PREVALENCE AND CHARACTERIZATION OF CIGUATOXIN IN FISHES ALONG INDIAN COAST

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*This is to certify that the thesis entitled "**Prevalence and Characterization of Ciguatoxin in Fishes along Indian Coast**" submitted by Ms. Rajisha R. (Reg. No. 5042) is an authentic record of research work carried out by her under my guidance and supervision at ICAR-Central Institute of Fisheries Technology, Kochi, Kerala in partial fulfillment of the requirement for the award of Ph.D. degree under the Faculty of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala and no part of this has previously formed basis for the award of degree or associateship in any University or Institution. I further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate has been incorporated in the thesis.*

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Declaration

*I, Rajisha R. do hereby declare that the thesis entitled "**Prevalence and Characterization of Ciguatoxin in Fishes along Indian Coast**" is a genuine record of research work carried out by me under the guidance of Dr. Ashok Kumar K, Principal Scientist & Head, Fish Processing Division, ICAR-Central Institute of Fisheries Technology, Kochi, Kerala in partial fulfillment for the award of Ph.D. degree under the Faculty of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala and no part of the work has previously formed the basis for the award of any degree, diploma, associateship or any other title or recognition from any University/Institution.*

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Dedicated to my Father in Law……..

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Rajisha R.

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Abbreviations

A:G ratio	:	Albumin Globulin ratio
ALT	:	Alanine Transaminase
APCI	:	Atmospheric Pressure Chemical Ionization
APPI	:	Atmospheric Pressure Photo-Ionization
ASP	:	Amnesic Shellfish Poisoning
AST	:	Aspartate Transaminase
AZP	:	Azaspiracid Shellfish Poisoning
CAD	:	Collision Gas
C-CTX	:	Caribbean Ciguatoxin
CE	:	Collision energy
CFP	:	Ciguatera Fish Poisoning
$CHCl_3$	:	Chloroform
Cps	:	Counts per second
CTX	:	Ciguatoxin
CUR	:	Curtain Gas
CXP	:	Collision cell Exit Potential
Da	:	Daltons
DA	:	Domoic Acid
DP	:	Declustering Potential
DSP	:	Diarrheic Shellfish Poisoning
EP	:	Entrance potential

ESI	:	Electron Spray Ionization
ESI	:	Electron Spray Ionization
EtOAc	:	Ethyl Acetate
FAB	:	Fast Atom Bombardment
FTIR	:	Fourier Transform Infra-Red spectrometer
GS1	:	Nebulizing Gas
HAB	:	Harmful Algal Bloom
HPLC	:	High Performance Liquid Chromatography
I-CTX	:	Indian Ocean Ciguatoxin
ip	:	Intra peritoneal
IS	:	Ion Spray voltage
KOH	:	Potassium Hydroxide
LC-MS/MS	:	Liquid Chromatography Mass Spectrometry
m/z	:	Mass to charge ratio
MALDI	:	Matrix-Assisted Laser Desorption Ionization
MATs	:	Marine Algal Toxins
MBA	:	Mouse Bioassay
MCA	:	Multi-Channel Acquisition
MeOH	:	Methanol
MRM	:	Multiple Reaction Monitoring
MU	:	Mouse Unit
NMR	:	Nuclear Magnetic Resonance

NSP	:	Neurotoxic Shellfish poisoning
OA	:	Okadaic acid
OD	:	Optical Density
PbTx	:	Brevetoxin
P-CTX	:	Pacific Ciguatoxin
PLTX	:	Palytoxin
PSP	:	Paralytic Shellfish Poisoning
PTFE	:	Polytetrafluoroethylene
PTX	:	Pectenotoxin
Q1 Q2 Q3	:	Quadrupole 1, 2 and 3
RT	:	Retention Time
SPE	:	Solid Phase Extraction
STX	:	Saxitoxin
TEM	:	Temperature of Ion Source
TIC	:	Total Ion Chromatogram
TTX	:	Tetrodotoxin
V_b	:	Bed volume
XIC	:	Extracted Ion Chromatogram
YTX	:	Yessotoxin

Chapter 1

General Introduction

Contents

1.1 Harmful algal blooms and biotoxins

Phytoplankton are the most important constituents of the marine food web and comprise 40 % of the total fixed global primary productivity (Falkowski, 1984; D'Silva et al., 2012). Around 5,000 marine phytoplankton species exist in the world and out of this 7% are responsible for algal blooms which include diatoms, dinoflagellates, raphidophyte, prymnesiophytes and silicoflagellates (Sona et al., 2003; Gopinathan et al., 2007; Padmakumar et al., 2012). Multiplication of algal cells to 10^5 to 10^6 cells per litre of seawater form a typical algal bloom (Smith et al., 1993). Around 60- 80 phytoplankton species are harmful or toxic known as Marine Algal Toxins (MATs), in which 75 % are contributed by dinoflagellates (Van Dolah, 2000; Roberts et al., 2004; Wells et al., 2015). Effects of Harmful Algal Bloom (HAB) include damage to marine ecosystems which results in economic losses and food borne illness in humans (Hallegraeff, 1995; Van Dollah, 2000; Shi, 2012). Reports of

HABs are increasing in frequency, intensity and geographic distribution due to climate change and increased rates of coastal eutrophication has been emerged as a global concern (Paerl, 1988; Smayda, 1989, 1992; Hallegraeff, 1993; Nixon, 1995; Richardson and Jorgensen, 1996; Daranas et al., 2001).

Definition for Harmful Algal Bloom (HAB) given by International Council for the Exploration of Seas (ICES, 1984) stated as "those which are noticeable, particularly to the general public, directly or indirectly through their effects such as visible discolorations of the water, foam production, fish or invertebrate mortality or toxicity to humans". Seafood borne intoxications, caused by marine biotoxins like Ciguatoxin (CTX), Saxitoxin (STX), Okadaic acid (OA), Brevetoxin (PbTx), Domoic Acid (DA), Palytoxin (PLTX), Pectenotoxin (PTX), Tetrodotoxin (TTX) and Yessotoxin (YTX) result from the ingestion of contaminated fish and shellfish with the MATs (Garthwaite, 2000; Botana, 2008; Lawrence et al., 2011; Shi, 2012). Marine biotoxins or the seafood toxins come under the category of naturally occurring chemical hazards (FDA, 2011). Figure 1.1 illustrates the classification of these toxins.

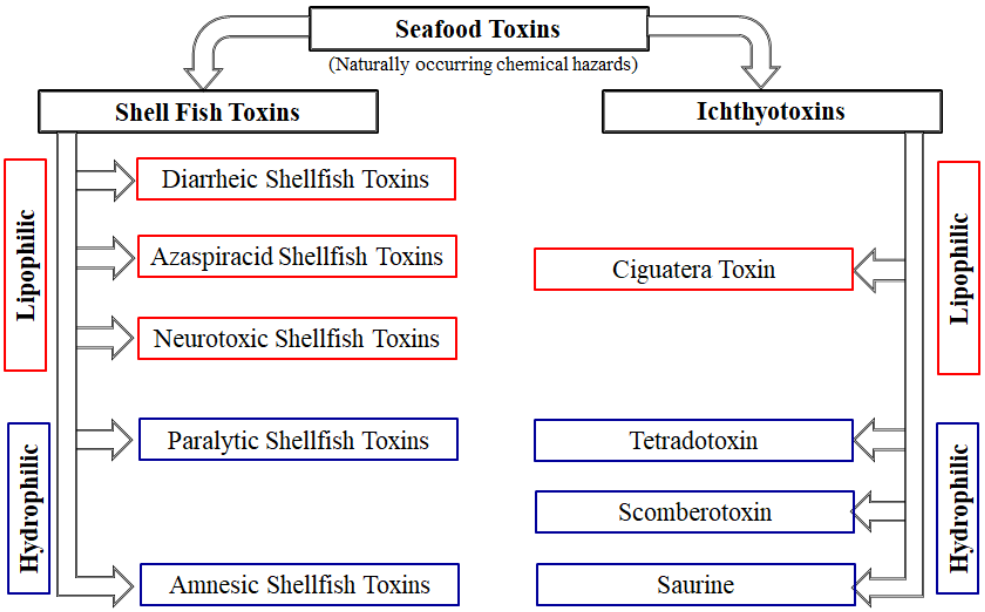


Fig 1.1: Classification of Marine biotoxin (FAO, 2004; FDA, 2011)

Seafood poisoning intoxications caused by contamination of seafood with MATs includes Amnesic shellfish poisoning (ASP), Neurotoxic shellfish poisoning (NSP), Paralytic shellfish poisoning (PSP), Diarrheic shellfish poisoning (DSP), Azaspiracid poisoning (AZP), and Ciguatera Fish Poisoning (CFP) (Garthwaite, 2000; FAO, 2004; EFSA, 2008a; 2008b; 2009a; 2009b; 2009c; 2009d; 2009e; 2010a; 2010b; Dickey & Plakas, 2010). These intoxications associated with gastrointestinal, neurological disorders, cardiovascular problems and in extreme cases can lead to death (FDA, 2011; Munday & Reeve, 2013).

Incidents of biotoxin related hazards are reported globally from Europe, Africa, North America, Central and South America, Asia, Oceania etc. (Yasumoto et al., 1978; Underdal et al., 1985; Perl et al., 1990; Rodrique et al., 1990; Morris et al., 1990; McMahon & Silke, 1998; De Schrijver et al., 2002; FAO, 2004; Aune et al., 2007). Human fatality also reported from all over the world on a global scale. Around 60000 intoxications, approximately 600 deaths per year and under reported cases also include around 10-50 times when compared to the known reports (FAO, 2004; FDA, 2011). Hence producers of shellfishes and finfishes have the responsibility to ensure their product must not contain the marine biotoxins in quantities that exceed 80µg/kg for PSP, 20 mg/kg of DA for ASP, 160 µg/kg of OA equivalents for Okadaic acid, Dinophysis toxins and Pectenotoxins in combination, 1mg of Yessotoxin equivalents per kilogram for YTX, 160 µg of Azaspiracid equivalents per kilogram for AZP and any detectable level per 100g of fish for Ciguatoxin (FDA, 2011). Table 1.1 describes the major seafood poisoning syndromes, sources, mechanism of action, clinical symptoms and treatments of poisoning syndromes.

Table 1.1 Marine Algal Toxins, source, clinical symptoms and treatments of poisoning syndromes (Caillaud et al., 2010; Shi, 2012; Friedman et al., 2017)

Toxin Group	Seafood Poisoning Syndromes					
	Fishes	Shell fishes				
	Ciguatera Fish Poisoning (CFP)	**Paralytic Shellfish Poisoning (PSP)**	**Diarrhetic Shellfish Poisoning (DSP)**	**Amnesic Shellfish Poisoning (ASP)**	**Neurotoxic Shellfish Poisoning (NSP)**	**Azaspiracid Shellfish Poisoning (AZP)**
	Ciguatoxin (CTX)	Saxitoxin (STX)	Okadaic Acid (OA)	Domoic Acid (DA)	Brevetoxin (BTX)	Azaspiracid (AZA)
Source	*Gambierdiscus toxicus*	*Alexandrium catenella* *A. minutum* *A. tamarense*; *Gymnodiniumcatenatum*; *Pyrodinum bahamense Alteromonas tetraodonis*; *Moraxella* spp.	*Dinophysis acuminata*; *D. acuta*; *D. fortii*; *D. norvegica*; *Prorocentrum lima*	*Pseudo-nitzschia multiseries* and *P. autralis*	*Karenia brevis*	*Protoperidinium crassipes*; *Azadinium spinosum*
Action on	Nerve, Muscle, Heart, Brain VGSC* openers	Nerve, Brain VGSC* blockers	Enzymes Protein phosphatases inhibitors	Brain Glutamate receptors stimulators	Nerve, Muscle, Lungs, Brain VGSC* openers	
Symptoms	***Mild case*** **After 3-5 hours** - Diarrhea, nausea, vomiting, and abdominal pain. **After 12-18 hours** -hot-cold inversion, muscular aches, tingling and numbness of lips, tongue, and perioral region Metallic taste, dryness of mouth, anxiety, prostration dizziness, chills, sweating, dilated eyes, blurred vision, and temporary blindness. ***Extreme Case*** Paralysis and death may occur in a few extreme cases.	***Mild case*** **Within 30 min** - Tingling sensation or numbness around lips, gradually spreading to face and neck, prickly sensation in fingertips and toes, headache, Dizziness, nausea, vomiting, diarrhea. ***Extreme Case*** Muscular paralysis, pronounced respiratory difficulty, choking sensation, death through respiratory paralysis may occur within 2-24 h after ingestion.	***Mild case*** **After 30 min to a few hours** (***seldom more than 12 h***): diarrhea, nausea, vomiting, abdominal pain. ***Extreme Case*** Chronic exposure may promote tumor formation in the digestive system.	***Mild case*** **After 3-5 hours** - nausea, vomiting, diarrhea, abdominal cramps. ***Extreme Case*** Decreased reaction to deep pain, dizziness, hallucinations, confusion, short-term memory loss, seizures.	***Mild case*** **After 3-6 hours** - chills, headache, diarrhea, muscle weakness, muscle and joint pain, nausea, vomiting ***Extreme Case*** Paresthesia, altered perception of hot and cold, difficulty in breathing, double vision, trouble in talking and swallowing	***Mild case*** Nausea, vomiting, severe diarrhea, and stomach cramps, similar to DSP and NSP ***Extreme Case*** Suspected carcinogen
Treatment	Symptomatic, Gut emptying and decontamination with charcoal is recommended.	Gastric lavage, artificial respiration. No lasting effects.	Recovery after 3 days, irrespective of medical treatment.	Supportive care	Supportive care	Supportive care

* Voltage Gated Sodium Channel

1.2 Marine algal toxins along Indian Coast

1.2.1 Shellfish toxins

Indian waters are regularly seen with algal bloom occurrences and a report stated that a total of 101 bloom incidents and 39 causative species responsible for blooms during the period from 1908 to 2009, of which *Noctiluca scintillans* and *Trichodesmium erythraeum* were the most common (D'Silva et al., 2012). Shellfish poisoning due to accumulation of toxic algae have been reported along the Tamil Nadu coast (Bhat, 1981), Karnataka coast (Karunasagar et al., 1984) and Kerala coast (Karunasagar et al., 1998). Bloom of *Noctiluca* spp. and associated mortalities has been reported by many researchers (Aiyar, 1936; Bhimachar & George, 1950; Prasad & Jayaraman, 1954; Devassy & Nair, 1987; Jugnu, 2006; Padmakumar et al., 2012; D' Silva et al., 2012; Sulochanan et al., 2014).

Bloom of the dinoflagellate *Gonyalaux polygramma* was reported by Prakash & Sharma (1964) along the south west coast of India. Bloom of the cyanophycean algae *Trichodesmium erythaeum* with associated reduction in fish catches (Prabhu et al., 1965; Naghabushanam, 1967; Devassy et al., 1978) and that of the marine rapidophyte *Hornellia marina* and related fish mortality along Malabar Coast has been reported by Bhimachar & George (1950).

