IMPACT OF HEAVY METALS ON THE TISSUES OF FRESH WATER FISH *Oreochrmis niloticus* – A SYSTEMATIC APPROACH

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Abstract:

Exposure to heavy metals is an important environmental problem resulting from anthropogenic activities. So, in the present study, the bioaccumulation of heavy metals (Pb, Cd, and Cu) in the muscles of fresh water edible fish *Oreochrmis niloticus* caught from the Theroor wetland was carried out as they remain present in some or the other form harmful for the human body and its proper functioning and to ascertain its impact on the protein levels in muscle of selected fish *Oreochrmis niloticus*. The results of the current study indicated that heavy metals in the edible parts of the investigated fish are not in the permissible safety levels for human consumption (FAO/WHO). Thus, this paper gives an overview of the manipulation of fish as a biomarker of heavy metals through proteomic studies which have proven to be very useful in the environmental pollution monitoring.

Keywords: **Heavy metals, Protein, Muscle, *Oreochrmis niloticus***

I. INTRODUCTION

Aquaculture and fresh water fisheries are gaining additional emphasis due to our concern in sustainability, greener solutions, conservation and food security. For centuries fresh water fishes has been recognized as an excellent food source for human beings and is preferred as a perfect diet not only due to its excellent taste and high digestibility but also because of the presences of unsaturated fatty acids, essential amino acids, minerals for the formation of functional and structural proteins (Kumar, 1992) and also due to the significant positive impact it brings in improving the quality of dietary protein by complementing the essential amino acids that are often present in low quantities in vegetable based diets (Sargent *et al.* 1995).The fresh water bodies, the habitat of freshwater fishes are according to the current scenario getting

very much highly polluted due to unawareness of rural and urban people intervention and activities. So being the major aquatic organism and being accumalatized to all the zones of living medium they respond to a wide range of natural and man-made environmental stressors, which can lead to molecular changes within their tissues especially muscles which contribute about 34–48 % of the total body mass in fish.

As of today, consumers are more aware off to know about the food they eat and also, they have so many methods to get clarified. Currently proteomics a bioinformatics tool plays an increasing role in food authentication. Fish, the globally accepted cheapest food but with rich source of protein, minerals and vitamins was used by all people. But there is in need of provenance of the food that can be guaranteed from farm to other bio indicator tools like water quality, by aquatic diversity, biochemical analysis etc. Moreover, the key aspect of aquaculture is characterization of the muscle proteome as muscle plays a central role in whole-body protein metabolism, encompassing physiology and growth. Since proteins can be used as bio molecular tool for many food property analyses like Food safety, food authentication, food quality, traceability, shelf-life, recently many molecular biologists started focusing on proteomic works in relation to environmental stress on fish to determine, evaluate and improve of the fish species production and management. Bostock *et al*., 2010, in his view stated that while aquaculture industry is growing at a higher rate than any other animal food-producing sector nutrition, welfare and health management have been thought to be the main limitations to an efficient production in aquaculture systems.

Rodrigues *et al*., 2012 explained that one of the main goals of aquaculture industry is to produce fish with an optimal growth performance and health status. Rodrigues *et al*., 2012 in his view stated proteomics has emerged as a powerful tool towards a deep understanding of aquatic organism's biology and provides data at a mechanistic level. Molecular interaction network visualization is one of the most user-friendly features developed for the simulation process of biological interactions. According to Ian Craig *et al*. (2005); Doran *et al*. (2007); Ohlendieck, (2011) presently researchers are trying to improve the yield of fish by looking at protein expression profiles and biochemical characterization of the entire protein complement of tissues in both normal and pathological specimens using informatics tools so that the functional genomics information generated could act as the baseline data for further molecular research on this species. Thus, computational study will allow muscle proteins to be studied at greater level in detail using a variety of bioinformatics tools that are available for making detailed comparative study and visualization of amino acid sequences, which provides knowledge about molecular evolution and variety of information related to structure and function of protein. Besides adding to the existing knowledge base on comparative muscle proteomics, the information generated would also serve as the baseline proteogenomic information on this exotic species O*reochromis niloticus* the freshwater fish of the family: Cichlidae inhabiting in the tropical wetland water bodies of Indian subcontinent, is an important food fish with high nutritive value. This species and is also considered as a species of choice in wetland pollution monitoring because it is a commercially important species contributing a major share to freshwater aquaculture production in the Indian

subcontinent; however, little omics information is available on this species. So, the main aim of this present analysis was to examine the changes in the muscle protein expression profiles of common freshwater fish *Oreochromis niloticus*, of Theroor wetland, a key model species for investigating environmentally induced physiological plasticity that are seriously lacking in the literature.

