# CHAPTER 6

# DISCUSSION

## 6.1. Oxidative stress, altered antioxidant and immune defense system upon individual exposure to mercuric chloride and cadmium chloride *in vivo* in macrophages of fish *Channa punctatus*

In recent years, there has been an emergent concern over the increase in heavy metals contamination affecting the terrestrial and aquatic environments and ultimately affecting human health. The release of pollutants, especially heavy metals, into the aquatic environment is known to cause detrimental effects to the environment and to the living organisms, giving a significant impetus to the study of oxidative stress responses in aquatic organisms induced by toxic metals (Soares *et al*., 2008). Due to tendency of bio-accumulation and bio-magnification in the food chain heavy metals are severe in their action. Fish accumulates these heavy metals in higher concentrations in their tissues, mainly through ingestion of contaminated food or by environmental absorption along the gill surface (Kraal *et al*.,1995), with metals being accumulated mainly in metabolically active tissues (Kock and Hofer, 1998), such as the kidney, liver, gills, intestine and digestive tract (Miliou *et al*., 1998). In fact, several works have reported that some fish species are more sensitive to heavy metals toxic effects than mammals (Kelly *et al*., 1998). Our results showed significant level of mercury and cadmium accumulation in liver, gills, muscle, kidney and intestine when treated singly. These results significantly correlate with the altered antioxidant as well as the non-specific immune functions of the intestinal macrophages.

Heavy metals not only affect the physico-chemical equilibrium of the aquatic body, but also disrupt the food web and, bring about morphological, physiological and cytogenetical changes in the aquatic inhabitants (Scalon *et al*., 2010). The functions of teleostean digestive tract include digestion, nutrients absorption, hormone secretion, immune protection and water and salt transfers for hydro-mineral homeostasis. It

regulates energy and material exchange between the environment and the internal medium (Khojasteh, 2012). Thus knowledge of the intestinal ultrastructure and morphology is essential for understanding the related functional mechanisms. The intestinal tract can be broadly divided into three sections morphologically: the anterior intestine which contains a number of blind-sacs known as pyloric caecae, the mid intestine which is narrower and free of caeca, and the posterior intestine which has a larger diameter, visible external annular folds and internal mucosal folds. It is believed that the presence of pyloric caecae in the anterior intestine increases the surface area for nutrient digestion and absorption (Buddington and Diamond, 1986), while the internal mucosal folds in the posterior intestine increases absorptive surface areas as well as increasing retention time of food (Ezeasor and Stokoe, 1980). In general, cellular uptake of nutrients across the intestine involves three steps: (1) absorption of nutrients through the apical membrane into the epithelial cells, (2) intracellular trafficking of nutrients to the basolateral membrane, and (3) extrusion of nutrients from the cells into the bloodstream. Scanning electron microscopy (SEM) is an important tool that reveals information on the surface ultrastucture and morphological changes that may occur in biological tissues. Mucosa is pivotal in digestion, absortion and metabolic processes. It represents a selective barrier to nutrients and avoids several toxins and pathogens. Moreover, it plays an important role in the electrolytic balance, immune response and endocrine functions (Bakary and Gammal 2010). Mercury and cadmium induced severe inflammatory damage in intestinal epithelium, disarrangement and fragmentation of mucosal folding, as observed in the present study, causes impairment of tissue organs which in turn has an obvious effect on its defense system. In the present study, TEM of the control group showing regular microvilli contained cores made up of fine filaments forming thick bundles which extended deeply in to the terminal web of the apical

cytoplasm. Invaginations of the apical plasma membrane were frequently seen among microvilli; vesicles and channel, which appeared to be continuous in the superficial cytoplasm. The TEM of control group also showed large goblet cells, which were filled with numerous mucous droplets of low electron density between surface epithelial cells. The transmission electron micrograph (TEM) of intestinal tissue showed disrupted microvilli, denudation in the goblet cells and disrupted endoplasmic reticulam, lack of gap junction and zona occludens and less electron dense material after 4 days and 7 days of mercury and cadmium exposure.

Macrophages play a significant part in immunity and immune responses. They assume a defensive role exhibited by their ability to carry on phagocytosis of parasites and microbes. They regulate lymphocyte activation and proliferation and they are essential in the activation process of T- and B-lymphocytes by antigens and allogenic cells (Mohamed and Elhelu 1983). Macrophages also participate in cellular immunity as antigen presenting cells; Cytokines released from macrophages harness the dual branches of immune status viz. humoral and cell mediated immunity.

Phagocytes upon stimulation with various agents, produce reactive oxygen species (ROS) through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (Forman and Torres, 2002). The respiratory burst of 'professional' phagocytes (granulocytes monocytes and macrophages) involves reduction of oxygen ($O_2$) to the anionic radical, superoxide ($.O_2^-$). This process is catalyzed by an NADPH oxidase localized in the plasma and phagosomal membranes (Bols *et al.*, 2001). NADPH oxidase is consists of the membrane (gp91PHOX and p22PHOX) and the cytosolic (p47PHOX, p67PHOX, and p40PHOX) components (DeLeo and Quinn, 1996; Babior, 1999). In addition, small G-proteins such as rac1, as well as kinases including PKC, regulate its activity (El-Benna *et al.*, 1997). Under normal

circumstances, activation of cells of nonspecific immunity may be beneficial to the host cell; particularly the reactive intermediates released during phagocytic respiratory burst activity possess bactericidal activity. Significant elevation in the respiratory burst activity upon mercury and cadmium exposure after 4 days and 7 days, as observed in the present results, may suggest over activation of the superoxide-producing enzyme NADPH oxidase and generation of large amount of ROS. Further, it could also be assumed that mercury and cadmium in macrophage might have suppressed the activity of the regulatory proteins leading to uncontrolled enzyme activity which is destined to cell damage when treated singly.

