, BM-basement membrane, v-vacuole; and D, Treated midgut tissue with DEHP (0.2g/Kg) (D) 10x resolution showing damaged structures of Lu-Lumen, BM-basement membrane, g-goblet cells, v-vacuole, BC-Basal cavity respectively. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

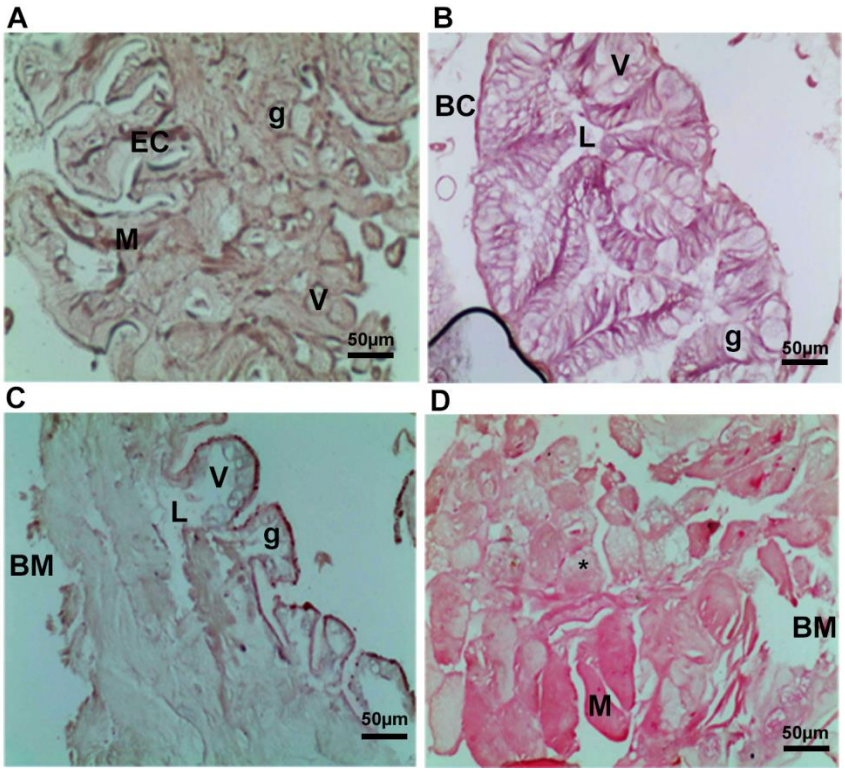


**Figure 20**. Histopathology of the hindgut in the fourth-instar larvae of silkworm under DEHP stress. A, Control showing normal structures of EC-epithelial cells, M-muscle v-vacuole, g-goblet cells; B, Treated hindgut tissue with DEHP (0.05g/Kg) showing damaged structures of BC-basal cavity, g-goblet cells, v-vacuole, L-Lumen; C, Treated hindgut tissue with DEHP (0.1g/Kg) showing damaged structures of L-Lumen, g-goblet cells, v-vacuole, BM-basement membrane; and D, Treated hindgut tissue with DEHP (0.2g/Kg) **(D)** 10x resolution showing damaged structures of M-Muscle, BM-basement membrane, An asterisk (*) denotes cellular damage and displacement into the lumen respectively. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

