CHAPTER 1

INTRODUCTION

Environmental health is of great concern, as aquatic environment is exposed to a variety of xenobiotics mainly heavy metals in marine water ecosystems (Barkhordar et al. 2013). Heavy metals in aquatic organisms tend to accumulate in the human tissues and organs by way of food chain. One of the ways to regulate the level of metals in the cells is to immobilize the heavy metals by binding to biological molecules such as Metallothionein (MT). Cadmium (Cd) is a ubiquitous trace metal, biochemically classified as a non-essential element. It occurs naturally in the aquatic environment and is released as a result of anthropogenic activities and natural processes (Ma et al. 2007). It has become a problem of higher magnitude because of the toxic nature of the pollutant in seas, rivers and estuaries waters (Fingerman et al. 1996, Sumit et al. 2013). The rapid industrialization, industrial waste discharge, and mining have contributed to widespread Cd contamination; aquatic organisms are exposed to elevated levels of heavy metals (Kalay and Canil 2000). Cd is naturally occurs combined with other elements such as Zn and Cu. Cd is primarily used for electroplating with other metals and in nickeled batteries because of its relative resistance to corrosion and high electrical and thermal conductivity. These inputs may results in increased Cd levels of in the aquatic ecosystems, which can be potentially toxic to organisms such as fish (Park 2001).

1.1 Cadmium

Cd was discovered by German chemist Friedrich Stromeyer (1776 - 1835) in 1817. It is commonly found in ores of Zinc Carbonate in the earth's crust (Morrow 2010). Cd is a silver-white, blue-tinged, transition metal, which
melts at 321°C and boils at 767°C. Cd is located in the d-block and 12th group of the periodic table. This divalent element has an atomic weight of 112.4 g/mol and an atomic number of 48. Table 1.1 shows properties of Cd.

### Table 1.1 Properties of Cd

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Name and Symbol</td>
<td>Cadmium, Cd</td>
</tr>
<tr>
<td>2</td>
<td>Atomic number</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td>Atomic mass</td>
<td>112.4 g/mol</td>
</tr>
<tr>
<td>4</td>
<td>Element category</td>
<td>Transition metal</td>
</tr>
<tr>
<td>5</td>
<td>Density</td>
<td>8.7 g/cm³ at 20°C</td>
</tr>
<tr>
<td>6</td>
<td>Melting point</td>
<td>609.93°F, 321.07°C, 594.22 K,</td>
</tr>
<tr>
<td>7</td>
<td>Boiling point</td>
<td>1413°F, 767°C, 1040 K,</td>
</tr>
<tr>
<td>8</td>
<td>Isotopes</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>Electronic shell</td>
<td>[ Kr ] 4d¹⁰ 5s² or 2, 8, 18, 18, 2</td>
</tr>
<tr>
<td>10</td>
<td>Energy of first ionization</td>
<td>867.8 kJ/mol</td>
</tr>
<tr>
<td>11</td>
<td>Energy of second ionization</td>
<td>1631.4 kJ/mol</td>
</tr>
<tr>
<td>12</td>
<td>Energy of third ionization</td>
<td>3616 kJ/mol</td>
</tr>
</tbody>
</table>

The consumption pattern of Cd in its various applications has increasingly shifted away from the traditional market areas of pigments, stabilizers and coatings to rapidly growing applications in Ni-Cd batteries (Figure 1.1).
Cd hydroxide is utilized as one of the two principal electrode materials in Ni-Cd batteries which have extensive applications in the aircraft industry for starting: emergency power and in consumer applications such as portable computers, cellular telephones, cordless power tools, portable household appliances and toys (Morrow and Keating 1997).

Cd sulphoselenide and sulphide are utilized as bright yellow to deep red pigments in plastics, ceramics, glasses, enamels and artists colors (Cook 1994). Cd bearing stabilizers retard the degradation processes in polyvinylchloride which occur upon exposure to heat and UV light. The stabilizers contain organic Cd salts, usually carboxylates such as Cd stearate or Cd laurate, which are incorporated into polyvinylchloride before processing and arrest any degradation reactions after subsequent processing and ensure a long life (CACC 1991).
Cd coatings are utilized in steel, aluminum, and certain other non-ferrous metal fasteners and moving parts to provide the best available combination of corrosion resistance, especially in alkali media and salts, and low coefficient of friction or lubricity. It's also widely employed in many electrical or electronic applications where a good combination of corrosion resistance and low electrical resistivity are required. Cd coatings exhibit excellent plating characteristics on a wide variety of substrates that have good galvanic comparability with aluminum, and are readily solderable (Morrow 1996).

