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A low cost microbiotest for screening behavioural and ecotoxicological responses of *Paramecium caudatum* and *Oxytricha fallax* to azadirachtin

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ABSTRACT

In the present study experiments were conducted to evaluate the toxic effects of azadirachtin in ciliated models Paramecium caudatum and Oxytricha fallax, which are considered as ideal organisms for investigating water quality fluctuations and risk assessment. Cell viability, cell morphology, cytopathological responses and macronucleus tests were performed using Paramecium caudatum and Oxytricha fallax to different concentrations of azadirachtin. The calculated LC_{50} value of azadirachtin against mortality curve for 3hrs exposure was found 239.44±13.94 and 263.006±13.1ppm to Paramecium caud atum and Oxytricha fallax respectively. Paramecia were more sensitive than Oxytricha to azadirachtin. Azadirachtin affected cell bahaviour, locomotion and cellular morphology of both the organisms. After a short period of exposure (20min to 30 min), there was an increase in the number of necrotic cells with typical features like blackening of cytoplasm, blebbing, macronuclear changes and leaking of internal contents leading to cell lysis. Morphological changes occured in the shape of macronucleus which were dependent on concentration. The macronuclear changes were significant showing deformities such as rod shape, marginalization of nucleus, fragmentation, uneven division and total diffusion of nucleus. The present findings indicate a possible necrotic and genotoxic effect of azadirachtin on both the organisms and these assays suggest the potential of ciliates for ecotoxicological studies.

Key words: *Paramecium caudatum*, cytopathological responses, blebbing, acute toxicity, macronucleus,

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INTRODUCTION

The fresh water ecosystems are greatly exposed to different toxicants from wastewaters. In recent times, pesticides are of major concern finding their way into the environment (Gupta 2004). Pesticides are chemical products used to eliminate the harmful organisms, but most of them will affect the non-target or beneficial organisms also by means of absorption, ingestion, respiration etc. The toxic effects of pesticides on protozoa, especially on ciliates are undeniable. Among the microbial communities, ciliate protozoa are valuable organisms for toxicological investigations, possessing a number of desirable features. The ciliates have been proved to be fascinating little animals being exponent of single cells and of whole organisms (Morange 2006). Since ciliates are unicellular and sensitive; they can be in very close contact with the environment and thus respond intimately to any kind of unfavorable stress. Ciliates have many advantages such as ubiquitous distribution, high reproductive rate, ease of culturing and accessibility of experimental manipulation which render ciliates to be used as test organisms in laboratory experiments (Weisse 2006). Ciliates showed high conservation of genes and better matches of coding sequences to those of humans and hence can be used as alternative models to eukaryotic organisms in ecotoxicological studies (Gutierrez *et al*. 2003). Ciliates also play a significant role in the ecosystem as they can provide an early warning indicator of changes in the environment and also perform key functions in energy flow and elementary recycling in ecosystems. Water quality is essential for the maintaining the health of aquatic organisms, the presence of toxic substances in water is a threat to organisms that are living there (Martin-Gonzalez et al. 2006). The present study was undertaken to better characterize the mechanism of action of azadirachtin in non target protozoan organisms. They allow to specify the interactions between a test pesticide and target cells and to identify the disturbed mechanism, altered or induced by the azadirachtin. An attempt has also been made to look into macronuclear deformities caused by azadirachtin in *Paramecium caudatum* and *Oxytricha fallax*.

