CYPERMETHRIN INDUCED CYTOPATHOLOGICAL, PHYSIOLOGICAL AND CYTOCHEMICAL CHANGES IN *PARAMECIUM CAUDATUM* AND *BLEPHARISMA INTERMEDIUM*

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Abstract

The main aim of the present work is to study the acute toxicity, physiological and cytochemical effects of Cypermethrin to freshwater ciliate protozoa *Paramecium caudatum* and *Blepharisma intermedium*. Acute toxicity tests were done for 3 hours and threshold, LC_{50} and LC_{100} values were derived. The calculated LC_{50} values were 60.23ppm and 93ppm for *Paramecium caudatum* and *Blepharisma intermedium* respectively. The Cypermethrin induced significant decrease in contractile vacuole activity and food vacuole activity with that of controls at sub-lethal concentrations such as 5ppm, 10ppm, 15ppm & 20ppm to *Paramecium caudatum* and 12ppm, 18ppm, 24ppm & 30ppm for *Blepharisma intermedium*. Cytochemical studies revealed that Cypermethrin induced various macro nuclear changes such as unevenly divided, fragmented, vacuolization, karyolysis and rod shaped in tested organisms. Based on bioassay tests, *Paramecium caudatum* found to be more sensitive than *Blepharisma intermedium*. In conclusion, *Paramecium caudatum* and *Blepharisma intermedium* could be used as complementary models to assess cytotoxic potential of Cypermethrin invitro studies.

Keywords

In vitro studies, Morphological deformities, Cytophysiology, DNA damage

Introduction

Insecticides have played a major role in the drastic increase of agricultural yield in India over the past 50 years but many of these insecticides may be harmful to the natural environment and non-target organisms through surface run-off from the treated area (Singh et al., 2008; Stueckle et al., 2008). To determine acute and chronic effects, bioassay has been considered to be a hopeful approach, to predict potential biological effects (Bae and Park, 2014). The best suited organisms for such bioassays are determined by the substances to be assessed and the sensitivity of the organism (Fawell, J.K., and S. Hedgecott, 1996). Ciliates are capable water quality bioindicators and can be used in evaluating the toxicity of pollutants (Paiva and silva-Neto, 2004; Madoni 2006; Morange, M.,2006). Also, they have high taxonomic density and sensitive to various concentrations of pollutants provide response to different pollutant levels (Pearl et al., 2003). Ciliates exhibit high conservation of genes and better matches of coding sequences to those of humans, hence can be used as model in ecotoxicological studies as an alternative to eukaryotic organisms (Gutierrez J.C., Gonzalez A.M., Diaz S. and Ortego R., 2003). Ciliates show minimum epigenetic variability and a single clone can be preserved for years together generation after generation (Amanchi N.R. and Masood hussain 2012). In the present studies, *Paramecium caudatum* and *Blepharisma intermedium* were selected as test species to evaluate toxic effects of Cypermethrin.

Material and methods

Test compound: Cypermil is a commercial grade, synthetic pyrethroid insecticide, used to control many pests, including moth pests of cotton, fruit and vegetable crops. It contains 20% w/w Cypermethrin, 8.8% w/w Emulsifier and 71.2% w/w solvent and manufactured by Insecticides India Limited. Stock solution of 1000ppm and experimental concentrations of Cypermethrin were prepared freshly as recommended by APHA (2007).

Organisms selected for the present studies: The freshwater samples were collected from different water bodies in Hyderabad for identification and isolation of *Paramecium caudatum*. Pureline stock culture of *Blepharisma intermedium* was supplied from Carolina Biological suppliers, NC, USA, for present studies.

Ciliate culture: The organisms were cultured separately using hay infusion and distilled water in 1:1 ratio at room temperature. Boiled okra was added as nutrient supplement for *Paramecia* and for *Blepharisma*, two grains of boiled wheat was added. Dilution technique was used to isolate *Paramecium caudatum* and pure line cultures were developed. Sterile conditions were maintained in all the experiments.

Acute toxicity tests: acute toxicity tests were conducted to measure immediate responses under stress conditions caused by Cypermethrin, as suggested by Apostol (1973). 0.5ml known concentration of pesticide solution was added to 4.5ml culture medium containing 50 organisms in a cavity block to achieve desired concentration of Cypermethrin. Three observations were made for all test concentrations. Controls were maintained simultaneously with the same number of organisms. Counting of number of organisms was done for every 10mins interval for the first 1hr and then for every 20mins interval during the next 2hrs using binocular microscope. Threshold, LC_{50} and LC_{100} values were calculated using probit analysis (Finney 1953).