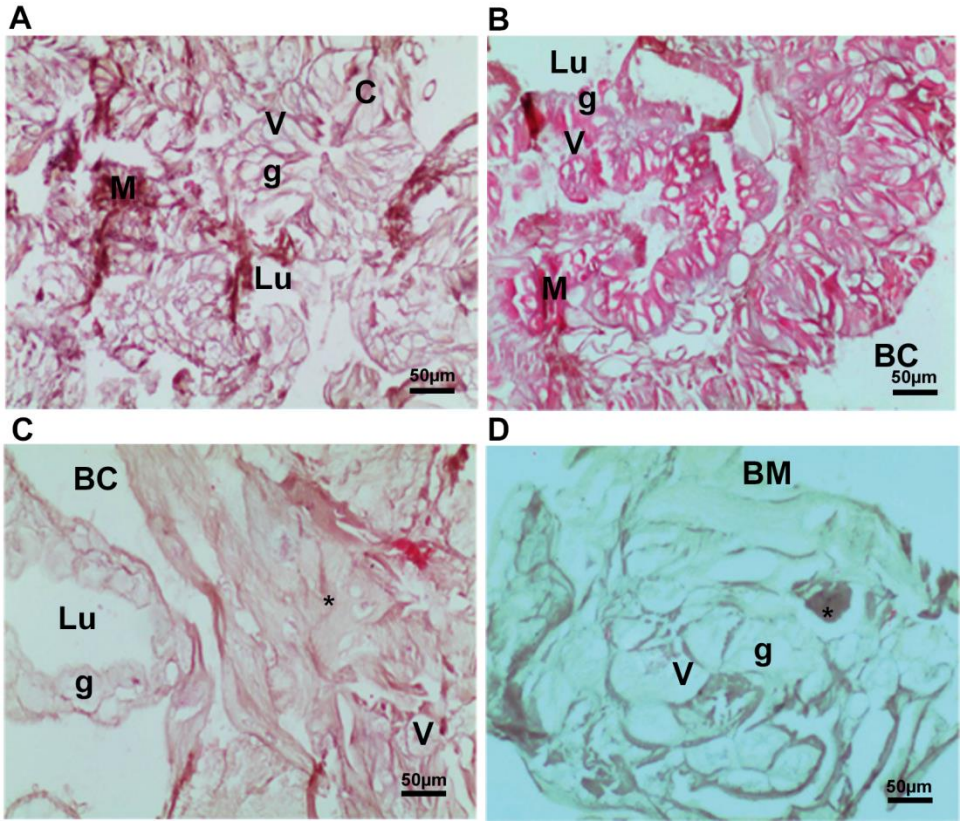


**Figure 21**. Histopathology of the hindgut in the fifth instar larvae of silkworm under DEHP stress. A, Control showing normal structures of Lu-Lumen, C-columnar epithelial cells, M-muscle v-vacuole, g-goblet cells; B, Treated hindgut tissue with DEHP (0.05g/Kg) showing damaged structures of BC-basal cavity, g-goblet cells, v-vacuole, Lu-Lumen, M-muscle; C, Treated hindgut tissue with DEHP (0.1g/Kg) showing damaged structures of Lu-Lumen, g-goblet cells, v-vacuole, BC-basal cavity, An asterisk (*) denotes cellular damage and displacement into the lumen; and D, Treated hindgut tissue with DEHP (0.2g/Kg) showing damaged structures of BM-basement membrane, An asterisk (*) denotes cellular damage and displacement into the lumen, g-goblet cells, v-vacuole respectively. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

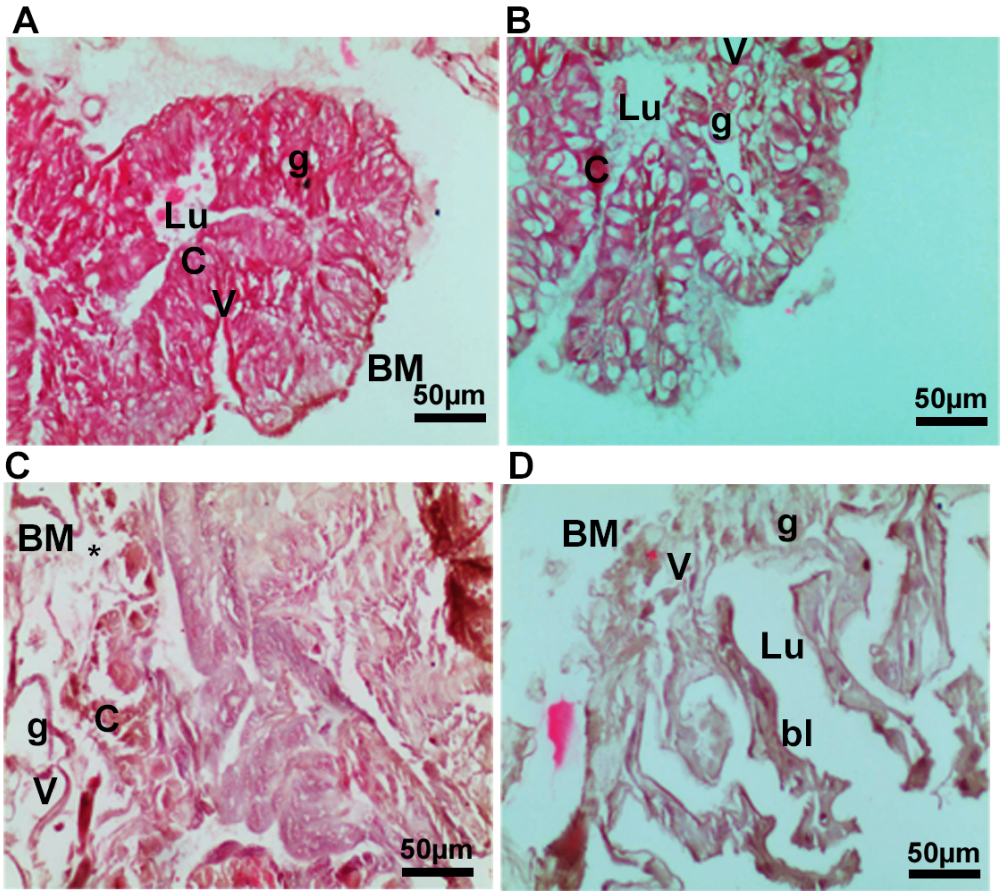


**Figure 22**. Histopathology of the hindgut in the spinning larvae of silkworm under DEHP stress. A, Control showing normal structures of Lu-Lumen, C-columnar epithelial cells, v-vacuole, g-goblet cells, BM-basement membrane; B, Treated hindgut tissue with DEHP (0.05g/Kg) showing damaged structures of C-columnar epithelial cells, g-goblet cells, v-vacuole, Lu-Lumen; C, Treated hindgut tissue with DEHP (0.1g/Kg) showing damaged structures of g-goblet cells, v-vacuole, BM-basement membrane, An asterisk (*) denotes cellular damage and displacement into the lumen, C-columnar epithelial cells; and D, Treated hindgut tissue with DEHP (0.2g/Kg) showing damaged structures of BM-basement membrane, Lu-Lumen, g-goblet cells, v-vacuole, bl- blebbing/blistering of the cells respectively. Histological studies were performed five times (n=10/group). All histological images were taken in 10X resolution.