MATERIALS AND METHODS

The commercial grade sample of azadirachtin was supplied by Hyderabad chemical suppliers Ltd, Hyderabad, India. Azadirachtin ($C_{35}H_{44}O_{16}$) is a widely used natural insecticide known to affect many species of insects by acting mainly as an antifeedant and growth disruptor. It is a chemical compound belonging to limnoids and is a secondary metabolite present in the neem tree seeds. *Paramecium caudatum* and *Oxytricha fallax* were selected as test species for present studies because of their noticeable abundance in aquatic environment and easy to culture and maintain in the laboratory. They were collected from fresh water pond within the vicinity of Osmania University, Hyderabad, India. Six grams of dried hay was boiled in one liter of distilled water, cooled and filtered. It was then sterilized in an autoclave for fifteen minutes at fifteen pounds pressure and preserved for the future use. Meat extract was supplemented to boost the bacterial multiplication. The log phase cultures were used for the present studies (Fig.1) (Shiny *et al*. 2005). The hay infusion medium was found as most appropriate culture medium for raring ciliates. For culturing the organisms, hay infusion medium was diluted with distilled water in the ratio of 1:1 and was poured into different cavity blocks. It gives the ciliates an environment nearest to their own habitat and therefore can maintain their normal metabolic activities even after continuous culturing. Sterile precautions were maintained throughout the study.

The toxicity effect at lower concentrations has been evaluated on the cell behaviour, morphology and cell viability. Stock solution and experimental concentrations of azadirachtin were prepared as recommended by APHA (1995). Stock solution of 1000ppm of azadirachtin was prepared using double distilled water. After preliminary rough dose finding experiments, the appropriate stock solutions and the test concentrations were selected, prepared afresh and used for the toxicity studies as suggested by Hussain *et al*. (2008). Acute toxicity test was conducted for 3hrs. In acute experiments 0.5ml of pesticide solution was added to 4.5ml of culture medium to achieve desired concentration of pesticide. Triplicates were maintained for all test concentrations. 50 organisms were introduced in each cavity block. Each cavity block, after adding pesticide was placed under binocular microscope and counting was done at 10min interval during first 1hr and thereafter 20min interval during the next 2hrs. LC_{50} value and lethal concentration were calculated against the mortality curve for 3hrs. Controls devoid of pesticide, with same number of organisms were run simultaneously. Nuclear staining was done to demonstrate and study the macronuclear changes in both the organisms on exposure to selective concentrations of azadirachtin using fuelgen fast green technique as suggested by Hardie *et al*. (2002). Around 50 organisms of both Paramecia and Oxytricha were exposed separately to the selected concentrations for 72 hrs duration with three replicates each. The treated and control cells were air dried. Fixation of cells was done by using 4% formalin for a period of 6 min followed by a short rinse with distilled water. The cells were then hydrolyzed first briefly in 1N HCl maintained at room temperature and then incubated 1N HCl at 60^{o}C exactly for 8min. The slides were then rinsed and washed with double distilled water. The hydroloysis was followed by transferring the slides to Schiff's reagent for a period of one hour. Then the slides were immersed in three changes of sulfurous acid salt solution for 6min, again rinsed in water, dehydrated in graded alcohols, cleared in xylene and mounted in DPX.

Statistical analysis

All the results were presented with suitable statistical interpretation such as test of significance, Mean and SD using Origin 6.1 software.

RESULTS AND DISCUSSION

Toxicity assessment and cytopathological studies

Paramecia and Oxytricha cells exposed to azadirachtin underwent a series of behavioural and morphological changes such as alteration in size and shape. The calculated LC_{50} value of azadirachtin against mortality curve for 3hrs exposure was found to be 239.44±13.94 and 263.006±13.1ppm to *Paramecium caudatum* and *Oxytricha fallax* respectively ($P < 0.001$). Concentration of 600ppm and above caused lethality in all the Paramecia within 5min of exposure. 650ppm and above concentrations of azadirachtin killed the Oxytricha instantly. The Oxytricha showed somewhat swollen and rounded body shape with blebbing of cytoplasm at higher concentrations. In Oxytricha death was equated with the cessation of cytoplasmic movement rather than with the rupture of cells. At the concentration of 100 and 150ppm Paramecia showed abnormal and disturbed movements. In first five minutes, rocking movements were observed, which are progressively retarded and in another 15min their ciliary movements were totally arrested. Shortening of longitudinal axis, formation of cytoplasmic vacuoles, blebbing and blackening of cytoplasm contents due to diffusion and mixing of the vacuolar contents were observed at 150 and 200ppm concentrations (Fig. 2 & 3). No visible

morphological and cytotoxic effects were observed at 50ppm concentration but the cells aggregated around the corners of cavity block. The present work shows that the Paramecia are more sensitive to azadirachtin than Oxytricha and are potential bioindicators of water pollution. They are also tools to study both pesticide toxicity and its possible necrotic effects on non-target organisms.