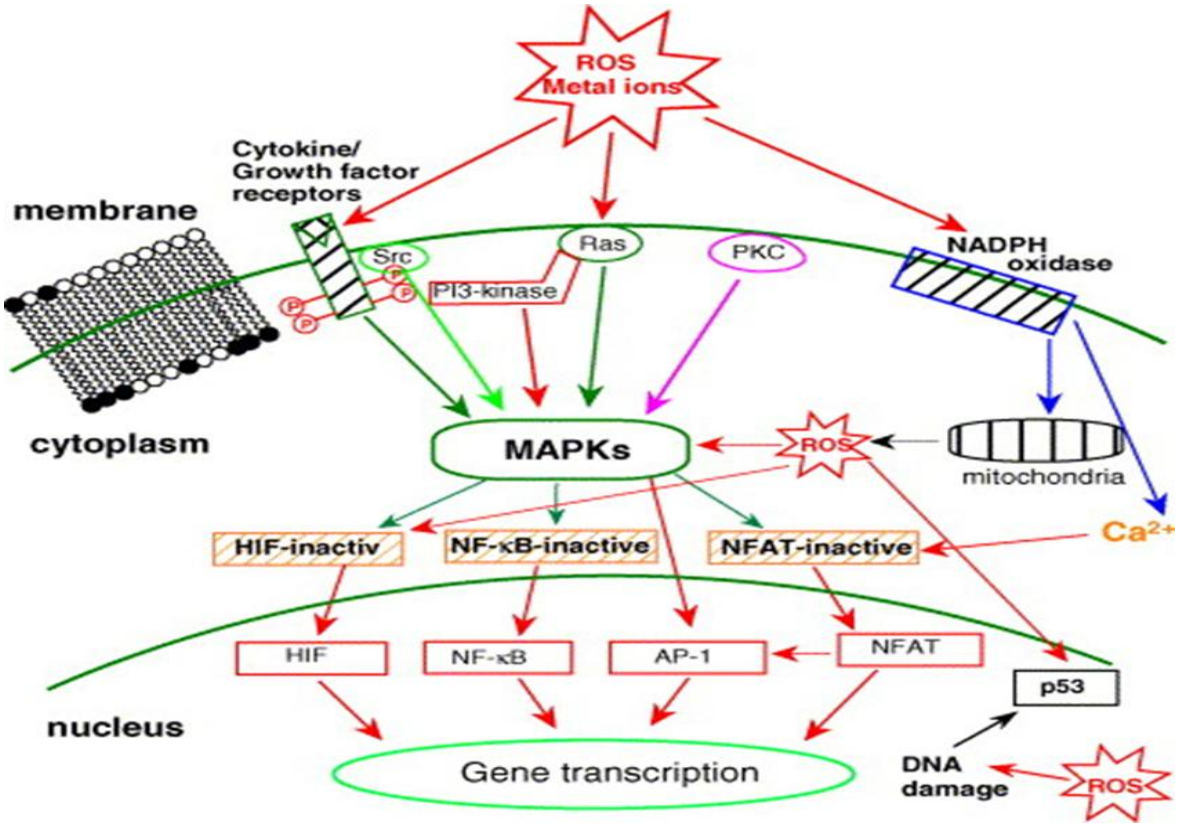


Fig. 6.1: Mechanism showing how metal ions induce oxidative stress by altering MAPKs and other signaling molecules within cells of living organisms.

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and can be used as an indicator of oxidative stress in cells and tissues (Quig

1998) (Halliwel 1993). Heavy metals, particularly lead, mercury and cadmium being members of the sulfhydryl reactive group promote the formation of hydrogen peroxide and enhance the subsequent iron and copper-induced production of lipid peroxides and the highly reactive hydroxyl radicals (Quig 1998). Thus, determination of heavy metal accumulation in tissues of aquatic or and the levels of malondialdehyde (MDA) as a measure of lipid peroxidation could serve as a radical way to establish relationship between metal toxicity and the stress response resulting from heavy metal accumulation under field conditions as well as provide a rational use of biomarker for oxidative stress in biomonitoring of aquatic pollution. Heavy metals, mercury and cadmium induced elevation in lipid peroxidation has been reported in the present work. When the animal's defenses are insufficient to neutralize ROS, oxidative damage may occur, and one of the most serious damages is formation of membrane lipid peroxides (Sancho *et al*., 2000). The integrity of plasma membrane is essential for cell viability, and as a consequence of over activation of phagocytes, its fluidity seems to be affected in fish (Dimitrova *et al.,* 1994). Thus it may be hypothesized that mercury and cadmium may stimulate the peroxidation of lipids by acting as catalysts in the formation of oxygen radicals when treated singly.

Many aquatic organisms including freshwater teleost have been shown to possess defense pathways to protect them against damages induced by oxyradical production (Secombes, 1990). Oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or excessive accumulation of ROS, or both, leading to cellular damage (Santos *et al*., 2004). ROS can attack multiple cellular constituents, including proteins, nucleic acids, and lipids. To cope with the damaging actions of ROS, organisms have evolved multiple systems of antioxidant defense (Tetyana *et al*., 2005). The enzymes that provide the first line of

defense include super oxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Reduced glutathione (GSH) is the primary cellular antioxidant (non- enzymatic) and plays an important role in the antioxidation of ROS and free radicals (Kavitha and Rao 2009). SOD catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by the CAT activity (Dimitrova *et al*., 1994). Our results showed significant decrease in the activity of CAT and significant increase in SOD activity when treated singly with mercury and cadmium. Low levels of catalase in mercury treated and cadmium treated fishes after 4 days and 7 days of exposure could be attributed to high production of superoxide anion radicals, which has been reported to inhibit catalase activity. The increase in SOD activity in mercury treated and cadmium treated fishes may be due to increased generation of reactive oxygen species.

