Flow Cytometric Analysis of Cell Population Alteration and Ros Generation in Spleen and Thymus of *Labeo rohita* under Experimentally Induced Azadirachtin Toxicity

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Abstract - Fishes exposed to environmental contaminants through agricultural runoff show morphological, biochemical and physiological changes along with cellular population alteration and ROS generation at subcellular level. In this study, the impact of No Observed Effect Limit (NOEL) dose of Neemshield, an azadirachtin based biopesticide has been assessed on the haemopoietic cell population of spleen and thymus of Labeo rohita Flow cytometry technique has been utilized as it allows a cell by cell qualitative and quantitative analysis of cell functions and offers great potential for multiparameter assay at sub-cellular level even in a minimal exposure condition. In a time dependent and dose independent experiment, flow cytometric analysis clearly subdivided the entire cell population of both the tissues in two separate groups, viz. granulocytes and lymphocytes. In both the cases, two populations showed significant variations (P Value : .002 and .012 respectively) over time. Pearson correlation suggested that both the splenic and thymic cell population are positively correlated showing a correlation value of .305 and .365 respectively. Result of flow cytometric measurement of ROS production in spleen showed a linear positive regression throughout experimental tenure with 2.24, 1.49, 1.37 and 1.08 fold increases from control group. In contrast thymus showed a negative linear regression of ROS production with 1.12, 1.18, 1.22 and 1.56 fold increase from the control group .Thus this study establishes that in assessment of azadirachtin toxicity, besides morphological and biochemical parameter analysis, it is important to emphasize on cellular responses to evaluate the precise and accurate effect at minimal concentration.

Key words: Flow cytometry, ROS, spleen, thymus, azadirachtin, Labeo rohita.

I. INTRODUCTION

Extensive use of inorganic, synthetic even biopesticides` for control of agricultural pest community has created problems related to physiological resistance to vectors, adverse environmental effects and high operational cost (Murthy et al., 2013). A major risk involved in this course is environmental contamination, especially translocation within the natural system where pesticides might enter the water bodies causing various deleterious effect on non-target organisms of aquatic community and ultimately on human. Therefore, fish, specially the teleost group, can serve as good model of bio-indicator of environmental pollution and can play significant role in assessing potential risk associated with contamination in aquatic environment (Lakra and Nagpure, 2009).

Natural pesticides based on plant extracts like azadirachtin , a biologically active compound of neem (*Azadirachta indica* A Juss) has been promoted as a new insecticide that is considered more eco-friendly than synthetic ones towards non-target aquatic life (Martinez, 2002; Isman, 2006). Though many studies have been done to evaluate the morphological, biochemical and histopathological effect of this contaminant on fish community (Omoregie and Okpanachi, 1992; Omoregie and Okpanachi, 1997; Rábago-Castro et al., 2006; Winkaler et al. 2007; Kumar et al. 2010, 2011a, 2011b, 2013; Prasad et al. 2011), little attention has been paid to assess its effect on cellular population alteration at no observed effect limit (NOEL) condition, as it comes to the natural aquatic body in a minimal concentration through agricultural runoff.

In assessment of toxicity, the identification of cell population and its probable alteration in fish haemopoeitic organs need serious attention (Dhanapakiam and Premlatha, 1994; Deneer, 2000). Though head kidney has been considered as the primary haemopoietic organ of almost all the fishes, spleen and thymus also play a crucial role in the formation of erythropoietic and lymphopoietic cell lineages. The structure, abundance, location and co-location of haemopoietic cells present in these organs may be of significance in any given clinical situation. In hematopoiesis, as cells differentiate and mature, different subsets of molecules are expressed that reflect a specialized functional capacity for that unique cell type (e.g., granulocytes vs. lymphocytes). Flow cytometry allows a cell by cell qualitative and quantitative analysis of cell functions and cell activities and offers great potential

for investigations in the areas of fish haematology and immunology because multiparameter measurements of single cells can be easily and rapidly performed. On the other hand, beside cell population alteration, Oxidative stress develops when there is an imbalance between prooxidants and antioxidants ratio, leading to the production of Reactive oxygen species (ROS)(Ahmad et al. 2000; Li et al., 2003).ROS, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radical (OH·) can react with biological macromolecules potentially leading to enzyme inactivation, lipid peroxidation (LPO), DNA damage and even cell death (Hermes-Lima M, 2004; Scandalios, 2005). Agrocontaminant induced alteration in oxidative stress parameters due to free radicals has already been described for various fish species (Glusczak et al., 2007; Maheswari et al., 2014) and considered as the main mechanism of cellular destruction.