Contractile vacuole activity: Pulsatory activity was studied by arresting the movement of test models by using protamine coated slides as suggested by Marsot and Couillard (1973). 25 organisms of each type were exposed to different sub-lethal concentrations such as 5ppm, 10ppm, 15ppm & 20ppm for *Paramecium caudatum* and for *Blepharisma intermedium*, 12ppm, 18ppm, 24ppm & 30ppm for 10 and 20min. 5 replicates and controls were maintained. Single individuals normal in every visible respect were picked and the rate of pulsation of one vacuole was determined. Rate of pulsation is the time required for one complete pulsation. Observations were recorded on the cells in each concentration. The rate of pulsation for each individual is calculated and compared with the control.

Food vacuole activity: studies were carried out for one hour after exposure to sub lethal concentrations of Cypermethrin. The cells were divided into two groups, exposed and control. 25 treated cells from each concentration, were picked with the help of micropipette, mixed with 3% India ink and kept for 10min. 10 organisms from each concentration were taken, immobilized on protamine coated slides. Preparation of India ink and counting of food vacuoles were done by the method suggested by Donna M. Bozzone (2000).

Cytochemical changes: In Cytochemical studies, nuclear staining was done by Feulgen fast green technique and it was found to be the most suitable technique as suggested by Rizzo and Nooden (1973).
Schiff's reagent was prepared as suggested by De Tomasi (1936). 4.5ml of culture media containing log phase ciliate cultures were taken into cavity block and 0.5 ml of test concentration was added. After one hour exposure, 2-3 drops of culture media containg 25 treated ciliates were placed on slide and air dried. 10 observations were made for each test concentration to each organism followed by cell fixation in carnoy's fixative. The cells were hydrolyzed first briefly in 1N Hcl at room temperature and washed in distilled water. Hydrolysis followed by transfer of slides into Schiff's reagent and incubated for 1 hour. The cells were immersed in three changes of sodium metabisulfite solution, again rinsed with water, dehydrated in graded alcohols, cleared in xylen and mounted in DPX. These slides were observed under microscope at 100X magnification and nuclear changes were recorded.

Statistical analysis: Probit analysis was used to calculate the LC_{50} values from acute toxicity tests. Data on contractile vacuole activity, food vacuole activity and cytochemical changes were analysed by Paired t-test to determine significant difference. A value of $P < 0.05$ was considered statistically significant.

Results

Acute toxicity studies

The concentrations like 5ppm, 10ppm, 15ppm, and 20ppm were showed no visible morphological changes and above 20ppm concentration triggered behavioral changes in the *Paramecium caudatum such as* speedy, rotatory movements towards the corners of the cavity block. 40ppm, 50ppm, 60ppm concentrations, brought cytopathological changes like oval shape, blackening of cytoplasm and blebbing. 160ppm concentration brought immediate cytopathological changes leading to total lysis of cell. The calculated LC_{50} value for 3hrs duration was found to be 60.23ppm (Fig:1A). The resulting LC_{50} value for *Blepharisma intermedium* exposed to Cypermethrin was 93ppm (Fig:1B). *Blepharisma intermedium* showed various behavioral changes in lower concentrations of Cypermethrin. LC_{100} was found to be 200ppm where complete death of the cells was observed.