The extent of threat on human health from HABs in Indian waters remains unreported and unregulated. Until 1980s, the phenomenon of Paralytic Shellfish Poisoning (PSP) was virtually unknown in Indian waters. So far, four PSP cases have been recorded from the coastal waters of Kerala and Tamil Nadu (one case each) and Karnataka (two cases). However, the dinoflagellates responsible for PSP outbreak could not be identified. The first PSP outbreak was recorded from Tamil Nadu in 1981 that resulted in hospitalization of 85 people and 3 deaths after consuming bloom affected clams (*Meretrix casta*) (Silas et al., 1982). In 1983, an outbreak of PSP resulted in death of a boy and hospitalization of several individuals following the consumption of clams (*Meretrix casta*) harvested from Kumble estuary in Mangalore, Karnataka (Karunasagar et al. 1984). These clams were analyzed with High Performance Liquid Chromatography (HPLC) and the toxin profile obtained corresponded to a strain of *Alexandrium tamiyavanichii* isolated from Thailand (Karunasagar et al. 1990). Subsequently, low levels of PSP were recorded in shellfish from surrounding estuaries near Mangalore on two occasions during 1985 and 1986 (Segar et al., 1989). Planktonic and cyst forms of *Gymnodinium catenatum*, a PSP–producing dinoflagellate were recorded during a study in Kumta River, Mangalore (Godhe et al., 1996) and there was no toxicity in shellfish. This study highlighted the importance of regular monitoring of coastal waters, sediment and shellfish in that region. An outbreak of PSP was also reported

from Kerala in 1997, where 7 people died and over 500 were hospitalized after consuming bloom affected mussels *Perna indica* (Karunasagar et al., 1998). Another bloom that hit Kerala in 2004 resulted in nauseating smell emanating from the coastal waters (The Hindu, 2004; The Hindustan Times, 2004). This bloom resulted in large–scale fish mortality and hospitalization of 200 people especially children who suffered from nausea and breathlessness caused by nauseating stench from the bloom and putrefying fish. The causative organism reported during the stench event included *Cochlodinium polykrikoides* (The Hindu, 2004) and *Karenia brevis* (The Hindustan Times, 2004); and subsequent to this event, an unidentified holococcolithophore (Ramaiah et al., 2005) and *Noctiluca scintillans* (Sahayak et al., 2005) were reported.

The Indian fisheries economy heavily depends upon the coastal zone marine products, so it is especially sensitive to constraints from red tides and toxic microalgae. So far, there are 7 fish killing species such as *Cochlodinium polykrikoides*, *Karenia brevis*, *Karenia mikimotoi*, *Noctiluca scintillans*, *Trichodesmium erythraeum, Trichodesmium thiebautii* and *Chattonella marina* that form algal blooms and are responsible for massive fish mortality in Indian waters. Although studies are underway, regular efficient monitoring systems have to be established in order to minimize public health risks and damage to fisheries. This is critical as the people of the coastal areas in the

region are highly dependent on fishery and are thus vulnerable to any incident that might affect seafood availability. Prevalence of toxic and non-toxic algal species along Indian coast has been associated with a typical algal bloom formation, a visible surface phenomenon which is shown in figure 1.2 and 1.3, encountered during the study period of research work.

Fig. 1.2: Bloom sample (*Trichodesmiun* sp.) located onboard M V Bharat Darshan on April 2014 Latitude 09° 56' 256" Longitude 75 ° 52' 216"

Fig.1.3: Green patchy bloom (High occurrence of *Noctiluca* sp.) located FORV Sagar Sampada Cruise station No.336 on March 2015 latitude 21° 7' 31.8", Longitude 63° 17' 58.92"

1.2.2 Ciguatera Toxin

Existence of ciguatoxicity has not been indicated by any highly visible surface phenomenon such as red tide as seen in the case of Paralytic Shellfish Poisoning (DeFouw et al., 2001), hence an early warning to the alarm of CFP incidence is not possible. Until 2016, the occurrence of Ciguatera Fish poisoning was virtually unknown to fishes from Indian Coast. This region specific biotoxin has been reported very recently from Mangalore and Kerala

coast (Rajeish et al., 2016; Rajisha et al., 2017a; 2017b). In all reported CFP cases *Lutjanus bohar* commonly known *Chempalli* fish species detected as ciguatoxic from south west coast of India and caused intoxication in local population. Incidence of ciguatoxin from Indian Coast led to the fact that prevalence of ciguatera toxin in fishes from our reef ecosystems due to the bioaccumulation or biotransformation of toxic dinoflagellate *Gambierdiscus toxicus* through the food web.

Ciguatoxin (CTX) is an important biotoxin resulting from the consumption of coral associated fishes. A benthic dinoflagellate known as *Gambierdiscus toxicus*, is responsible for the production of Gambiertoxin. The biotransformation of Gambiertoxins in large fishes makes it more potent and significant in respect of human health (Lehane & Lewis 2000; Dickey & Plakas 2010; Friedman et al., 2017). CTX is a tasteless, colourless, odourless, heat and acid stable, lipophilic polyether compound which is stable at freezing temperature also (Abraham et al., 2012). CTXs which are secondary metabolites with numerous congeners having different molecular structure have been reported from different geographical origins namely Pacific (P-CTX), Caribbean (C-CTX) and Indian oceans (I-CTX) (Lewis & Sellin, 1992; Legrand et al., 1992; Lewis et al., 2000; Hamilton et al., 2002a; Pottier et al., 2002a; Caillaud et al., 2010). The geographical distribution of ciguatoxic fish poisoning (CFP)

affected globally in the tropical and subtropical regions and people suffered from this intoxication were reported to be ranging from 50,000 to 5,00,000 individuals annually (Lewis, 2001; Caillaud et al., 2010), even though it is difficult to ascertain the under-reporting cases of CFP (Friedman et al., 2017). A wide variety of 400 finfish species are implicated in CFP (Lewis, 2006) and it is responsible for the substantial economic loss because of the chronic health impacts after fish consumption.

It is felt that, there is an urgent need for the regular monitoring and development of ciguatoxin analysis in finfishes along Indian Coast. Chapters related with this are included so as to address this major seafood safety issue by developing and applying various research methods to detect the toxin. Specifically Chapter 2 reviews the fact and perspectives for the surveillance of ciguatoxin in fishes along Indian Coast and also discusses various methods available for detection. Chapter 3 details the collection and authentication of reef fishes along Indian Coast. Chapter 4 discuss about the screening of 262 reef associated finfishes for their accumulation of ciguatoxicity. Chapter 5 outlines a series of extraction methods based on mass Spectrometry detection of ciguatoxin and its molecular ions, which is being carried out for the first time from Indian Coast. Chapter 6 discusses about the isolation of purified ciguatoxin using a novel extraction and purification method and chapter 7

gives the investigation on the chemical structure of the purified toxin by spectroscopic techniques. Figure 1.4 depicts a flow chart indicating the chemical and structural investigation on prevalence and characterization of ciguatera along Indian coast.

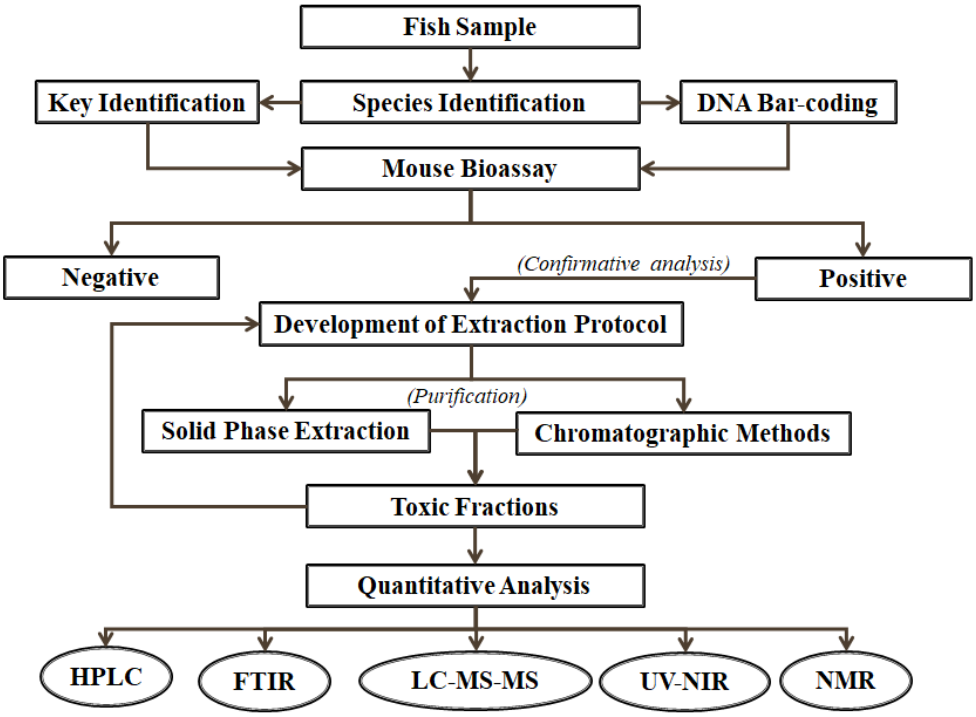


Fig.1.4: Flowchart representation of chemical and structural characterization of CTX

1.3 Research aims objectives and hypotheses

1.3.1 Research Aims

1. To understand the epidemiology and clinical recognition of ciguatera toxicity related to CFP case/outbreak analysis.

2. To develop a detailed understanding of Ciguatoxin associated with different reef-associated finfishes including their structures, toxicology and mode of action.
3. To improve understanding of the threats to the marine ecosystem through bioassays and ensuring food safety.

This study was a preliminary research in the area of investigation of Ciguatoxin in India. UNESCO (2016) declared a notification that there has been a shift in the distribution and occurrence of biotoxins around the world because of Climate Change. There has been a consecutive rejection from the European Union for Indian consignments only because of the presence of Ciguatoxin. Intoxication due to CTX was reported from fish samples collected from South West Coast of India (Rajeish et al., 2016; Rajisha et al., 2017a & 2017b). CFP is emerging as an important food safety concern which has to be addressed. Hence there is an urgent need to understand the prevalence, toxicity and detection methods for CFP.

1.3.2 Research Objectives

1. Identification of coral reef associated finfishes for the screening of CFP
2. Toxicological assessment of CFP by Mouse Bioassay and acute toxicology tests
3. Optimization of the established CTX extraction methods in Q-TRAP Liquid Chromatography Tandem Mass Spectrometry

4. Purification and chemical characterization of CTX using a Novel Extraction Protocol
5. Structural elucidation of CTXs using NMR Spectroscopic Techniques

1.3.3 Research Hypotheses

1. *Gambierdiscus toxicus* produces more potent Ciguatoxin (CTX) in reef associated fishes that can be extracted and purified from finfishes along Indian Coast by chromatographic techniques. This has been considered as a first validated study along Indian Exclusive Economic Zone.
2. The toxicity of Ciguatoxin can be investigated using in vivo bioassays and Mouse bioassay method has validated for the screening of CTX in reef fishes.
3. A sensitive detection method using Q TRAP Mass Spectrometry for determination of Ciguatoxin can be developed.
4. A novel extraction protocol for the determination of Ciguatoxin can be developed using AB Sciex 4000 Q TRAP Mass Spectrometer.
5. The chemical structure of CTX can be elucidated by Spectroscopic techniques (UV, LC- MS/MS, NMR and FTIR).

Chapter 2

Literature Review

Contents

Ciguatera Fish Poisoning (CFP) is a seafood-borne toxicity by consumption of toxin contaminated tropical and subtropical reef fishes which affects more than 50,000 people annually (FDA, 2011; Meyer et al., 2015; Friedman et al., 2017). Ciguateric fish accumulates complex, more polar and sodium channel activating ciguatoxin (CTX) orally through the food web (Lewis, 2001). Climate change and other environmental disruptions are the enhancing factors for the spreading of ciguateric fish around the world (Ruff, 1989; FDA, 2011) and now CFP is considered as a global disease. Ciguatera toxicity generates long lasting human health problems and at least 175 symptoms so far are recorded in humans (Sims, 1987; Lewis, 2001; Bradford & Joseph, 2008), which is categorized in to gastrointestinal, neurological and cardiovascular problems. Dinoflagellates responsible for the production of ciguatoxin exist within coastal waters between 35^0 north and 35^0 south of the equator (Lewis, 2001; FDA, 2011) and reviewers had documented the spread of ciguatera due to many reasons which include increased number of fishing community, consumption of contaminated

fishes associating oceanic oil rings habitats, increased number of travel and trade, increase in ocean temperature, importation of contaminated fish in to new areas where it is not reported earlier etc. (Frenette et al., 1988; Morton et al., 1992; Glaziou & Legrand, 1994; Moulignier et al., 1995; Bruneau et al., 1997; Lewis, 2001; Pottier et al., 2001; DeHaro et al., 2003; Sheppard & Rioja, 2005; Villareal et al., 2007). But the primary risk factor for ciguatera is the consumption of contaminated fish. CFP is reported from tropical and subtropical countries as a global disease (FDA, 2011). In recent times CTX toxicity reported from Indian Continental shelf also (Rajeish et al., 2016; Rajisha et al., 2017a; 2017b). Public health issues are reported from Kerala and Karnataka, major coastal fishing states in India and now there is responsibility for medical professionals to give proper attention to the symptomatic clinical data associated with this toxicity, and hence this well-timed observation would aid the research in the field of CFP and recovery of the toxic samples. There is no fatality reported so far, but the symptoms of ciguatera exist as a primary concern to the fisheries sector and export trade, because reef fishery contributed highly demanded, good tasted and priced fishes. CTX is a novel toxin to our ecosystem and there is a need for regulatory measures and authentic laboratory analysis for the monitoring and surveillance of ciguatera. A multidisciplinary research in the field of ciguatera has been initiated in the late 1950s by Banner and his team (Banner et al., 1960) and their interpretations has considered as a base study for the understanding of

complex nature and cause of ciguatera (Lewis, 2001). A detailed review regarding the occurrence of this toxin, its mechanism of action, chemical nature, clinical manifestations, detection and risk assessment will help in understanding the chronic impacts of Ciguatera Fish Poisoning. This literature review emphasis on the fact and perspectives for the surveillance of Ciguatera fish poisoning and its impact on seafood safety. It is organized in to three main sections; (1) A review of Ciguatera fish poisoning and its causative agent *Gambierdiscus toxicus* (2) Sample preparation and detection techniques of Ciguatoxin in fish and (3) Risk assessment of ciguatera for food safety.