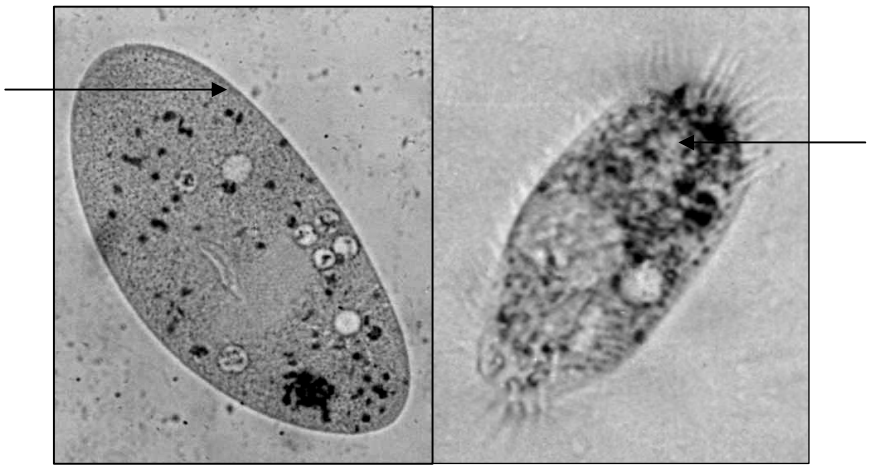

Fig 1: *Paramecium caudatum* and *Oxytricha fallax* showing contractile vacuole (Control), (A& B) (400X).

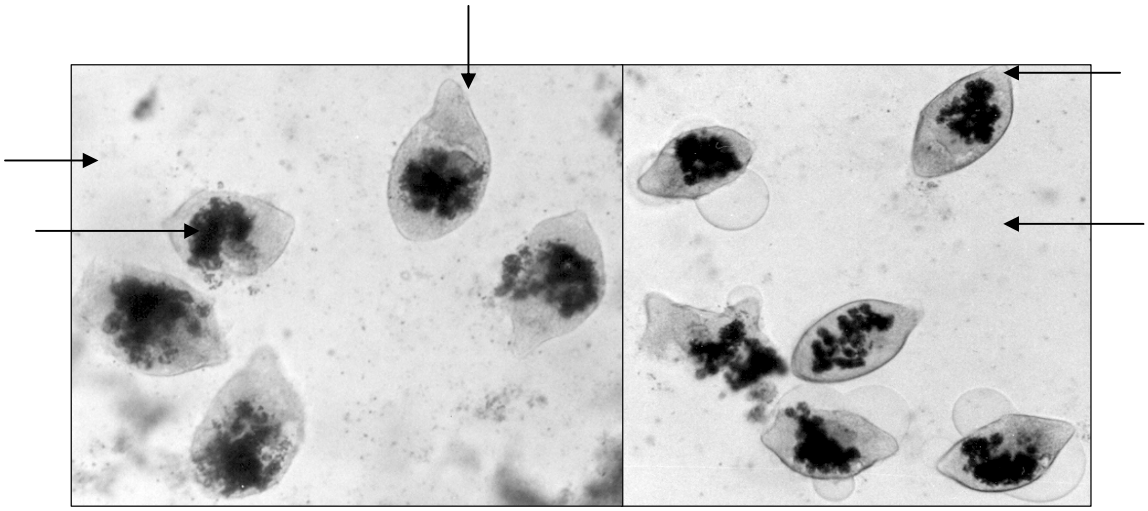

Fig 2: Azadirachtin induced morphological deformities in Paramecia. E: swelling, shortening of longitudinal axis, blackening of cytoplasm and narrowing of anterior (200X). D: Oval or spindle shape deformity, vacuolization of cytoplasm, blebbing and rupturing of cell membrane (200X).

