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Effect of cypermethrin on reproductive efficacy in zebrafish (*Danio rerio*): *In-vivo* and *in-silico* studies

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Abstract

Aim : The aim of the present study was to evaluate the reproductive efficacy of male and female zebrafish following cypermethrin exposure.

Methodology : The adult zebrafish (*Danio rerio*) of both sexes were exposed to cypermethrin at three selected concentrations 0.1, 1.0 and 10 µg l⁻¹ over a period of 21 days. After completion of experimental period, the reproductive endpoints such as fecundity, hatchability, testis and ovarian histology and plasma vitellogenin levels were selected and determined in this study.

Results : Cypermethrin exposure did not affect the cumulative fecundity rates in experimental fishes over controls. However, cypermethrin at 10 µg l⁻¹ showed a significant reduction in the sperm number in male fishes over control. On the other hand, the same concentration of cypermethrin did not show significant changes in the plasma vitellogenin levels of both male and female fishes over their respective controls. Analysis of testicular and ovarian architectures of male and female zebrafish exposed to cypermethrin at 10 µg l⁻¹ showed no marked differences over controls. In addition, molecular docking studies revealed that the binding energy between the cypermethrin and zebrafish estrogen receptor (zER) β1 was almost similar to the binding energies exhibited by reference molecules, estradiol and ethinyl estradiol with zERβ1. Further, binding energies between the ligands (cypermethrin and its metabolites phenoxybenzaldehyde and 3-phenoxybenzoic acid) with zERα were low as compared to the binding energies between the reference molecules and zERα.

Interpretation : *In-vivo* studies indicated that cypermethrin at 10 µg l⁻¹ leads to spermatotoxicity in zebrafish and *in silico* analysis showed that the cypermethrin at least in part interfere with the signalling of zERα.

Key words: Cypermethrin, Estrogen receptors, Spermatogenesis, Zebrafish

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Introduction

Pesticides are mainly used to protect the agricultural crops against pests. However, due to their wide usage and mismanagement they enter the environment via agricultural runoff water and contaminate drinking water. Several adverse effects, including reproductive health disorders have been reported in humans and wildlife (Mnif *et al.*, 2011) due to pesticide toxicity. Among a range of pesticides, Cypermethrin, [alpha-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylic acid], is one of the synthetic type II pyrethroid pesticides. Cypermethrin is widely used to control pests in various activities like wool processing and sheep dipping (USEPA, 1998), treating salmonids for sea lice (Ernst *et al.*, 2001), in forestry (Torstensson *et al.*, 1999), controlling pests of cotton and soybean, moths etc., (Carriquiriborde *et al.*, 2007). Like other pesticides, cypermethrin is not specific and the toxic effects of cypermethrin on non-target organisms, including fishes have been reported. In India, the aquaculture of edible fishes in paddy field is a common practice and thus, agricultural runoff water containing harmful pesticides can interfere with the reproductive performance of fish. Moreover, fishes are often used as bio-indicators of water pollution, as they respond to even low concentrations of toxic chemicals. Thus, studies encompassing the ecotoxicological impact of pesticides in fish are considered important. Previously, it has been reported that exposure of fishes to cypermethrin alters behaviour, biochemical and haematological variables, inhibits tissue antioxidant status and developmental toxicity, neurotoxicity and histological changes in the gills and intestine (Marino and Ronco, 2005; Prashanth and David, 2006; Carriquiriborde *et al.*, 2007; Prashanth *et al.*, 2011; Ullah *et al.*, 2018). Studies of Singh and Singh (2008) have shown that the cypermethrin-induced reduced steroidogenesis occurs via alterations at the level of hypothalamo-hypophyseal-gonadal axis in *Heteropneustes fossilis* (Bloch). Studies also indicated that the male parr exposed to cypermethrin impair sensory signals necessary to detect the priming pheromone released by female salmonids (Moore and Waring, 2001). However, the toxic effects of cypermethrin at low doses on cumulative fecundity, vitellogenin levels and sperm reproductive endpoints are not well defined.