II. MATERIALS AND METHODS

The proteins and enzymes in *Oreochromis niloticus* that are responsible for heavy metal assimilation were identified at the genome level. The proteins with already known function were explored by text mining whereas the functions of hypothetical and uncharacterized proteins were predicted. The KEGG Orthology (KO) system is a pathway based definition of orthologous genes (Moriya *et al*. 2007). The function of missing proteins was predicted according to the methods described by Chellapandi, (2011). Briefly, a protein whose function was not yet reported in databases or annotated function not compiled in KEGG pathway database, but which has separate KO system in the KEGG GENES database, their protein function was assigned to be predicted with KO.

2.1 Proteome retrieval The complete proteome of *Oreochromis niloticus* was retrieved from Uniprot nowledgebase. UniProt KB/Swiss-Prot is the manually annotated and reviewed section of the UniProt Knowledgebase (UniProt KB) (The Uniprot Consortium).

2.2 Metabolic Pathway Analysis The metabolic pathway analysis of *Oreochromis niloticus* was carried out using KEGG (Kyoto Encyclopedia of Genes and Genome) (Kanehisa *et al*. 2016). KEGG is a database resource that integrates genomic, chemical and systemic functional information. Further, Protein-Protein interactions of *Oreochromis niloticus* can be identified using STING database (Szklarczyk *et al*. 2015).

2.3 Cell simulation Simulation of intervention of heavy metals in the system of *Oreochromis niloticus* was carried out using Cell designer software (Funahashi *et al*. 2008). It is an application in which compartmental topology, geometry, molecular characteristics, and relevant interaction parameters can be used defined. It automatically forms a corresponding mathematical system of ordinary and/or partial differential equations from the given biological description.

III. RESULTS AND DISSCUSSION

The extensive text mining with the available databases resulted in the identification of the proteins involved in the assimilation of heavy metals in *Oreochromis niloticus* .

Lead assimilation system

A total of 5 proteins were identified in *Oreochromis niloticus* involved in Lead assimilation. The list of proteins is tabulated in Table 1, which signifies genes such as glo1, esd, Loc100695823, gstk1 and

unknown corresponding to proteins Lactoyl glutathione lyase, S-formyl glutathione hydrolase, S-(hydroxy methyl) glutathione dehydrogenase, Glutathione S-transferase and Glutathione peroxidase respectively. The KO assignment for these proteins reveals its role in Glutathione metabolism which is portrayed in Fig 1. Further, the rates of metabolic changes of the selected proteins in the presence of Lead at various concentrations were simulated using Cell designer and the results are presented in Table 2.

From Table 2 it can be inferred that the concentration of the proteins Lactoyl glutathione lyase (I3J4P3), S-(hydroxy methyl) glutathione dehydrogenase (I3IXM8), Glutathione S-transferase (I3K4Z5) increases with increase in the concentrations of Lead. On the other hand, the concentration of the proteins, S-formyl glutathione hydrolase (I3KGY7) and Glutathione peroxidase (I3KTP3) decreases with increasing concentration of lead at various time intervals.

Cadmium assimilation system

A total of 5 proteins were identified in *Oreochromis niloticus* involved in cadmium assimilation. The list of proteins is tabulated in Table 3 which signifies genes such as Loc100696572, Jun, Rad51, and unknown corresponding to all Uncharacterized proteins. Superoxide dismutase was also involved in cadmium assimilation with gene name Sod2. The KO assignment for these proteins reveals its role in Apoptosis which is portrayed in Fig 2. Further, the rates of metabolic changes of the selected proteins in the presence of cadmium at various concentrations were simulated using cell designer and the results are presented in Table 4.

From Table 4 it can be inferred that the concentration of the proteins I3KVU8, I3KYD0 and I3IYF0increases with increase in the concentrations of cadmium. On the other hand, the concentration of the proteins, I3K6D5 and I3IZK4 decreases with increasing concentration of cadmium at various time intervals.