Cd alloys include electrical conductivity alloys, electrical contact alloys, and heat conductivity alloys. Other small uses of Cd include Cd sulphide and Cd telluride in solar cells, and another semiconducting Cd compounds in a variety of electronic applications (CACC 1991). Cd metal discharged into the environment from the industries, such as petroleum, paint, textile, mining, electroplating, batteries, chemicals, fertilizer, tyre, detergent manufacturing etc. Most industrial effluents contain Cd metal, the types and concentration of these metals vary with industries (Varma et al. 1976, Duffus 1980, Mason 1991).

1.1.1 Lethal Concentration 50 (LC$_{50}$)

It is the standard measure to study the toxicity in the aquatic medium, where half of the sample populations (50%) of a specific test animal in a specified period die from exposure via inhalation or respiration or through intake of water as in fishes. LC$_{50}$ measurement is in micrograms or milligrams of material per litre or parts per million (ppm) of water.

Cd exposure of fish to very low concentrations of this metal may lead to an increased body concentration that can result in several toxic effects including tissue damages, vertebral alterations, and respiratory changes and ultimately death (Sorensen 1991, Singh et al. 2010). Gills are the major entry
site of metals and act as a transient store for accumulated metals (Kalay and Canil 2000). As heavy metals cannot be degraded, they are deposited, assimilated or incorporated in water, sediment and aquatic animals (Linnik and Zubenko 2000). Therefore, heavy metals can be bio-accumulated and biomagnified via the food chain and finally assimilated by human consumers resulting in health risks (Agah et al. 2009). Fishes are major part of the human diet due to high protein content; various studies have been underway worldwide on the contamination of different fish species by heavy metals (Singh et al. 2008, Kumar et al. 2011, Sen et al. 2011, Sumit et al. 2013). Cd will not break down in the environment and can be bio-accumulated for many years, after the exposure to low levels (Suedel et al. 1997). The toxic pollutant affects water quality, feeding, and swimming behavior of fish, delays the hatching and maturation period (Atif et al. 2005). One of the ways to regulate the level of metals in the cells is to immobilize the heavy metals by binding to biological molecules such as MT.

1.2 Histology

Histological techniques provide a visual means for the examination and analysis of cell/tissue physiology and morphology at the microscopic level. Histology represents a broad technology, invaluable for studying and understanding the microscopic three-dimensional organization, structure, and function of cells and tissues and is especially useful for the diagnosis and understanding diseases at cellular level. The process first involves isolation and fixation of cells/tissue of interest. Fixation preserves the structure and morphology of the specimen throughout the harsh conditions of dehydration, clearing, embedding, sectioning and staining.

Liver is an abdominal organ which plays a vital role in detoxification and excretion of many exogenous and endogenous substances (Van-Dyk 2003),
it accomplishes these detoxification action by means of three mechanisms as summarized below:

1. A filtering system of large macrophages called kupffer cells lining the blood sinusoids,
2. Phase I detoxification pathway,
3. Phase II detoxification pathway (Cabts 2000).

The liver is characterized by polygonal shaped hepatocytes with granular cytoplasm and centrally placed round nuclei. Hepatocytes are arranged in a well-organized hepatic cords and separated by narrow blood sinusoids. The light microscopic examination of the control fish liver shows normal architecture (Rappaport et al. 1954, Munishi and Dutta 1996, Mumford et al. 2007, Ahmad et al. 2011, Dar et al. 2011, Amin et al. 2013).

Hepatocytes constitute about 80% of the liver cell population. The liver has a great number of functions, most of which is performed by the hepatocytes. Light microscope observation shows that it is not possible to distinguish, hexagonal subdivisions of hepatic parenchyma (hepatic lobules) in fish livers (Rappaport et al. 1954, Dar et al. 2011, Amin et al. 2013). The normal structure of the hepatic parenchyma in fish (Munishi and Dutta 1996) has been depicted in Figure 1.2. The fish liver shows normal hexagonal shaped hepatocytes with clear distinguishable cell membranes. The hepatocytes are cohesively arranged and constitute the majority of the liver tissue. Each hepatocyte contains a single, spherical nucleus and a clear single nucleolus. The nuclei are mostly centrally located within the hepatocytes with some nuclei tending to occur closer to the cell periphery bordering the sinusoids.
A prominent characteristic feature observed in most hepatocytes of fish is vacuolated cytoplasm, shown as clearly unstained spherical vacuolar structures within the cells. The cell membrane of individual hepatocytes is clearly visible through light microscopic analysis (Munshi and Dutta 1996).