Reproduction is controlled by several hormonal factors including estrogens in vertebrates (Bondesson *et al.*, 2015). It is well known that estrogens exert their genomic actions via members of nuclear receptor family, the estrogen receptors (Lucas *et al.*, 2011; Bondesson *et al.*, 2015). Estrogen signalling is well conserved among the vertebrates including teleost fishes and studies have shown that the reproductive tissues of zebrafish at least in part express different isoforms of estrogen receptors at varying levels (Lu *et al.*, 2017). Like other vertebrate estrogen receptors, zebrafish estrogen receptors comprises of two major domains in its typical structure: DNA binding domain (DBD) and ligand binding domain (LBD) wherein the DBD and LBD play an important role in recognition of response elements on DNA and

ligand binding, respectively, and eventually regulates the gene expression. Thus, compounds with estrogenic properties can able to interfere with the estrogen mediated signalling physiological events including reproduction in vertebrates. Further, it has been shown that up-regulation and down-regulation of genes which are under the control of estrogen receptors depend on the agonistic and antagonistic binding of ligands (Begam *et al.*, 2017). The basic difference between the agonistic or antagonistic activity relies on the conformational changes of the receptors, particularly at helix 12, resulting in trans-activation or trans-repression of estrogen target genes (Begam *et al.*, 2017). *In-vitro* studies have shown that pyrethroid pesticides including cypermethrin exhibits estrogenic properties (Chen *et al.*, 2002). Given that cypermethrin is able to bind estrogen receptors (McCarthy *et al.*, 2006) and estrogen signaling is one of the pre-requisites for reproduction in zebrafish; it is conceivable that the cypermethrin-induced repro-toxic effects may underlie disruption of estrogen signaling. To answer this pertinent question, *in-silico* studies have been employed in this study. Now-a-days, the computational studies are widely accepted and used to compliment the experimental data in biological systems (Hong *et al.*, 2002; Ng *et al.*, 2014). In fact, *in-silico* screening is not only helpful in identification and prioritization of compounds to be screened but also reduce the timeline and also the number of compounds to be tested (Ribay *et al.*, 2016). Moreover, such screening through *in-silico* studies might be helpful in desining comprehensive testing strategies via *in-vivo* and high throughput *in-vitro* studies.

Zebrafish is well accepted as a model system for ecotoxicological studies, including the reproductive toxicity analysis and endocrine-disrupting effects of environmental pollutants (Rubinstein, 2006). Moreover, the small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates of zebrafish have also added advantages (Segner, 2009). Zebrafish have asynchronus ovaries, containing follicles at all the stages of development as well as mature eggs (Selman *et al.*, 1993). Most strikingly, zebrafish can absorb small molecules from surrounding waters through chorion, skin and gill, the administration of drugs in minute quantities is straight forward (Parng, 2005). Based on these aspects, zebrafish was selected as test organism in this study. In view of the above, the objective of this study was two fold: first to investigate the effect of cypermethrin on the reproductive performance of male and female zebrafish by considering the reproductive endpoints such as fecundity, plasma vitellogenin levels, and histology of both testis and ovaries and second *in-silico* analysis to evaluate the interaction between cypermethrin and their metabolites with zebrafish estrogen receptors (ERs).

Materials and Methods

Fish maintenance and breeding : Adult zebrafish were purchased from a local pet shop (Anantapur, Andhra Pradesh, India) and reared in animal house throughout the year. Fish were

kept in constant aerated 100 l glass aquaria containing dechlorinated tap water at $26 \pm 2^\circ\text{C}$ under a photoperiod of 12:12 hr light and dark cycles and the same water were used in the experiments. Fish in stock were fed twice a day, with alternating diet of freshly hatched brine shrimp (Sanders Brine Shrimp Co, Utah) and dry flake food (Tetra, Germany).

Experimental design : Adult zebrafish in equal rations ($n=8$) of male and female (6 months old) were exposed to cypermethrin at 0.1, 1 and $10\mu\text{g l}^{-1}$ for a period of 21 days in 20 l full glass aquaria with constant aeration. Simultaneously, control (untreated) and solvent control (treated with solvent alone) fish were maintained in separate tanks. About 50% of water in the aquaria was changed daily, however the concentration of cypermethrin and absolute alcohol at the desired levels were maintained by fresh additions accordingly. The concentrations of cypermethrin was selected based on its presence in surface water (0.078 to $2.8\mu\text{g l}^{-1}$) and rain water ($0.376\mu\text{g l}^{-1}$) as reported by USEPA, (1998) and Laabs *et al.* (2002), respectively.

Fecundity studies: Zebrafish initiates spawning after onset of light. Spawned eggs were collected from the spawning trays using a strainer and the collected eggs were counted for fertilized and unfertilized eggs.