2.1 Ciguatoxin

Ciguatera Fish Poisoning (CFP) is a seafood-borne illness associated with a wide variety of gastrointestinal, neurological and cardiovascular symptoms in humans. The term "cigua" given for the intoxication caused by the ingestion of coral reef fishes was first used by Don Antonio Para in Cuba in 1787 as a trivial name in Spanish to represent a univalve mollusk *Turbo livona pica* (Juranovic & Park, 1991; Scheuer, 1994; DeFouw et al., 2001). The incidence of ciguatera has been depicted from centuries back, since the time of Alexander the Great (356-323 B.C) (Scheuer, 1994; Pearn, 2001; Wong et al., 2014) and Homer's Odyssey (800 B.C) (Ragelis, 1984). A benthic dinoflagellate known as *Gambierdiscus toxicus*, growing predominantly in tropical and sub-tropical areas associated with coral reefs (Friedman et al.,

2008). This dinoflagellate responsible for the production of less polar toxin precursors known as Gambiertoxins. It is transferred and metabolized into the more polar Ciguatoxin (CTX) by the fish itself through the food web at higher level (Holmes et al., 1991; DeFouw et al., 2001; Friedman et al., 2017). CTXs are bio accumulated and concentrated in the food chain and both herbivorous and carnivorous fish can become toxic. Small fish ingest the toxin and then are eaten by larger fish, so that fish higher in the food web contains high CTX concentrations which are in turn consumed by humans (Banner et al., 1960; Gillespie et al., 1986; Crump et al., 1999; Lehane & Lewis 2000; Dickey & Plakas 2010). Yasumoto et al. (1977) firstly considered *G. toxicus* as the responsible species for CTX accumulation based on the hypothesis of Randall (1958). FAO (2017) clearly posturized the bioaccumulation process of CTX through the marine food chain which is shown in figure 2.1

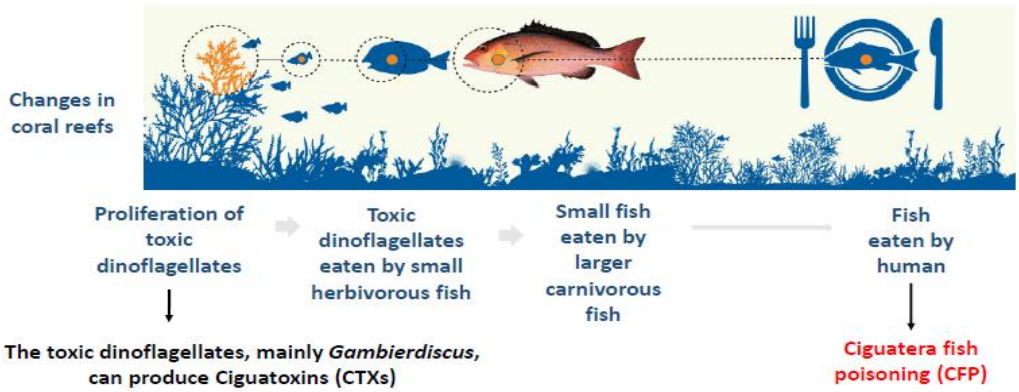


Fig.2.1: Bioaccumulation of Ciguatoxin through the food web (Source: FAO, 2017)

CFP is not associated with any external surface phenomenon or symptoms in the marine ecosystem such as red tide formations as in the case of other toxic algal proliferations (DeFouw et al., 2001), therefore an early warning to the alarm of CFP incidence is not possible (Scheuer, 1994). Estimated number of people suffered from this intoxication has been reported to be ranging from 10,000 to 50,000 annually as per previous references (Baden et al., 1995, Lewis, 2001) and now the range has changed to 50,000 to 5,00,000 individuals annually (Lehane & Lewis, 2000; Caillaud et al., 2010) which shows the intensity of occurrence even though it is difficult to ascertain the under reporting cases of CFP (Tester et al., 2010, Skinner et al., 2011, Friedman et al., 2017). Figure 2.2 shows the current global distribution of CFP according to FAO, 2017.

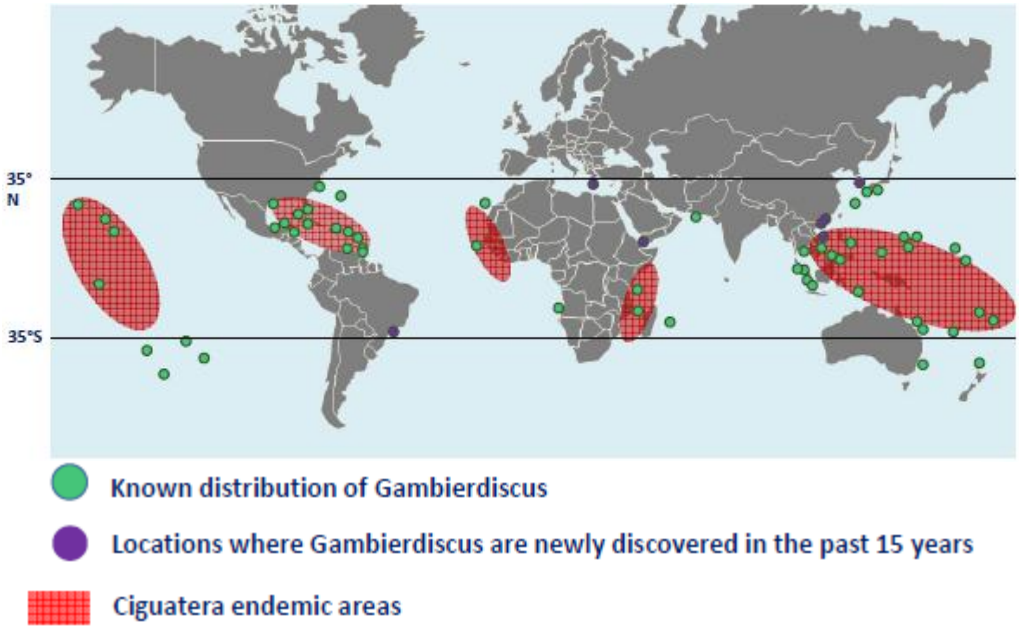


Fig.2.2: Current distribution of toxins (Source FAO, 2017)

CFP may be recognized worldwide as CTX group toxins, classified in to three major categories according to their occurrence as Caribbean (C-CTXs), Pacific (P-CTXs) and Indian Ocean (I-CTXs) origin and it is also identified for the first time in fish caught from Europe (EFSA, 2010a) and hence the geographical distribution of CFP affected globally in the tropical and subtropical regions. Around 400 reef associated finfish species (Halstead, 1978, Caillaud et al., 2010) are found to be ciguatoxic due to the process of biotransformation in the food web, but comparatively small number of species are regularly implicated in ciguatera (Lehane & Lewis, 2000). Most of these ciguateric fishes are coming under the category of top selling, good tasting and highly demanded food fishes in the world market (Friedman et al., 2017). FDA (2011) listed common reef associated finfishes implicated in CFP (Table 2.1).

Table 2.1: Common reef associated fin fishes with Ciguatera toxicity (FDA, 2011; Friedman et al., 2017)

Species	Family
Barracuda	Sphyraenidae
Amberjack	Seriola
Grouper	Serranidae
Snapper	Lutjanidae
Po'ou (*Cheilinus* spp.)	Labridae
Jack	Carangidae
Trevally (*Caranx* spp.)	Carangidae
Wrasse	Labridae
Surgeon fish	Acanthuridae
Moray eel	Muraenidae
Roi (*Cephalopholis* spp.)	Serranidae
Parrot fish	Scaridae

Other different ciguatoxic fish species are also reported around the world from various researchers (Blythe et al., 1992; Vernoux & Lejeune, 1994; Hokama

et al., 1998; Lewis et al., 1999; Hsieh et al., 2009; Azziz et al., 2012; Chan, 2013). Gillespie et al. (1986) reported narrow-barred Spanish mackerel, *Scomberomorus commersoni* ciguatoxic from Australian coastline. Chinain et al. (2010a) reported Scarids (Parrotfish) and Acanthurids (Unicorn fish) were rated as high-risk ciguatoxic fish species from French Polynesia.

Friedman et al, (2017) reviewed a number of case definitions used to describe the human health effects and diagnosis related to CFP (EFSA, 2006; Lampel, 2012; CDCP, 2016) and illustrates a modified universal case definition according to clinical, laboratory and epidemiological criteria. Clinical criteria include a wide array of symptoms characterized into three major groups. Preliminary symptoms start with gastrointestinal (e.g. nausea, diarrhea and vomiting, abdominal pain) problems which begin within 6-12 hours of fish consumption and resolve spontaneously within 1-4 days. Secondarily, the neurological symptoms which affects the central and peripheral nervous systems (e.g. paresthesia in the extremity and circumoral regions, pruritis, dysuria, myalgia, hallucinations, depression, cold allodynia, giddiness, vertigo, visual, balance and behavioral disturbance, loss of consciousness) were prominent after the gastrointestinal symptoms but in some cases they may starts simultaneously with the initial symptoms (DeMotta & Noceda 1985; Arcilla-Herrera et al.,1998; Pearn, 2001; Arena et al., 2004; Friedman et al., 2007; Stewart et al., 2010;

Zimmerman et al., 2013). Third category includes the cardiac symptoms (e.g. hypotension, bradycardia) at the early stage of toxicity and proceeds in combination with the initial two categories of symptoms (Chateau-Degat et al., 2007a; Katz et al., 1993). All these symptoms start within 2-30 hr after toxic fish consumption (Caillaud et al., 2010) and may persist from weeks to months and years. According to Chan (2016), CFP is rarely fatal (<0.1% fatality) and Lewis (2000) reported that it may be higher in the Indian Ocean. Certain foods and behaviours reactivate the ciguatoxic symptoms like nuts, caffeine, pork, chicken, alcohol consumption, tobacco smoking, fish consumption etc. (Gillespie et al., 1986; Glaziou & Martin, 1993; Lewis, 2000; Lewis, 2001; Chateau-Degat et al., 2007b). Clinical diagnosis of ciguatera fish poisoning is considered as a challenge to emergency physicians because of the patient's exhibit or present with a mixture of gastrointestinal, neurocutaneous and constitutional symptoms (Cheng & Chung, 2004). There is no effective treatment for this poisoning syndrome and the available remedy is based on acute symptomatic and supportive care for the patients (Friedman et al., 2017). Intravenous mannitol (one gram/kilogram body weight over a 30 to 45 minute period) and atropine (0.5 mg every 3-5 minute) was administered as dosage (Pearn et al., 1989; Baden et al., 1995; Lewis, 2001). CFP diagnosis is done in suffered individuals based upon the visible symptoms, time of onset and previous history of fish consumption (whether reef associated or its toxic history). There are no reliable biomarkers or documentation symptoms yet

discovered to confirm the exposure of this toxicity. Distinct differences are present in case of CFP symptoms according to various geographical distributions. In the Pacific CTX, neurological symptoms are dominated, whereas in the Caribbean, gastrointestinal problems are highly dominated (Lewis, 2001). Indian Ocean CTX pause a group of symptoms like hallucinations, mental depression, lack of coordination etc. along with typical ciguatera symptoms (Lewis, 2001). Table 2.2 describes common CFP associated symptoms in humans.

Table 2.2: Clinical diagnosis of ciguatera symptoms (Morris et al., 1982; Coleman, 1990; Lewis, 2001; Arena et al., 2004; Chateau-Degat et al., 2007a; Baumann et al., 2010; Friedman et al., 2017)

Category	Symptoms
Gastrointestinal	Abdominal pain , Vomiting , Diarrhoea , Nausea
Neurological	Generalised weakness , Vertigo Lingual paraesthesia , Extremities Paraesthesia, Circumoral Paraesthesia Arthralgia and Myalgia Dental pain , Ataxia Paradoxical Temperature sensation Respiratory paralysis Coma, Weakness in the extremities, Headache Myalgia and Arthralgia
Cardiovascular	Dizziness Hypotension (systolic BP <100 mmHg) Bradycardia (pulse rate <60 beats/min) Chest pain
Others	Chills Sweating Shortness of breath Itching (two to three days) Nightmares, mental depression, hallucinations Lack of coordination and loss of equilibrium

2.2 Sample preparation and Ciguatoxin detection in fish

Ciguatoxin emerging as a new toxin from our coast and the absence of purified standards and complex nature of CTX in fish tissue will be a major concern in the development of a laboratory analytical method. USFDA, NOAA, laboratories in Japan and Australia have been developed *in-vitro* assay protocol for determination of ciguatoxin in fish (Dickey, 2008). Traditional methods are practiced among local population, which include animal testing, observing the bleeding at the tail of the fish fillet (Figure 2.3), observing silver coins turning black on a hypothetical cooked fish, feeling a sensation on tingling when rubbing the liver on gums etc. (Banner et al., 1963 and Chinain et al., 2010b). These methods are practically not suitable for the determination of CTX toxicity.

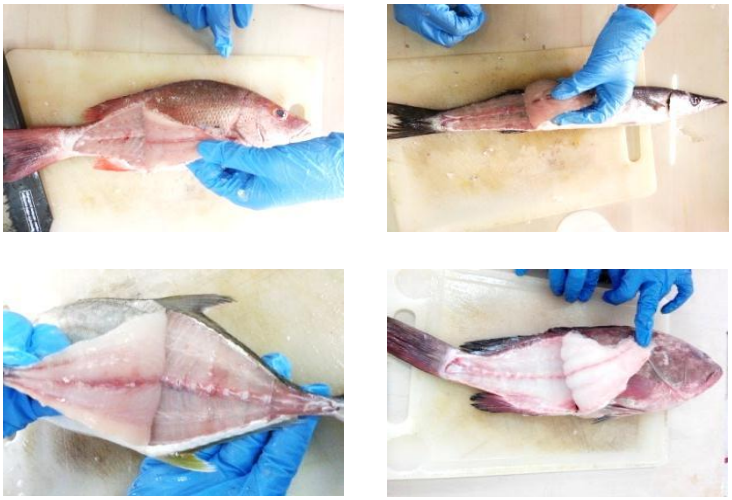


Fig. 2.3: Traditional method of observing the bleeding line at the tail of fish fillet

An ideal or recognized official method for CTX detection in fish is not yet established (Caillaud et al., 2010; Friedman et al., 2017). Sample preparation is a key concern in the development of CTX associated fish extraction and purification methods. Because of the low concentrations or trace quantity of toxin present in fish samples, association of toxin in complex form of tissue matrix and the presence of numerous congeners found in a single sample, sample preparation and development of simple extraction protocols are difficult in case of ciguatoxin analysis (Friedman et al., 2017). Results of CFP diagnosis in humans with clinical symptoms and the fish remnants of implicated fishes helps in the analysis of associated CTX toxicity and extraction. Bioassays, immunoassays, cell based assays and physico-chemical instrumentation analysis are developed for the determination of CTX. But there are no pure standard reference material and biomarkers available for the authentication and confirmation of exposure to CTX in humans and fishes. Quillam, (2003) suggested an order of criteria for a typical investigation of an entirely new and unknown toxin through association of many different techniques (Figure 2.4).

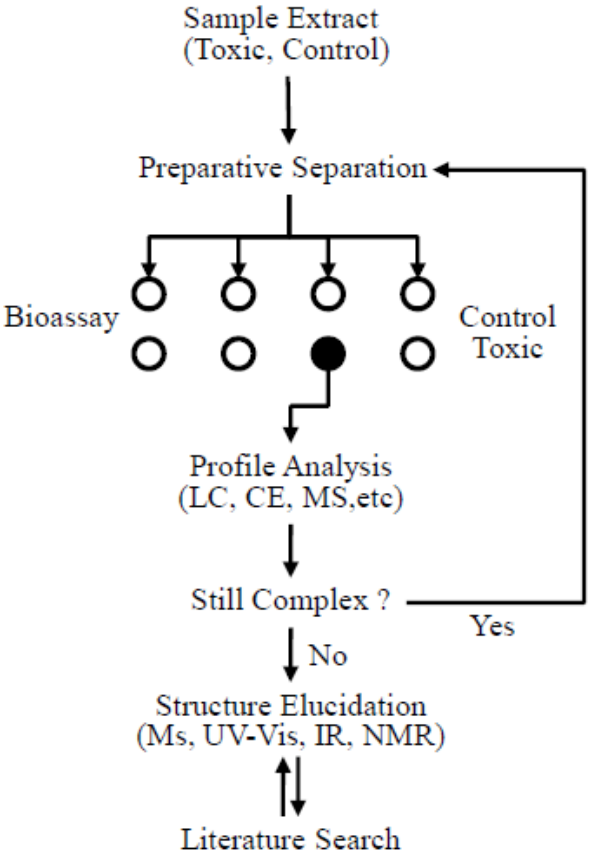


Fig. 2.4: Flow chart indicating general principles of toxin isolation through integration of multiple separation and assay techniques (Quilliam, 2003)

Ciguatoxin is a lipid soluble compound and most of the sample preparation methods are based on Acetone or Methanol extraction (Caillaud et al., 2010). Since it is a lipophilic compound, fatty acids should be eliminated by using separate solvent extraction and liquid-liquid partitioning methods using n-hexane, Ethanol, Diethyl ether, Chloroform etc. These solvents will reduce the excessive fatty acid content present in fish samples and increase the toxin recovery in ether extract (Lewis, 1995). But using these solvent partitioning methods, the purity of

toxin is not attained and chances of contamination are higher while doing the flash evaporation steps under vacuum conditions. Solid Phase Extraction (SPE) clean-up methods are better option to overcome this difficulty during extraction procedure and it also helps to improve the purity of CTX extract. SPE is the most appropriate method for extraction of sample which is present in trace quantities in a complex matrix. The major struggle while conducting the SPE extraction is the understanding of proper eluting fraction or ratio of solvents to be used, in which the recovery of toxin is high. For attaining the correct elution programme, a series of solvent ratio should be followed and each fraction has to be collected and analyzed. Various SPE clean up procedures are developed for Mouse Bioassay (MBA), Cell Based assay, Receptor Binding assay and LC-MS/MS analysis of CTX samples. Wong et al. (2009) developed SPE clean up method using Florisil Cartridge for use with MBA. Silica gel and Aminopropyl SPE method is followed for purification of CTX from fishes for use with Cell Based Assay and LC-MS analysis (Dickey, 2008). A rapid purification of CTX samples through Sep Pak C_{18} cartridges to be used with Receptor binding assay was conducted by Darius et al. (2007). Ciguatoxin Rapid Extraction Method (CREM) developed using only two gram of fish flesh through C_{18} and Silica Sep Pak SPE cartridges and the toxin recovery was found comparatively high when compared to other clean-up methods (Lewis et al., 2009). Yogi et al. (2014) followed Florisil and Primary Secondary Amine (PSA) Sep Pak Cartridges for the purification of CTX samples

from Japan. SPE is also practiced for the purification of CTXs from wild and cultured *Gambierdiscus* sp. using Florisil SPE cartridge, which is followed by further purification steps using Size Exclusion Chromatography method in HPLC (Chinain et al., 1999; 2010b; Darius et al., 2007).