The sinusoid is an irregularly dilated vessel whose diameter is larger than the diameter of regular capillaries. Between the cords of hepatocytes is a three-dimensional network of cylindrical blood sinusoids (Elias and Bengelsdorf 1952). Sinusoids are lined by two kinds of cells: squamous cells similar to regular endothelium and Kupffer cells (Ross et al. 1989).

The nucleus of fish hepatocytes is generally a single, centrally located, spherical nucleus with a clear, dark nucleolus. Binuclear cells have been observed in normal liver histology (Geyer 1989).
The kidney of fish is usually located in a retroperitoneal position against the ventral side of the vertebral column. It is light or black or dark brown organ normally extending throughout the length of the body cavity. It is usually divided into anterior or head kidney, which is largely composed of hematopoietic elements, and posterior or excretory kidney (Cutler 2006). The ureters, which collect urine from the collecting ducts to the urinary papilla, may fuse at any level and may be dilated, after fusion, to form a bladder. The urinary ducts open to the outside that appraises posterior to the anus. The primary function of the kidney in fish is osmoregulations of water and salts rather than excretion of nitrogenous wastes (Cutler 2006, Onyeanusi et al. 2007). In fish, majority of nitrogenous wastes are excreted by the gills. In water, the kidney is adapted to conserve salt and eliminate excess water. This is accomplished by a high glomerulus filtration rate, re-absorption of salts in the proximal tubules, and dilution of urine in the distal convoluted tubule (Mumford et al. 2007).

The kidney is the main organ responsible for excretion of toxic substances and detoxification (Eisler 1998). Toxicants reach kidney via circulation. The kidney is a vital organ that maintains body fluid homeostasis (Charmi et al. 2009). The kidney is the main target organ of Cd accumulation in many fishes as shows by recent toxicological investigations carried out by different authors (Farombi et al. 2007, Kumar et al. 2008). The fish nephron varies considerably between marine, euryhaline and freshwater forms mirroring a significant difference between their respective function. Though this is a fact, the basic cellular architecture is similar. Each nephron consists of several segments with specific structure and function (Guyton and John 2006).

Renal corpuscle acts as an ultra-filrate of plasma. This filtrate then passes into the renal tubule where it is mixed with non-threshold substances to form urine. Proximal convoluted tubule (PCT) is the first segment of the
nephrons that resorbs 85% of the water, sodium and chloride; glucose, amino acids, proteins, vitamin C, and inorganic ions are absorbed. Proximal convoluted tubule has tall columnar cells with brush border, large spherical, pale-staining, basally located nuclei. In the distal convoluted tubule more water is resorbed, and urine is concentrated or diluted. Distal convoluted tubule shows short columnar cells with oval, basally located nuclei and no brush border, stain less intense than PCT. In the collecting tubules there is collection of non-threshold substances for excretion, and there is more water resorption (Mumford 2004).

1.3 Cellular DNA Damage by Cd – DNA Fragmentation and Apoptosis

DNA fragmentation was first documented by Williamson (1970) when he observed discrete oligomeric fragments occurring after cell death in primary neonatal liver cultures. He described the cytoplasmic DNA isolated from mouse liver cells after culture was characterized by DNA fragments with a molecular weight consisting of multiples of 135 kDa. This finding was consistent with the hypothesis that these DNA fragments were a specific degradation product of nuclear DNA damage (Williamson and Robert 2013).

Mitochondria are involved in a variety of key events including release of caspase activators, changes in electron transport etc. (Gottlieb 2000). Alterations in mitochondrial structure and function have been shown to play a crucial role in caspase-9 dependent apoptosis (Green and Kroemer 1998) by releasing apoptotic factors from mitochondria including cytochrome c. In this manner, released cytochrome c interacts with Apaf-1 and pro-caspase-9 to form apoptosome. Then caspase-9 cleaves and activates caspase-3, the executioner caspase, which cleaves poly (ADP-ribose) polymerase (PRAP) and activates endonucleases leading to DNA fragmentation (Cai et al. 1998). DNA breakdown by Ca$^{2+}$ and Mg$^{2+}$ dependent endonucleases also occurs, resulting in DNA fragments of ~180 bp (Bortner et al. 1995). A characteristic “DNA
ladder” can be visualized in agarose gel electrophoresis with an ethidium bromide stain under ultraviolet illumination.