The aim of this study was to observe the cell population alteration of spleen and thymus of *Labeo rohita* and to investigate the ROS generation by flow cytometric analysis by scattered light flow cytometry in the mode FSC/SSC after the exposure to experimentally induced azadirachtin solution through bath treatment in a sublethal dose (NOEL-1/7th of 96hr LC_{50}).

II. MATERIALS AND METHODS

- Fish maintenance and toxicity test for LC_{50} determination

Healthy fish specimens of rohu, *Labeo rohita* with an average weight of 25.0 ± 3.00 g were procured from a local fish farm, Matsyajibi Samabay Samity, Anandapur, Kolkata, India during non-breeding period (early March) and was acclimatized for a period of 7 days and was fed with commercial pelleted diet of fish meal. The range finding test to assess toxicity was conducted to ascertain LC50value. The stock solution and working test solution was prepared in organic solvent DMSO as described by Kumar et al., (2012). The commercially available Azadirachtin EC 66 % (Neemsheild organic manure, Fatehpur, Delhi, India) was used for preparation of stock solution. The LC50value was calculated for 96 hour of exposure to azadirachtin. NOEL concentration is also determined, which is 1/7th of the final LC_{50} value.

In the present study, fishes were kept in groups of 10 in 30 L aquaria. The fishes were not fed 24 hours before and during the experimental exposure as few reports are available on the effect of feeding in toxicant exposed animals. This study was approved by the Animal Research Ethical Committee of Zoology Department, University of Calcutta.

- Sampling procedure

Samples of thymus and spleen tissue were collected at day 0 (before administration of contaminant), and at 24hr, 48hr, 72hr and 96hr after exposure period. At each collection time, four individuals from the challenge group and two from the control group were considered. Sampling was performed randomly, and no attempt was made to select fish showing clinical symptoms. Both the tissues were collected from the live fish for flowcytometric analysis.

- Cell isolation and flowcytometric analysis

The spleen and thymus cells were isolated using the method previously described by O'Halloran et al. (1998). Briefly, cells were disrupted from the tissues and passed through a 250 mm nylon mesh. Red blood cells were separated from the cell suspension by density gradient centrifugation i.e. 4 mL of cell suspension layered over 3 mL of Histopaque (Sigma Chemical Co., St Louis, MO, USA) and centrifuged at 400X *g* for 30 min at 22°C. The cells collected from above the Histopaque layer, were washed twice, counted microscopically (using a haemocytometer in the presence of trypan blue) and diluted with tissue culture media (i.e. TCM, consisting of RPMI 1640 with 20 mM HEPES, 300 mg /L glutamine and 100 µgm/L gentamycin sulphate, Sigma Chemical Co., St Louis, MO, USA; supplemented with 10% fetal calf serum (FCS), CSL, Melbourne, Vic., Australia) to the desired cell concentration of $1X10^6$ cells/mL. Flow cytometry was used to measure the population distribution of spleen and thymus cells and their probable fluctuation throughout the experimental tenure according to the procedure of Harford et al. (2006). The flow cytometer was set to collect 10,000 events and gated regions were placed around three distinct populations, i.e. granulocytes (greater size and granularity indicated by high "forward scatter" and low "side scatter"—FS and SS), lymphocytes (lower FS and SS) and debris (low FS, high SS and propidium iodide (PI) positive). Data were also collected on the percentage of granulocytes and lymphocytes emitting fluorescence (i.e. FITC-positive granulocytes) at 520 nm.