Copper assimilation system

A total of 5 proteins were identified in *Orochromis niloticus* involved in copper assimilation. The list of proteins is tabulated in Table (5) includes Copper-transporting ATPase, Cytochrome c oxidase, Amine oxidase, Superoxide dismutase [Cu-Zn] and Tyrosinase-related protein 1. The KO assignment for these proteins reveals its role in Tyrosine metabolism which is portrayed in Fig 3.

From Table 6 it can be inferred that the concentration of the proteins F5C7J6 and I3JAL8 exhibit positive correlation with the concentration of copper. But, the concentration of the proteins, D2Y6D3, I3K1J1 and Q6IUZ2 decreases with increasing concentration of copper at various time intervals.

Heavy metals occur naturally in the earth's crust at various levels. Recently due to latest technological and industrial developments many dangerous chemicals have been released without any pre-treatments directly are indirectly into inland water bodies. Nonetheless, the problems arises when they are

released at chronic levels into the environment due to rapid urbanization, anthropogenic activities, modern agricultural practices, using huge amount of pesticides and chemicals in lands in and around water bodies, getting contaminated with heavy metals due to over use of municipal waste, compost waste, influx of heavy metals from industries all together resulting in accumulation of heavy metals in the environment, which in turn causes toxicity to living organisms in particular- fish (Miransani, 2011). This current situation has further got worsened by increasing population growth leading to inherent food demand specifically protein rich food i.e. fish.

As of today the consumption of fish, the poor man's lobster by human beings have increased globally to 80-90% because, biologically fish muscle proteins contain all essential nutrients, so fish holds prime importance in food industry. Furthermore, it is very nutritious part of human diet because it is rich in vitamins, minerals and all essential amino acids in right proportion. In addition, fish populations tend to be stable and easy to collect and they were widely used in bio monitoring of environmental pollutants, heavy metals in specific. So, heavy metals contamination of water causing stress to organisms has become one of the important constraints to fish productivity and fish food quality (Protein). Moreover majority of human population especially rural people living within the surrounding of freshwater bodies which often acts as sinks for such pollutants arising from anthropogenic materials in environment in unprecedented levels consume fish harvested from them. Xenobiotics (i.e.) heavy metals bio accumulated in aquatic inhabitants are subsequently transferred to humans through the food chain.

The movement of toxicants through a transported medium may significantly impact all immune organisms ie. fauna, flora as populations or as ecological communities. Also a variety environmental process may alter the chemical structure of pollutants which could challenge organisms in particular locations by altering their community in a particular location and by influencing their ability to with stand further challenges posed by anthropogenic activities and pollutant. So, therefore very complex system called metal assimilation system was taken up to analyze the fish muscle protein using different computational tool as protein various in amount in polluted environment. So biomarkers with in sentinel organism (*Oreochromis niloticaus)* have been extensively used to evaluate changes in the environment and habitat.

According to Liu *et al.* (2013) proteomics is a well-established technique in the post- genomic era, which deals with large scale expression of proteins in an organism. This being a powerful tool not only describes complete protein changes in any organisms but also helps in comparing variations in protein profile of an organisms at organ, tissue, cell and organelle levels under different stress condition including heavy metals. Ge *et al*. (2013) too stated that omics technology, a robust molecular biomarker measurement of protein would allow early detection of environmental stress upon exposure of chemicals. It is also well known that proteins are the important bio molecule that directly takes part in any organism's stress response and moreover organisms adapting to heavy metals stress are always accompanied with proteomic changes.

Thus proteomic analyses offer a new platform for identifying target proteins which take part in heavy metals detoxification, and in studying complex biological processes and interactions among the possible pathways that involves a network of proteins. Thus, proteomic technique has be exploited in this current chapter for deciphering the possible intervention between protein abundance and stress adaptation as it can contribute to better understanding of physiological mechanisms under heavy metal stress and further signaling cascade that leads to changes in the expression to large number of genes in metabolic profile under heavy metal toxicity.