DAPI (4, 6-diamidino-2-phenylindole) was first synthesised in 1971 in the laboratory of Otto Dann as part of a search for drugs to treat trypanosomiasis. Although it was unsuccessful as a drug, further investigation indicated it bound strongly to DNA and became more fluorescent when bound. This led to its use in identifying mitochondrial DNA in ultracentrifugation in 1975, the first recorded use of DAPI as a fluorescent DNA stain. DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells. Because it passes through the membrane less efficiently in live cells the effectiveness of the stain is lower (Kapuscinski 1995).

Apoptosis, also known as selective cell suicide or programmed cell death, is a physiologically normal and an important process for normal embryogenesis, maintenance of tissue homeostasis and for proper functioning of the immune system in multicellular organisms (Ellis et al. 1991, Jacobson et al. 1997). Apoptosis itself plays an important role in the development of various diseases (Fisher 1994, McConkey et al. 1996).

A family of intracellular cysteine proteases known as caspases (cysteine aspartyl-specific proteases) collaborates in a proteolytic cascade where caspases activate themselves and others that induce apoptosis (Cryns and Yuan 1998, Thornberry and Lazebnik 1998). Caspases are classified as upstream initiators or downstream effectors of apoptosis. These proteins give key cellular components that are required for normal cellular function such as the structural proteins cytoskeleton and nuclear proteins. This controlled and energy dependent process shrinks the cells, condenses the chromatin and finally removes the apoptotic bodies by macrophages without leaving trace behind. Apoptotic cells often undergo plasma membrane changes that trigger and
promote the macrophage response. One such change is the translocation of phosphatidylserine from the inside to the outer surface of the cell (Thornberry and Lazebnik 1998).

Apoptosis are triggered by activation of the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, results from activation of members of cysteine protease family called caspases (Miller 1999, Fan et al. 2005). Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (Nemes et al. 1996).

1.4 Metallothionein

MT was first isolated from horse kidney by Margoshes and Vallee in 1957. MT are low molecular weight (6000 - 14000 Daltons) cysteine-rich, cytosolic and metal binding protein that is found in eukaryotic, animals, higher plants and some prokaryotes. MTs are widely expressed in organisms; such as eukaryotes are responsible for essential metal metabolism and heavy metals detoxification (Kagi and Schaffer 1988, Kagi 1991). MTs functions are still unclear, but experimental data suggest that MT protect against metal toxicity (Peterson et al. 1996). MTs are non-enzymatic and heat stable protein, MT functions by way of primary metal storage, transport and detoxification to the respective organs of the first 40 residues of the polypeptide wraps around the metal by forming two large parallel loops separated by a deep cleft containing the metal cluster. MT does not contain aromatic amino acid and one third of its residues are cysteine (Hamer 1986, Kagi and Schaffer 1988, Kagi 1991, Peterson et al. 1996, Duncan 2009).

The MT family consists of four isoforms designated MT-1 MT-2, MT-3 and MT-4. MT-1 and MT-2 was expressed in liver and kidney tissues, MT-3 is expressed predominantly in the brain and MT-4 in differentiating stratified
squamous epithelial cells (Roesijadi 1994). Many reports have addressed MT structure and function, but despite increasing experimental data the function of this elusive protein have yet to be identified (Vergani *et al.* 2003). MTs induction by various metals implies an essential role in heavy metal detoxification; they could facilitate the accumulation of toxic heavy metals, such as Cd, by chelating metal ions within the cell. However, the mechanisms for excretion of metal ions are not known (Roesijadi and Fellingham 1987, Roesijadi 1994, Vergani *et al.* 2003).