### 5.6. Chronic reproduction assay

A chronic reproduction experiment was carried out to evaluate the toxic effect of DEHP on $f_1$ generation. For fecundity analysis, we mated 10 different pairs. 1, control female with control male, 2, 3, and 4 control female with DEHP treated males (0.05 g/kg, 0.1 g/kg, and 0.2 g/kg), and 5, 6, and 7 control male with treated females (0.05 g/kg, 0.1 g/kg, and 0.2 g/kg)), and followed by 8, 9, and 10 treated male (0.05 g/kg, 0.1 g/kg, and 0.2 g/kg) with treated female (0.05 g/kg, 0.1 g/kg, and 0.2 g/kg). The mean fecundity was reduced significantly ($p < 0.01$) in DEHP treated mated pairs compared to the control pair. The mean fecundity in the control group was 540 ± 10.25 whereas, in control female with treated male groups (0.05, 0.1, and 0.2 g/kg DEHP), the mean fecundity was 450 ± 9.27, 400 ± 8.75, and 184 ± 5.74 respectively. A similar degree was observed in control males with treated females 424 ± 8.81, 450 ± 9.27, and 300 ± 7.84 respectively, or treated male with the treated female also showed a significant ($p < 0.01$) reduction in fecundity (420 ± 8.83, 225 ± 5.78, and 130 ± 4.82 respectively). Therefore, DEHP effected fecundity in both males and females (Figure 23).

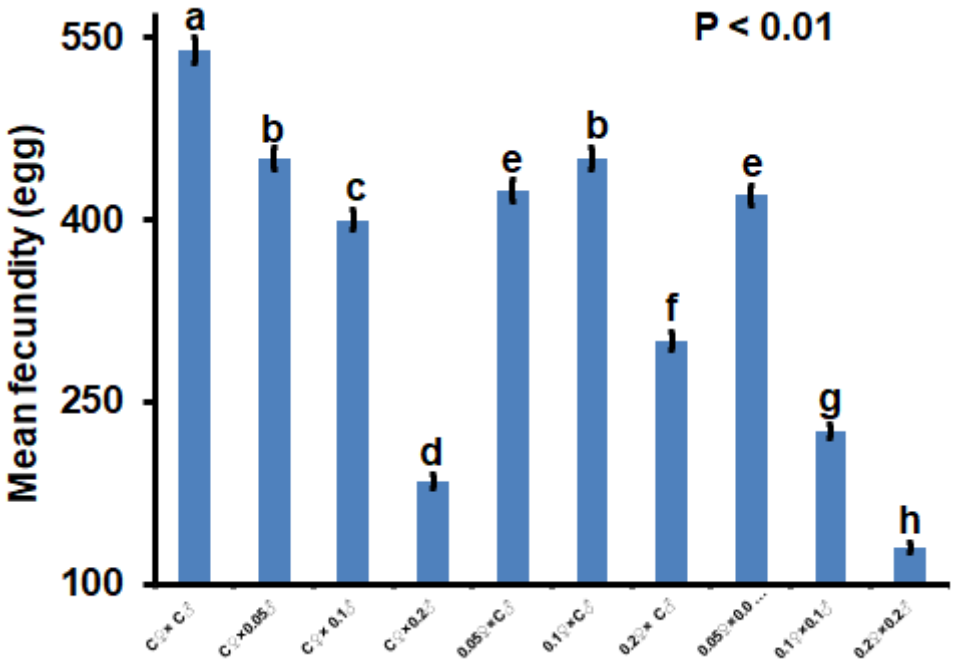


**Figure 23**. Reproductive toxicity of DEHP on fecundity. ♂ indicates male and ♀ symbol indicates female. The first bar indicates control male with female, followed

by control female with treated male, control male with treated female, and treated male with treated female. Data were expressed as mean ± S.D. Significant differences were found between treated pairs with control pair $p < 0.01$. Bars with different alphabets determine the significance between studied groups.

Further, we have measured the hatching rate (%) to determine the population decay. Hatching rate (%) was significantly reduced in DEHP treated groups, compared to the control group (Figure 24). Chronic reproduction assay was performed five times (n=80/group).