So in the present work muscle protein of *Oreochromis niloticus* has been analyzed for intervention of metals which will be beneficial for humans in drug designing and maintain food care from consumer point of view. The heavy metals considered most toxic and apparently most poisonous to freshwater life which are reviewed in the present study include zinc, copper, nickel, lead, cadmium and mercury. Fish and people are primarily exposed to heavy metals like lead, cadmium, zinc, nickel, copper and mercury by food ingestion and breathing. These heavy metals get accumulated in the muscles, bones, blood and fat. Neonates and young children are especially delicate to even low levels of heavy metals. As recently pointed out, in several areas of the freshwater wetlands, high concentrations of these elements are present in many types of commercial important fish, *Oreochromis niloticus* (WHO, 2005; Erdoayrul Az, ateay DA (2006); Nduka *et al*. 2010; Hosnia Abdel-Mohsien and Manal Mahmoud, 2015). Long term consumption of foodstuff (i.e. fish) contaminated with metals may lead to the accumulation of toxic metals in several vital organs resulting in perturbation of biochemical processes, which may cause liver damage, kidney damage, cardiovascular problems, nervous problems, bone disorders, memory loss, mental retardation, low IQ, learning deficits in particular among young children and also in people of other age too.

The toxic effects of heavy metals can affect the individual growth rates, physiological functions, mortality and reproduction in fish (Amundsen *et al*. 1997). Lead deplete major antioxidants in the cell, especially thiol-containing antioxidants and enzymes, and can cause significant increases in a reactive oxygen species (ROS) production, followed by a situation known as ‘‘oxidative stress’’ leading to various dysfunctions in lipids, proteins and DNA (Ercal *et al*. 2001). This is further validated from the present study, that the lead assimilation system is a part of glutathione metabolism. Similarly, sublethal effects such as decreased growth, inhibited reproduction, and population alterations may occur after chronic exposure to cadmium (Eisler, 1985). This has been further proved from the present study on the involvement of cadmium assimilation system with apoptotic pathway. On the other hand, copper forms a part of many enzymes and glycoprotein in fishes, it is important for nervous system function and is necessary for hemoglobin synthesis Sorensen, (1991); Nordberg *et al*. (2007). In line with the findings, the present study confirms its role in tyrosine metabolism. In contrast, the present study reveals the role of nickel in calcium signaling pathway, it was already confirmed that nickel exposure results in decreased blood parameters in fishes (Gochfeld, 2003). But mercury contamination causes irreversible damages, such as neurological

impairment and lesions, behavioral and cognitive changes, ataxia, as well as convulsions, in addition to its harmful effect on reproduction which supports the present finding of the role of mercury assimilation system in cancer (Oliveira, 2006). In contrast, zinc exposure has been shown to induce histopathological alterations in ovarian tissue of *Tilapia nilotica* (degeneration and hyperaemia) (Abd, 1999) and liver tissue of *Oreochromis mossambicus, but the present study shows its involvement in protein processing as it's a cofactor for many enzymes.* Thus, the *In-silico* approach made in the present study revealed that fish proteins have functional significance and this study can be further taken up to next stage in exploring proteins of which can be incorporated in designing effective vaccines- drug designing approach.

The present study gives an overview of water quality, eco-biology of micro and macro organisms, the diversity status and impact of pollutants especially xenobiotic on fish biomolecules and their health effects on human beings. As pieces of continuity the next part of the present study was focused on protein molecule of *Oreochromis niloticus* because the proteome varies among organs with time and reflection of organisms to environment due to their adaptive nature. Because there is a general consensus about today's industrial and human activity impacts in the fresh water which is the cause of the depletion of a varied range of living organisms. It is well agreed with the present results that the current situation of wetland water seems to be further exacerbated by risk derived from the continuous incoming of industrial pollutant in form of heavy metals which in turn alters the biomolecules of the major aquatic life like fish. So the analyses of the impact and it level on *Oreochromis niloticus* muscle proteomic analysis was a carried out as it gives the snapshot of selected organisms state of being and map the entirety of its adaptive potential and mechanisms. On analyzing the impact of xenobotics on fish muscle using system approach-abioinformatics tool resulted in the identification of theproteins involved in the assimilation of heavy metals in *Oreochromis niloticus*. There are 5 proteins involved in Lead, Cadmium, Copper assimilation in *Oreochromis niloticus*; 4 proteins involved in Zinc assimilation in *Oreochromis niloticus*; 3 proteins involved in nickel assimilation in *Oreochromis niloticus*; 1 protein involved in mercury assimilation in *Oreochromis niloticus* was identified using Uniport. Each and every heavy metal had its own impact on the biomolecule pathway like metal lead following the situation known as oxidative stress leads to various dysfunction in fish. Similarly, copper, nickel and mercury exposures resulted in decrease in blood parameter level and dysfunction of nervous system. Zinc exposure will induce histopathological. Finally, the functions and status of the interested proteins are analysed using Uniport, bioinformatics tool and discussed to obtain a better understanding of the molecular mechanisms in combination with other investigations. And most of the metals are present in edible portion of fish. Humans are also affected by eating fish and can cause a few of health problems. The levels of toxic elements in different fishes depend on the fish sex, age, season and place. The pollution of waterways with anthropogenic activities are the major cause of aquatic loss and imbalanced food chain. To eliminate and avoided the aquatic life loss there is need to use the advanced technologies generating less heavy metal pollution to environment