One of the most remarkable effects of mercury and cadmium exposure on macrophages was a time-dependent decrease in GSH. Glutathione, a tripeptide thiol consisting of glycine, cystein and glutamic acid moieties, presents in high concentration virtually in all types of living cells. Glutathione plays an important role in the protection of the cells from oxidative damage by reducing disulphide groups of proteins and other cellular molecules, or by scavenging free radicals and active oxygen species (Ahmed 2005). GSH is the most well studied antioxidant molecule in fish. Heavy metal cations are characterized by an extremely high affinity to –SH residues (USPHS, 1997) resulting in decrease of GSH level (Sandhir *et al*., 1994) established that glutathione reductase, the enzyme responsible for recycling of glutathione from the oxidized form (glutathione disulfide; GSSG) to the reduced form (reduced glutathione; GSH) is deactivated by mercury and cadmium when treated singly, resulting in low levels of GSH. The results of the present study clearly show depleted levels of GSH in both mercury treated and cadmium treated group of fish as compared to control after 4 days and 7 days of

exposure, and may contribute to the above facts. Antioxidant enzymes that have often been studied as oxidative stress biomarkers link detoxification of ROS with the metabolism of reduced glutathione (Stegeman et al., 1992; Viarengo and Nott, 1993). These include glutathione peroxidase (GPx), an enzyme removing hydrogen peroxide by the simultaneous oxidation of reduced GSH to its oxidized form glutathione disulfide (GSSG) and glutathione reductase (GR), an enzyme catalyzing the conversion of GSSG back to its reduced bioactive form thus maintaining GSH/GSSG equilibrium (Paskerova *et al*., 2012). Glutathione S- transferase (GST), an enzyme involved in the detoxification process and in protecting against peroxidative damage, is ubiquitous in the cytosol and microsomes of eukaryotes (Sreejai and Jaya, 2010). The results of the present study reveal significant depletion of GPx, GR and GST activity indicates an impaired detoxification mechanism of the fish upon low concentration of mercury and cadmium exposure for 4 days and 7 days of exposure.

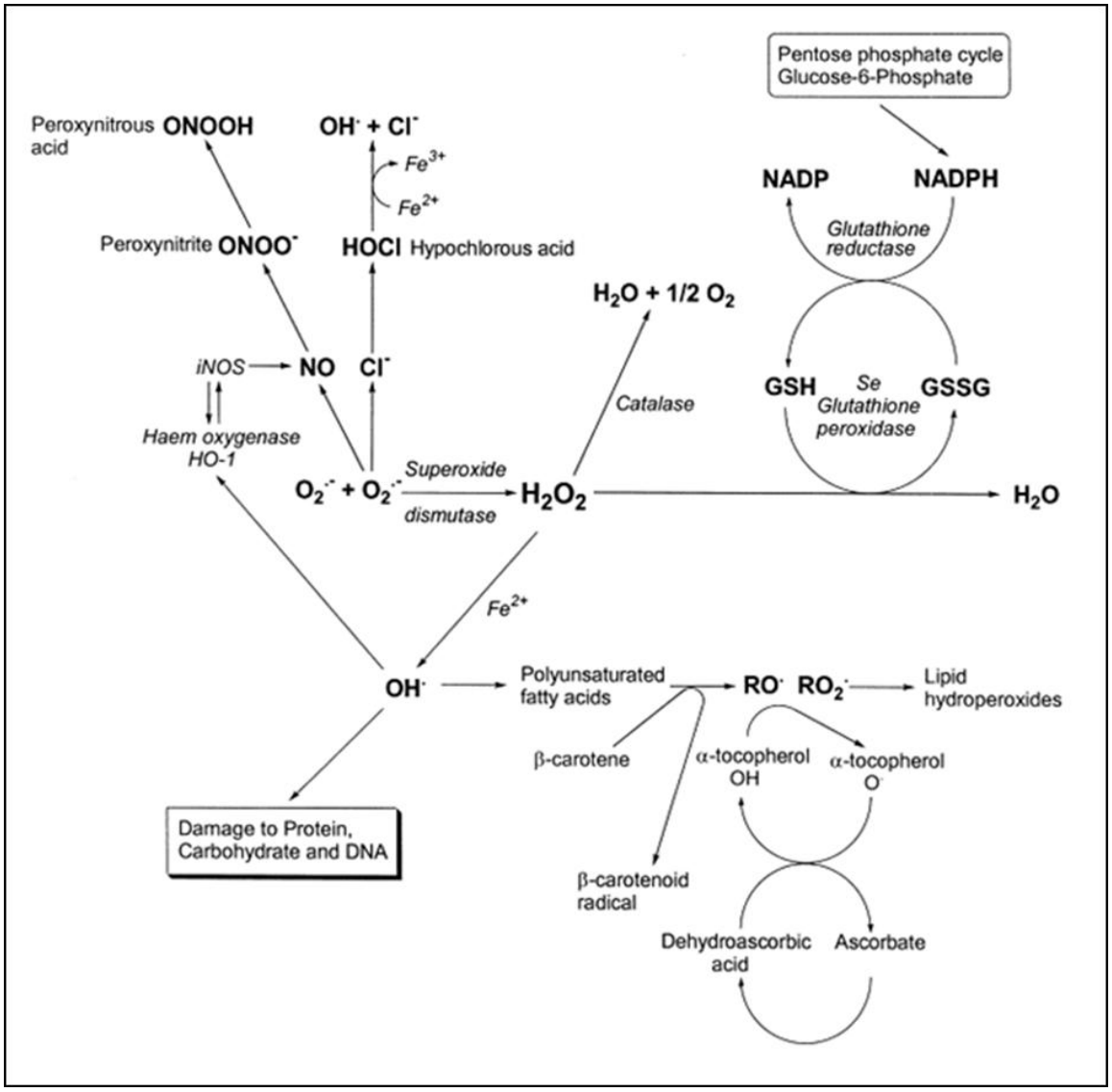


Fig. 6.2: Mechanism showing the role of enzymatic and non- enzymatic antioxidant system in combating free radicals produced in living organisms

Macrophages have been regarded as an important part of the cellular immune system of fish and function to protect the host by phagocytizing foreign materials including disease causing agents and any other toxicants. Therefore changes in their phagocytic abilities have been of interest to ascertain the effect of environmental pollutants on the normal function of macrophages (Shim *et al.*, 2002). Macrophages recognize and engulf bacteria into phagosomes, which subsequently fuse with lysosomes. These phagosomes mature into phagolysosomes upon vesicle-mediated delivery of various antimicrobial

effectors. Reduction of phagocytic capacity by mercury exposed and cadmium exposed group may suggest that the biological activity of alternate complement pathway is altered which is an important pathway in the defense mechanism of fish (Ellis, 2001), and the C3 component of it is likely to be inhibited, making the host cells prone to infection and diseases.

Prolonged exposure to mercury and cadmium tends to inhibit the killing capacity of macrophages when treated singly as seen from the findings that show a significant increase in bacterial viability within the macrophages. This indicates that heavy metal exposed leucocytes are somehow less potent to kill the bacteria efficiently that allows pathogens to easily gain access to the host tissues.