Blood collection and plasma preparation: Blood was collected by puncturing the caudal vein in heparin coated micro-capillary tubes. Plasma was separated by centrifuging the capillary tubes at 1,500 rpm for 3 min. After centrifugation, the tubes were immediately frozen dried by dipping in liquid nitrogen and stored at -80°C until further analysis.

Necropsy and gonad histology: Both male and female zebrafish were dissected and gonads were taken out. The isolated gonads were weighed to their nearest milligram using Shimadzu weighing balance. The gonads of both control and experimental groups were fixed in Bouin's fluid for a period of 24 hr followed by dehydration steps through a graded series of ethanol and embedded in paraffin wax (Leica Histowax, Leica Microsystems, Wetzlar, Germany). Serial paraffin sections were cut at $5\mu\text{m}$, collected onto glass slides, and stained with Periodic-Acid Schiff's or hematoxylin-eosin. Analysis of slide preparations was performed with an Olympus BX 60 microscope.

Vitellogenin analysis: The plasma VTG levels were analyzed based on the protocol described earlier (Fenske *et al.*, 2001) using ELISA technique. The antibodies raised against the zebrafish VTG were used to analyze the levels of VTG in the plasma samples of zebrafish in control and experimental groups. The antigen-antibody complex bound to the plate was detected by a horse-radish peroxidase conjugated secondary antibody. The antigen-antibody interactions were assessed calorimetrically at 450 nm in terms of enzyme activity (Microtitre plate reader: Spectra Max250) by adding a suitable substrate 3,3' diaminobenzidine-tetrachloride and hydrogen peroxide (Fenske *et al.*, 2001).

Homology modelling: The retrieved amino acid sequences of zebrafish estrogen receptor (zER) α (GenBank ID: NP_694491), zER $\beta 1$ (GenBank ID: CAC93848) and zER $\beta 2$ (GenBank ID: AAN60793) were used for homology modeling studies. Based on the blastp analysis, four templates were selected to model the selected zebrafish estrogen receptors. Protein with PDB ID: 1ERE (human estrogen receptor (hER) ligand-binding domain in complex with 17β -estradiol) acts as agonistic conformation template for zER α , while protein with PDB ID: 1ERR (human estrogen receptor ligand-binding domain in complex with raloxifene) acts as the antagonistic conformation template for zER α (Brzozowski *et al.*, 1997). Proteins with PDB ID: 1L2J (hER β ligand binding domain in complex with (R, R)-5, 11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol) acts as a template for both zER $\beta 1$ and zER $\beta 2$. After alignment studies (ClustalW2: Larkin *et al.*, 2007), modelling was performed using the modeller software (Modeller 9v8) using CHARMM force field which uses conjugate gradient method with a convergence gradient of 0.01. At least, ten models were generated, and the modeled structures were subjected to validation analysis using PROCHECK (Laskowski *et al.*, 1993) and ProSA web server (Wiederstein *et al.*, 2007). After selection of suitable model, the modeled structure was subjected to active site prediction analysis using PDBsum (Laskowski, 2009).

Ligand preparation and docking: For molecular docking studies, six ligands were selected: 17β -Estradiol (CID: 5757), 17β -Ethinyloestradiol (CID: 5991), Cypermethrin (CID: 2912), Cyclopropanecarboxylic acid (CID: 91658), 3-Phenoxybenzaldehyde (CID: 38284), and 3-Phenoxybenzoic acid (CID: 19539). Each selected ligand was individually docked to zER α (agonist), zER α (antagonist), zER $\beta 1$ and zER $\beta 2$ using the AutoDockTools (Sanner *et al.*, 1999). The resulting docking conformations were clustered into families of similar conformation, with root mean square deviation (RMSD) clustering tolerance as $1.0\text{ \AA}$. As a rule, the lowest docking energy conformations were included in the largest cluster. Each ligand was docked with each of the zERs to obtain the best binding conformation.

Statistical analysis: The data were expressed as mean $\pm$ S.D. of six individual zebrafish. Student-t-test (SPSS version 16.0) was used to analyze the data statistically. The significance was set at $p < 0.05$.

Results and Discussion

No clinical signs of toxicity were noticed and none of the animals were excluded from this study. No changes were recorded in the courtship and mating behavior in control and experimental fishes at all selected doses over a period of 21 days. However, cypermethrin at a dose of $10\mu\text{g l}^{-1}$ showed lethargic movements and immediate cessation in spawning fishes. The results indicated that the cumulative fecundity (total eggs spawned/treatment) and percent hatchability were similar for all treatment groups before and after exposure to toxicant (Fig. 1).