**Table 1.2 Classifications of MT and their expressed regions**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Based on</th>
<th>Classifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Metals present</td>
<td>Major- MT-1 &amp; MT-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minor- MT-3 &amp; MT-4</td>
</tr>
<tr>
<td>2</td>
<td>Proteins are encoded by a family of genes which are located on chromosome</td>
<td>MT-1a, MT-1b, MT-1c etc.</td>
</tr>
<tr>
<td>3</td>
<td>Biological system</td>
<td>MT-1, MT-2 &amp; MT-3</td>
</tr>
<tr>
<td></td>
<td>Central nervous system</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neurons</td>
<td>MT-3</td>
</tr>
<tr>
<td>4</td>
<td>Family of eukaryotes and prokaryotes</td>
<td>MT-1</td>
</tr>
<tr>
<td></td>
<td>Proteins with sequences related to MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proteins with sequences not related to MT</td>
<td>MT-2</td>
</tr>
<tr>
<td></td>
<td>Peptides that are not genetically encoded</td>
<td>MT-3</td>
</tr>
<tr>
<td>5</td>
<td>Expressed in tissues</td>
<td>MT-1 &amp; MT-2</td>
</tr>
<tr>
<td></td>
<td>Liver and kidney</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain and to male reproductive organs</td>
<td>MT-3</td>
</tr>
<tr>
<td></td>
<td>Specific to stratified squamous epithelia</td>
<td>MT-4</td>
</tr>
</tbody>
</table>
Another classification based on a large family of eukaryotes and some are in prokaryotes refers to three classes: (i) proteins with sequences related to MT, (ii) proteins with sequences not related to MT, and (iii) peptides that are not genetically encoded (Maret 2009). All above classification are tabulated in 1.2.

More specifically, fish MT stores Cd and protect the cell against Cd toxicity by tightly chelating Cd ions (Peterson et al. 1996). MTs have since been reported in many vertebrates including many species of fish (Olsson et al. 1998, Roeva et al. 1999). MTs can be induced by the essential metals Cu and Zn and the non-essential metals Cd, Ag and Hg in both vertebrates and invertebrates, but their induction is variable. Such variation is intra-specific and inter-specific, and is down to a variety of reasons mainly environmental and physiological conditions (Jourdan 2001, Amiard et al. 2006).

MTs are also transcriptionally induced by various physiological and toxicological stimuli, such as oxidative stress, suggestion that in vivo they may neutralize hydroxyl radicals, cytokines, chemicals, and heat as well as heavy metals (Bauman et al. 1991, Dalton et al. 1997, Viarengo et al. 2000). That is of increased MT synthesis is associated with increased metal binding capacity, and increased resistance to metal toxicity (Amiard et al. 2006).

In vertebrates, particularly in fish, metal detoxification processes depend mainly on metal binding to MTs. As a consequence, any MT metal exposure relationship is easier to demonstrate in fish than in invertebrates, which are common in invertebrate metal detoxification (Mason and Jenkins 1995). Furthermore the sampling of fish often seems to be less controllable than that of invertebrates, particularly as regards presence/absence, selection of size, age or weight categories, and the influence of these factors on the inducibility of fish MT is well-recognized (Hamza-Chaffai et al. 1997, Tom and Auslander 2005).
MT has been documented to bind a wide range of metals including Cd, Zn, Hg, Cu, As, Ag, etc. (Freisinger and Vasak 2013). MT binds three metal ions in its beta domain and four in the alpha domain. Cysteine is a sulfur-containing amino acid, hence the name "-thionein". However, the participation of inorganic sulfide and chloride ions has been proposed for some MT forms. By binding and releasing zinc, MTs may regulate zinc levels within the body. Zinc, in turn, is a key element for the activation and binding of certain transcription factors through its participation in the zinc finger region of the protein (Huang et al. 2004).

In the MTs N-terminal is in the beta domain, while the C-terminal is in the alpha domain, there are no disulfide bonds present in the primary structure, and secondary structures such as alpha helix and beta sheets are not present. Thus, the three dimensional structure in metal free MT is essentially a random coil (Stillman 1995, Klaassen 1999). The MTs act like a chelating agent with each Cd atom binding to sulfur. Thus, the Cd is tetrahedral coordinated to the sulfur, and so the bonding is particularly strong. This would mean that the protein is more flexible and the entropy would be larger. The only fixed relationship in a random coil is the peptide bond between the amino acid (Romero-Isart and Vasak 2002).

1.5 Western Blot

The Western blot techniques are very sensitive and allow detection of very low amount of proteins due to the use of specific antibodies. Western blot is a widely used analytical technique used to detect specific proteins in a sample of tissues homogenate or extract (Eslami and Lujan 2010). It uses gel electrophoresis to separate native proteins or denatured proteins by the length of the polypeptide. Proteins are transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein (Kapadia et al. 2003).
1.6 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara and Miller 2014). The procedure was conceptualized and first implemented by Coons et al. (1941). IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue (Ramos-Vara and Miller 2014).