Numerous substances generated during inflammation, like cytokines and chemokines, have the capacity to enhance the speed of macrophage and orient the movement in the direction of an increased concentration gradient of the agent. Mercury and cadmium when exposed singly on macrophages, may lead to altered the morphology of the macrophages and expression of cell adhesion molecules. On the macrophage surface which further alters the shape and orientation of macrophages so that they migrate slowly and adhere and engulf bacteria poorly.

Adherence of antigen to the macrophage cell membrane is a vital step in phagocytosis. Phagocytosis first requires attachment of the bacteria to the surface of the phagocyte, which may involve hydrophobic interactions or sugar/lectin interactions (Secombes, 1996). However, this property of cell adherence is significantly inhibited upon mercury exposure and cadmium exposure when treated singly as it is evident from a fall in absorbance at 570 nm with respect to control suggesting that these heavy metals may

either suppress enough production of lectins or may reduce its opsonising activity, which may have obvious effect on the host defense mechanism.

In recent years, NO has been shown to be a very important molecule in regulating immune functions as well as having a direct antimicrobial effect (Gunasegaran *et al*., 1993; Liew *et al*., 1990).It exhibits a wide range of important functions in vivo, acting as a releasing factor mediating vasodilation, as a neuronal messenger molecule, and as a major regulatory molecule and principal cytotoxic mediator of the immune system (Beckman and Koppenol, 1996; Dimmeler and Zeiher, 1997). When macrophages are activated with bacterial cell wall lipopolysaccharide, they begin to express high level of nitric oxide synthase, which oxidizes L-arginine to yield citrulline and nitric oxide (NO). Nitric oxide plays a significant role in killing of phagocytized pathogens within macrophages can combine with the superoxide anion to yield even more potent antimicrobial substances (Chakraborty and Sengupta 2014). Reactive nitrogen species (RNS), a family of antimicrobial molecules derived from nitric oxide (NO), are produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) (Fang, 2004; Nathan and Shiloh, 2000). The present results illustrate that the amount of NO production by the mercury and cadmium singly exposed group is strikingly less as compared to the control group. This finding may probably signify suppression of NOS2expression and subsequent decrease in antimicrobial activity of macrophage.

Myeloperoxidase (MPO) produced by macrophages are known to play an important role in cellular defenses against various bacterial infections. MPO can generate oxidants from $H_2O_2$ and a range of co-substrates, like chlorine and nitrite (Paul *et al*., 2014). Myeloperoxidase is usually considered as a catalyst of hypochlorous acid formation and therefore as a source of another potentially toxic neutrophil metabolite, but it could also be considered as a catalyst of peroxide removal. Its kinetic properties are such that it

breaks down hydrogen peroxide regardless of hypochlorous acid formation, functioning as a catalase or peroxidase depending on the conditions under which it operates (Winterbourn *et al*., 1985).

Activated phagocytes produce a number of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates and during phagocytosis, a metabolic process known as the respiratory burst occurs in activated macrophage. As the lysosomes fuses with the phagosome, the activity of myeloperoxidase produces hypochlorite from hydrogen peroxide and chloride ions (Chakraborty and Sengupta 2014). HOCl that is produced by MPO is strongly bactericidal (Klebanoff, 1967) and markedly increases the antibacterial potency of ROS (Rosen and Klebanoff, 1979; Rosen *et al*., 1990). This might indicate that MPO is important in host defense in fish. Insufficient release of MPO from mercury and cadmium treated macrophages may indicate that its bactericidal potency has been suppressed resulting in poor host defense mechanism.

Cytokines as modulators of the immune response have been little studied in fish. A significant number of cytokines are functionally active in teleost (Secombes 1996). Macrophages play an essential role for the initiation and activation of the innate immune system, by recognizing and releasing a large number of cytokines such as tumor necrosis factor- a (TNF- α), interleukin-1 (IL-1) and IL-6 (Nathan, 1987). Intestinal macrophages have been described a possible source of TNF-α and IL-6. Normally, a rise in the TNFα and IL-6 causes heightened immune activity. The up regulation of secreted inflammatory cytokines by macrophages occurs upon the selective recognition and interaction of the pathogen-associated molecular patterns (PAMPs) that are conserved in bacterial species by the pattern recognition receptors (PRRs) such as the Toll-like receptor (TLR) proteins. However, contrary to this, we found that mercury and cadmium caused a rise in TNFα and IL-6 levels and

inflammation, leading to immunosuppression, thus indicating multiple targets viz., PAMP recognition, receptor dysfunction and/or by affecting the cell-signaling network rendering the fish to an immune compromised and inflammatory state. The inflammatory as well as the functional loss of immune surveillance may well be attributed to oxidative stress induced changes from an increased TNF-α and IL-6 titer. Low levels of TNF-α and IL-6 may preferentially regulate normal intestinal homeostasis; whereas, elevated levels influence or initiate pathological conditions in the intestine.

Thus, it can be concluded from this study that mercury and cadmium has a deleterious effect on the immune functions of fish, specifically on its intestine. The in vivo exposure to sub-lethal concentration of mercuric chloride and cadmium chloride caused alterations in the antioxidant defense system and induced oxidative stress in fish. The in vivo exposure to sub-lethal concentration of mercury and cadmium also caused suppression of the immune parameters (phagocytic activity, intracellular killing, cell adhesion, nitric oxide release and myeloperoxidase release) measured in the intestinal macrophage of *C. punctatus*. After 4 days and 7 days of mercuric chloride and cadmium chloride exposure levels of TNF-α and IL-6 were also found to be significantly more in the treated group than that of control group. These results suggest that the alterations in the phagocytic activity and the oxygen dependent killing mechanisms as well as the antioxidant defense mechanisms can be used as potential biomarkers for risk assessment in aquatic ecosystem.