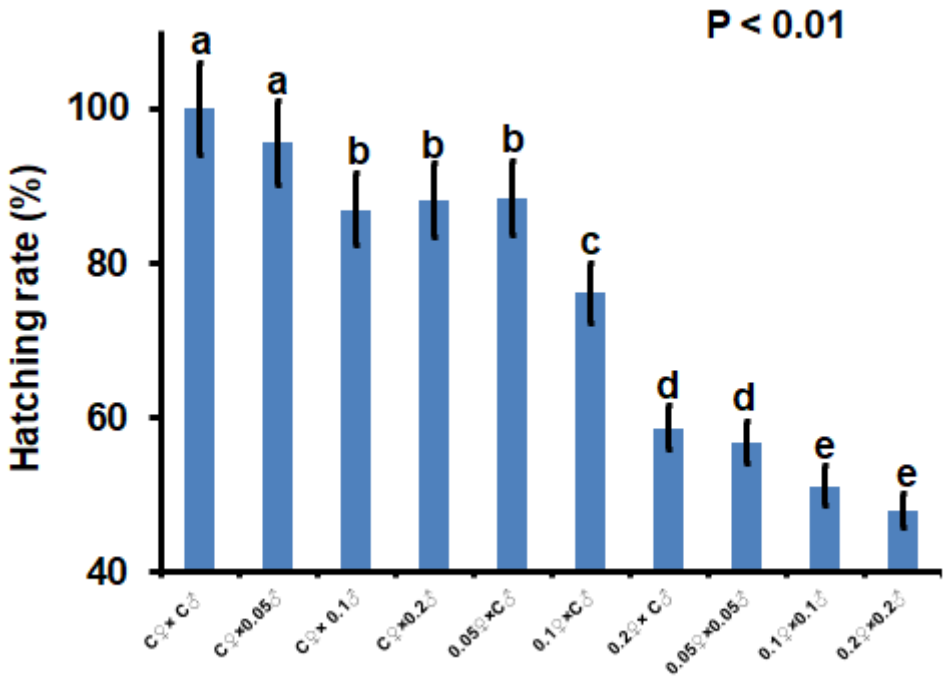


**Figure 24**. Reproductive toxicity of DEHP on hatching rate (%). ♂ indicates male and ♀ symbol indicates female. The first bar indicates control male with female, followed by control female with treated male, control male with treated female, and treated male with treated female. Data were expressed as mean ± S.D. Significant differences were found between treated pairs with control pair $p < 0.01$. Bars with different alphabets determine the significance between studied groups. The chronic reproduction assay was performed five times (n=80/group).

### 5.7. Real-time quantitative PCR (qRT-PCR)

In RT-PCR, the vitellogenin gene expression was observed in fat bodies of three life stages of silkworm (spinning larvae, pupae, and eggs) with exposure to different doses of DEHP in both sexes (male and female). The significant induction of the Vg gene was observed in females treated with DEHP but it was silent in control males. At the high dose of DEHP (0.2 g/kg), the vitellogenin was increased significantly in females ($p < 0.001$). Besides, Vg gene expression was also observed in the male fat body at the doses of 0.1 and 0.2 g/kg DEHP (Figure 26). Vitellogenin (Vg) gene expression in pupae increased significantly in treated male and female pupae ($p < 0.001$). But the Vg gene expression was reduced significantly in egg compared to the control ($p < 0.001$). The ecdysone receptor (EcR) was also reduced significantly in DEHP treated groups ($p < 0.001$). Each gene was amplified five times in triplicate (n=20/group).