Characterization of CTX using Mass Spectrometry and NMR methods required a large quantity of CTX extract and Size Exclusion chromatography using preparatory HPLC columns and other column chromatographic methods was followed for the purification of Pacific and Caribbean CTXs. Highly toxic and large sized fish samples or pure culture of *Gambierdiscus* sp. were subjected to different chromatographic purification steps and recovered milligram quantities of pure toxin extracts (Lewis et al., 1991; Lewis & Jones, 1997; Vernoux & Lewis, 1997; Hamilton et al., 2002a). Florisil, Sephadex LH20 and TSK Fractogel chromatography showed good recovery of I-CTX toxin extracts, but significant loss of toxicity is observed during preparative HPLC; hence isolation to purity was not possible in case of Indian Ocean ciguatoxin (Hamilton et al., 2002b). Hence characterization of ciguatoxin depends on an efficient extraction and purification method of samples using different chromatographic approaches.

2.2.1 Mouse Bioassay and other *in vivo* and *in vitro* methods

Mouse bioassay (MBA) has been widely used for the selective determination of ciguatoxicity in fishes introduced by Banner et al. (1960) and refined by Yasumoto et al. (1984). In this method the lethality observed in Mouse Units (MU), followed by intra peritoneal injection of crude fish ether extract into mice and up to 24 hour observation of toxicity and relationship between dose and time to death is used to quantify toxicity (Lewis, 1995). Other *in vivo* assays for the detection of CTX include Chicken assay (Kosaki et al., 1968), Brine Shrimp assay (Granade et al., 1976; Bienfang et al., 2008), Mosquito Larvae assay (Bagnis et al., 1985), Diptera Larvae assay (Labrousse & Matile, 1996) etc. These assays are not recommended for CTX quantification, hence not widely used in laboratories for screening of ciguatoxin (Caillaud et al., 2010). MBA is followed as an official testing method for Paralytic and Diarrheic shellfish toxins as per European Union and FDA guidelines (FDA, 2011; AOAC, 2012). In case of CFP, MBA is used for the screening of ciguatera implicated reef fish samples. Yasumoto et al. (1984) and Caillaud et al. (2010) suggested that any fish containing above 2.5 Mouse Unit (MU)/100g should be avoided as food, since it has long term neurological effects. Sub lethal doses were in the range between 0.18 and 0.45 MU per 20mg of ether extract (Wong et al., 2005). The utility of MBA method is

limited by the requirement of dose response curve because of the lapse of purified CTXs for accurate quantification; hence the curve is not linear (Hoffman et al., 1983; Lehane & Lewis, 2000; Lewis, 2003). Lewis (1995 & 2003) revised the MBA extraction protocol and Wong et al. (2005; 2009) developed a Solid Phase Extraction (SPE) clean up method for CTX fish extract for MBA. Routine analysis of samples by mouse bioassay cannot be recommended since it is non-specific and ethically objectionable (Abraham et al., 2012). Sodium channel specific cytotoxicity (Manger et al., 1993; 1995) and sodium channel receptor binding in rat brain synaptosomal preparations (Lombet et al., 1987; Lewis et al., 1991; Poli et al., 1997) were developed as an alternative to *in vivo* assay. *In vitro* mouse Neuroblastoma assay was used as a screening procedure, using an ouabain-veratridine dependent method by Dickey et al. (1999) and Manger et al. (1995). EFSA (2009c) recommended *in vitro* assay as an alternative to *in vivo* animal assays for monitoring and investigation of Marine Algal Toxins (MATs).

2.2.2 Physico-chemical detection of CTX in fish

FDA applied a two- tiered protocol for monitoring of CFP which includes *In vitro* assay and Mass Spectrometry analysis (FDA, 2011; Friedman et al., 2017).

2.2.2.1 Mass Spectrometry

The main physico-chemical methods used in toxin analysis are chromatographic methods with optical (UVD and FLD) or mass spectrometric detectors (Quilliam, 2003). A typical LC-MS system comprises HPLC for analyte separation, an atmospheric pressure ionization interface to produce ionised molecules and MS in which ions are separated and detected in a high vacuum environment. Analyte separation by HPLC requires a suitable column and mobile phase. Atmospheric pressure ionization is the key step for detection of toxins by MS. There are several types of ionizations including Electron Spray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI), Atmospheric Pressure Photo-Ionization (APPI), Fast Atom Bombardment (FAB) and Matrix-Assisted Laser Desorption Ionization (MALDI). ESI is commonly used for marine toxins because of its simplicity and robustness, sensitivity and suitability for compounds with widely ranging polarities and molecular weight (Quilliam, 2003; Núñez et al., 2005). Figure 2.5 shows an internal structure of a Triple quadrupole AB Sciex 4000 Mass Spectrometer.

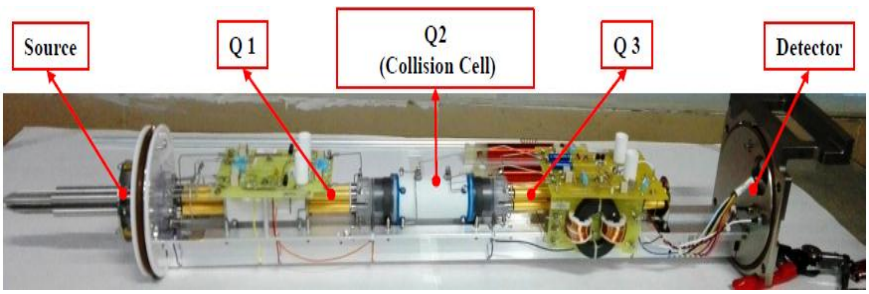


Fig.2.5: Internal structure of QTRAP MSMS (AB Sciex 4000 Instrument)

A rapid extraction method using solid phase extraction procedure using silica and C_{18} cartridges was developed by Lewis et al. (2009) in order to reduce the time of sample preparation. Various ciguatoxin congeners were quantified using Mass spectrometry method for Pacific, Caribbean and Indian Ocean forms. For Pacific ciguatoxin, P-CTX-1, P-CTX-2 and P-CTX-3 congeners with molecular masses identified as 1111.6 & 1095.5 Da were isolated from carnivorous fishes (Lewis et al., 1991, Lewis et al., 1993, Lewis & Jones, 1997). Another two congeners for P-CTX are CTX-3B (49-epi-CTX-3C) and CTX-3C with molecular ions 1023.6 Da and M-seco-CTX-3C with m/z 1041.6 Da were isolated from *Gambierdiscus toxicus* (Satake et al., 1993a; Chinain et al., 2010b; Roeder et al., 2010). Molecular mass of m/z 1061.6 Da identified for CTX-4B (GT-4B) isolated from *Gambierdiscus* sp. and herbivorous fish as source organisms and also for 52-epi-ciguatoxin-4B (CTX-4A; GT-4A) isolated from *G. toxicus* (Murata et al., 1990; Satake et al., 1996; Yasumoto et al., 2000; Roeder et al., 2010). $[M+H]^+$ ions 1057.6 Da for CTX-2A1 congener isolated from both *G. discus* and carnivorous fish and 1039.5 Da for CTX-2C1 from *G. toxicus* as source organism (Satake et al., 1998; Roeder et al., 2010). Almost 10 congeners are identified for Pacific ciguatoxin as fish and algae as causative species and six congeners for Caribbean ciguatoxin (C-CTX) isolated only from carnivorous fishes as source organisms. C-CTX having a m/z 1141.6 Da for C-CTX-1 and C-CTX-2,

1127.6 Da for C-CTX-1127, 1143.6 Da for C-CTX-1143, 1157.6 Da for C-CTX-1157 and 1159.6 for C-CTX-1159 has been identified as congeners by various researchers (Vernoux & Lewis, 1997; Lewis et al., 1998; Lewis et al., 1999; Pottier et al., 2002a & 2002b; Pottier et al, 2003). Indian Ocean ciguatoxin (I-CTX) isolated from carnivorous fishes with molecular masses m/z 1141.6 Da for I-CTX-1 & I-CTX-2 and 1157.6 Da for I-CTX-3 & I-CTX-4 congeners (Hamilton et al., 2002a & 2002b). Mass Spectrometry (MS) is an excellent tool for the identification and characterization of ciguatoxin congeners, since it is provided enhanced sensitivity and selectivity by measuring accurate masses or a series of fragment ions. Lewis et al. (1994) introduced Ion Spray (IS) as ion source for MS analysis. Yogi et al. (2014) carried out an LC-MS/MS analysis using Triple Quadrupole Mass Spectrometry, in which 14 reference toxins were used and pure CTX-1B and CTX3C were prepared from fish samples collected from Japan.

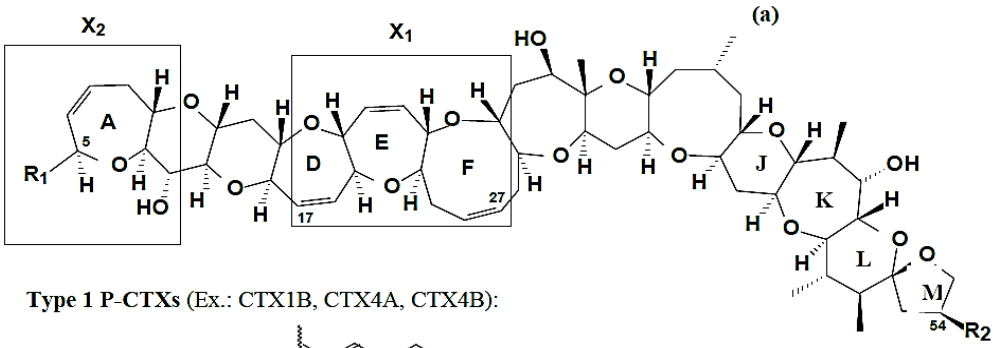
2.2.2.2 Nuclear Magnetic Resonance (NMR)

Modern NMR has proved as a vital technique for the full elucidation of the chemical structures of novel biotoxins (Shi et al., 2012). Proton (^{1}H) NMR and Carbon-13 (^{13}C) enables determination of the proton environment (number and configuration of neighbouring protons) and identification of the number and type of carbon atoms in an organic molecule respectively. The

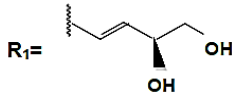
combination of ^{13}C and ^{1}H NMR in 2D experiments along with FT IR and UV Visible NIR allows the elucidation of the carbon connectivity and 3-dimensional chemical structure of complex organic molecules (Shi et al., 2012). Pacific and Caribbean CTXs are structurally elucidated so far and NMR is the key technique used for this purpose. CTX is a group of highly oxygenated and cyclic polyether molecules and structurally related with the Brevetoxin (PbTx) group (Lewis, 2001). Murata et al. (1989) started the pioneer work in the structural confirmation of Pacific CTX and its precursor from *G. toxicu*s using NMR methods. From the Pacific P-CTX-1(m/z 1111 Da), P-CTX-2 and P-CTX-3 (both has m/z 1095 Da) were structurally isolated from carnivorous fish and P-CTX-3C (m/z 1045) isolated from *G. toxicus* (Lewis et al., 1991, 1993; Satake et al., 1993a, 1996, 1998). Caribbean C-CTX-1 and C-CTX-2 with molecular mass 1141 Da are structurally elucidated from carnivorous fish (Vernoux & Lewis, 1997; Lewis et al., 1998). P-CTX-4A and P-CTX-4B with molecular mass 1061 Da has been structurally elucidated from *G. toxicus* and herbivorous fishes (Murata et al., 1990). CTX are structurally distinct from other biotoxins (Yasumoto & Murata, 1993) and using Mass Spectrometry and NMR techniques, several minor toxins are also detected (Lewis & Jones, 1997; Vernoux & Lewis, 1997). Around 20 ciguatoxin congeners are structurally elucidated by Yasumoto et al. (2000) using high energy Mass Spectrometry and NMR techniques.

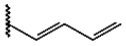
2.2.2.2.1 Chemical and Structural Properties of CTX

Investigators in the field of marine biotoxins defined ciguatoxin as tasteless, colourless, odourless, heat and acid stable, non-protein, lipophilic polyether compound which is stable at freezing temperature also (Vernoux & Lewis, 1997; Lewis et al., 1998; Lewis, 2001; Pottier et al., 2002b; Abraham et al., 2012). Pacific CTX was divided in to Type I and Type II based on number of carbons 60 and 57 respectively and the structure of the ether ring (Murata et al., 1990; Legrand et al., 1998). Caribbean CTXs contains 62 number of carbon and 14 numbers of E rings. Vernoux and Lewis (1997) first isolated and structurally identified two C-CTXs and later Pottier et al., 2002a; 2002b identified additional congeners. Hamilton et al. (2002b) isolated four I-CTXs, but their structural characteristics were unidentified. Structure of P-CTX (Type I and Type II) and C-CTX elucidated by different researchers were given in figure 2.6. (Murata et al., 1990; Lewis et al., 1991; Satake et al., 1996; Lewis & Jones, 1997; Vernoux & Lewis, 1997; Lewis et al., 1998; Yasumoto et al., 2000; Lewis, 2001; FAO, 2004; Caillaud et al., 2010, FDA, 2011).