## 6.2. Oxidative stress, altered antioxidant and immune defense system upon simultaneous exposure to mercuric chloride and cadmium chloride *in vivo* in macrophages of *Channa punctatus*

Next to air, water is an essential constituent of life support system and its quality plays a pivotal role in the maintenance of health. A variety of contaminants including toxic heavy metals like, cadmium, copper, mercury and zinc are found to be ubiquitously present in rivers, reservoirs and are disadvantageous for aquatic organisms (Olsson, 1998). In general, the heavy metals are not biodegraded and therefore, their bioaccumulation in fish, oyster, mussels, sediments and other components of aquatic ecosystems have been reported from all over the world. It appears that problem of heavy metals accumulation in aquatic organisms including fish needs continuous monitoring and surveillance owing to biomagnifying potential of toxic metals in human food chain (Kumar and Singh 2010). Heavy metals are accumulated in fish body in different amount. These variations result from different affinity of metals to fish tissues, different uptake, deposition and excretion rates. Accumulation of metals in various organs of fish may cause structural lesions and functional disturbances (Jezierska and Witeska, 2006). The present results showed significant level of both mercury and cadmium accumulation in liver, gills, muscle, kidney and intestine when treated simultaneously with mercury and cadmium as compared to the control. These results significantly correlate with the altered antioxidant as well as the non-specific immune functions of the macrophages, and also contribute to the above facts.

The mucosa of teleosts provides a protective physical barrier that is important in terms of both osmoregulation and pathogen defense. Simultaneous exposure to mercury and cadmium induces severe inflammatory damage in intestinal epithelium, disarrangement

and fragmentation of mucosal foldings, as observed in the present study, causing impairment of tissue organs which in turn has deleterious effects on its defense system.

The apical surface of the normal epithelial cell is characterized by the presence of regular microvilli and intact RER, frequent invaginations of the plasma membrane at the base of microvilli. It is well known that goblet cells secrete mucous which is used for lubrication and for protection of the mucosa against chemical and physical damage. But after 4 days and 7 days of simultaneous exposure of mercury and cadmium it was found from transmission electron micrograph (TEM) of fish intestine that there is a abnormalities in mitochondria reduction in the number of goblet cells in the stomach. The cells were irregularly shaped with electron-dense nuclei and the nuclei were also irregular in shape with deep indented margins. This may lead to damage of the epithelial cells and other tissues (Omar *et al*., 1998). Lack of apical cytoplasm with unifom microvilli, junctional complexes like zona occludens, desmosomes and gap junctions between epithelial cells were found in the multi metal treated group as compared to the control group. Further, the presence of excess number of granular cells (eosinophil?) in the intestinal tissue in multi metal treated group as compared to the control showed the characteristics of infection and inflammation.

The generation of reactive oxygen species (ROS) and other free radicals during metabolism is a necessary and normal process that ideally is compensated for by an elaborate endogenous antioxidant system. However, due to many environmental, lifestyle, and pathological situations, excess radicals can accumulate, resulting in oxidative stress. Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the cell's ability to reduce ROS, detoxify reactive intermediates and repair damage that may occur in cellular molecules. Reactive oxygen species (ROS) are a cluster of highly unstable compounds that are generated during

inflammation and normal cellular metabolism via incomplete reduction of molecular oxygen. They consist of molecules with unpaired electrons such as superoxide anions ($O_2^{-\cdot}$) and hydroxyl radical ($OH^{\cdot}$), and molecules that have oxidizing propensity but do not possess free electrons, for example hydrogen peroxide ($H_2O_2$) and hypochlorous acid (HOCl) (Abid et al., 2005) . The ROS can rapidly attack all types of biomolecules such as nucleic acids, proteins, lipids, and amino acids (De Vos and Schat, 1991; Mehta *et al*., 1992; Luna et al., 1994), leading to irreparable metabolic dysfunction and cell death. Elevation in respiratory burst activity may indicate that simultaneous treatment of mercury and cadmium potentiates the activity of superoxide-producing enzyme NADPH oxidase generating large amount of ROS thus, exerting a variety of damaging effects.

When the animal's defenses are insufficient to neutralize ROS, oxidative damage may occur, and one of the most serious damages is membrane lipid peroxidation (Scandalios 2005). The integrity of plasma membrane is essential for cell viability, and as a consequence of over activation of phagocytes, its fluidity seems to be effected in fish (Elferink 1987). The level of malonaldialdehyde (MDA) in the tissue is considered a measure of lipid peroxidation status. Lipid peroxidation is linked to the production of $O^{-}_{2}$. Metal-mediated formation of free radicals causes augmentation of lipid peroxidation. Lipid peroxides, formed by the attack of radicals on polyunsaturated fatty acid residues of phospholipids, can further react with redox metals, finally producing mutagenic and carcinogenic MDA (Reyes *et al*., 2013). The results of the present study clearly show that simultaneous intoxication of fishes with mercury and cadmium may enhance formation of MDA when treated with that of the single metal treated groups. Thus it may be hypothesized that mercury and cadmium simultaneously may stimulate the peroxidation of lipids by acting as catalysts in the formation of oxygen radicals.

The SOD-CAT system provides the first defense against oxygen toxicity and represents a cellular defense mechanism to counteract toxicity of ROS. Superoxide Dismutase (SOD) is a metalloenzyme acting as primary preventive inhibitor by catalyzing the conversion of superoxide anion ($O_2^{\bullet-}$) to $H_2O_2$ and $O_2$ (Symons and Gutteridge 1998). Under normal conditions, superoxide anions can undergo spontaneous dismutation in a pH- and concentration dependent reaction to yield $H_2O_2$ and $O_2^-$, the reaction being catalysed by superoxide dismutases. Further, $H_2O_2$ is detoxified by the enzyme catalase. Catalase is a metal-containing enzyme, is the most efficient enzyme that promotes redox reaction. Both $O_2^-$ and $H_2O_2$ can damage a variety of biomolecules (Anjem *et al*., 2009; Imlay, 2009). The results of the present study showed significant decrease in the activity of CAT, in contrast to SOD activity that significantly increased upon simultaneous treatment of fishes with mercury and cadmium. The heme groups of catalase lie deeply buried and are accessible to solvent by way of a narrow channel lined with hydrophobic residues (Reid *et al*., 1981). $O_2^-$, overproduced upon heavy metal intoxication, is small enough to gain access to the heme group of catalase and would convert the resting enzyme to the ferro-oxy state, rendering the enzyme in an inactive form. On the other hand, increased activity of SOD may lead to uncontrolled production of free radicals like $H_2O_2$ thus exerting cellular toxicity.