In short term acute toxicity studies, first visible changes to occur at lower concentrations were irregularities in ciliary beating, which often resulted in spinning movements and swimming away. Under pesticidal stress, many cells could not move normally in a straight line but were spinning around themselves. The motility of cells was progressively decreased and became erratic until movement completely ceased. At higher concentrations, the movement of the cilia became weaker and irregular and after a while the organism died. Stress egestion of food vacuoles and pulsatory changes in contractile vacuole activity were also observed. Similarly, Nilsson (2005) reported loss of cell shape and enlargement of contractile vacuole in Tetrahymena on exposure to higher ethanol concentration. Mahboob *et al*. (1998) reported lethal dose of vepacide *Azadirachta indica* was 1566.85±134.06 mg/kg in rat by the oral route. The rats showed symptoms such as dullness, irritation, lacrimation and diarrhea. It also altered food intake, body weight, and hematological and biochemical parameters in rats (Rahman & Siddiqui 2004). The cell morphology, viability, uptake, metabolism and accumulation of parathion were studied at lower concentrations using *Tetrahymena pyriformis* (Solanki & Paliwal 2007). The possibility of utilizing *Euplotes crassus*, for the cell viability, fission rate, lysosomal membrane stability, and cholinesterase (ChE) activity tests were evaluated using certain pesticides, mercury and different mixtures of these compounds (Francesca *et al.* 2007). Ciliates proved to be excellent models for assessing xenobiotic compounds like pesticides especially in the environmental domain (Rouhabi *et al.* 2008). The effects of hexavalent chromium on the cell growth and accumulation ability of paramecium and *Paramecium bursaria* were investigated successfully by Golam *et al* (2009). Libor *et al.* (2009) used the *Paramecium caudatum* successfully in silver nanoparticles toxicity study and it was proven that certain protozoan ciliates are useful tools in nanotechnology.

Macronuclear changes

Cytochemical studies were carried out in *Paramecium caudatum* and *Oxytricha fallax* under pesticidal stress. Numerous aberrations in the shape such as rod shaped nucleus, marginalization of nucleus, fragmentation, uneven division, diffusion of nucleus and total absence of nucleus have been found. Such abnormalities are related to cell division failures, cell death processes and to genotoxicity or mutagenicity. The results obtained from the data are clear that the deformities were concentration dependent. In *Oxytrcha fallax*, diffusion of nucleus is the highest recorded abnormality followed by fragmentation and disappearance of nucleus. Unevenly divided nuclear forms are commonly found in *Paramecium caudatum* followed by rod shaped forms to different concentrations of azadirachtin. Interestingly, numerous cytoplasmic vacuoles were also observed in the cytoplasm of paramecia when exposed to azadirachtin. The highest total abnormalities (61.6±2.792) were recorded when *Paramecium caudatum* exposed against 300ppm of azadirachtin for 72hrs. In concentrations of 300, 100, 30 and 3ppm the percent abnormal forms recorded were 61.6±2.792, 53.2±1.923, 49.2±2.387 and 22±0.707 respectively. Whereas in the second set of experiment *Oxytricha fallax* showed 53.2±1.923, 43.4±2.408, 37±1.581 and 19.2±1.483 abnormalities to 300, 100, 30 and 3ppm of azadirachtin respectively (Table 1 & 2).