Thus, Uniport being a very promising application in search and detection of different proteins and the characterization of biologically active proteins and the impact of contaminants in aquatic organisms have been applied in the present study to enhance the aquaculture proteomics for the global analysis of protein expression in freshwater fishes of commercial importance for the benefit of consumers. Form consumer point of view regarding the food quality and health assurance aquacultural industries in-spite of following traditional methods many different instrumental techniques have been proposed for food authentication one such new approach called proteomics has been applied to analyze the muscle composition of *Oreochromis niloticus* of Theroor wetland wetland for creating awareness and data base for future generations to conserve the natural resources like wetland ecosystem and their components especially freshwater fishes and their feeds.

Table 1 Proteins involved in Lead assimilation in *Oreochromis niloticus*

S. No	Uniprot ID	Protein	Gene name	Reaction	Function
1	I3J4P3	Lactoyl glutathione lyase	Glo1	(R)-S-lactoyl glutathione = glutathione + methylglyoxal	Catalyzes the conversion of hemimercaptal, formed from methylglyoxal and glutathione, to S-lactoylglutathione.
2	I3KGY7	S-formyl glutathione hydrolase	Esd	S-formyl glutathione + H_2O = glutathione + formate	Serine hydrolase involved in the detoxification of formaldehyde
3	I3IXM8	S-(hydroxy methyl) glutathione dehydrogenase	LOC100695823	S-(hydroxy methyl) glutathione + $NAD(P)^+$ = S-formyl glutathione + NAD(P)H.	S-(hydroxymethyl)glutathione dehydrogenase activity
4	I3K4Z5	Glutathione S-transferase	gstk1	RX + glutathione = HX + R-S-glutathione	Glutathione Transferees activity
5	I3KTP3	Glutathione peroxidase	-	-	glutathione peroxides activity; response to oxidative stress