- Estimation of intracellular reactive oxygen species (ROS)

Single cell suspensions were prepared from spleen and thymus by treatment with collagenase 1 (2mg ml^{-1}) at 37°Cwith constant shaking. The cells were then stained for 30 minutes at room temperature in dark with cell permeable fluorescent and chemiluminescent probes, 2´, 7´dichlorofluorescein-diacetate (DCFH-DA) in a Ca^{2+} enriched binding buffer and analyzed by a FACS caliber flow cytometer. For each set, a total of 10,000 even counts were taken. ROS was detected in the FL-1 channel using an excitation and emission filter at 488nm and 530nm respectively. The mean values are considered only

and data were analyzed using Cell Quest from Becton Dickinson (Acharya et al., 2009).

- Statistical analysis

Statistics were performed using the computer package SPSS 17.0 (SPSS Inc., Chicago, IL, USA). An independent sample's t- test and bivariate correlation test were also performed to analyze the significance of population fluctuation and to determine homogenous subsets.

III. RESULTS AND DISCUSSION

- Quantitative changes of cellular composition and determination of granulocytes and lymphocyte subpopulation counts by FACS analysis

Neem pesticides are now extensively used in agro ecosystems due to their target specificity against insect pests and minimal effects towards non-target biota (Goektepe et al., 2004) at effective dose. However, the effect on fish tissues should be estimated as the aquatic bodies are also contaminated through precipitated water from the adjacent landscapes. . In the present study, cell sorting based upon cell size and granularity resulted in construction of cytograms for two discrete subpopulations viz. granulocytes and lymphocytes which represent total cellular composition of both the spleen and thymus tissue as corroborated in the study of Basiji et al. (2007) which described cellular composition of haemopoietic organs. Figure 1 (A1-5 and B1-5) and Figure 2(A1-5 and B1-5) represent cytograms of fish spleen and thymus, respectively, exposed to azadirachtin solution at NOEL concentration ($1/7^{th}$ of the LC_{50} value) in 0, 24, 48, 72 and 96 hours. The X axis shows the measure of forward scattering, proportional to cell size (FSC) while the Y axis shows side scattering property, proportional to cell granularity (SSC). For each exposure, 10 individual fish tissues were separately taken and for each set a total of 10,000 event counts were recorded. In the experiment, using *Labeo rohita* splenic cells, granulocytes (P1) appeared to be the main target of azadirachtin toxicity. In a time dependent and dose independent experiment, exhilaration in the number of granulocytes with beads (i.e. $FITC^+$ granulocytes) reached statistical significance at 0.01 (*P* value .002). Granulocytes exposed to the azadirachtin showed a regression equation of $y= 8.1327ln(x) + 15.885$ signifying the steady increase of the population percentage over the time. It should be noted that the flowcytometer counts are a proportion of 10,000 events, and a reduced lymphocyte number (due to bioagrocontaminant toxicity) can result in a concomitant apparent rise in granulocytes counts. The increase in numbers of granulocytes was associated with decreased cell concentrations within samples, which was noticeable in a lengthening of the amount of time to collect 10,000 events (data not shown). The significant decrease in lymphocyte counts (P2) is apparent due to specific fluctuation in the granulocyte subpopulation indicating a lesser proportion of lymphocytes within the 10,000 event count. The reduction in the number of lymphocytes with beads (i.e. $FITC^+$ lymphocytes) reached statistical significance at 0.01 (*P* value .012). Lymphocyte population exposed to the azadirachtin showed a regression equation of $y= 0.948ln(x) + 15.37$signifying almost linear propagation of the population percentage over the time. The Pearson correlation suggests that the granulocyte and lymphocyte populations are positively correlated (0.305) but statistically it is non-significant (*P* value .157) (Fig 3). Small number of other cells was distributed randomly in the cytogram and was not considered as part of any particular population due to lack of distinct scattering pattern. In contrast, the haemopoeitic subpopulation alteration pattern is distinctively different in thymus tissue. Over the experimental tenure, reduction in the number of granulocytes and lymphocytes with beads (i.e. $FITC^+$ granulocytes) reached statistical significance at 0.05 (*P* value .012 and .043 respectively). Here, Granulocytes showed a regression equation of $y= -14.09ln(x) + 43.306$, whereas lymphocyte population depicted an equation of $y = -15.45ln(x) + 40.82$, signifying the gradual degradation of the population percentage over the time. The Pearson correlation suggests that the granulocyte and lymphocyte populations of thymus are positively correlated (0.278) but statistically it is non-significant (*P* value .367) (Fig 4). This study establishes a novel approach towards characterization of fish spleen and thymus cell population and can be exploited to compare with the pattern of cell subpopulation alteration in the primary fish haemopoietic tissue, the head kidney (Kondera et al., 2012) when exposed to external contaminants. Stress factors including starvation, diseased condition and change of salinity or pH of water may induce modifications at cellular level which can also be detected from the study of cytogram, providing a powerful and rapid tool to understand such changes and studied by many researchers (Scapigliati et al., 2000; Scapigliati et al., 2002; Chilmonczyk et al., 2002).Because of the lack of phenotypic markers, the study of the fish cellular immune response involves the screening of morphological characteristics and functional mechanisms and interesting data are obtained from the direct study of non-labeled cells, on the basis of cell autofluorescence. Thus the heterogeneity occurring in the cytogram profiles is correlated with the health status of the fish and could represent a significant part of the inter-species and intra-species variability occurring in the results. The result here depicted that though thymus is comparatively uninfluenced by the effect of azadirachtin solution and may serve as secondary haemopoietic tissue in *L. rohita*; spleen cell populations, both granulocytes and lymphocytes, showed