Type 1 P-CTXs (Ex.: CTX1B, CTX4A, CTX4B):

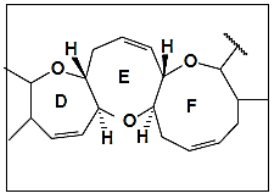
CTX1B  R_1= OH R_2= OH

CTX4B  R_1= R_2= H

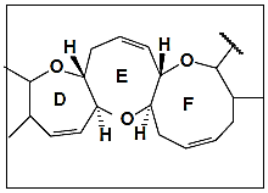
CTX4A: epimer of CTX4B at C52

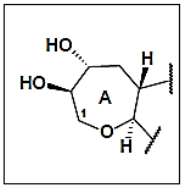
Type 2 P-CTXs (Ex.: CTX3C, CTX2A1):

- CTX3C

$R_1 = R_2 = H$ X_1 = 

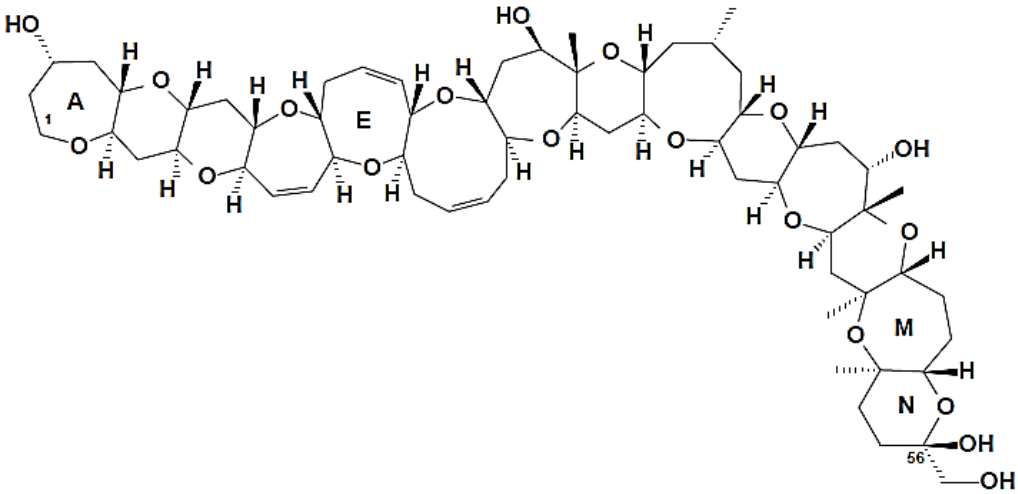
- CTX2A1 (2,3-dihydroxyCTX3C)

R_2=H X_1= 

X_2= 

a) P-CTX Type I with 13 number of E rings and 60 number of carbons & Type II with 57 number of carbons

(b) C-CTX-1 (C-CTX2 is an epimer of C-CTX-1 at C-56).



b) Caribbean CTX 1 & CTX 2 with 14 number of E rings and 62 number of carbons

Fig. 2.6: a) Structure of Pacific Ciguatoxin (Type I & II) and b) structure of Caribbean Ciguatoxin (Lewis et al., 1998; Lewis, 2001; FAO, 2004; Caillaud et al., 2010)

2.3 Risk assessment of CFP for food safety

EFSA (2010a) panel on contaminants in the food chain assessed, Ciguatoxin as an emerging biotoxin for which widely screened toxicity assay MBA has found limitations due to insufficient detection and ethical concerns. In vitro assay and Receptor binding assay have been developed as an alternative, but they need further development and only few laboratories got the needed facility for cell based assays. Hence LC-MS/MS Tandem Mass Spectrometry is only considered as valued method, in which reference standards need to be developed according to the distinctive nature of CTX due to various geographical regions. The major preventive measures for ciguatera

include, avoiding ciguateric fish, proper surveillance and reporting of incidence based on clinical data, community outreach and education to avoid misdiagnosis and under reporting of cases (Friedman et al., 2017). The ciguatera transmission from person to person which include effects on the embryo/ fetus via placenta and breast feed infant via mothers milk showed the risk of ciguatera toxicity in humans (Bagnis & Legrand, 1987; Blythe & De Silva, 1990; Ruff & Lewis, 1994; Karalis et al., 2000). In the present scenario, where food safety is becoming prime concern of all people, it is felt that ciguatera poisoning will assume and will be a major concern for the marketing of reef associated finfishes. The amount of toxins is directly correlated to the size of the fish and results indicated that large sized fishes had more ciguatoxin in comparison to small fishes (Pottier et al., 2001). Hence it is advisable for the consumers to take only fishes of small size. Ban or size restrictions on certain reef fish species can be taken as an initial safety measure to protect the consumers from the lethal effects of this toxicity.

European Union regulations states CFP that "Fishery products containing biotoxins such as ciguatoxin or muscle-paralyzing toxins must not be placed on the market" but there is no reference analytical method suggested for CFP samples, which restricts the implementation of regulatory safety limits (Caillaud et al., 2010).

2.3.1 Analysis of Ciguatera case/Outbreak samples

Due to climate and other environmental changes, the geographic distribution of *Gambierdiscus* spp. expanded to the under reported CFP regions and thereby stimulated the expansion of ciguatoxic fishes (Friedman et al., 2017). Our maritime resources including highly demanded and good tasted reef associated finfishes and their exports are the key to the fishery economy. In case of CFP toxicity observed from Mangalore Coast during September 2016, the persons were hospitalized by consuming fish head curry of Red snapper or Chempalli for supper (MTNN, 2016; Times of India, 2016). The workers of nearby exporting fish firm were hospitalized due to consumption of fish heads, which was considered as a waste in the fish export factory. The fish might be poisonous and the export should be allowed after proper testing of the fish parts, if the head caused poisoning then the remaining parts will definitely contain the toxicity. This incident showed the failure of hazard identification from the industry, and most of the industries handling the export of reef fishes have the responsibility for the proper checking and diagnosis of their consignments, so as to ensure the export safety of our products. Reef fishery mainly contributed major species coming under the group of snappers, reef cods, croakers, trevally, barracuda etc., are highly needed species in the foreign markets. To ensure these seafood items safety at the initial level of

marketing and exporting was an important measure. Reports on the existence of ciguatera from our coasts are rarely occurred, but some incidents came recently and toxic species are being screened out from the laboratory analysis (Rajeish et al., 2016; Rajisha et al., 2017a, 2017b). Incidence or report on ciguatera might cause ban of many species which subsequently affect the food security and trade. Climate change and globalization of trade has led to an increase in the spread of ciguatera, hence guidance is needed for those countries where there is CFP risk management programmes not yet implemented (FAO, 2017).

Chapter 3

Ciguatoxin Implicated Reef Fish Sample Collection and their Authentication from Indian Coast

Contents

3.1 General Introduction

The term "cigua" is given for the intoxication caused by the ingestion of CTX contaminated reef dwelling fishes (DeFouw et al., 2001). CTX is accumulated in the flesh of predatory finfishes directly by eating toxic marine algae and other fishes containing the toxins. These fishes are harvested either commercially or by recreational methods and hence consumers who eat the fish that contain ciguatoxin are at risk for getting CFP (Friedman et al., 2017). It is difficult to identify a contaminated ciguateric fish because the toxin does not have any effect on fish and is devoid of changes in texture, smell or taste when compared to non-toxic fishes (Lehane & Lewis, 2000). More than 400 fish species have been reported to be potentially ciguateric (Caillaud et al., 2010). Larger fishes accumulate toxins due to bioaccumulation making it more

potent in comparison to those in small fishes (FAO, 2004; Farrell et al., 2016). Common Ciguatoxic Fish Species belongs to the family of Muraenidae, Sphyraenidae, Serranidae, Carangidae, Lutjanidae, Acanthuridae, Scaridae, Labridae, Scombridae, Balistidae etc. (FDA, 2011; Friedman et al., 2017). USFDA guidance for fish and fishery products hazards and controls provide details of hazards which are "reasonably likely to occur" in particular fish and fishery products under ordinary circumstances. Occurrence of natural toxins in fish not previously associated with that toxin is considered as a new or emerging problem in the case of fish and fishery products. According to FDA (2011), CFP is now described as being associated with consumption of toxin contaminated fish found in tropical or subtropical areas around the world between 35^0 north latitude and 35^0 south latitude, particularly the Caribbean, Pacific and Indian Ocean and in the Flower garden Banks area in the northern Gulf of Mexico. Action levels for CFP are now listed as 0.01ppb for Pacific and 0.1ppb for Caribbean ciguatoxin. CFP is considered as a natural toxin and FDA listed out some of the responsible potential vertebrate species under ciguatera hazard category (Table 3.1).

Table 3.1 Ciguatera Fish Poisoning Hazard (Source FDA, 2011)

Sl No	Common name	Species
1.	Amberjack	*Seriola* spp.
2.	Barracuda	*Sphyraena* spp. *S. barracuda* *S. jello*
3.	Moray Eel	*Gymnothorax funebris* *Lycodontis javanicus*
4.	Grouper	*Cephalopholis* spp. *C. argus* *C. miniata* *Epinephelus* spp. *E. fuscoguttatus* *E. lanceolatus* *E. morio* *Mycteroperca* spp. *M. venenosa* *M. bonaci* *Variola* spp. *V. louti*
5.	Grouper or Coral Grouper	*Plectropomus* spp.
6.	Grouper or Gag	*Mycteroperca microlepis*
7.	Grouper or Hind	*E. guttatus* *E. adscensionis*
8.	Hogfish	*Lachnolaimus maximus*
9.	Jack or Trevally	*Caranx* spp. *C. ignobilis* *C. melampygus* *C. latus* *C. lugubris* *C. ruber* *Carangoides bartholomaei* *Seriola rivoliana*

10.	Jack or Blue Runner	*C. crysos*
11.	Jack or Rainbow Runner	*Elagatis bibinnulata*
12.	Job fish	*Aphareus* spp. *Aprion* spp. *Pristipomoides* spp.
13.	Mackerel, Narrow-Barred Spanish	*Scomberomorus commerson*
14.	Mackerel, Spanish or King	*S. cavalla*
15.	Parrot fish	*Scarus* spp.
16.	Snapper	*Lutjanus* spp. *L. bohar* *L. gibbus* *L. sebae* *L. bucanella* *L. cyanopterus* *L. jocu* *Symphorus nemaptophorus*
17.	Tang	*Acanthurus* spp. *Ctenobaetus* spp. *Naso* spp.
18.	Triggerfish	*Balistes* spp.
19.	Wrasse	*Cheilinus undulatus*

Indian capture fisheries sector comprises snappers, reef cods, barracudas, croakers and carangids as commercially exploited finfishes mainly for the export purpose from different coastal states (MPEDA, 2017). Bigger sized reef fishes are highly demanded category in the fish exporting industries because of their taste and market value. CTX accumulated toxicity is higher in large sized fishes than smaller ones (Oshiro et al., 2010). Hence fish samples were collected from fishing harbours along different coastal states and also

from different exporting industries involved in the export of reef fishes. These samples were purchased only for research purpose and most of the exporters provided filleted, degutted and head portions of bigger sized fish samples for the study. Therefore accurate identification of fish samples is needed for the toxicological analysis. So beside morphological key identification, also carried out DNA barcoding of some samples for the confirmation. This chapter discuss about the data based on sample collection and authentication of fish specimens.

3.2 Materials and methods

3.2.1 Sampling area and species

Sampling stations were fixed based on the availability or abundance of reef associated finfishes. Samples collected along the Indian coast including major fishing harbours and landing centers, local fish suppliers and exporters. Reef fish samples were randomly collected from May 2015 to August 2017 for surveillance of ciguatoxin contamination (sampling locations with latitude and longitude given in Figure 3.1). Finfishes from different groups of species including snappers, barracuda, croakers, groupers, carangids etc. were collected for analysis. Fish samples collected were immediately frozen at -20^{0}C in laboratory until extraction. The samples were collected from

Thoppumpady (Ernakulam),

Neendakara (Kollam),

Sakthikulangara (Kollam),

Vizhinjam (Trivandrum) - Kerala Coast

Tuticorin,

Kanyakumari - Tamil Nadu Coast

Mangalore - Karnataka Coast

Veraval - Gujarat Coast

Mumbai – Maharashtra Coast.

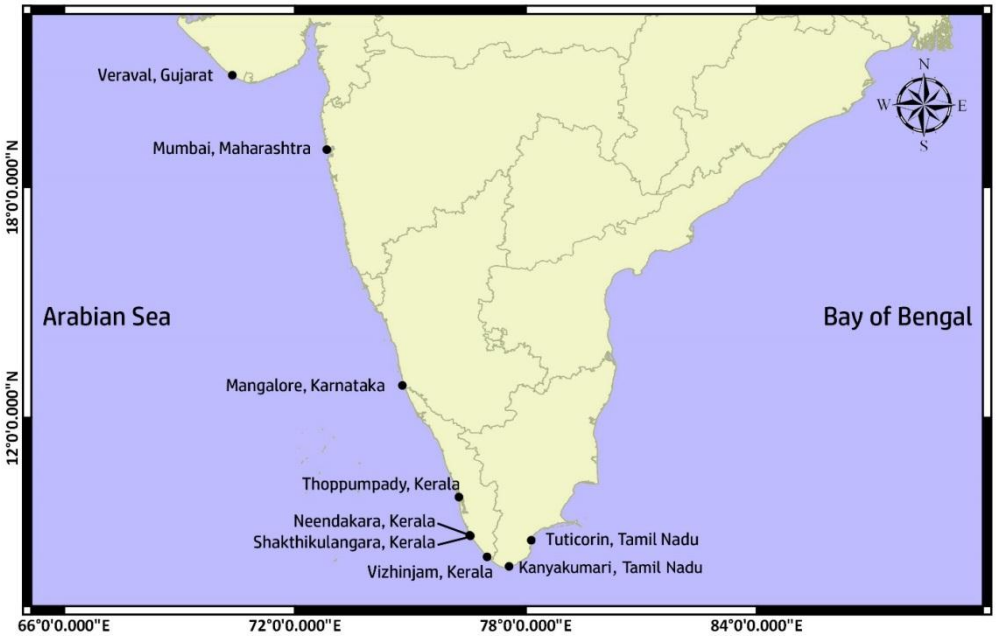


Fig.3.1: Map showing location of sampling sites

3.2.2 Species identification

Fish identification is done based on Morphological characteristics (Smith & Heemstra, 1986) and DNA barcoding method (Ward et al., 2005). DNA barcoding based on Cytochrome Oxidase Subunit I (COI) gene is adopted for species level fish identification. For barcoding the total genomic DNA of the samples was isolated using DNeasy Blood & Tissue Kit (Qiagen, Germany), as per the manufacturer's instruction and the COI gene was amplified using universal primer pair (Ward et al., 2005). Partial COI fragments were amplified using primers is given in Table 3.2. Final DNA concentration and purity was estimated by Optical Density (OD) reading using a bio spectrometer (Eppendorf, Germany) at A 260 nm and A 260/280.

Table 3.2 Primers used for identification of fish

Primer		Reference
FishF1	5'- TCAACCAACCACAAAGACATTGGCAC -3'	Ward et al. (2005)
FishR1	5'- TAGACTTCTGGGTGGCCAAAGAATCA -3'	

The reaction was carried out in 25µl volume, containing mixture of 1x Taq buffer, 2.5 mM Magnesium Chloride and 50 µM of each primer, 200 µM of each dNTP, 0.5 U Taq DNA polymerase, 75 ng of template DNA and autoclaved double distilled water to make up the volume to 25 µl. The reaction mixture was thermal cycled for 35 cycles of 30 s at 94°C, 30 s at 52°C, 45 s at 72°C and final extension of 10 min at 72°C, with an initial denaturation step at

95°C for 4 min. The PCR products were sequenced bidirectionally using ABI 3730 capillary sequencer in the sequencing facility. The raw DNA sequences obtained were edited and aligned using BioEdit version 7.0.5.2 (Hall, 1999). The edited partial sequences of COI gene were analyzed for species identification using the NCBI BLAST search engine and the sequences were submitted to GenBank database (NCBI, 2017). The identity of the experimental nucleotide sequences to known sequences in GenBank was performed using the NCBI-BLAST program (http:/www.ncbi.nlm.nih.gov). Phylogenetic tree was generated by the neighbour-joining method using MEGA 4.0 software (Tamura et al., 2007).

3.3 Results and Discussion

3.3.1 Sample collection

A total of 262 reef associated finfish samples were collected from different sources across Kerala, Tamil Nadu, Maharashtra, Gujarat and Karnataka during the study period from May 2015 to August 2017 for the screening of ciguatoxicty. Screened samples comprised of eight species of snappers *viz., Lutjanus argentimaculatus, L. fulvus, L. bohar, L. gibbus, L. johnii, Pinjalo pinjalo, Aprion virescens, Pristipomoides filamentosus,* two species of Barracuda *viz., Sphyraena putnamae and S. jello;* seven species of groupers *viz., Variola louti, Epinephelus bleekeri, E. coioides, E. diacanthus, E. merra, E. polylepis and E. chlorostigma;*

and other species include, *Otolithoides biauritus* (croakers), *Caranx ignobilis* (carangids), *Lethrinus nebulosus* (emperor fish) etc (Figure 3.2). Table 3.3 categorizes the twenty fish species and their earlier report on toxicity from Pacific, Caribbean and Indian Ocean regions. Total number of fish specimen collected, average length and weight, area of collection etc. are given in Table 3.4.