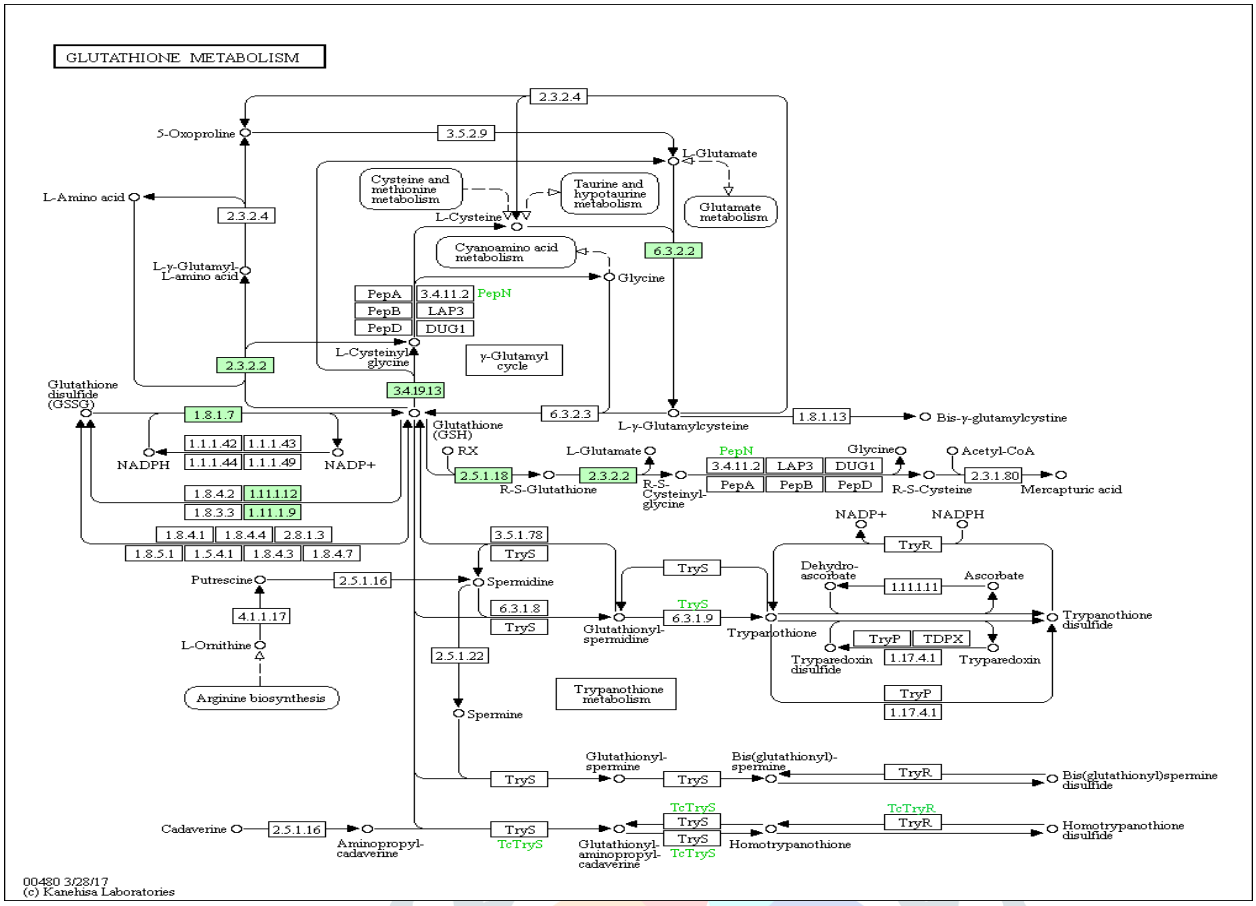

Fig 2 Network of Glutathione metabolism in *Oreochromis niloticus*

Table 2 Rate of change of Lead assimilation system at various concentrations of Lead in *Orochromis niloticus*

Conc. of Lead	Conc. in mM					Time in Min
	I3J4P3	I3KGY7	I3IXM8	I3K4Z5	I3KTP3	
2.1	50	25	70	80	15	5
2	45	32	60	75	22	10
1.9	42	35	52	45	25	15
1.7	33	42	45	35	32	20
1.4	29	45	40	30	35	25
1.2	23	50	35	25	40	30
0.9	20	55	30	20	45	35
0.8	15	60	20	10	50	40
0.7	0	70	10	5	60	45
1.9	42	35	52	45	25	50
1.8	35	40	48	48	30	55
1.7	33	42	45	35	32	60
Correlation	0.961956	-0.97014	0.961119	0.903499	-0.97014	

Table 3 Proteins involved in cadmium assimilation in *Oreochromis niloticus*

S. No	Uniprot ID	Protein	Gene name	Reaction	Function
1	I3KVU8	Uncharacterized protein	LOC100696572		ATP Binding; response to cadmium ion
2	I3K6D5	Superoxide dismutase	Sod2	2 superoxide + 2 H^+ = O_2 + H_2O_2	superoxide dismutase activity; response to cadmium ion
3	I3KYD0	Uncharacterized protein	JUN	-	transcription factor activity; response to cadmium ion
4	I3IZK4	Uncharacterized protein	Rad51	-	recombines activity; response to cadmium ion
5	I3IYF0	Uncharacterized protein	-	-	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process; response to cadmium ion