marked alteration throughout the experimental tenure, conforming its role as primary haemopoietic organ along with head kidney and suggesting that it performs a major role in erythropoeitic and leucopoeitic cell lineage regulation. Thus the present result is in agreement with the earlier investigations (Gultekin et al., 2000; Sayeed et al., 2003; Monteiro et al., 2006) to establish the fact that fish splenic tissue is also influenced by the toxic effects of azadirachtin along with other targeted organs viz. gill, liver and pronephros at even NOEL dosage.

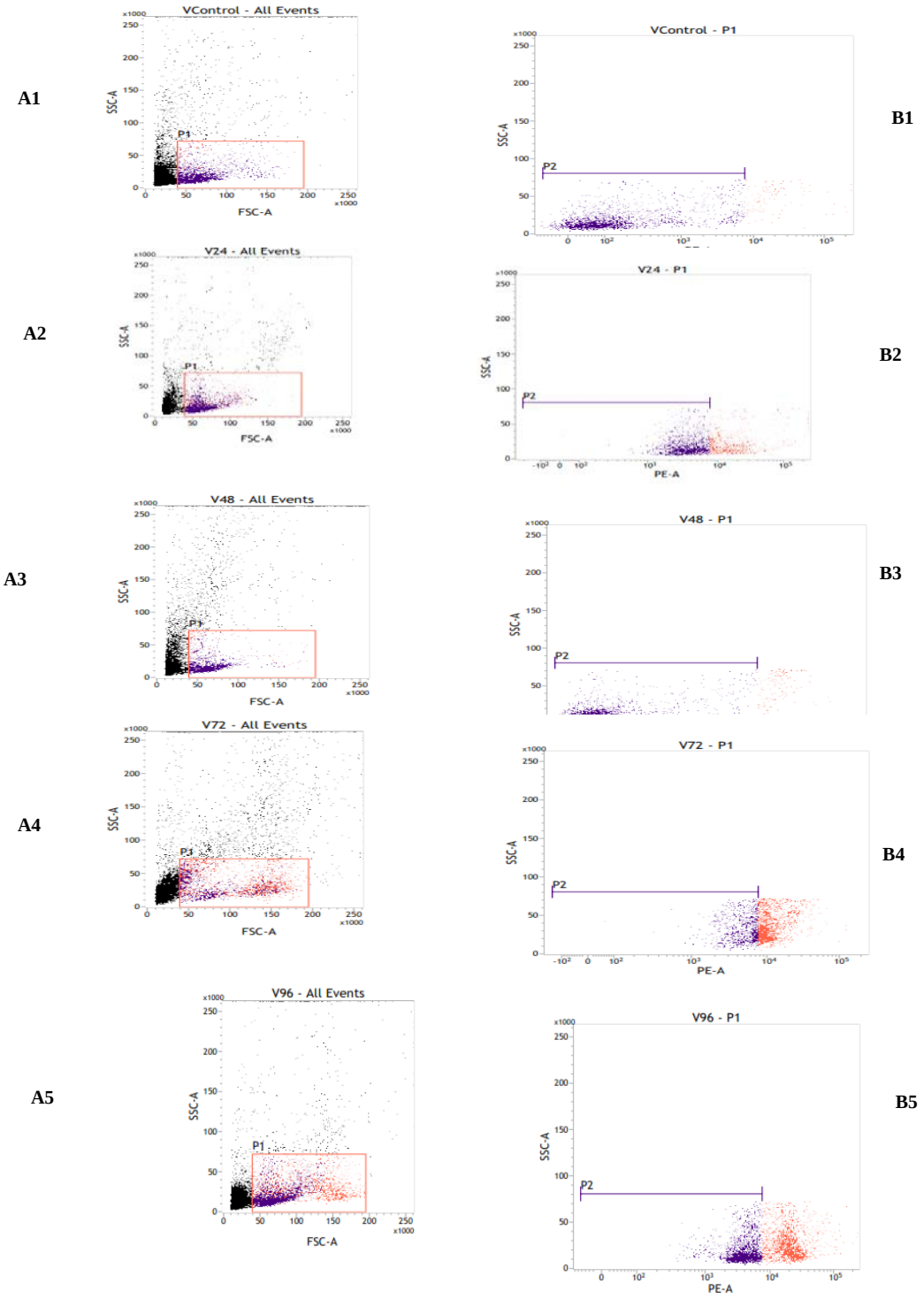

Fig 1 (A1-5 and B1-5) : Erythropoietic and lymphopoietic population profiles of *Labeo rohita* spleen tissue treated with azadirachtin solution at NOEL concentration ($1/7^{th}$ of the LC_{50} value) at different exposure periods. A1/B1, A2/B2, A3/B3, A4/B4 and A5/B5 represents the control group, 24 hours, 48 hours, 72 hours and 96 hours of exposure respectively. The scattering diagrams show two distinct population viz. granulocytes (P1) and lymphocytes (P2) respectively