Table 3.3 Common coral reef fishes found in Indian Coast and previous reports on their CTX toxicity

Sl. No	*Common Name*	*Scientific Name*	*Citation on toxicity*
	Snappers		
1.	Mangrove red snapper	*Lutjanus argentimaculatus (Forsskål, 1775)*	Leung et al. (1992) Wong et al. (2014) Chan (2014)
2.	Black tail snapper	*Lutjanus fulvus (Forster, 1801)*	Wu et al. (2011)
3.	Two spot red snapper	*Lutjanus bohar (Forsskål, 1775)*	Oshiro et al. (2010) Wu et al. (2011) FDA (2011) Wong et al. (2014)
4.	Humpback red snapper	*Lutjanus gibbus (Forsskål, 1775)*	Lehane & Lewis (2000) FDA (2011) Wu et al. (2011)
5.	John's snapper	*Lutjanus johnii (Bloch, 1792)*	FDA (2011)
6.	Crimson job fish	*Pristipomoides filamentosus (Valenciennes, 1830)*	Lewis (2000) Oshiro et al. (2010) FDA (2011)
7.	Pinjalo	*Pinjalo pinjalo (Bleeker, 1850)*	FDA (2011)
8.	Green job fish	*Aprion virescens (Valenciennes, 1830)*	FAO (2004) Wong et al. (2014)
	Groupers		
9.	Yellow-edged lyre tail	*Variola louti (Forsskål, 1775)*	Oshiro et al. (2010) FDA (2011) Yogi et al. (2013)
10.	Brown spotted grouper	*Epinephelus chlorostigma (Valenciennes, 1828)*	Lehane & Lewis (2000) FDA (2011)
11.	Duskytail grouper	*Epinephelus bleekeri (Vaillant, 1878)*	Wong et al. (2014) FDA (2011)
12.	Orange-spotted grouper	*Epinephelus coioides (Hamilton, 1822)*	Lehane & Lewis (2000) Wong et al. (2005) FDA (2011)
13.	Spinycheek grouper	*Epinephelus diacanthus (Valenciennes, 1828)*	Gillespie et al. (1986) FDA (2011)

14.	Honeycomb grouper	*Epinephelus merra (Bloch, 1793)*	Lehane and Lewis (2000) FDA (2011)
15.	Small scaled grouper	*Epinephelus polylepis (Randall & Heemstra, 1991)*	Lewis (2000) FDA (2011)
	Croakers		
16.	Bronze croaker	*Otolithoides biauritus (Cantor, 1849)*	Oshiro et al. (2010) FDA (2011)
	Carangids		
17.	Giant Trevally	*Caranx ignobilis (Forsskål, 1775)*	Oshiro et al (2010) FDA (2011)
	Barracuda		
18.	Sawtooth barracuda	*Sphyraena putnamae (Jordan & Seale, 1905)*	DeFouw et al. (2001) Stewart et al. (2010) FDA (2011)
19.	Pick handle barracuda	*Sphyraena jello(Cuvier, 1829)*	FDA (2011)
	Emperor		
20.	Spangled emperor	*Lethrinus nebulosus (Forsskål, 1775)*	FDA (2011)

Table 3.4 Details of fish specimens screened for investigation of CFP

Sl No	Species	No. of sample	Average weight (g)	Average Length (cm)	Area of collection
1.	*L. argentimaculatus*	16	1204.89±132.64	42.34 ±8.48	GU, KL, TN
2.	*L.fulvus*	8	973.83±89.83	39.56±6.85	GU, TN
3.	*L.bohar*	27	6072.22±187.2	51.65±5.10	KL, TN, KR
4.	*L. gibbus*	18	775.00±130.54	36.93±4.03	KL,TN, KR
5.	*L. johnii*	6	3052.14±158.34	61.65±6.87	KL, KR
6.	*P. filamentosus*	13	493.94±125.98	32.12±5.21	KL, TN, KR
7.	*P. pinjalo*	8	1332.37±143.76	43.69±5.75	GU,KL,TN
8.	*A.virescens*	12	1294.08±139.58	36.21 ± 4.25	KL, TN, KR
9.	*V.louti*	9	2275.80±245.56	51.56±5.50	GU,KL,TN
10.	*E. chlorostigma*	11	1263.73±184.32	42.97±471	GU, KR, TN
11.	*E.bleekeri*	12	1168.53±234.29	41.94±3.64	GU, KL, TN
12.	*E. coioides*	14	2229.12±382.30	51.23±4.64	GU, TN
13.	*E.diacanthus*	17	1192.99±164.85	42.21±5.30	GU, MA
14.	*E.polylepis*	13	1470.99±139.34	45.04±2.91	GU, KR
15.	*E. merra*	8	554.41±60.72	33.29±5.91	GU, TN
16.	*O.biauritus*	12	2738.06±392.43	54.60±6.62	GU, MA
17.	*C.ignobilis*	18	406±42.60	58.77±4.80	KL, GU, MA
18.	*S. putnamae*	15	712.27 ± 84.9	53.4±5.58	KL, TN, GU
19.	*S. jello*	11	4906 ± 3.56	52.5±3.55	KL, TN
20.	*L. nebulosus*	14	2658±145.45	59.73±5.78	GU, MA

KL- Kerala, TN- Tamil Nadu, KR- Karnataka, GU- Gujarat, MA- Maharashtra

Lutjanus argentimaculatus

Lutjanus fulvus

Lutjanus bohar

Lutjanus gibbus

Lutjanus johnii

Pristipomoides filamentosus

Pinjalo pinjalo

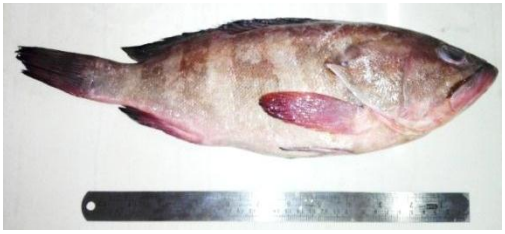
Aprion virescens

Variola louti

Epinephelus chlorostigma

Epinephelus bleekeri

Epinephelus coioides



Epinephelus diacanthus

Epinephelus merra

Epinephelus polylepis

Otolithoides biauritus

Caranx ignobilis

Sphyraena putnamae

Lethrinus nebulosus

Sphyraena jello

Fig. 3.2 Images of Fish Specimens used for toxicological screening of CFP

3.3.2 Species authentication

True identification of species is pre-requisite for any toxicological study. Fish identification is traditionally based on morphological characteristics. Few samples collected for the analysis contains mostly the caudal peduncle part of the fish, head portions, filleted fishes; hence absence of morphological characteristics and identification marks made it difficult to identify the fish using taxonomic keys. These samples were subjected to DNA barcoding. Sequenced amplified products deposited in Genbank with accession numbers and key identification reference are given in Table 3.5. In fisheries, DNA barcoding has found application in determining the taxonomic identity of unknown fish species, damaged fishes and mislabeled fishery products (Maralit et al., 2013). The genes commonly recommended in DNA barcoding are the cytochrome C oxidase 1 (COI-1) and the 16S rRNA gene (Zheng et al., 2014). A Blast analysis of COI sequence showed identity of fish species (Figure 3.3, 3.4 & 3.5). Phylogenetic tree based on COI amplification of gene sequences together with respective sequences of other known species showed it to be similar to the identified species and thus confirmed the identity of unknown fish samples in this study. Accession numbers not allotted for *L. gibbus, L. johnii, P. pinjalo, E. chlorostigma, E. merra* and *S. jello.* These samples were identified using morphological methods and when suspected fish samples among this were subjected to nucleotide sequencing the similarity showed to other species in the same genus.

Table 3.5: Species Authentication using DNA barcoding with accession numbers and Key Identification method

Sl No	Species	Accession Number/Key Identification Ref.
1.	*L. argentimaculatus*	MF383183 MF383184
2.	*L.fulvus*	MF383186
3.	*L.bohar*	MF383185 KY057337
4.	*L. gibbus*	Smith & Heemstra (1986)
5.	*L. johnii*	Smith & Heemstra (1986)
6.	*P. filamentosus*	MF383189 MF383190
7.	*P. pinjalo*	Smith & Heemstra (1986)
8.	*A.virescens*	MF383168 MF383169
9.	*V.louti*	MF383196
10.	*E. chlorostigma*	Smith & Heemstra (1986)
11.	*E.bleekeri*	MF383177
12.	*E. coioides*	MF383175 MF383176
13.	*E.diacanthus*	MF383172 MF383173 MF383174
14.	*E.polylepis*	MF383178 MF383179
15.	*E. merra*	Smith & Heemstra (1986)
16.	*O.biauritus*	MF383187 MF383188
17.	*C.ignobilis*	MF383170 MF383171
18.	*S. putnamae*	MF383191 MF383192 MF383193 MF383194 MF383195
19.	*S.jello*	Smith & Heemstra (1986)
20.	*L. nebulosus*	MF383180 MF383181 MF383182

L. argentimaculatus is a reef dwelling subtropical fish distributed along the Indo West Pacific and Eastern Mediterranean region with a maximum published weight of 8.7kg (Allen, 1985) and ciguatoxicity occurrence reported by Lewis, (1986); Dalzell, (1991); Leung et al. (1992); Wong et al. (2014) and Chan (2014). *L. fulvus* is a reef associated tropical fish (Allen, 1985) and Halstead et al. (1990) reported the fish as ciguatoxic. *L. bohar* is a reef-associated tropical fish distributed along the Indo-Pacific region with earlier reports of ciguatera poisoning (Halstead et al., 1990) and large fishes from oceanic areas in the western Pacific are often ciguatoxic (Dalzell, 1991). According to Oshiro et al. (2010), *L. bohar* weighing less than 4 kg are found to be non-toxic and 11.9% of the species exhibit CTX toxicity. Dalzell, (1991); Lieske & Myers, (1994); FDA, (2011) and Wu et al. (2011) reported *L. gibbus* coming under the group of snappers as ciguatoxic from different regions. *L. johni* and *P. pinjalo* are tropical fishes coming under the family of lutjanidae and FDA, 2011 listed the fishes coming under the family of groupers are susceptible to ciguatoxic accumulation. *P. filamentosus* is a subtropical fish with ciguatoxicity reported by Olsen et al. (1984). *A. virescens* is a tropical fish and Myers, (1999) reported that large individuals of this species may be ciguatoxic. Groupers like *V. louti* and *Epinephelus* sp. associated with coral ecosystem were reported to be ciguatoxic from different regions and croakers, carangids, barracudas, emperors etc. also were

mentioned as ciguatoxic fish species (FDA, 2011). From Indian Ocean region, CTX was characterized initially from *L. bohar* and *L. sebae* fish samples using HPLC-MS (Hamilton et al., 2002b). This region specific biotoxin has been reported very recently from Mangalore Coast along Indian EEZ by Rajeish et al. (2016). Hence it is estimated that there is a need for regular monitoring and testing of the reef fish samples from Indian Coast. An investigation based on clinical and epidemiological data of CFP and routine laboratory analysis of fish samples before being export or import to local and foreign markets will be taken as an initial precautionary approach in the area of CTX research. The main focus of this research was to establish a good surveillance system and analytical methods for the identification of ciguatoxicity.

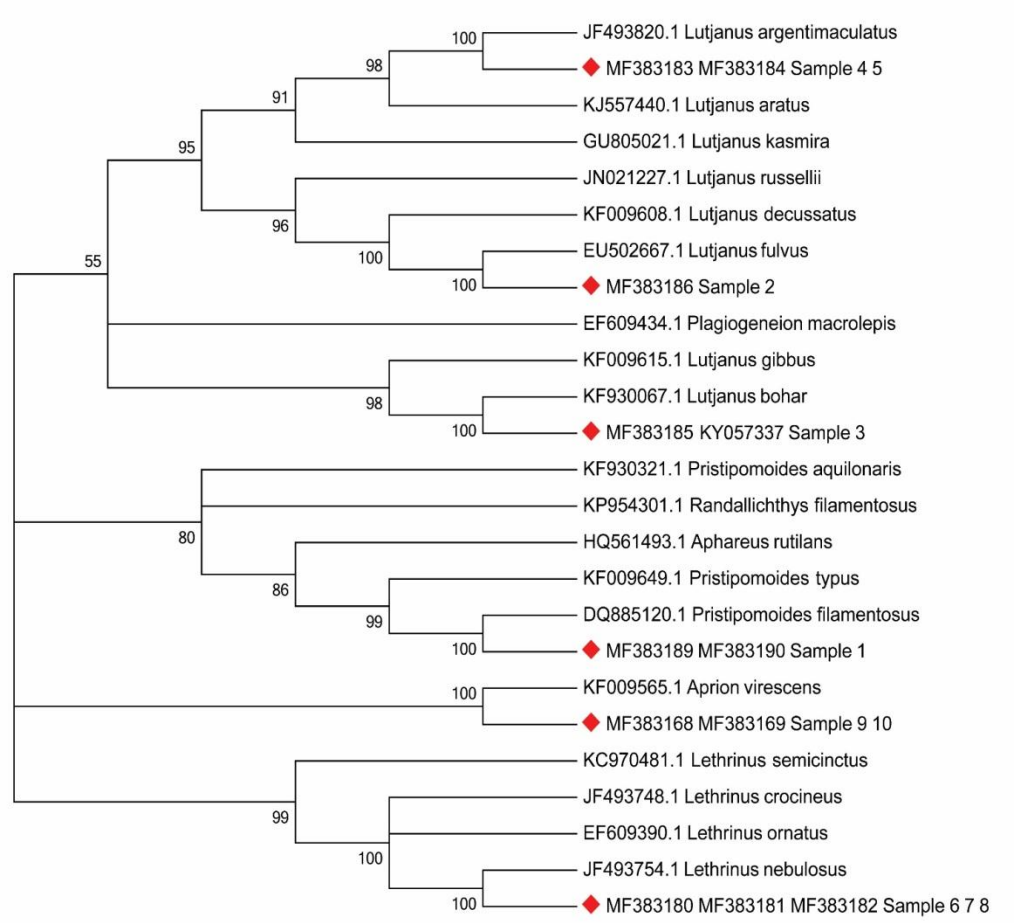


Fig. 3.3. Phylogenetic trees of DNA Barcode generated samples based on partial COI gene sequences (99 - 100% similarity of sequences), *L. argentimaculatus, L. fulvus, L. bohar, P. filamentosus, A. virescens* species based on 641bp and *Lehrinus nebulosus based on* 651bp.

◆ Refers to COI gene sequence from this study.