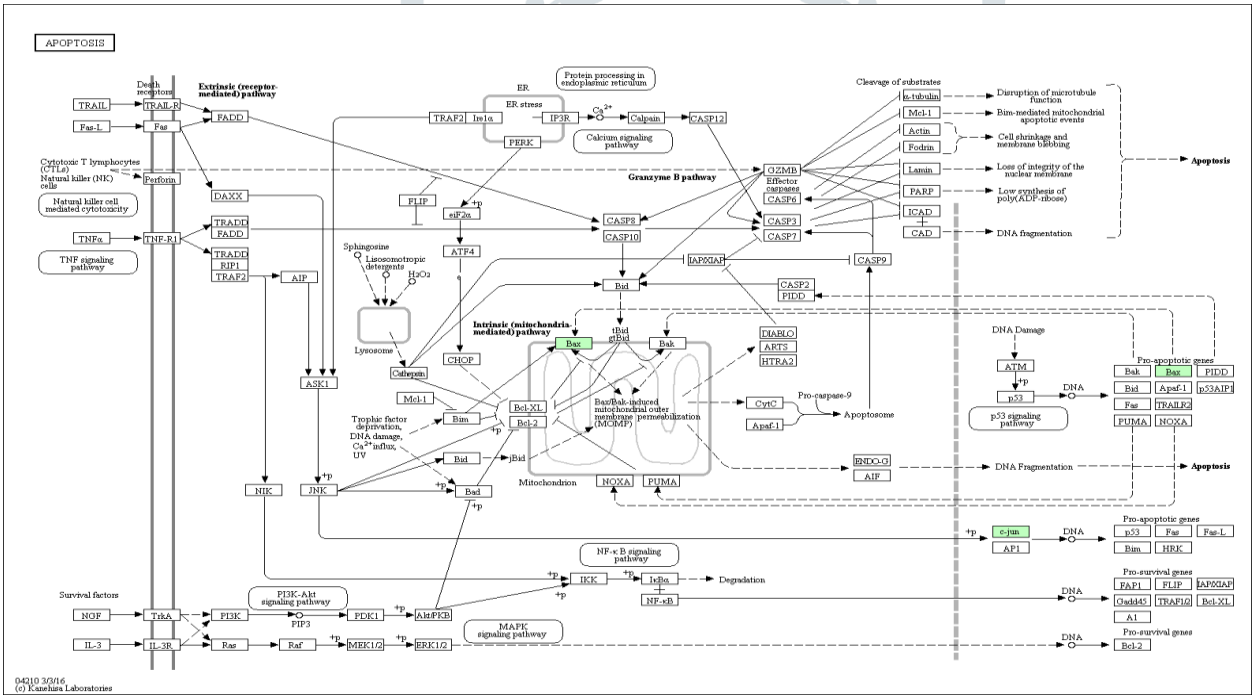

Fig (3) Network of apoptosis in O*reochromis niloticus*

Table 4 Rate of change of cadmium assimilation system at various concentrations of cadmium in *Orochromis niloticus*

Conc. of Cadmium	Conc. in mM					Time in Min
	I3KVU8	I3K6D5	I3KYD0	I3IZK4	I3IYF0	
2.05	70	5	89	5	56	5
2.04	65	12	76	7	54	10
2.05	68	15	65	5	58	15
0.92	63	19	54	12	59	20
0.85	61	25	48	15	49	25
0.79	55	36	42	13	47	30
0.71	43	42	37	14	39	35
0.62	39	56	33	25	37	40
0.53	35	63	29	27	34	45
1.03	50	77	25	32	29	50
1.02	51	89	21	35	24	55
1.03	52	95	19	37	15	60
Correlation	0.807288	-0.55473	0.7945	-0.57952	0.507741	

Table (5) Proteins involved in copper assimilation in *Orochromis niloticus*

S. No	Uniprot ID	Protein	Gene name	Reaction	Function
1	F5C7J6	Copper-transporting ATPase 1	-	-	copper-exporting ATPase activity
2	D2Y6D3	Cytochrome c oxidase			
3	I3K1J1	Amine oxidase	LOC100705916	-	copper ion binding
4	I3JAL8	Superoxide dismutase [Cu-Zn]	Sod1	2 superoxide + 2 H^+ = O_2 + H_2O_2.	Binds 1 copper ion per subunit.
5	Q6IUZ2	Tyrosinase-related protein 1	-	-	copper ion binding