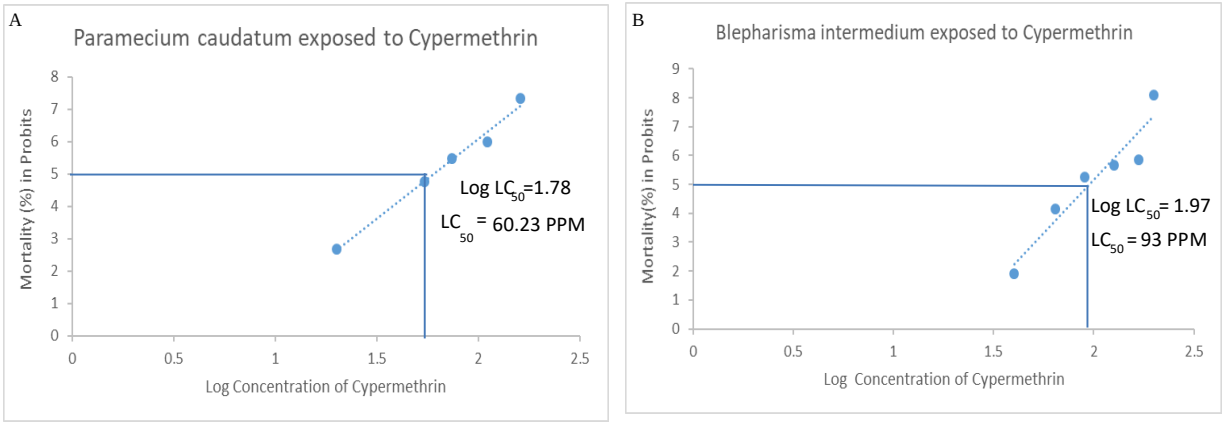

Figure: 1. Acute toxicity effect of Cypermethrin on A. *Paramecium caudatum* B. *Blepharisma intermedium*

Contractile vacuole activity

Cypermethrin had extreme inhibitory effect on contractile vacuole activity of *Paramecium caudatum* at 20ppm concentration for 20 minutes exposure, where the average time for one pulsation was recorded as 33.3 seconds. It is much higher than the control value which is 12.5 seconds (Table: 1). Least inhibition was observed at 5ppm, where 17.64 seconds were required for 20minutes exposure. In *Blepharisma intermedium* the mean & SD value of pulsation per minute was 2.4±0.49 in control, where as in treated groups like 12ppm, 18ppm, 24ppm, & 30 ppm, the mean & SD values of pulsations per minute recorded were 1.8±0.4, 1.4±0.49, 1.2±0.4, 1±0 for 20 minutes exposure (Table: 2).

Table 1: Effect of Cypermethrin on Contractile vacuole activity of *Paramecium caudatum*

Concentration in ppm	Exposure time in minutes	Average time for one pulsation in seconds	Average pulsations per minute and SD Values
control		12.5	4.8±0.4
5	10	15.78	3.8±0.4
	20	17.64	3.4±0.49
10	10	20	3±0
	20	21.4	2.8±0.4
15	10	23.07	2.6±0.49
	20	25	2.4±0.49
20	10	27.27	2.2±0.4
	20	33.3	1.8±0.4

The values Mean±SD of five observations; Student t-test was performed, and values are significant $P < 0.05$

Table 2: Effect of Cypermethrin on Contractile vacuole activity of *Blepharisma intermedium*

Concentration in ppm	Exposure time in minutes	Average time for one pulsation in seconds	Average Pulsations per minute and SD Values
control		25	2.4±0.49
12	10	33.33	1.8±0.4
	20	33.33	1.8±0.4
18	10	37.5	1.6±0.49
	20	42.85	1.4±0.49
24	10	42.85	1.4±0.49
	20	50	1.2±0.4
30	10	50	1.2±0.4
	20	60	1±0

The values Mean±SD of five observations; Student t-test was performed, and values are significant $P < 0.05$

Food vacuole activity

The food vacuole activity in *Paramecium caudatum* has shown significanct variation with respect to different treatment groups such as 10ppm, 15ppm & 20ppm (Figure:3). When compared to control, highest negative impact on food vacuole activity was seen in 20ppm exposed for one hour with 60% decrease in the food vacuole activity ($p<0.05$) followed by 15ppm with 37.6% decrease and 10ppm with 23.8% decrease. In *Blepharisma intermedium* the retardation in food vacuole activity was significant at 18ppm, 24ppm & 30ppm (Figure: 4). 55% reduction in 30ppm, 34% reduction in 24ppm and 21% reduction in 18ppm was observed compared to control food vacuole activity ($p<0.05$).