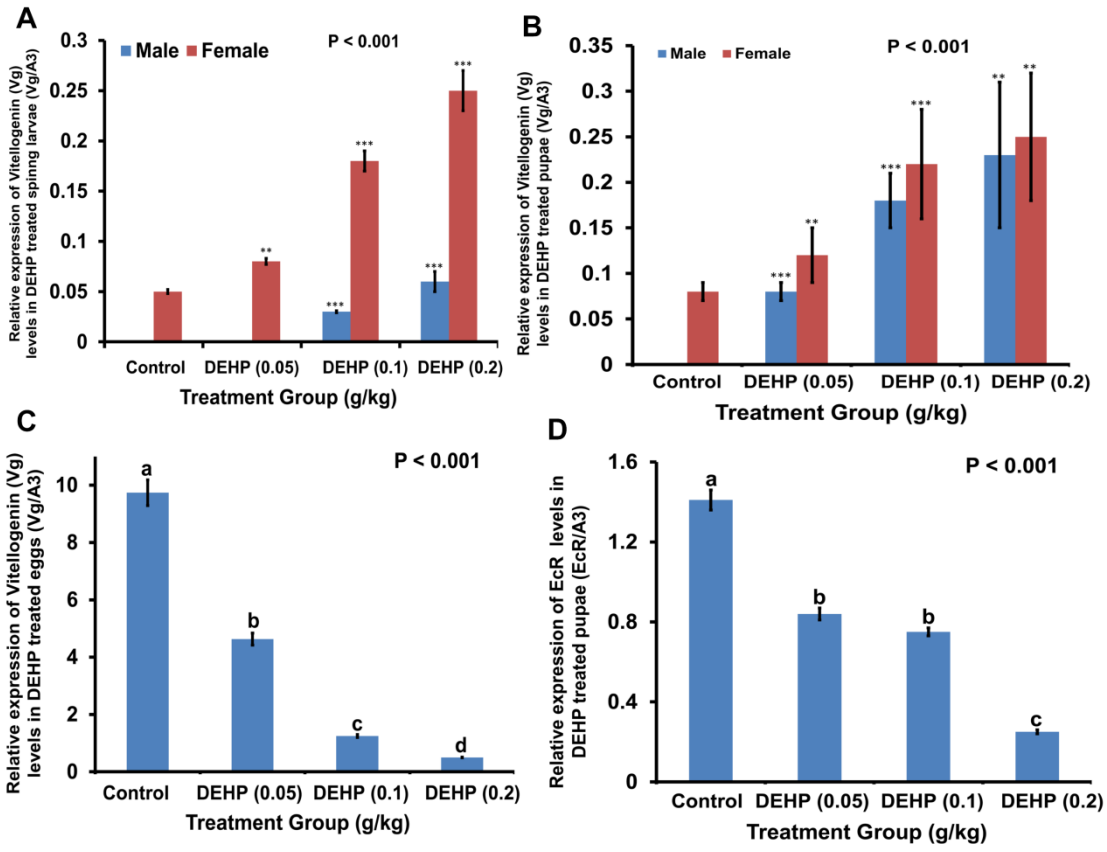

**Figure 25**. Real-time PCR analysis of Vitellogenin (Vg) gene expression in different life stages. A, relative Vg gene expression in spinning larvae; B, pupae of male and female silkworms; C, Vg expression in eggs; and D, ecdysone receptor (EcR) expression in female pupae. * and different alphabets indicate significance in studied groups. One way ANOVA was performed and significant difference between groups was $p < 0.001$. Data were expressed as a mean ± S.D. Each gene was amplified five times in triplicate (n=20/group).

In this study, we were studied Morphological, biological and economic characteristics, oxidative stress markers, antioxidants, and biochemical parameters, histological damages, gene expression studies to determine the toxicity of DEHP. Silkworm (*Bombyx mori*) is a good insect model to study reproductive toxicity (Yuan et al., 2013).

# CHAPTER-6

# DISCUSSION

### 6.1. Acute toxicity studies and abnormalities

DEHP is one of the environmental contaminants and less study has been done on reproduction toxicity. Based on LD50 data (Table 4), DEHP has greater toxicity properties compared to the other chemicals (Roh et al., 2006). In the current investigation, the acute toxicity of DEHP was assessed using Morphological, biological and economic characteristics, oxidative stress markers, antioxidants, and biochemical parameters, histological damages, and induction of gene expression studies to the silkworm (Dhawan et al., 2000; Anderson et al., 2001, 2004; Kohra et al., 2002; Tominaga et al., 2003; Roh et al., 2006; Kalita et al., 2016). The current study displayed mortality was increasing while doses of DEHP was increasing that means mortality and DEHP concentration were proportional to each other (Figure 7). We also observed intoxication symptoms in DEHP treated silkworms like the black color spot on the skin, upward face movement, agony movements, oozing out hemolymph through the anus, and vomiting. This might be due to the neurotoxicant effect of DEHP (Zhang et al., 2008; Wang et al., 2015).

### 6.2. Morphological, biological and economic characteristics

To determine, the toxic effects of any xenobiotic on growth and reproduction of experimental animal models are the most accepted parameters and found to be very sensitive parameters. Long term changes occur in growth and reproduction due to a decrease in body length and egg number after DEHP exposure but unable to correlate between these xenobiotics and physiological stress of test animal model because of lesser availability of xenobiotics in the environment. The biological characters like body length, width, and weight of silkworm were decreased significantly in DEHP treatment groups. The development, growth, and reproduction was depended on the economical parameters of the cocoon. In this study, the cocoon parameters were also decreased significantly. The DEHP treated larvae food consumption very less due to appetite loss. This suggested that starvation or feeding behavior could play a major role in the developmental stage as well as in reproduction (Etebari et al., 2005). Which in turn decreases the economical parameters of the silkworm.