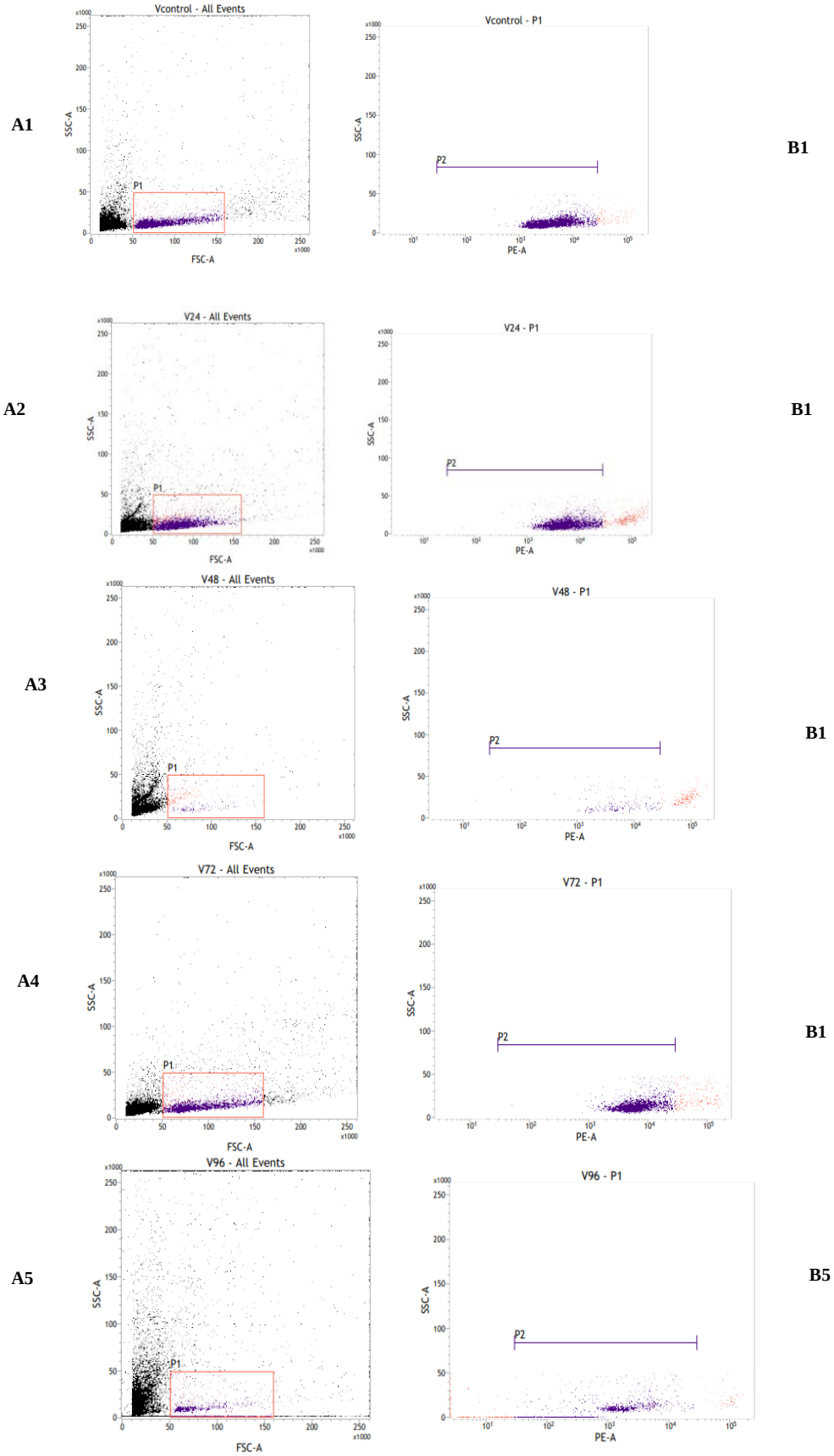

Fig 2 (A1-5 and B1-5) : Erythropoietic and lymphopoietic population profiles of *Labeo rohita* thymus tissue treated with azadirachtin solution at NOEL concentration ($1/7^{th}$ of the LC_{50} value) at different exposure periods. A1/B1, A2/B2, A3/B3, A4/B4 and A5/B5 represents the control group, 24 hours, 48 hours, 72 hours and 96 hours of exposure respectively. The scattering diagrams show two distinct population viz. granulocytes (P1) and lymphocytes (P2) respectively.

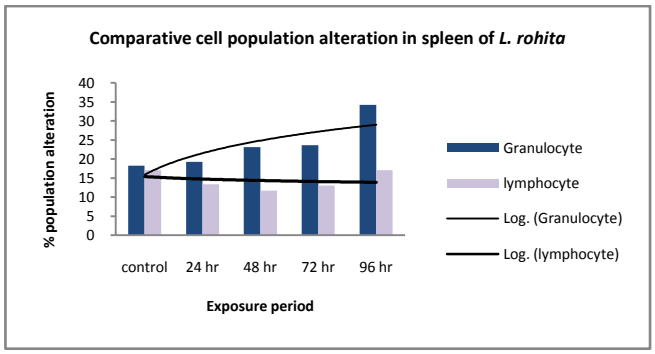

Fig 3: Graph representing the % population fluctuation in spleen tisuue of *Labeo rohita* treated