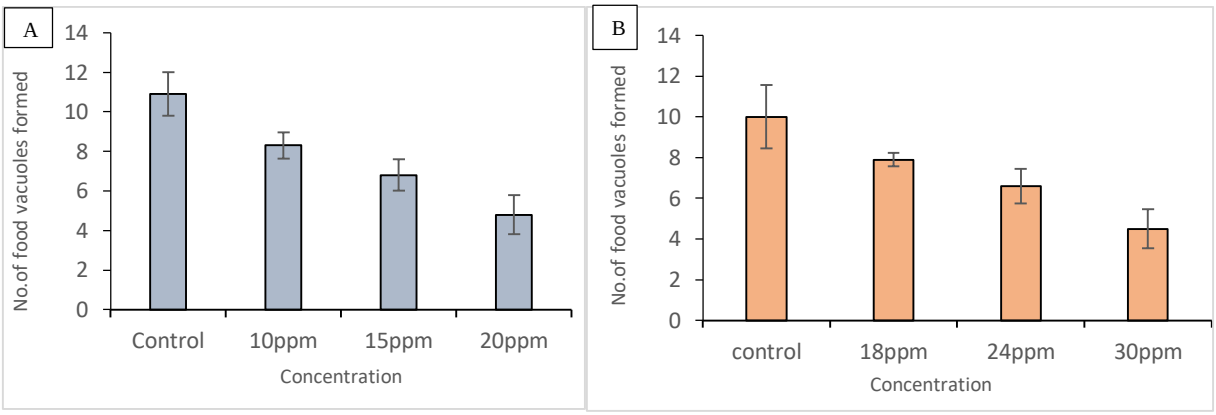

Figure:2 Effect of Cypermethrin on food vacuole activity of A. *Paramecium caudatum* B. *Blepharisma intermedium*

The values Mean±SD of ten observations; Student t-test was performed, and values are significant $P < 0.05$.

Cytochemical studies

The treated *Paramecium caudatum* underwent various macronuclear changes under insecticidal stress. The nuclear abnormalities were categorized into Rod shaped, unevenly divided, vacuolated, karyolysis and other deformities. In higher concentration of Cypermethrin that is at 20ppm *Paramecium* showed 60.2% total nuclear abnormalities, in which rod shaped 11.8%, unevenly divided 5.7%, vacuolated 10.3%, karyolysis ↑20%, other abnormal forms 12.9% (Figure:3). When *Blepharisma intermedium* exposed to Cypermethrin, cells exhibited various nuclear changes like unevenly divided, vacuolated, karyolysed, fragmented and other deformities. Unevenly divided forms were found more in number than the others. Highest percent of total abnormalities were observed at 30ppm (42%) and lowest was at 12ppm (19.9%) (Figure:4).