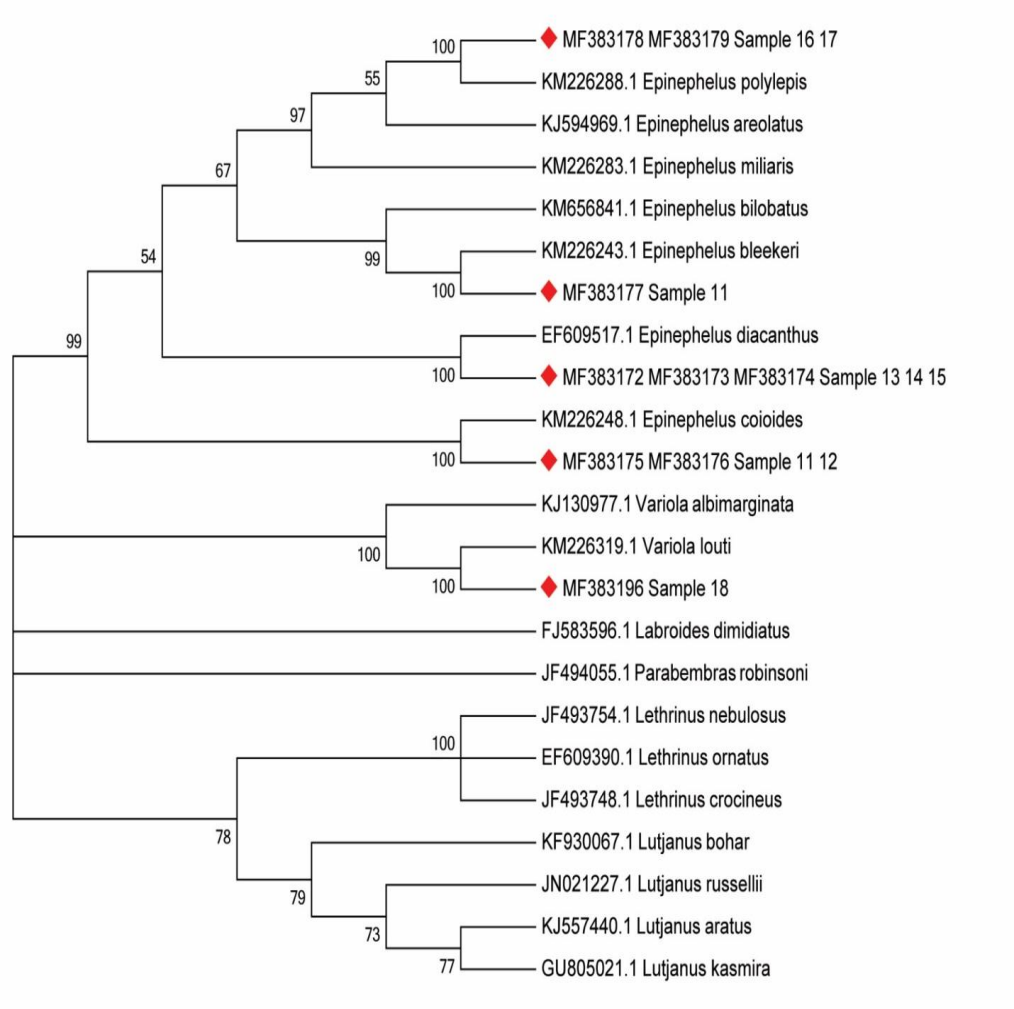


Fig. 3.4. Phylogenetic trees of DNA Barcode generated samples based on partial COI gene sequences (99 - 100% similarity of sequences), *E.polylepis, E. bleekeri* species based on 641bp*, E. diacanthus* based on 651bp and *E. coioides, V. louti* based on 641bp.

◆ Refers to COI gene sequence from this study.

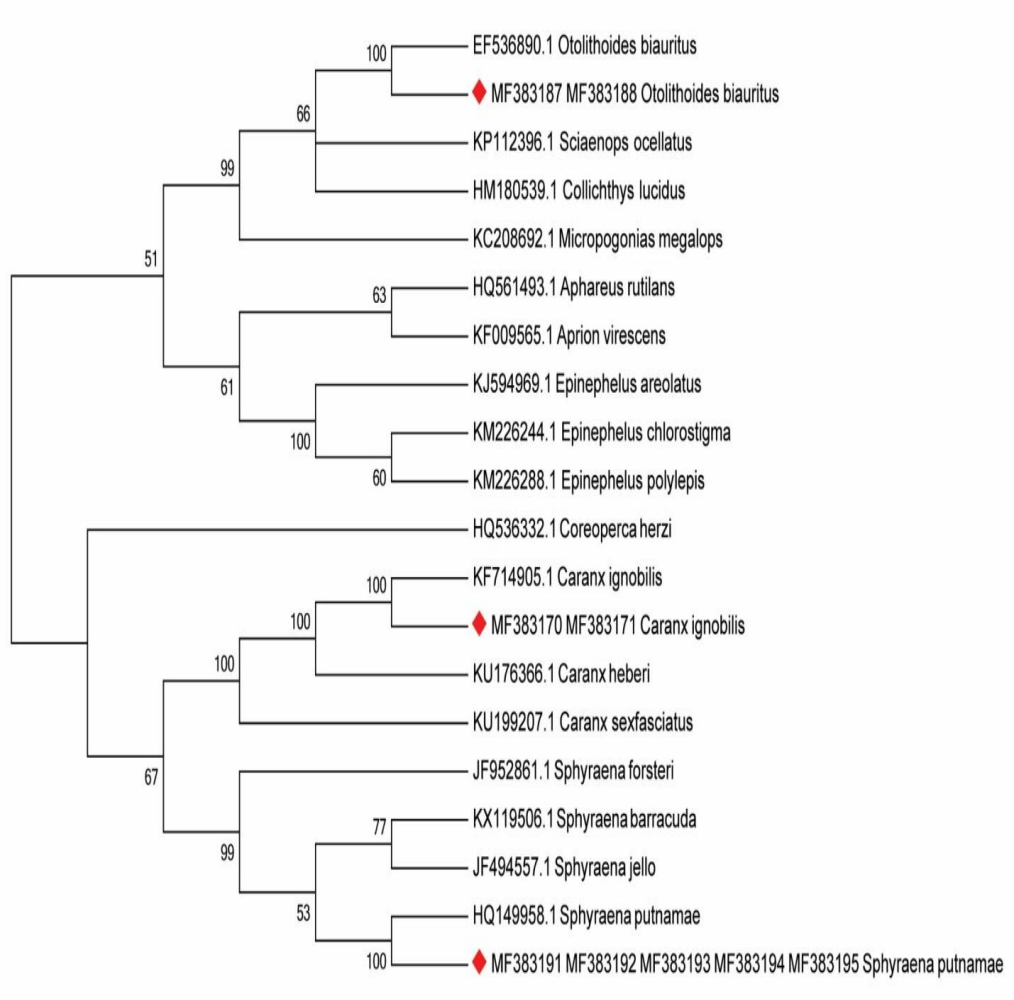


Fig. 3.5. Phylogenetic trees of DNA Barcode generated samples based on partial COI gene sequences (99 - 100% similarity of sequences). *O. biauritus, C. ignobilis* species based on 641bp and *S. putnamae* based on 651bp.

◆ Refers to COI gene sequence from this study.

3.4 Conclusion

A total of 262 samples were collected and investigated for screening of ciguatoxicity along Indian Coast. CTX related toxicity records from the

selected sampling sites are very rare. Rajeish et al. (2016) reported an incidence of Ciguatoxin related human intoxication in June 2015 from Mangalore region, in which the samples were purchased from a local fish market in Mangalore and two persons from a family were hospitalized. This is the only report on CFP syndrome from Indian Coast. Species identification through DNA barcoding helps to identify the samples collected in degutted, filleted forms and fish body parts (head, tail, viscera, etc.). Fish species of maximum weight has taken for screening of toxin because of fish higher in the food web tend to contain the highest CTX concentrations (Dickey & Plakas 2010; Oshiro et al., 2010; FDA, 2011). Data based on previous reports of ciguatoxicity showed that among the collected specimens *L. bohar* reported to be the most common fish implicated in ciguatoxicity (Froese & Pauly (2017); Hamilton et al., 2002b; Oshiro et al., 2010; FDA, 2011; Wong et al., 2014; Rajeish et al., 2016). DNA barcoding helps to confirm the species level identification of unknown samples among the 20 different finfish species. 30 numbers of fish samples were sequenced and deposited in GenBank NCBI with accession numbers. Phylogenetic tree of species based on COI gene showed its similarity to other respective sequences.

Chapter 4

Toxicological Investigation of Reef Fishes along Indian Coast through an *In Vivo* Mouse Bioassay Method

Contents

4.1 General Introduction

Ciguatera fish poisoning, a foodborne illness associated with consumption of finfishes around the world, generates acute gastrointestinal, neurological and cardiovascular problems in humans, including vomiting, diarrhea and abdominal pain, severe localized itching, tingling of extremities and lips, and thermal dysesthesia, as well as other chronic symptoms (Lewis, 2001 & 2006). Incidence of Ciguatoxin (CTX) toxicity affects approximately 50,000 to 5,00,000 people per year (FDA, 2011; Meyer et al., 2015; Friedman et al., 2017). CTX is a colorless, odorless, heat stable, lipid soluble polyether compound and remains unaffected by freezing, drying or cooking process (Lewis, 2006; Abraham et al., 2012). CTXs are secondary metabolites with numerous congeners having different molecular structure and had been

reported from different geographical origins namely Pacific, Caribbean and Indian Ocean (Caillaud et al., 2010).

In vivo bioassays are semi quantitative and sensitive because ciguatoxin induce characteristic signs of toxicity, but cost and ethical issues are major shortcoming for animal experimental studies. The Mouse bioassay is presently the most widely used assay for detection of ciguatoxin in fish. A number of other animal assays have been reported for the detection such as chicken assay, mongoose and cat assay, brine shrimp assay, mosquito assay or dipteral larvae assay. Even though these bioassays are used in few laboratories, but only the mouse bioassay has been validated (Lewis, 1993; Ojeda et al., 2008). Fish specimens were screened using mouse bioassay method for ciguatoxicity and were followed as a practical test to monitor the level of toxicity. The test consists of intraperitoneally injecting the toxic extract into mice and observing the symptoms over 24hr, until death occurs. Mouse Bioassay method for ciguatoxin was first introduced by Banner et al. (1960), which can be considered as an initial attempt for discriminating ciguatoxic fish. As trace quantities of CTX congeners in extract and its structurally complex nature greatly troubled the development of reliable detection methods. An ideal universal analytical method for the routine monitoring of CFP was still lagging. Another important limitation for the study is the non-availability of

pure CTX standards, since ciguatoxin is quite distinctive in nature (Lewis, 2001) and it varies according to various geographical regions. Incidence of ichthyotoxicity along Indian coast delivers a need for good surveillance system and analytical confirmatory methods for the protection of consumers along with exporters. The widely recommended method for CTXs testing in coral fish samples is still based on the mouse bioassay of fish ether extracts (Hoffman et al. 1983; Vernoux & Lejeune, 1994; Lewis, 1995; Ting & Brown 2001; ANSES, 2016). Until establishment of a validated alternative analytical method for CTXs detection (Hungerford, 1993), the mouse bioassay will still play an important role in ciguatera research and public health protection. Continuous development and improvement of the existing methodologies for this toxicity bioassay are needed for enhancing detection and accurate determination of CTXs in the fish tissues.

G. toxicus responsible for CTXs also produced many bioactive compounds like water soluble maitotoxins (MTXs) (Holmes et al., 1990; Yasumoto et al., 1977), gambierols (Satake et al., 1993a) and gambieric acid (Nagai et al., 1992 & 1993). Yasumoto et al. (1993) isolated a toxic constituent Gambierol from the cultured cells of the ciguatera causative dinoflagellate, *Gambierdiscus toxicus*. Gambierol exhibits potent lethal neurotoxicity against mice (minimum lethal dose 50µg/Kg, ip), and the

symptoms resemble those caused by ciguatoxins, implying that gambierol is also involved in ciguatera toxicity. Symptoms caused in mice make gambierol and ciguatoxin to be similar compounds causative of ciguatera (Satake et al., 1993b). Lethal dose value reported for Ciguatoxin from different regions including Pacific, Caribean and Indian Ocean has been given in Table 4.1.

Table 4.1: LD_{50} dose reported for Ciguatoxin

Toxin	LD_{50} in Mice (µg/kg)
P-CTX-1	0.25
P-CTX-2	2.3
P-CTX-3	0.9
C-CTX-1	3.6
C-CTX-2	1.0
I-CTXs	5.0

(Source: Lewis et al., 1991; Caillaud et al., 2010)

Ito et al. (2003) studied pathological effects on experimental mice after administration of Gambierol at 60-150µg/kg and Terao et al. (1991) tested pathological changes induced in mice receiving 0.7µg/kg of ciguatoxin. After ip administration, the organs affected by gambierol were liver, lung and secondary heart and in case of ciguatoxin, the histopathological changes were observed in liver, heart, medulla of adrenal glands and autonomic nerves. Hence histopathological analysis was done to establish exposure of toxicity in the organ systems in mice. As mammalian toxicology is almost related to humans, these changes in organ systems can be also attributed to human body systems also. The details of toxic effects in human body and the symptoms

observed during the toxicity can be correlated with the mice symptoms and it can prove the relevance of CTX exposure.

In case of humans, symptoms of ciguatera toxicity observed during intoxication are used to diagnose and differentiate CFP from other seafood borne toxicity. Confirmation of CFP cases/outbreak of ciguatera toxicity depends on the detection of ciguatoxin from the remaining meal remnants and plasma collected from affected patients (Dechraoui et al., 2007). Hence complete clinical data and monitoring is necessary for the analysis of CFP cases from the medical professionals and it will help the researchers to find out and develop a reliable laboratory analytical method for CTX determination. CFP cases are underreported because sometimes it gets escaped from the observations of medical professionals, which cause difficulty to assess accurately the epidemiology CFP cases (Ting et al., 1998). About 400 million people live globally in areas where CFP is present (Caillaud et al., 2010). CFP is rarely fatal with fatalities estimated to be <0.1%; however, fatalities may be higher in the Indian Ocean (Lewis, 2000). Clinical manifestations appear 2–30 hr after consumption of ciguateric fish. The symptomatology of CFP is quite complex; there are many toxicological manifestations at different levels. The symptoms of CFP involve general (e.g., weakness, joint pains, back stiffness, myalgia, headache, chills, faintness, dizziness, oliguria and itching), digestive

(e.g., nausea, vomiting, diarrhea, abdominal pain, cramps and dehydration), cardiovascular (e.g., low arterial pressure, irregular heartbeat and bradycardia) and neurological (e.g., dysesthesia, temperature reversal, paresthesia, superficial hyperesthesia, mydriasis and absence of the patellar and achillean reflex) pathologies (Randall, 1958; Bagnis et al., 1979; Hokama, 1988). Some neurological symptoms are characteristic of CFP, such as dysesthesia (reversal of cold and hot sensation/hypersensitivity to cold) and paresthesia (lack of sensitivity in the extremities). CFP neurological effects can also last for months, and occasionally, years (Ting et al., 1998; Caillaud et al., 2010)

Presently, there is no generalized screening procedure for identifying ciguateric fish. Risk management approaches taken for avoiding ciguateric fish at the distributor or individual level and marketing/industry or government level will minimize the lethal effects of CFP (Lewis, 2001). Risk assessment of the marine biotoxin was performed by the recognized International bodies, such as Joint FAO/WHO committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) (Caillaud et al., 2010). These organizations put some restrictions on the sale of fish of certain species or size from a given area.

The standard Mouse Bioassay (MBA) performed by intraperitoneal (ip) injection of the diethyl ether fraction obtained after liquid partition of fish

extracts is often used for the surveillance and determination of CTX-contaminated fish (Lewis, 1995 & 2003; Wong et al., 2005; ANSES, 2016). This chapter discusses about the method of screening of reef fish samples for the presence of ciguatera toxicity through Mouse bioassay. The investigation of toxicity also focuses on the outbreak/case analysis of ciguatoxic samples. Effects of CFP on humans were also investigated as part the study. Effects of toxicity in mice were continuously monitored and the symptoms of toxicity studies were also observed. The root of toxicity exposure in mice were understood by histological and blood serum sample analyses.

4.2 Materials and Methods

4.2.1 Sample collection

4.2.1.1 Reef Fish sample collection

A total of 262 reef associated finfish samples were collected from different sources across Kerala, Tamil Nadu, Karnataka Maharashtra and Gujarat, for the determination of ciguatoxicity using MBA. Details of sample collection are discussed in the previous chapter (Table 3.4). Screened samples for ciguatoxicity comprised of 20 different finfish species including snappers, groupers, barracudas, carangids, reef cod etc.