with azadirachtin solution at NOEL concentration ($1/7^{th}$ of the LC_{50} value) at different exposure periods. Regression line of granulocyte subpopulation showing a positive acceleration whereas lymphocyte subpopulation showing an almost linear propagation throughout experimental tenure.

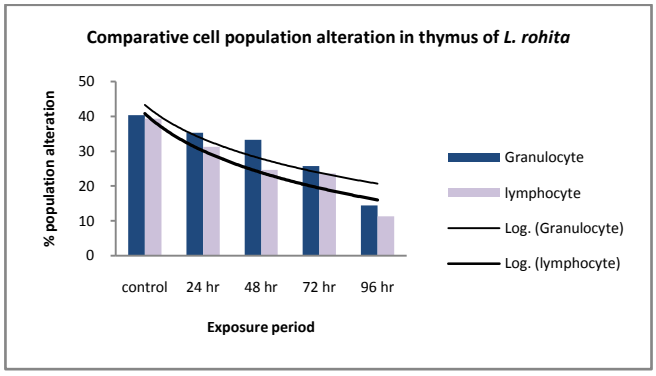

Fig 4: Graph representing the % population fluctuation in thymus tissue of *Labeo rohita* treated with azadirachtin solution at NOEL concentration ($1/7^{th}$ of the LC_{50} value) at different exposure periods. Regression line of both granulocyte and lymphocyte subpopulations showing negative throughout experimental tenure.