Fig. 1: Effect of cypermethrin on cumulative fecundity of zebrafish. Graph indicates fecundity during 21 days of exposure in control, solvent control and 0.1, 1.0, 10 µg l⁻¹ concentration of cypermethrin.

Fig. 2: Effect of cypermethrin on plasma vitellogenin concentrations of control and experimental male and female. Bars (mean ± S.D.) represent vitellogenin values, expressed as ng ml⁻¹ of blood plasma (n = 8).

Fig. 3: Effect of cypermethrin on structural integrity of testis and ovaries of adult zebrafish. Phase contrast micrograph pictures of control testis (A); testis of cypermethrin treated fishes (B); control ovary (C) and ovaries of cypermethrin treated fishes (D); [Abbreviations: SG-A: Spermatogonia-A, SG-B: Spermatogonia-B, SPC-A: Spermatocyte-A, SPC-B: Spermatocyte-B, SD: Spermatid, SZ: Spermatozoa; O: Oogonia, PO: Perinucleolar oocytes, CAO: Cortical alveolar oocyte, EAO: Early vitellogenic oocyte, MO: Mature/Spawning oocyte].

The results revealed that the male and female zebrafish exposed to cypermethrin at 0.1, 1 or 10 $\mu\text{g l}^{-1}$ did not affect cumulative fecundity (total eggs spawned/treatment) and hatchability rate (%), suggesting normal fertilization efficacy and spawning of eggs (Wang and Ge, 2003a, b). Previously, it has been shown that fathead minnows exposed to fipronil, a phenylpyroazole insecticide did not cause changes in the testicular index, ovarian index, fertilization rate and fecundity (Bencic *et al.*, 2013). In contrast, Shi *et al.* (2011) suggested that exposure of zebrafish embryos to 25 $\mu\text{g l}^{-1}$ cypermethrin showed a suite of morphological abnormalities such as body-axis curvature, pericardial edema and large yolk sac.

Significant reduction in fecundity and hatchability was observed in fishes as a result of dimethoate toxicity (Ansari *et al.*, 2011). It was also shown that exposure of female zebrafish to environmentally relevant doses of di-2-ethylhexyl phthalate (DEHP) significantly reduce oogenesis thereby embryos (Carnevali *et al.*, 2010). Exposure studies conducted with other pyrethroids like deltamethrin, permethrin and fenvalerate showed

dose response decrease in the hatching success, and hatching time associated with few eggs failing to hatch, thereby reducing fecundity in fishes (Koprucu and Aydin, 2004; Gonzalez *et al.*, 2003; Tanner and Knuth, 1996; Barry *et al.*, 1995). Published reports also indicated that exposure of salmon eggs and milt to 0.1 $\mu\text{g l}^{-1}$ and 0.5 $\mu\text{g l}^{-1}$ resulted in reduced fertilization rates by 47% and 39%, respectively (Moore and Waring, 2001). The results of the present study corroborates with previous reports and suggest that the fecundity and hatchability rate of fishes may vary with pesticides. In this study, zebrafish showed behavioural abnormalities such as lethargic movements in male fishes exposed to cypermethrin at 10 $\mu\text{g l}^{-1}$. In addition, adverse effects on reproductive behavior of male *Salmo trutta* L. (parr) at 1 $\mu\text{g l}^{-1}$ cypermethrin was also reported (Jaensson *et al.*, 2007).

The average plasma vitellogenin levels in untreated male and female zebrafish were 193.85 and 361831.12 ng ml^{-1} respectively. No significant changes were observed in the plasma vitellogenin levels in male zebrafish exposed to cypermethrin at 10 $\mu\text{g l}^{-1}$ as compared to controls (Fig. 2). Similar trend in the

Fig. 4: Ramachandran plots for the homology modeled zfERα (agonist), zfERα (antagonist), zfER β1 and zfER β2.