### 6.3. Oxidative stress markers, antioxidants, and biochemical parameters

Oxidative stress markers such as hydrogen peroxide ($H_2O_2$), lipid peroxidation (LPX), and protein carbonyl content were increased in DEHP treated groups. $H_2O_2$, LPX, and PC content are good oxidative stress markers to assess the oxidative damage (Davies et al., 1999; Krishnan and Kodrik, 2006). Later, we found that antioxidants (SOD, CAT, and GSH) were decreased in DEHP treated groups. Hence, fewer antioxidants in hemolymph against higher $H_2O_2$ might be a reason in higher levels of LPX and PC content (Sahoo et al., 2015). Our current findings were agreed with previous studies, the PC content was increased in *Spodoptera littoralis* due to oxidative stress (Krishnan and Kodrik, 2006). Based on these findings, we suggested that oxidative tissue damages play a significant role in oxidative stress markers increment. Numerous previous studies support that growth, development, and physiological responses modulate by $H_2O_2$ is a strong oxidative stress marker (Zhao and Shi, 2010). Lower levels of SOD and CAT suggested that lower levels of metabolism in hemolymph aginst ROS levels and we know that hemolymph is well known reactive oxygen species generator (Ahmad, 1992; ToRU, 1994). GSH is required for aerobic cells to fight against ROS species and which acts as a buffer inside the cell (Allen and Sohal, 1986; Dolphin et al., 1989). Depletion in GSH, shown an increase in LPX and which leads to oxidative stress damage (Maellaro et al., 1990). Chemicals caused tissue damages in mammals which leads to severe injury. Hence, it is very important to evaluate the tissue damages caused by drugs (Inagaki et al., 2012). Toxic substances cause an increase in hepatic marker enzymes such as ALT, AST, and ALP in damaged tissue (Ozer et al., 2008). In mammalians blood, ALT, AST, and ALP activities are increased due to tissue damage (Lindblom et al., 2007). ALT and AST are playing a vital role in amino acid catabolism by transferring an amino group to the keto group. These enzymes shared a link between carbohydrates and protein metabolism (Martin et al., 1981). ALP is a hydrolytic enzyme and involved in numerous physiological conditions of the midgut. The ALP was significantly increased due to oxidative stress and tissue damage (Miao, 2002).

### 6.4. Chronic reproduction assay and induction of vitellogenin (Vg)

There are a number of studies on the toxic effect on reproduction parameters. In the present study, the toxic effect of DEHP chemical on silkworm reproduction was observed. For reproductive toxicity assays, we used fecundity and hatching rate. Fecundity and hatching rate of the egg was decreased significantly in DEHP treated groups. Our results suggest that abnormal maturation of either ovary or testis in DEHP treated groups. Hence, which leads to abnormal egg development. Our results were related to the previous study showed no eggs were laid by emerged chironomids exposed to 4-nonyl phenyl (Bettinetti and Provini, 2002) at 250 µg/g 4-NP.

Vitellogenin gene (Vg) induction studies have been reported in invertebrates (Hahn et al., 2002; Matozzo and Marin, 2005). Vg expression has been shown in a specific tissue, sex, and stage. In the current study, Vg gene expression was increased in females at doses 0.05 g/kg, 0.1 g/kg, and 0.2 g/kg DEHP respectively. Our study results were related to previously studied reported that the Vg gene induction was increased dose-dependently in *Chiromonus riparius* (Hahn et al., 2002). We found interesting results of Vg gene expression in males exposed to DEHP. Generally, Vg gene expression was absent in males. Induction of the Vg gene in males may suggest that it is an indicator to assess the reproduction toxicity of DEHP and other related phthalates. Ecdysone (20-OH ecdysone) is a vital molecule in vitellogenesis. It involves development, oocyte maturation, and growth of invertebrates. Impairment in the Ecr receptor leads to abnormalities in normal ovarian, oocyte and egg growth (Hodin and Riddiford, 1998; Takeuchi et al., 2007). In our study, we observed the impairment in the EcR receptor in DEHP treated groups. In summary, our data suggested that the reproductive toxicity of DEHP in *B.mori*. DEHP caused impairment in morphological and economic characteristics, antioxidant enzymes and also in reproduction which led to the decrease in fecundity and hatching rate. The vitellogenin gene expression in males by DEHP ca be used as a good biomarker to assess the reproductive toxicity in *B.mori*.

# CHAPTER-7

# SUMMARY

## 7. Summary

1. We investigated the physiological and reproductive effects of DEHP on silkworm and it has a wide range of effects.

2. The present study showed the effect of DEHP on growth and economical important parameters of silkworm as well as genotoxicity.

3. The exposure of sub-lethal concentration of DEHP showed behavioral changes which lead to the increase of oxidative stress (LPx, PC and H2O2) and biochemical parameters (ALT, AST and ALP), inhibition of antioxidant enzymes activity (SOD, CAT, GSH).

4. DEHP exposure leads to tissue damage, gut leakiness, and REDOX imbalance in insect larvae. This mimics closely the pathological symptoms.

5. The molecular target of ecdysteroids is known to be the EcR, a 20-OH ecdysone receptor. Any impairment of normal EcR function will lead to aberrations in normal ovarian, oocyte and egg growth. Our study showed that DEHP indirectly caused impairment of EcR expression relating to ecdysteroid activity.

6. In summary, our data demonstrated the reproductive toxicity of DEHP in B. mori. DEHP exposure caused impaired reproduction, which was associated with altered expression of the Vg gene in the eggs and fat body of the male and female pupae and consequently poor accumulation of egg yolk in developing eggs, which may have led to a reduction in egg numbers and a decrease in hatching rate. The induction of Vg expression by DEHP is a potential biomarker for monitoring reproductive toxicity in *B. mori* (Figure 26).