- Flow cytometric measurement of ROS production

Result of flowcytometric measurement of ROS production showed a fluctuation of the DCF mean peak in case of fish splenic and thymus tissues treated with azadirachtin in different exposure period (Fig 5 and Fig 6). It demonstrated a linear positive regression of the ROS production in case of spleen tissue with 2.24, 1.49, 1.37 and 1.08 fold increases from the control group. However in thymus, it showed a negative linear regression of ROS production with 1.12, 1.18, 1.22 and 1.56 fold of increase from the control group (Fig7). Surprisingly, though the ROS generation is somehow negligible in the initial days of experiment, it accelerated as the exposure period increases suggesting delayed effect of the contaminant in the thymus tissue. Thus there was a clear indication of higher H_2O_2 production in the exposed fish community in comparison to control in a time dependent and dose independent experiment.Many environmental pollutants, including synthetic and organic agrocontaminants, are capable of inducing oxidative stress in fish (Hincal et al., 1995; Dorval et al., 2003; Pandey et al., 2003; Sayeed et al., 2003; Monteiro et al., 2006). This event resulted in the formation of highly reactive compounds such as free radicals or oxyradicals (O_2^-, H_2O_2 and .OH) in the haemopoeitic tissues that frequently react with cellular macromolecules, leading potentially to enzyme inactivation, lipid peroxidation, DNA damage and even cell death (Van der Oost et al., 2003).

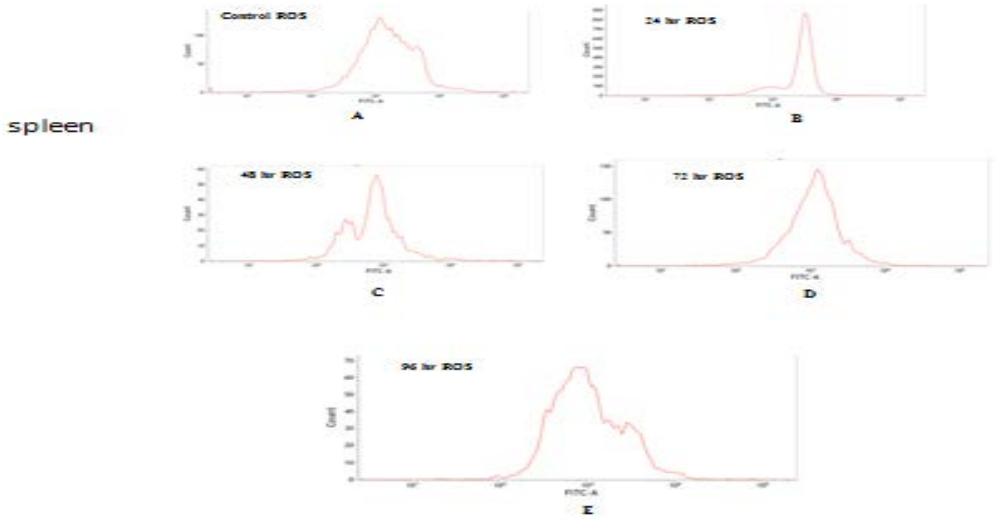

Fig 5: Effect of immune response on azadirachtin induced ROS production in spleen tissue of *Labeo rohita*. Intracellular accumulation of ROS measured using an excitation and emission setting of 488 and 530 nm respectively. A, B, C, D and E represents the control group, 24 hours, 48 hours, 72 hours and 96 hours of exposure.