There are a number of molecules that function as scavengers of free radicals. One of the most abundant and most important molecular antioxidants in cellular cytoplasm is glutathione (GSH). GSH is a low molecular weight thiol. It can react directly with ROS species, thereby detoxifying them (Almoth 2008). Glutathione related enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR) function either directly or indirectly as antioxidant and glutathione-S transferase (GST) plays an important role in metabolic detoxification.GR catalyzes the formation of GSSG (oxidized form) to GSH

(reduced form) thus maintaining the GSSG/GSH ratio in cells. Decreased levels of glutathione in the cytoplasm or an increase in the ratio of GSSG to GSH resulting from an increase in ROS can lead to transcription of redox sensitive genes. This occurs via initiation of a signal cascade resulting in assembly of transcription factor subunits in the nucleus (Almroth 2008). The present study revealed that simultaneous exposure to mercury and cadmium decreased the glutathione related enzyme activity and the antioxidant molecule GSH significantly.

Phagocytosis is one of the most important parameters associated with immune reaction in both invertebrates and vertebrates which normally mobilizes lysosomes for the invading phagocytosed materials (Gopalakrishnan *et al*., 2011). Phagocytosis starts when bacteria have adhered to the surface of the phagocyte. It involves several steps like recognition and attachment of a foreign particle, engulfment and digestion. A particle attached to the surface membrane initiates the ingestion phase by activating an actin-myosin contractile system which extends pseudopods around it. As adjacent receptors attach to the surface of the foreign particle, the plasma membrane is pulled around the particle until it is completely enclosed in a vacuole (phagosome). Then, cytoplasmic granules fuse with the phagosome and discharge their contents around the micro-organism, which is subjected to a considerable battery of microbicidal mechanisms. Upon 4 days and 7 days of simultaneous exposure of mercury and cadmium, macrophages fail to perform its normal functions, probably by deactivating the actin-myosin contractile system, thereby allowing foreign particles or pathogens to gain access to the body which is clearly depicted in the results of the present study.

Professional phagocytes have evolved and are known to internalize and kill as many antigens as possible. Normally, bacteria within macrophages are rapidly killed and degraded in the phagolysosome with the secretion of enzymes and toxic peroxides,

making it difficult to dissect the mechanism of death (Slauch, 2011). The present study shows that macrophages upon simultaneous treatment of mercury and cadmium fails to kill pathogens efficiently when compared with that of the control group. The present study clearly illustrates a greater percentage of viable bacteria within the multi-metal treated cells as compared to the control and the single metal treated groups which suggest that both mercury and cadmium limits its intracellular killing activity after internalization of the pathogen.

Nitric oxide (NO•) synthesized by inducible nitric oxide synthase (iNOS) in activated macrophages is an important host defence mechanism; mediates "non-specific" immunity. NO• contributes to the inflammatory response; leading to tissue leakage and damage thereby increasing vascular permeability (Kulkarni and Madrasi 2008). The results of the present study shows a significant decrease in NO release upon simultaneous exposure to mercury and cadmium after 4 days and 7 days on macrophages as compared to control and the single metal treated group. Inhibitory influence of toxic heavy metals like mercury and cadmium might be exerted on the catalytic site of NOS by direct binding or by interference with electron transfer during catalysis, thus, suggesting the possibility of iNOS being sensitive to mercury and cadmium. Huang et al., (2002) established that in the absence or too low a level of NO, IL-12 signaling in NK cells is blocked, thus reducing the IFN-γ release of NK cells (Diefenbach et al., 1999) and rendering the innate defense ineffective against invading pathogens. However, when NO escapes from the phagocytic milieu due to morphological damage to macrophages exposed to heavy metals like mercury and cadmium, it reacts with singlet oxygen species to form peroxynitrite radicals that cause DNA fragmentation in the cells.

The myeloperoxidase (MPO) is a highly basic heme enzyme which is released extra cellularly by activated neutrophils, monocytes and macrophages. MPO catalyses the oxidation of halide ions by $H_2O_2$ (hydrogen peroxide) to the corresponding hypohalous acids (HOCl, HOBr etc), which are strongly bactericidal and markedly increases the antibacterial potency of ROS. Along with oxidants from $H_2O_2$, MPO can generate a range of co-substrates, most notably chlorine (Foote *et al*., 1983) and nitrite (Eiserich, 1998). Upon simultaneous treatment of mercury and cadmium the enzyme poorly performs its activity as it is clearly evident from the present study which depicts a significant fall in percentage of enzyme release when compared to control and single metal treated group.