Immunohistochemical analyses might be of great importance allowing the detection of the specific cell types expressing MTs in organs with great complexity (Coyle et al. 2002). MT induction has been studied in major organs such as liver and kidney in different fish species (Kito et al. 1982, Roesijadi 1992). Hepatocyte cells are mainly found in liver, it makes up to 70 - 85% of liver cytoplasm mass in all organisms. Nephrons are mainly found in fish kidney, it makes up to 85% of kidney cytoplasm mass in all organisms (Eisler 1998, Charmi et al. 2009).

Major components in a complete IHC experiment: 1). primary antibody binds to specific antigen; 2). the antibody-antigen complex is formed by incubation with a secondary, enzyme-conjugated, antibody, 3). with presence of substrate and chromogenic, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding.

1.7 Protein Purification

Protein purification is a series of processes intended to isolate one or a few proteins from a complex mixture and cells, tissues or whole organisms. Purification is vital for the characterization of the structures, functions and interactions with the protein of interest. The purification processes separate the
protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins.

Affinity chromatography is a separating biochemical mixtures based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligands. HiTrap™ columns may used with a peristaltic pump or a syringe, and are supplied with a detailed protocol to ensure optimum results. All molecules, particularly proteins, have a complex 3D structure. Ligands that have the ability to recognize one of the structural motifs of a molecule are used in affinity chromatography. This interaction can be extremely specific and the ligands will interact with only one type of molecule, or the ligands can be designed so that it recognizes a group of structurally similar molecules.

Affinity chromatography involves 3 main steps:

1. Incubation of a crude sample with the affinity support to allow the target molecule in the sample to bind to the immobilized ligands.

2. Washing away non-bound sample components from the support.

3. Elution of the target molecule from the immobilized ligands by altering the buffer conditions so that the binding interaction no longer occurs.

The affinity chromatography is so selective; it is a very powerful tool in isolating and purifying proteins from a mixture of proteins. The most well-known application of affinity chromatography is as a capture step in the purification of monoclonal antibody-based pharmaceuticals. Although affinity chromatography is also used as an analytical technique; it has not reached the same popularity as in process chromatography (Lowe 1996).
1.8 MALDI TOF MS

MALDI-TOF MS is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. The time of flight method of measuring particle m/z ratio is done as follows. An ion of known electrical charge and unknown mass enters a mass spectrometer and is accelerated by an electrical field of known strength. This acceleration results in any given ion having the same kinetic energy as any other ion given that they all have the same charge. The velocity of the ion will depend however on the mass-to-charge ratio. Basically, MALDI is talking about the ionization technique of the sample in the source, while TOF is discussing about the distinguishing technique for the ions in the analyzer (Baldwin 2004).

MALDI-TOF is an efficient process for generating gas-phase ion of peptides and proteins for mass spectrometric detection. TOF is a mass analyzer in which the flight time of the ion from the source to the detector is correlated to the m/z of the ion. MALDI MS is an attractive approach for determination of accurate molecular weight of intact proteins (Dave et al. 2011).

Proteins are digested with trypsin (cleaves at R-X and K-X except when X is Pro) and a peptide mass fingerprint produced by analyzing the digested protein with a MALDI-TOF. The mono-isotopic masses of the peptides seen in the TOF mass spectrum are software selected and used to search a protein database. The database has been theoretically digested with trypsin and the experimentally generated mass list is compared to theoretically digested database. The match is scored on number of factors, depending on the search program utilized. For identification of known purified protein the listed
programs are used to search MALDI-TOF Peptide Mass Fingerprinting data: Mascot, MSiFit, Aldente, ProFound etc.

Mascot database search program was used for protein identification using peptide mass by MALDI TOF-MS Peptide Mass Fingerprinting.

1.9 Homology Modelling

1.9.1 Bioinformatics Software’s

Homology modeling also called template-based modeling or comparative modeling or, refers to modeling a protein 3D structure using a known experimentally determined structure of a homologous protein as a template.

1.9.1.1 SWISSPROT

UniProtKB/SwissProt is the manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB). It is a high quality annotated and non-redundant protein sequence database, which brings together experimental results, computed features and scientific conclusions. It is maintained by the UniProt consortium and is accessible via the UniProt.