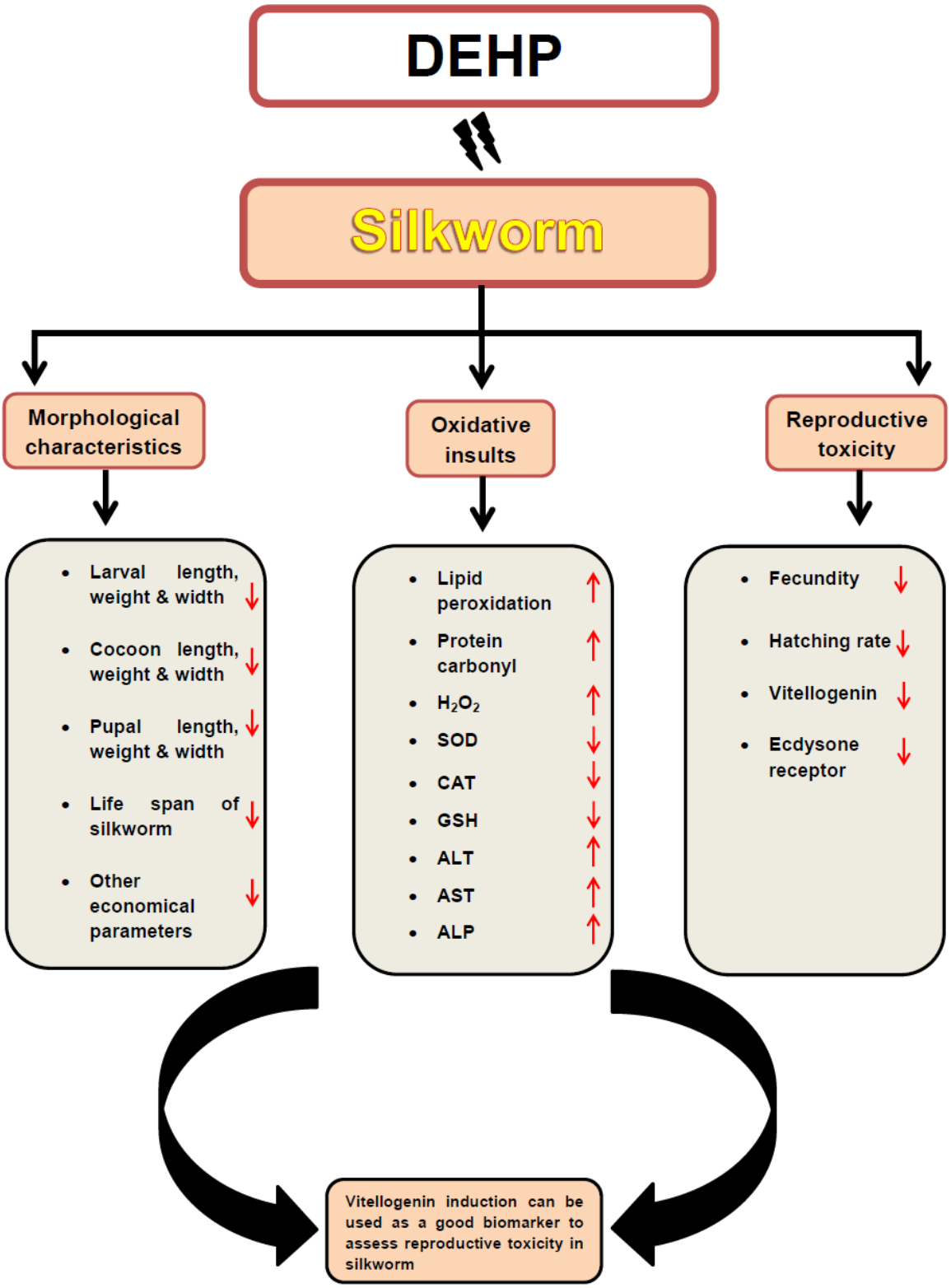


**Figure 26**. Summary of the present study.

## APPENDIX

### LIST OF ACRONYMS

| ABBREVIATED | FULL FORM |
|---|---|
| ˚ | Degree |
| % | Percent |
| µL | Micro litre |
| mg | Mili gram |
| µg | Micro gram |
| ng/ mL | Nano-grams per milli Liter |
| kg | Kilo gram |
| C | Celsius |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| dNTPs | Deoxinucleotide Triphosphate |
| EDTA | Ethylene-diamine tetra-acetic acid |
| Kb | Kilo base pair |
| PCR | Polymerized Chain Reaction |
| rpm | Rotation per minute |
| TAE | Trisbase acetic acid EDTA |
| SDS | Sodium Dodecyl Sulfate |
| h | Hour |
| M | Molar |
| Min | Minutes |
| bp | Base pair |
| RFLP | Restriction fragment length polymerphism |
| CAT | Catalase |
| GSH | Reduced glutathione |
| SOD | Superoxide dismutase |
| MDA | Malondialdehyde |
| GSTM1 | Glutathione-s-transferase mu1 |
| GSTT1 | Glutathione-s-transferase theta1 |
| GSTP1 | Glutathione-s-transferase para1 |
| pg/ml | Picogram per milli litre |
| MCV | Mean cell volume |
| PCV | Packed cell volume |
| EtBr | Ethidium bromide |
| DEHP | Di(2-ethylhexyl) phthlate |
| QRTPCR | Quantitayve realtime PCR |
| cDNA | Complemenatray DNA |