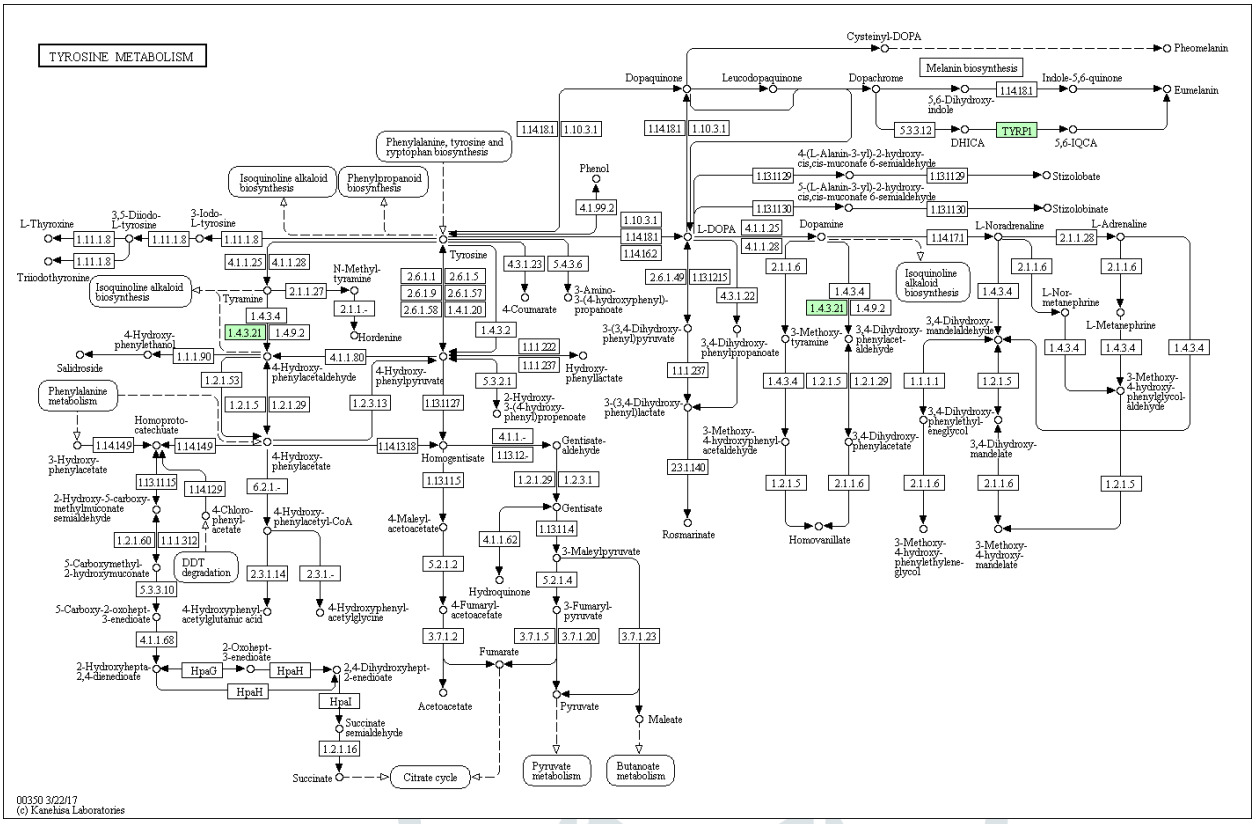

Fig (4) Network of tyrosine metabolism in *Oreochromis niloticus*

Table 33 Rate of change of copper Assimilation System at various concentrations of copper in *Oreochromis niloticus*

Conc. of Copper	Conc. in mM					Time in Min
	F5C7J6	D2Y6D3	I3K1J1	I3JAL8	Q6IUZ2	
3.28	75	25	4	65	18	5
3.21	71	36	6	59	26	10
3.30	69	42	7	57	34	15
2.47	67	49	9	56	45	20
2.42	63	57	13	55	57	25
2.31	54	68	15	43	63	30
2.19	49	74	18	41	69	35
2.09	38	81	19	39	71	40
1.9	34	89	25	35	75	45
3.09	23	91	31	27	81	50
2.97	21	93	35	23	85	55
2.89	18	90	40	19	90	60
Correlation	0.200492	-0.43849	-0.12811	0.19827	-0.43405	

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