vitellogenin levels of control and experimental female zebrafish was observed (Fig. 2). Vitellogenin is a process through which the developing oocyte is nourished. Vitellogenin is a female specific marker and also a yolk protein which is transported from its target site, liver to ovaries in fishes. Several studies have demonstrated conflicting results with regard to vitellogenin level in fishes exposed to varied range of xenoestrogens. Exposure of male and female zebrafish to chemicals such as butachlor, DEHP (Carnevali *et al.*, 2010) and β -endosulfan (Han *et al.*, 2011) showed increased levels of vitellogenin. While, disturbances in

the reproductive efficacy in male zebrafish as indicated by alterations in spermatogenic stages and a decrease in the vitellogenic oocytes of female zebrafish was observed during dehydroabetic acid and betulinol exposure (Christianson-Heiska *et al.*, 2008). Few studies also demonstrated that exposure of zebrafish embryos to bisphenol A modulates the expression levels of vitellogenin gene (Naderi *et al.*, 2014). Conversely, zebrafish juveniles exposed to prochloraz at high concentrations diminished vitellogenin levels whereas at low concentrations enhanced the levels of vitellogenin (Kinnberg *et al.*, 2007). On the

Fig. 5: Docking images of A: zER α (agonist), B: zER α (antagonist), C) zER β 1 and D) zER β 2 with selected ligands [Estradiol (a), Ethinyl Estradiol (b), Cypermethrin (c), Cyclopropanecarboxylic acid (d), 3-Phenoxybenzaldehyde (e) and Phenoxybenzoic acid (f)] The hydrogen bonds are represented with yellow dotted lines.

other hand, up-regulation of vitellogenin gene expression was noticed in male and female zebrafish at low concentrations of perflouroctanesulfonate (Du *et al.*, 2009). Exposure of fathead minnows to fipronil did not show significant change in the VTG levels as compared to controls (Bencic *et al.*, 2013). However, cypermethrin at of $10 \mu\text{g l}^{-1}$ did not show significant changes in the plasma vitellogenin levels of control and experimental fishes (either males or females).

Histological analysis of testis and ovaries directly reflect the toxicological effects of xenoestrogens, and thus believed to be valuable endpoints of reproduction. In the current study, the testicular architecture of control zebrafish exhibited an intact epithelium as basement membrane with all stages of spermatogonia, spermatocytes, spermatid and spermatozoa (Fig 3A) on the other hand, no structural differences were observed in the testis of cypermethrin exposed fishes (Fig 3B). However, the relative frequency of spermatozoa was deteriorated by 12% ($p < 0.01$) in cypermethrin exposed zebrafish over controls. This in turn might suggest that the selected dose of cypermethrin was able to induce spermatotoxic effects without disturbing the histological architecture of testis in zebrafish. In female zebrafish,

the ovarian histology exhibits oogonia, perinucleolar oocyte, corticle alveoli, matured oocyte and post ovulatory follicles (Fig. 3C) on the other hand, similar ovarian histological observations were noticed in cypermethrin exposed zebrafish, suggesting cypermethrin did not affect the structural integrity of ovaries (Fig. 3D). Previously, it has been shown that cypermethrin at 0.02 ppb showed no histological changes in the testis of freshwater fishes, *Esomus danricus* (Saravanan *et al.*, 2016). Surprisingly, these authors reported that 0.02 ppb cypermethrin showed cytoplasmic agglutination in the ovaries of *E. danricus*. Studies also indicated that zebrafish exposed to combination of pesticides including cypermethrin showed disruption of ovary, liver, kidney and gills architecture (Rajini *et al.*, 2015). Previous reports reveal that bluegill fishes, *Lepomus macrochirus* exposed to $1 \mu\text{g l}^{-1}$ endosulphan over a period of two weeks showed deterioration of testicular architecture (Dutta *et al.*, 2006). Similarly, zebrafish exposed to dioxin 50 pg ml^{-1} showed reduction in the thickness of testicular germinal epithelium (Baker *et al.*, 2016). Piecing the results, we hypothesize that the toxic effect of chemicals in zebrafish might be at least in part depend on the type of chemical, duration of experiment, quantity of chemical used, stage of life cycle and gender. Based on the present findings and studies of

Shi *et al.* (2011), reported that cypermethrin at $10 \mu\text{g l}^{-1}$ could induce spermatotoxic effects as indicated by reduced sperm number in zebrafish but the same dose of cypermethrin was ineffective to interfere with the fertility efficacy. The exact mechanism underlying deterioration of sperm number in fishes exposed to $10 \mu\text{g l}^{-1}$ cypermethrin could not be determined in this study. However, spermatogenesis is controlled by several factors including estrogens and their signaling in vertebrates including fishes (Schulz and Miura, 2002). Previously, the estrogenic properties of cypermethrin have been demonstrated *in-vitro*. Thus, we hypothesize that cypermethrin and their metabolites were able to interfere with male reproduction in zebrafish via estrogen receptor signaling. To test this hypothesis, *in silico* analyses was also performed in this study.