# CHAPTER-9

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## BRIEF BIO-DATA OF THE CANDIDATE

## CURRICULUM VITAE

**2.1.** Name (Mrs): **Pratima Khandayataray**

**2.2.** Designation: **Research Scholar**

**2.3.** Complete Postal Addresses and PIN: **Department of Zoology, Mizoram Central University, Aizawl-796004, Mizoram**

Telephone Number(s), E-mail: **8729894324**, **9777853279**, **pratimapbk@gmail.com**

**2.4.** Date of birth: **17/06/1995**

**2.5. Educational Qualification: Degrees obtained (Begin with Bachelor's Degree)**

| Degree | Institution | Field(s) | % of marks | Year |
|---|---|---|---|---|
| B.Sc (Biotechnology) | Utkal University | Biotechnology | 61.72 | 2012-2015 |
| M.Sc | North Odisha University | Biotechnology | 67.00 | 2015-2017 |

**2.6**. **Research Experience**

Department of Zoology, Mizoram University, Mizoram

Project entitled as "**Effect of DEHP (diethylhexyl phthalate) on Vitellogenin (Vtg) expression in *Bombyx mori* (silk worm)".**

**2.7. Conferences/Presentations**

1) The work shop on **"*Human Genetics*",** held during February 27-28, 2018. Organized at: Department of Human Genetics, Tripura University, Agartala,Tripura.

**2.8.** Other information, if any: **Nil**

## PARTICULARS OF THE CANDIDATE

| | | |
|---|---|---|
| NAME OF THE CANDIDATE | : | **PRATIMA KHANDAYATARAY** |
| DEGREE | : | **MASTER IN PHILOSOPHY** |
| DEPARTMENT | : | **ZOOLOGY** |
| TITLE OF DISSERTATION | : | **Induction of vitellogenin gene expression as a marker to assess the reproductive toxicity effects of di(2-ethylhexyl)phthalate (DEHP) in *Bombyx mori*** |
| DATE OF ADMISSION | : | **24.07.2018** |

COMMENCEMENT OF DISSERTATION: **January 2019 to December 2019**

APPROVAL OF RESEARCH PROPOSAL

1. BOS : **08.04.2019**
2. SCHOOL BOARD : **17.05.2019**

REGISTRATION NO. & DATE : **MZU/M.Phil./530 of 17.05.2019**

DUE DATE OF SUBMISSION : **December**

EXTENSION (IF ANY) : **NA**

HEAD
Department of Zoology

## LIST OF PUBLISHED PAPERS

| S.No. | Paper |
|---|---|
| **1** | Lalrinzuali Sailo, Meesala Krishna Murthy, **Khandayataray Pratima**, Vikas Kumar Roy and Guruswami Gurusubramanian (2018). Monosodium Glutamate Toxicity and the Possible Protective Role of L–Carnitine. **Science and Technology**. 6 (1). 45-56. |

## LIST OF CONFERENCE/SEMINAR/WORKSHOP ATTENDED AND PARTICIPATED

| S.No. | Conference/Seminar/Workshop attended and participated | Date |
|---|---|---|
| 1 | The work shop on "*Genetic analyses of compex traits"*, February 27, 2018. Organized at: Department of Human Physiology, Tripua University, Agartala, Tripura. | 27-28, February 2018 |
| 2 | National Seminar on *"Conservation and sustainable use of medicinal and aromatic plants"* 13th-14th September 2018, Mizoram University. | 13th-14th September 2018 |
| 3 | International conference on "*Biodiversity, environment and human health: Innovations and Emerging trends*" 12-14 November 2018, Mizoram University. | 12-14 November 2018 |
| 4 | International conference on "*Chemistry and environmental sustainability*" 19-22 February 2019, Mizoram University. | 19-22 February 2019 |
| 5 | Workshop/ Training on *"Bioinformatics for zoologists"* held at Departet of Biotechnology, Mizoram University held during 26-31st August 2019. | 26-31st August 2019 |
| 6 | National workshop on *"Ethics in research and preventing plagiarism"* 03rd October 2019, Department of Physics, Mizoram University. | 03rd October 2019 |
| 7 | International conference on "*Recent advances in animal science*" 06th – 08th November 2019, Department of zoology, Pachhunga University College, Mizoram University. | 06th – 08th November 2019 |