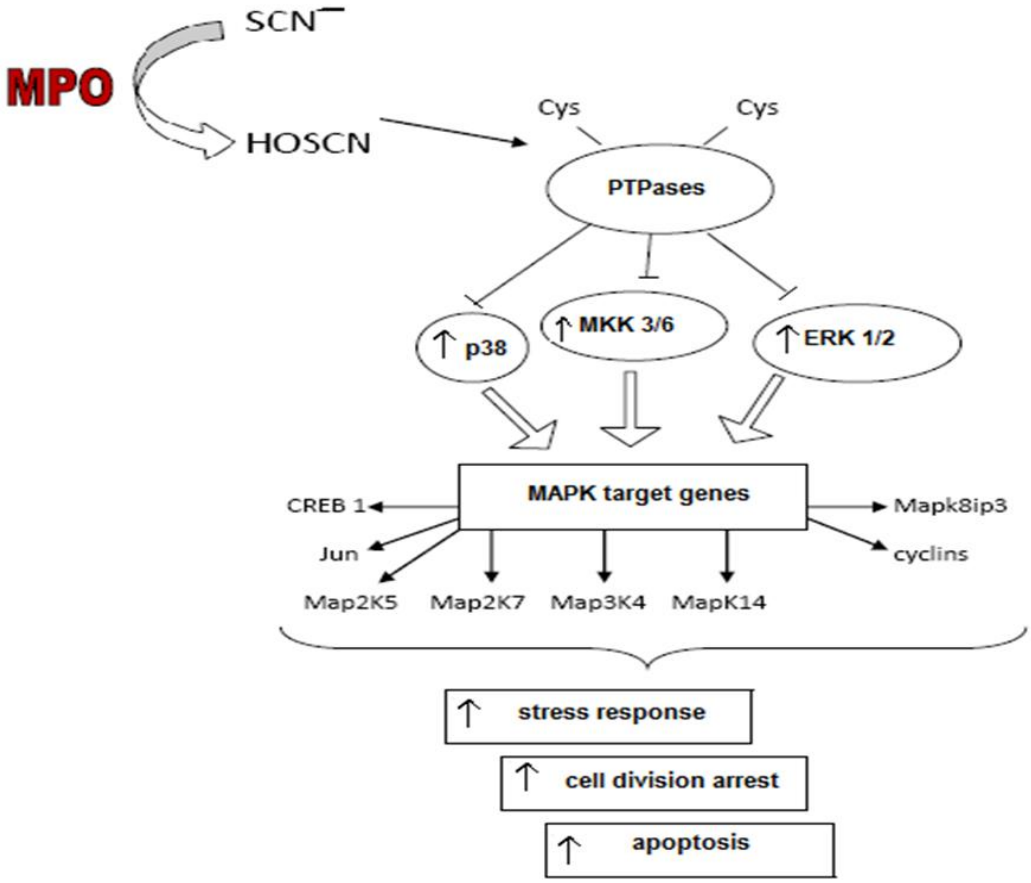


Fig. 6.3: A flowchart showing how the activity PTPase is stimulated upon MPO catalysed $SCN^-$ion and thereby altering the functions of MAPK target genes leading to stress response.

Monocytes and tissue macrophages are the most important cells in the immune response of fish. Macrophages are not only important in the production of cytokines (Clem *et al*., 1985) but are the primary cells involved in phagocytosis and killing of pathogen upon first recognition and subsequent infection (Shoemaker *et al*., 1997). Vallego *et al*.,*(*1992) suggested that macrophages as being the primary antigen presenting cells in teleost, thus linking the non- specific and acquired immune responses. By releasing a large number of cytokines such as tumor necrosis factor- a (TNF-α), interleukin-1 (IL-1) and IL-6, macrophages play an important role in the activation of innate immune system. Levels of TNF-α and IL- 6 was found to be significantly more in multi- metal treated groups than that of control group and single metal treated group. Cytokine expression becomes upregulated, probably by the action of both the heavy metals at multiple levels viz., PAMP recognition, receptor dysfunction and/or by affecting the cell-signaling network rendering the fish to an immunocompromised and anti-inflammatory state. The following figure summarizes the effects of mercury and cadmium exposure in the fish macrophages:

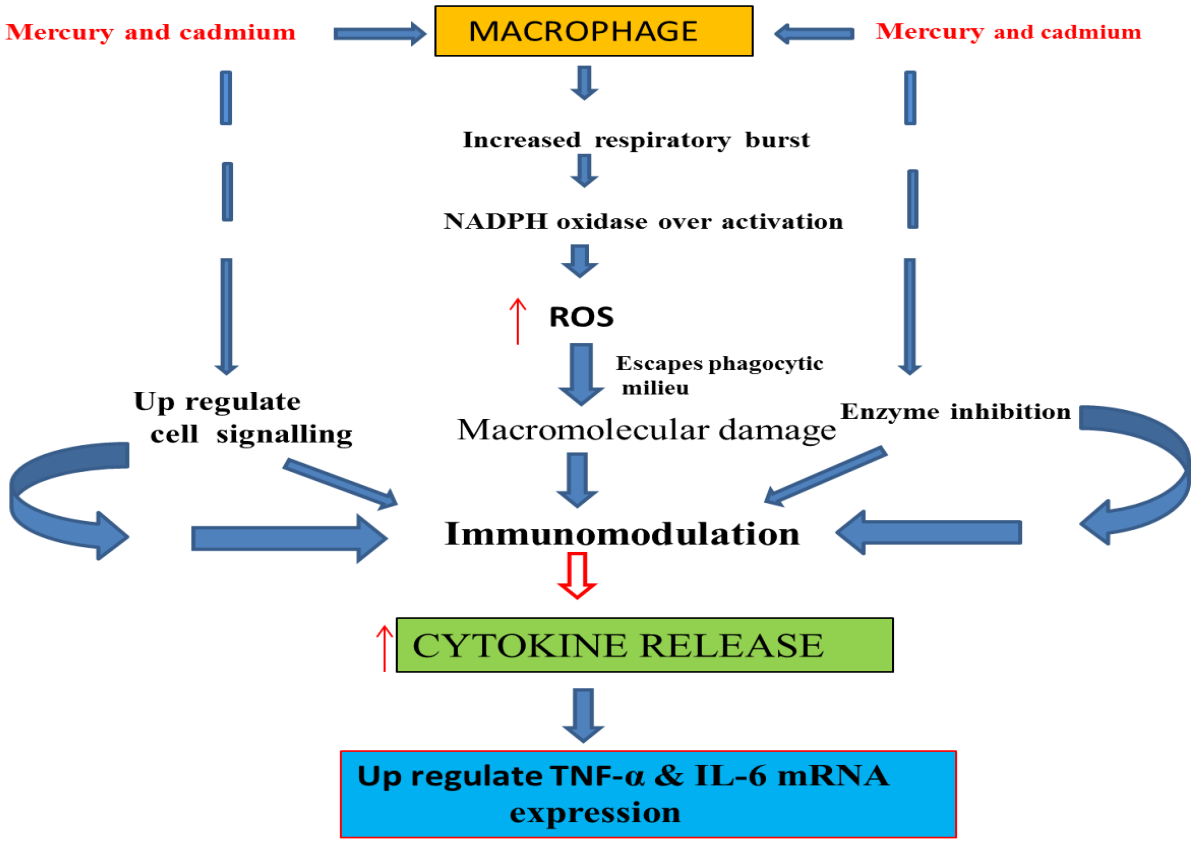


Fig. 6.4: An overview of the general mechanism of action of mercury and cadmium exposure in fish intestinal macrophages.

### 6.3.1: One way ANOVA

In addition to 'Student t' test, one way classified ANOVA was conducted to test whether there is significant difference of NO and TNF-α release levels across the dose levels of mercury (Hg) and cadmium (Cd), refer to table no.5.3, 5.4, 5.9, 5.10 (page no. 121, 124). The dose levels of Hg (mg/L) are respectively, 1.2, 0.6, 0.3, 0.15, 0.075 and the dose levels for Cd (mg/L) are respectively, 7.84, 3.92, 1.96, 0.98, 0.49. For each dose level, four observations were recorded. The mean NO and TNF-α for each dose is presented in the last row. Thus, the one–way classified ANOVA was estimated on the basis of these 5 doses keeping the control also as a dose category. Computed F-statistics was found to be highly significant even at 0.001 percent, which implies significant variations of NO and TNF-α across the levels of Hg and Cd. The same

ANOVA was repeated after dropping control as a separate dose level. The high F- value in this case is suggestive of statistically significant variation of NO and TNF-α releases across doses.