Table 1: Azadirachtin induced macronuclear changes (%) in *Paramecium caudatum* exposed for 72hrs

Conc/ppm	Total abnormal forms	Unevenly divided	Vacuolated	Fragmented	Rod shape	Other deformities
300	61.6±2.792	15.8±1.923	7±1.581	7±2.549	25±1.870	6.2±1.303
100	53.2±1.923	14.2±1.788	6±707	8.2±0.836	17.4±1.516	5.8±1.643
30	49.2±2.387	12±1.870	5.2±1.303	5±1.224	19.6±1.341	7±1.581
3	22±0.707	5±1.581	2.2±1.483	2.8±0.836	8±1	4.2±1.303
Control	0.246±0.429	----	----	----	----	0.246±0.429

Mean and SD values are significant at P<0.05, (n= 5)

Table 2: Azadirachtin induced macronuclear changes (%) in *Oxytricha fallax* exposed for 72hrs

Conc/ppm	Total abnormal forms	Diffusion	Disappeared	Fragmented	Rod shape	Other deformities
300	53.2±1.923	19±1.87	3±1	8.8±1.483	10±1.870	12±1.870
100	43.4±2.408	16.4±2.073	1.8±0.836	6.6±1.140	7.6±1.516	9.2±1.483
30	37±1.581	3.8±1.483	2±0.707	5±1.581	7.2±1.303	9±1.224
3	19.2±1.483	8.8±1.303	----	2 ±0.707	4.2±1.303	4.2±0.836
Control	0.052±0.083	----	----	----	----	0.052±0.083

Mean and SD values are significant at P<0.05, (n= 5).

Paramecium caudatum and *Oxytricha fallax* have been employed to analyze possible toxic impact of azadirachtin in the shape, structure and size of the nucleus. Hussain *et al.* (2008) found that when *Paramecium caudatum* was exposed to different concentrations of carbofuran, it caused macronuclear abnormalities such as fragmentation, uneven division and vacuolization. In related experiments, Amanchi and Hussain 2008 demonstrated cytotoxic effects of delfin insecticide (*Bacillus thuringiesis*) on cell behavior, morphology, phagocytosis, contractile vacuole activity and macronucleus in *Paramecium caudatum*. The macronuclear aberrations like marginalization, fragmentation, vacuolization and complete diffusion of macronucleus increased with increasing concentrations of delfin up to 100ppm. Present studies of nuclear deformities in Paramecia and Oxytricha showed similar findings and are in agreement obtained in earlier studies on different ciliates with different toxic agents (Nilsson 1974; Nilsson 1999; Madoni and Bassanini 1999; De Lorenzo *et al.* 2001; Miyoshi *et al.* 2003; Jha 2004; Amanchi and Hussain 2007; Amanchi and bhagavathi 2009). Rao *et al* (2007) observed blackening of cytoplasm, blebbing and macronuclear changes in *Paramecium caudatum* exposed to monocrotophos. Dias *et al.* (2003) reported cytoskeletal alterations in *Tetrahymena pyriformis* when treated with Triton X 100. It was reported that after 1hr of treatment, cell started to become round in shape with the nucleus moving to the cell periphery. Genotoxic potential of azadirachtin has been reported to have mitotic poisoning effect on mouse chromosomes (Awasthy *et al.* 1995) and caused chromosomal aberrations in fish (Chandra and Khuda-Bhuksh. 2004). Similarly

contamination to standing water bodies of azadirachtin near 10mg/L or higher caused significant adverse effects on zooplankton communities (Kruetweizer *et al.* 2004). The metabolism of azadirachtin results in production of electrophilic ions and radicals, interacting with the nucleophilic sites of DNA leading to breaks and other damages in the organism (Klopman *et al.* 1985). Therefore, the action of azadirachtin in the present study on the test organisms may perhaps be explained in terms of its interaction and binding with nuclear DNA and causing damage to it. Extrusion of nucleus and hypertrophy in Oxytricha and Paramecium has suggested that such forms are degenerative forms, nearing to death.

CONCLUSION

After considering the experimental results, it was concluded that both *Paramecium caudatum* and *Oxytricha fallax* are responsive bioindicators of stress conditions and are sensitive to azadirachtin. The sensitivity of both the organisms has made them as alternative models to eukaryotic organisms for biomonitoring studies and assessment of pesticide toxicity.

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