URL: http://www.uniprot.org/

1.9.1.2 PDB

The Protein Data Bank (PDB) is a repository for the 3D structural data of large biological molecules, such as proteins and nucleic acids. (See also crystallographic database). The data typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are freely accessible on the internet via the websites of its member organizations.

URL: www.rcsb.org/
1.9.1.3 BLAST

The Basic Local Alignment Search Tool (BLAST) is a widely used tool for finding matches to a query sequence within a large sequence database, such as genbank. BLAST is designed to look for local alignments, i.e. maximal regions of high similarity between the query sequence and the database sequences, allowing for insertions and deletions of sites. Although the optimal solution to this problem is computationally intractable, BLAST uses carefully designed and tested heuristics that enable it to perform searches very rapidly (often in seconds). For each comparison, BLAST reports a goodness score and an estimate of the expected number of matches with an equal or higher score than would be found by chance, given the characteristics of the sequences. When this expected value is very small, the sequence from the database is considered a "hit" and a likely homologue to the query sequence. Versions of BLAST are available for protein and DNA sequences and are made accessible in MEGA via the web browser.

URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi

1.9.1.4 PROTPARAM

PROTPARAM is a tool which allows the computation of various physical and chemical parameters for a given protein stored in SwissProt or TrEMBL or for a user entered sequence. The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

URL: http://web.expasy.org/protparam/

1.9.1.5 CFSSP

CFSSP (Chou & Fasman Secondary Structure Prediction Server) is an online protein secondary structure prediction server. This server predicts
regions of secondary structure from the protein sequence such as alpha helix, beta sheet, and turns from the amino acid sequence. The output of predicted secondary structure is also displayed in linear sequential graphical view based on the probability of occurrence of alpha helix, beta sheet, and turns. The method implemented in CFSSP is Chou-Fasman algorithm, which is based on analyses of the relative frequencies of each amino acid in alpha helices, beta sheets, and turns based on known protein structures solved with X-ray crystallography. CFSSP server is written in Perl, which runs through CGI (Common Gateway Interface). CFSSP is freely accessible via ExPASy server or directly from BioGem tools at http://www.biogem.org/tool/chou-fasman.

1.9.1.6 MODELLER9v8

MODELLER is used for homology or comparative modelling of protein 3D structures. The user provides an alignment of a sequence to be modelled with known related structures and MODELLER automatically calculates a model containing all non-hydrogen atoms. MODELLER implements comparative protein structure modelling by satisfaction of spatial restraints and can perform many additional tasks, including de novo modelling of loops in protein structures, optimization of various models of protein structure with respect to a flexibly defined objective function, multiple alignment of protein sequences and/or structures, clustering, searching of sequence databases, comparison of protein structures etc.

1.9.1.7 SPDBV

SWISS-PDB Viewer can load and display several molecules simultaneously. Each molecule is loaded into its own layer. Each molecule is composed of groups (i.e. amino acids, nucleotides, substrates). Each group is composed of atoms, whose coordinates are taken directly from a PDB file. DEEPVIEW is a friendly but powerful molecular graphics program. It is
designed for full compatibility with computing tools available from the Expert Protein Analysis System, or ExPASy, Molecular Biology Server in Geneva, Switzerland. While DEEPVIEW is simple to use for viewing structures and creating vivid illustrations, it also shines as an analytical tool. DEEPVIEW allows you to build models from scratch, simply by giving an amino-acid sequence. DEEPVIEW can find hydrogen bonds within proteins and between proteins and ligands. It allows you to examine electron-density maps from crystallographic structure determination, to judge the quality of maps and models, and to identify many common types of problems in protein models. It allows you to view several or many models simultaneously and superimpose them to compare their structures and sequences. It computes electrostatic potentials and molecular surfaces, and carries out energy minimization. For proteins of known sequence but unknown structure, DEEPVIEW submits amino acid sequences to ExPASy to find homologous proteins, onto which you can subsequently align your sequence to build a preliminary three-dimensional model. Then DEEPVIEW submits your alignment to ExPASy, where the SWISS-MODEL server builds a final model, called a homology model, and returns it directly to DEEPVIEW.

1.9.1.8 RASMOL

RASMOL is a program for molecular graphics visualization originally developed by Roger Sayle. This site is provided for the convenience of users of RASMOL and developers of open source versions of RASMOL.

Knowledge on the characterization of MT protein in marine catfish Arius arius (A. arius) is minimal. Hence this lacuna prompted the current pursuit to isolate, purify and characterize the MT protein in marine catfish, A. arius.