For NO release :

- F = 394.58 (in case of Hg without control as separate group)
- F = 413.54 (in case of Hg with control as separate group)
- F = 137.86 (in case of Cd without control as separate group)
- F = 197.94 (in case of Cd with control as separate group)

For TNF-α release:

- F = 65.62 (in case of Hg without control as separate group)
- F = 64.73 (in case of Hg with control as separate group)
- F = 55.29 (in case of Cd without control as separate group)
- F = 54.80 (in case of Cd with control as separate group)

### 6.3.2. Isobologram: A mathematical model to study interactivity

Multimetal synergism has been proved by applying a multivariate anova to the experimental results of TNF-α release and constructing an isobologram running an ordinary least squares regression between effects (TNF-α release) and dose levels of metals (single and multimetal) in log-linear form. In the isobologram, on plotting the concentrations of mercury against the concentrations of cadmium at which effect (in this case TNF-α release) remains constant a convex line showing synergism is demonstrated, refer fig.5.47. (page no. 127). Here, shortfall of TNF-α release for treated group values (both single and multimetal) from the mean TNF-α release of the control group is supposed to reflect the impact of heavy metal exposure. The figure below portrays that a simultaneous exposure to mercury and cadmium has a synergistic effect

as compared to the effects of independent exposure to them. Mercury and cadmium are present as a fraction of their minimal effective concentrations.The exact mechanism of this multi-metal interaction on fish immune functions is yet to be elucidated.

### 6.4. Heavy metals (Mercury and Cadmium) alters the relative gene expression of tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) genes in the intestine of *Channa punctatus* after the exposure for 7 days

Macrophages are multifunctional cells of the immune system with extended functional roles including clearance of microorganisms, xenobiotic material, and apoptotic cells to the regulation of both innate and acquired immune responses. They are involved in phagocytosis, antigen presentation and release of antimicrobial and antitumor agents, as well as in the inflammatory reaction by secreting different types of cytokines and chemokines (Mackenzie *et al*., 2006). Several fish cytokine genes have been isolated and characterized in recent years and its mRNA expression can be used as a tool for measuring immune responses. In particular, pro-inflammatory cytokines, including interleukin-1β (Zou *et al*., 1999), TNF-α (Laing *et al*., 2001), and IL-6 (Kishimoto *et al*., 1988) are commonly used immune-regulatory genes in fish. Interleukin-1b (IL-1β) is a key mediator of host response infections and a primary cause of inflammation (Dinarello 2002), identified in 13 teleost species with a role similar to that in mammals (Mathew *et al*., 2002). TNF-a is an inflammatory cytokine as well, and, in mammals, it is produced by macrophages, neutrophils, monocytes, natural killer cells and T cells after their stimulation by bacterial lipopolysaccharide. It is probably the most studied cytokine in fish, in species such as rainbow trout (Laing *et al*., 2001) carp (Saeij *et al*.,2003), catfish (Zou *et al*., 2003), red seabream (Cai *et al*., 2003), Atlantic salmon (Ingerslev *et al*., 2006), Gilthead seabream (Castillo *et al*., 2002) and the mandarin fish (Xiao *et al*., 2007)

IL-6 is mainly secreted by macrophages and T cells to stimulate the immune system as a response to inflammation (Hirano, 1998; Kaplanski *et al*., 2003; Inoue *et al*., 2005) and has been described in Fugu (Bird *et al*., 2005), rainbow trout (Iliev *et al*., 2007) as well as in the seabream (Castellana *et al*., 2008).

Cytokines, such as TNF-α and IL-6 have a fundamental role in the regulation of the fish immune response (Engelsma *et al*., 2002). In the present study, the exposure of *Channa punctatus* to mercury and cadmium for 7 days caused a general increase in TNF-α and IL-6 mRNA indicating a stimulatory action upon proinflammatory processes. Mercury and cadmium interact positively, inducing an increase in cytokine expression suggesting a possible increase in inflammatory status.

One of the most potent sources of superoxide anions is the phagocyte respiratory burst NADPH oxidase system. Six homologs of the cytochrome subunit of the phagocyte NADPH oxidase were found: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2. The homologs are referred to as the NOX family of NADPH oxidases. These enzymes have the capacity to transport electrons across the plasma membrane and to generate superoxide and other downstream reactive oxygen species (ROS). The physiological functions of NOX family enzymes include host defense, post translational processing of proteins, cellular signaling, regulation of gene expression, and cell differentiation. NOX enzymes also contribute to a wide range of pathological processes (Bedard and Krause 2007). Exposure to environmental contaminants provides an exogenous source of superoxide. To counteract the potential damage caused by oxyradicals, cells have developed a number of protective mechanisms that includes antioxidant enzymes, such as SOD, CAT etc. In the present study, RB, LPO and SOD were increased in *Channa punctatus* after 7 days of exposure to mercury, cadmium and mercury plus cadmium. These results support that the heavy metals as an inducer of ROS production in fish and

the results also indicate that the antioxidant defenses of the cells were activated to counteract the pro-oxidative potential of mercury, cadmium and the mixture of both. The present study showed an increase in TNF-α and IL-6 mRNA expression after 7 days of exposure. In our study the differences between the TNF-α and IL-6 mRNA expression level caused by mercury or cadmium alone and those obtained after exposure to the mixture, revealed an synergistic interaction, possibly due to a cumulative effect of both stimulus and subsequent ROS overproduction, impairing cell metabolism. Thus it may be hypothesized that mercury and cadmium simultaneously somehow suppressed the translation of the regulatory proteins as several studies showed that the increase in the expression of a given gene is not always accompanied by the correspondent translation into protein (Teles *et al*.,2011), leading to uncontrolled enzyme activity, elevation in the respiratory burst activity as a result of which there is an over activation of the superoxide producing enzyme NADPH oxidase and generation of large amount of ROS which is destined to cell damage.