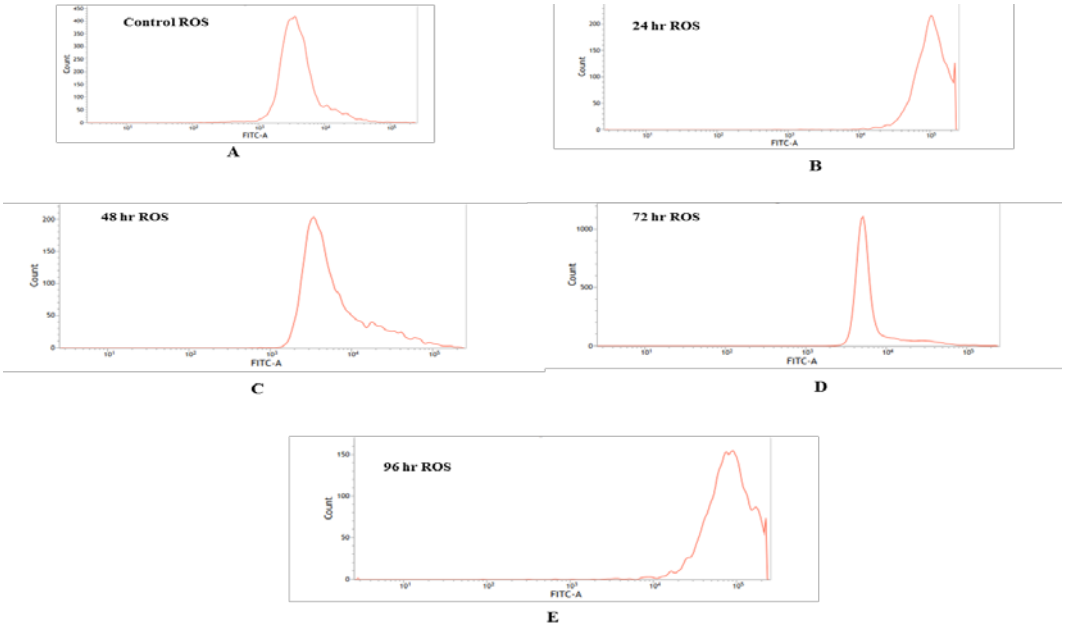

Fig 6: Effect of immune response on azadirachtin induced ROS production in thymus tissue of *Labeo rohita*. Intracellular accumulation of ROS measured using an excitation and emission setting of 488 and 530 nm respectively. A, B, C, D and E represents the control group, 24 hours, 48 hours, 72 hours and 96 hours of exposure.

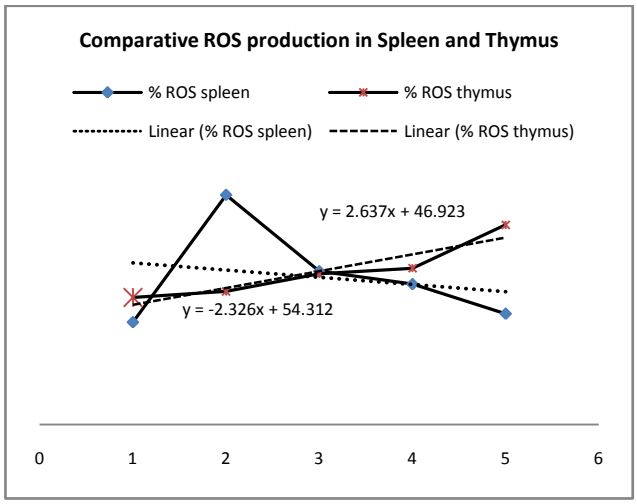

Fig 7: Graph representing comparative % population ROS production in spleen and thymus tissues in *Labeo rohita* following azadirachtin exposure in a time dependent and dose independent experiment.

IV. CONCLUSION

Thus the result reported in the present study indicates that the exposure of azadirachtin induces a definite oxidative stress in spleen and thymus, and the measurement of ROS by flowcytometry proves a sensitive method that succeeds in quantifying within the cell, with the advantage of an accurate determination.

These results indicate that in assessment of toxicity of agro contaminants through agricultural run offs, it is important to evaluate the cellular responses in fish exposed to sublethal($1/7^{th}$ of the LC_{50} value) concentrations in the aquatic ecosystems in addition to reliable morphological studies.

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