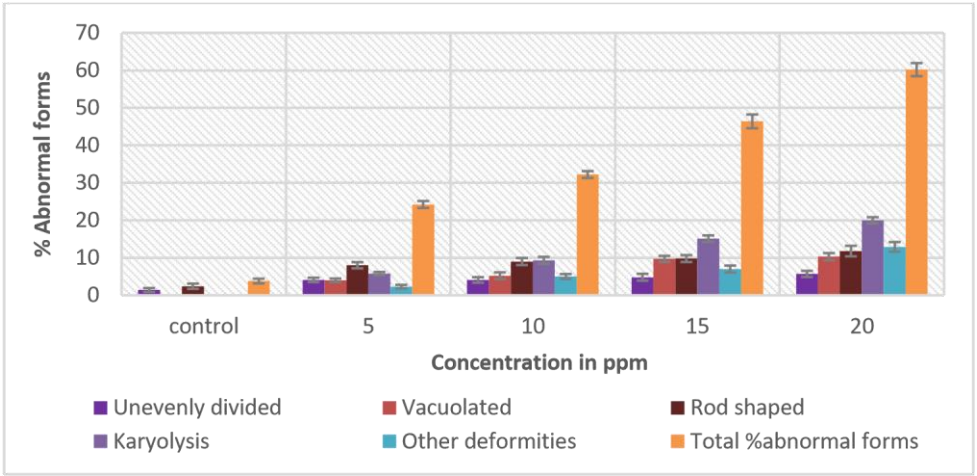

Figure: 3. Macronuclear changes in *Paramecium caudatum* exposed to Cypermethrin

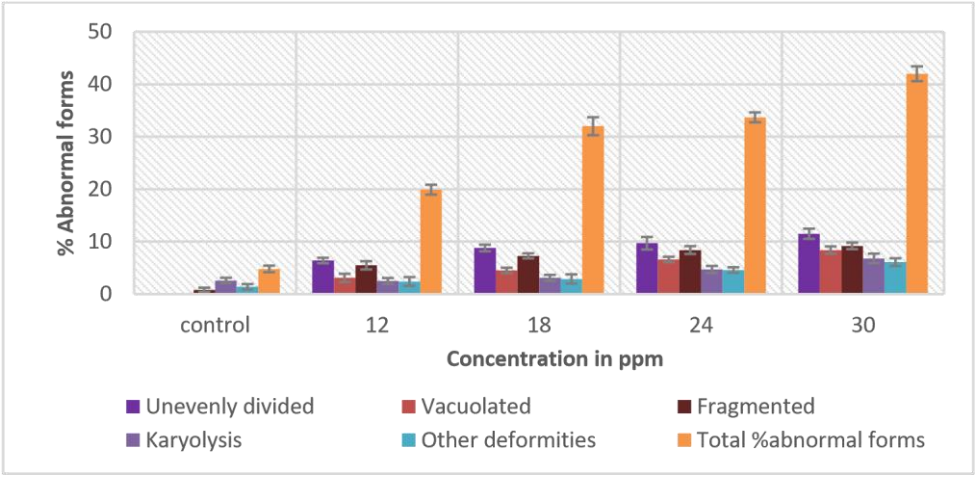

Figure: 4. Macronuclear changes in *Blepharisma intermedium* exposed to Cypermethrin

The values Mean±SD of ten observations; Student t-test was performed, and values are significant $P < 0.05$.

Discussion:

Cypermethrin is a hydrophobic molecule which can simply pass through the cell membrane and distracts the cell structure and cause leakage of cytoplasmic enzymes (Hussain et al., 2013). In the present studies contractile vacuole activity results showed a significant decrease in the mean values of pulsations per minute for 10 & 20 minutes exposure with that of controls. Kitching, (1956); Stock et al., (2001), (2002) explained that any change in the ionic composition of the surrounding environment responsible for the change in the ionic composition of cytoplasm plus the fluid in the contractile vacuole. nd osmolarity of the fluid in the contractile vacuole is always hypertonic to the cytoplasm and osmolarity of the cytoplasm is always hypertonic to exterior osmolarity. Toumani, (2002) reported the rate of water expulsion was reduced when external osmolarity was increased in *Paramecium*. Also, Masaki, (1993) reported concentration and time dependent decrease in the contractile vacuole activity of *Paramecium* exposed to mAB DS-I (secondary antibody). Similar changes were recorded by Amanchi, (2012) on other ciliate model *Euplotes patella* exposed to Delfin insecticide. Damage to contractile vacuole apparatus under high stress conditions caused by Cypermethrin may be another reason for significant reduction in pulsatory output of tested organisms.

The average food vacuoles formed in control *Paramecium caudatum* and *Blepharisma intermedium* were 10 and 10.9 respectively, whereas in treated groups formation of food vacuoles was decreased in concentration dependent manner. Cypermethrin might have caused damage to cell membrane which in turn causes damage to cilia. Nilson, 1981; 2005, Masood et al., 2008, Ouissem (2016) demonstrated that alterations in food vacuole formation in ciliates under stress conditions caused pesticides. Changes in external environment reduce the ability of contractions of cytostome, which in turn causes diminish in food vacuole number. The present results agreed with those of Jaleel (2002) and Amanchi et al., (2012). They stated that inhibition in food vacuole formation could be due to damage in cell membrane and cilia.

Cypermethrin damages lipids, proteins and DNA in cells causing cell death (Ferrari, 2000). Concerning the present studies, nuclear changes such as rod shaped, unevenly divided fragmented and karyolysis were observed in both the experimental organisms to all the tested concentrations. This result is in agreement with Amanchi et al., 2008. Similar studies on higher animal models of rats revealed Cypermethrin induces nuclear changes in the liver cells such as ovoid nucleus and cytoplasmic vacuolation (Aldana et al., 1998; Manal Abdul- Hamid et al., 2017).

Conclusion

The present results revealed that Cypermethrin caused damage to cell structure, morphology and damage to internal organells and their function. *Paramecium caudatum* found to be more sensitive to Cypermethrin than *Blepharisma intermedium*. In conclusion, ciliates found to be useful models for toxicity assays replacing higher animal models.

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