For molecular docking studies, a reliable structure of protein is necessary. Therefore, a modeled structure of zebrafish estrogen receptors were analyzed for their reliability in terms of structural aspects. Stability of models was analyzed based on the Ramachandran plot statistics (97.2%, 95.8%, 96.1% and 94.3% of residues were present in the allowed region for zFER α (agonist), zFER α (antagonist), zFER $\beta 1$ and zFER $\beta 2$, respectively (Fig. 4). ProSA analysis showed that the selected model of zFERs revealed the resemblance of native structures with a Z-score of -6.82, -6.78, -7.24 and -6.73 for zFER α (agonist), zFER α (antagonist), zFER $\beta 1$ and zFER $\beta 2$, respectively, conforming that the selected models generated by Modeller 9v8 were highly reliable. Accordingly, the stable structures were used for molecular docking studies.

The results of molecular docking analysis (Fig. 5) indicated that the selected ligands showed interactions with the zebrafish estrogen receptors with the following binding energies: zFER α (agonist), zFER α (antagonist), zFER $\beta 1$ and zFER $\beta 2$ were as follows: 17 β -estradiol ($-10.30 \text{ kcal mol}^{-1}$, $-9.92 \text{ kcal mol}^{-1}$, $-10.28 \text{ kcal mol}^{-1}$ and $-9.36 \text{ kcal mol}^{-1}$), 17 β -ethinyl estradiol ($-10.54 \text{ kcal mol}^{-1}$, $-9.92 \text{ kcal mol}^{-1}$, $-10.25 \text{ kcal mol}^{-1}$ and $-9.73 \text{ kcal mol}^{-1}$), cypermethrin ($-7.98 \text{ kcal mol}^{-1}$, $-8.72 \text{ kcal mol}^{-1}$, $10.40 \text{ kcal mol}^{-1}$, $8.84 \text{ kcal mol}^{-1}$), cyclopropanecarboxylic acid ($-7.37 \text{ kcal mol}^{-1}$, $-6.95 \text{ kcal mol}^{-1}$, $-6.39 \text{ kcal mol}^{-1}$, $-7.12 \text{ kcal mol}^{-1}$), 3-Phenoxybenzaldehyde ($-6.34 \text{ kcal mol}^{-1}$, $-7.16 \text{ kcal mol}^{-1}$, $-6.81 \text{ kcal mol}^{-1}$ and $-6.97 \text{ kcal mol}^{-1}$) and 3-Phenoxybenzoic acid ($-7.12 \text{ kcal mol}^{-1}$, $-8.07 \text{ kcal mol}^{-1}$, $-7.85 \text{ kcal mol}^{-1}$ and $-8.13 \text{ kcal mol}^{-1}$).

The basic difference between the ER α agonist and ER α antagonist lies in the conformation changes of LBD wherein the agonist-LBD interactions adopt an active conformation: H12 rests across H3 and H11, facilitating downstream activation process via accommodating the binding of a co-regulator. On the other hand, antagonist-LBD, H12 is displaced from this position resulting in distortion of this co-regulator binding groove and inhibition of receptor activation (Ng *et al.*, 2014). It has been reported that activation and/or inhibition of ER-LBD actions, at least in part depend on the agonist-ER-LBD and antagonist-ER-LBD interactions, respectively (Ng *et al.*, 2014). From the binding energies and interactions between the selected ligands and

estrogen receptors, this study proposes that cypermethrin, 3-Phenoxybenzaldehyde, 3-Phenoxybenzoic acid and 17 β -ethinyl estradiol might possess ER antagonistic properties, thereby directly (competitive effect) and/or indirectly (allosteric effect) preventing the binding of agonists.

The results of *in-silico* analysis demonstrated that the interactions of cypermethrin and its metabolites with estrogen receptors in zebrafish might provide a clue towards the cypermethrin induced reprotoxic effects. However, to gain further insights into the mechanism(s) underlying cypermethrin-induced inhibition of spermatogenesis in zebrafish, studies encompassing both biochemical and molecular aspects are required. Studies in this direction might also throw a light on epigenetic mechanisms. To summate, cypermethrin at $10 \mu\text{g l}^{-1}$ provokes inhibition of spermatogenesis. Furthermore, the interactions between cypermethrin and their metabolites with zebrafish estrogen receptors (*in silico*) might pose one of the important questions with regard to the mechanism(s) underlying cypermethrin-induced repro-toxic effects in zebrafish.

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