4.2.1.2 Human Intoxicated sample collection

During the study period, fish samples which caused human intoxication after consumption were traced out and taken for analysis at the laboratory. Toxic samples include fish remnants of caudal peduncle part of fish from Vizhinjam (Trivandrum, Kerala) and head, tail and filleted portions of red snapper from Mangalore (Karnataka) during January and September 2016 respectively. The analytical data of these samples based on mouse toxicity and clinical manifestations are depicted as two case studies. These samples were taken as a positive control for the validation of Mouse bioassay method.

All the Ciguatoxin related cases were monitored from January 2015 to December 2017 for three years. Clinical manifestations in affected Individuals were investigated with the help of medical professionals. The particulars of gastro intestinal, neurological, cardiovascular and other symptoms were documented on the basis of a Questionnaire (Annexure-I) prepared for the data collection and fish samples were collected as per availability.

4.2.2 Mouse bioassay analysis

4.2.2.1 Extraction of toxin

Extraction of fish samples was carried out as per IOC manuals and Guides No. 33 (Lewis, 1995) and European Union Reference method (ANSES, 2016) for Mouse Bioassay of Ciguatoxin. A flow diagram describing

the steps involved in the extraction of ciguatoxin ready for Mouse bioassay is shown in Figure 4.1. Fifty gram of fish sample was cooked at 70^0C for 15 min, and cooled to room temperature. The cooking step denatures the proteins which interfere with acetone during the homogenization step will reduce extraction efficiency. Tissue samples were then minced, diluted with acetone [3:1 V: W (ml g-1)] and homogenized for 5-15 min using a homogenizer (PRO Scientific Inc., USA) under iced condition. The homogenized samples were filtered using Whatman no.1 filter paper and the filtrate collected in a round bottom flask. Residual acetone and water were removed using a rotary evaporator (Heidolph, Germany) operated at 55^0C. The dried extract was transferred to a separatory funnel, added methanol:water (9:1), shaken well followed by extraction with 1:1 (v/v) n hexane and the upper hexane layer was discarded. This extraction process repeated twice. The residual methanol:water was removed using vacuum evaporator. Further, ethanol:water (1:3) was added and shaken with diethyl ether (1:1) to separate the layers and the ether layer was collected. Ether extraction was repeated twice and ether fractions were pooled at an elevated temperature of 40-55^0C. Collected dried ether extract were assumed to contain the CTXs. With this procedure, approximately 63% of ciguatoxin is recovered from fish flesh using routine methods (Lewis & Sellin, 1993). The ether soluble material contains toxic fractions and is ready for testing with the Mouse bioassay.

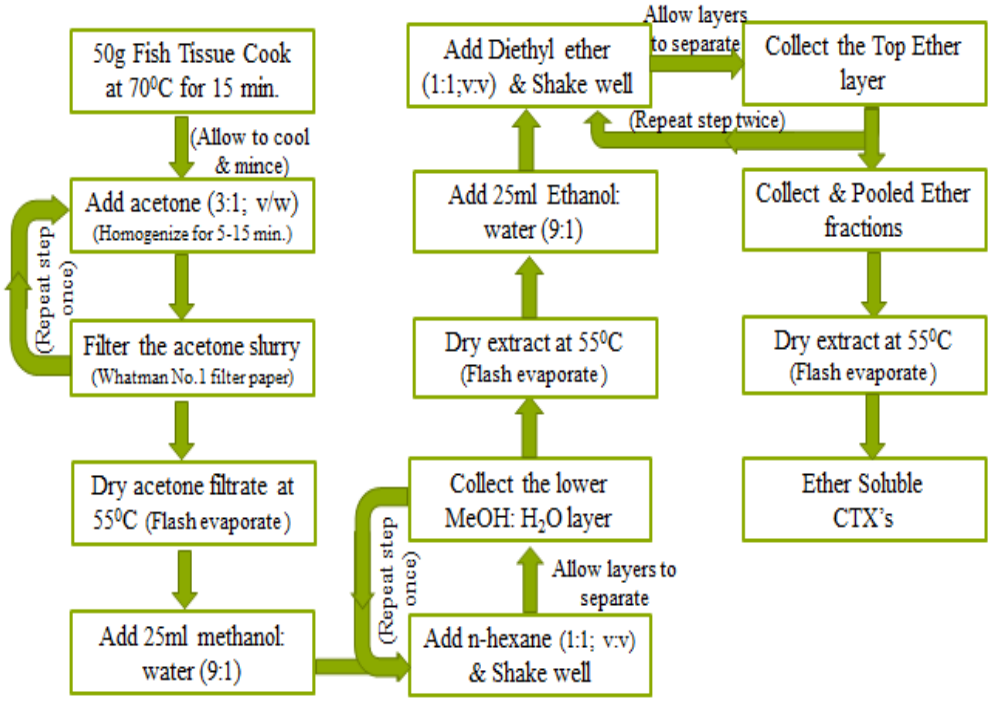


Fig.4.1: Flow diagram showing extraction and partial purification of the CTXs from fish tissue (IOC Manuals and EU Guidelines) (Lewis, 1995; ANSES, 2016)

4.2.2.2 Preparation of ether soluble extract for injection

Ether fractions collected and dried under nitrogen or using flash evaporator. Dried ether extract was dissolved in Chloroform:Methanol (97:3) mixture and dried under N_2 . The dried fraction was suspended in 1-5% Tween 60 in 0.9% saline, sonicated for 180 seconds and filtered through 0.45 PTFE membrane filter prior to ip injection. Figure 4.2 illustrates the preparation of ether soluble extract.

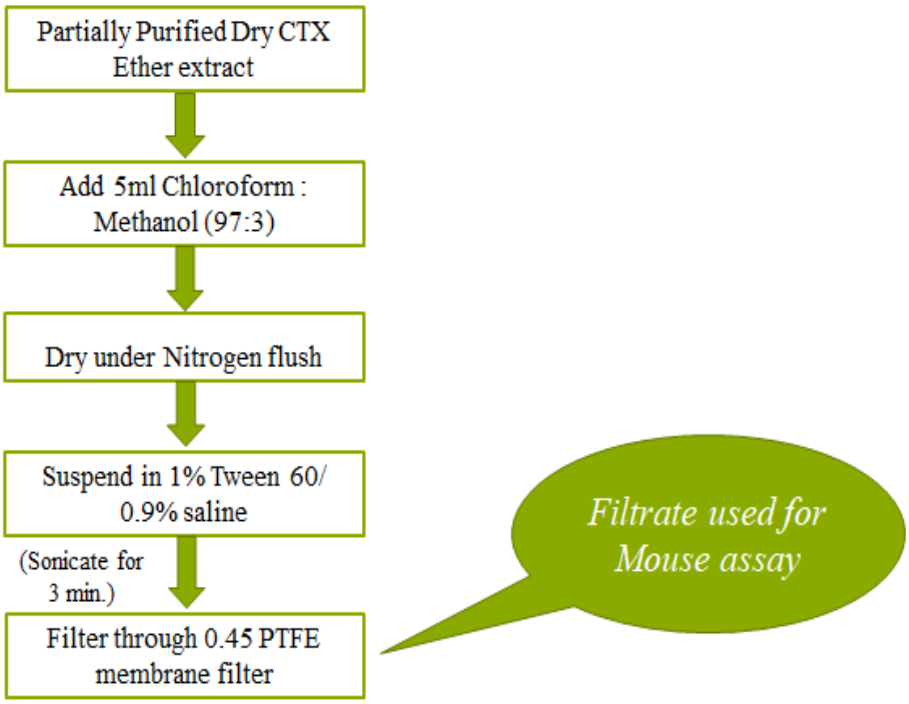


Fig. 4.2: Flow diagram showing preparation of ether soluble extract for injection

4.2.2.3 Animal study - Experimental Design

Animal experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC). Female albino mice weighing 20±2 g were assay in duplicate by intra peritoneal injection with 0.5ml of the prepared extract, whereas control mice were injected with only 0.5ml Tween 60 in 0.9% Saline. Time of injection, weight of mice, amount of extract (g) administered, time of onset and nature of signs and time of death was recorded for each

injection. The post-injection behaviour was observed and recorded for at least 24 hr (Check list of symptoms given in Annexure-II). Weight loss in injected mice was also recorded up to 24 hours at an interval of minimum 3 hours.

4.2.2.4 Studies on Dose v/s Death Time relationship

The relationship between dose and time to death is used to quantify each fraction. For the mix of ciguatoxin typically found in carnivorous fish (Lewis & Sellin, 1992) this relationship is approximated by:

$$\log MU = 2.3 \log (1 + T^{-1})$$

where, MU = number of mouse units of ciguatoxin injected and T = time to death in h (Lewis et al., 1992). One MU is the LD_{50} dose for a 20 g mouse which is equivalent to 5 ng CTX-1. For routine assay of ciguatoxin, a dose v/s time to death relationship should be established for each colony of mice and preferably for each species of fish to be assayed.

4.2.3 Histological and biochemical examination

After the onset of symptoms, animals were anesthetised and blood was collected by cardiac puncture. Serum was prepared by micro centrifuging blood at 6000 rpm for 5 min, and supernatants were transferred to an eppendorf and stored at -70ºC.

4.2.3.1 Histological examination

After blood serum collection, liver, kidney, heart, brain and intestine were removed for histopathological analysis. All the organs were immediately fixed in 10% buffered formalin and processed for histology with H&E staining.

4.2.3.2 Estimation of Total serum protein

Measurement of total serum protein is a useful test in a variety of disorders. It is calculated using photometric test according to biuret method as per the instructions given by the DiaSys Diagnostic quantitative test kit for in vitro determination of total protein in serum or plasma on photometric systems (Thomas, 1998; Johnson et al., 1999; Guder & Zawta, 2001).

4.2.3.3 Estimation of Albumin

Albumin is an important binding and transport protein for various substances in plasma. The method followed is photometric test using Bromocresol green as per the DiaSys Diagnostic quantitative test kit for in vitro determination of total protein in serum or plasma on photometric systems (Thomas, 1998; Johnson et al., 1999; Guder & Zawta, 2001).

4.2.3.4 Estimation Albumin/Globulin ratio

The albumin to globulin (A/G) ratio has been used as an index of disease state; however, it is not a specific marker for disease because it does

not indicate which specific proteins are altered. The normal A/G ratio is 0.9-2.0. The A/G ratio can be decreased in response to a low albumin or to elevated globulins.

It is calculated as the amount of albumin proteins that is found in the blood compared to the amount of other proteins. The A/G ratio should be above one. A value less than one is clinically significant.

$$\text{A/G Ratio} = \frac{\text{Albumin Level}}{\text{(Total protein - Albumin Level)}}$$

4.2.3.5 Estimation of Aspartate amino transferase (AST) and Alanine amino transferase (ALT)

AST and ALT were estimated by optimized UV test according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine, Modified) as per the instructions given by DiaSys Diagnostic Test Kit (Thomas, 1998; Guder & Zawta, 2001; Schumann et al., 2002).

4.2.3.6 Statistical analysis

The data were analyzed using one way ANOVA and the significant differences between the means were determined using Tukey multiple range comparison tests. The level of significance was set at $P<0.05$. All the analyses were performed using statistical software package IBM SPSS version 20.

4.3 Results and Discussion

During the study period from January 2015 to December 2017, there were 262 coral fish samples collected from different sources along Indian Coast. The fish specimens screened for toxicity comprised of *Lutjanus argentimaculatus, L. fulvus, L. bohar and L. gibbus, L. johnii, Pristipomoides filamentosus, Pinjal pinjalo, Aprion virescens, Variola louti, Epinephelus bleekeri, E. coioides, E. diacanthus, E. chlorostigma, E. polylepis, E. merra, Otolithoides biauritus, Caranx ignobilis, Sphyraena putnamae, S. jello* and *Lethrinus nebulosus.* The samples were collected in the form of whole fish, filleted, descaled, degutted ones, head and caudal part of large sized fishes etc.

In 2016, two incidents of human associated CFP syndromes were documented during the research period. Based on laboratory analysis the major fish species implicated in CFP along Indian Coast is *L. bohar*, locally known as "*Chempalli*". *L. bohar* is a coral reef inhabitant, being found at depths from 4 to 180 m, native to the Indian Ocean, but is widespread in the Indo-Pacific from the east African coast to the western Pacific Ocean, north to the Ryukyu Islands, and south to Australia (www.fishbase.org). Toxicity tests using mouse bioassay have reported that, 11.9% of *L. bohar* fishes are ciguatera toxic with individuals weighing less than 4 kg to be non-toxic (Oshiro et al., 2010).

4.3.1 Clinical diagnosis of intoxication

4.3.1.1 Case study 1: Vizhinjam (Trivandrum, Kerala)

Six individuals (one male and five females) were hospitalized after consuming fish dish (*Chempalli* curry). The fishes were purchased from a local market in Vizhinjam. The symptomatology in the hospitalised patients corroborated with earlier reports of ciguatera fish poisoning (Rajeish et al., 2016) with typical clinical signs like gastrointestinal, neurological and cardiovascular symptoms. All six patients (one male and five females) were admitted with CFP symptoms of vomiting, diarrhoea, paraesthesia of upper limbs and lower limbs. Out of this, five patients belonged to one family lived near Chakkipara Market, Trivandrum. Their symptoms started six hours after consumption of fish dish (*Chempalli* curry). Symptoms like vomiting, diarrhoea, circumoral paraesthesia and paraesthesia of limbs were common to all family members. One of the three daughters also had paradoxical temperature reversal (cold objects sensed as hot and hot objects sensed as cold). All the patients were haemo-dynamically stable except the husband who had sinus bradycardia (low heart rate). The sixth patient was a female and her major symptom was giddiness. She also had abdominal pain and paraesthesia of limbs. In the affected individuals, the onset of ciguatera toxicity started within 24 hr of consumption of fish curry and symptoms lasted for 1-4 days.

However, in case of one individual, it persisted for six months as reported previously by Glaziou & Martin (1993) and Pearn (1995). All the above said patients were treated by giving supportive measures like intravenous fluids and antiemetics. The husband's bradycardia improved and normal heart rate was restored after 2 days. Patients were in better condition at the time of discharge and the only symptom that persisted was paraesthesia of limbs.

Species authentication of collected caudal peduncle part of fish samples (Figure 4.3) on partial sequencing yielded an average length of 627 bp for COI gene (Accn. No. KY057337). The causative fish species identified as *Lutjanus bohar* is also known as two spot red snapper.

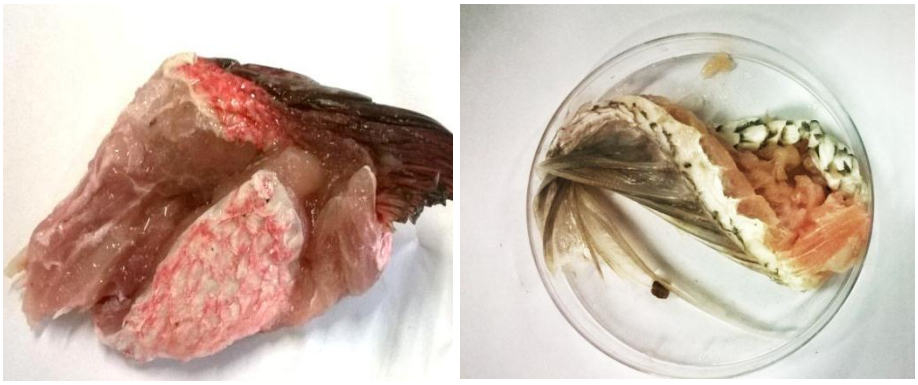


Fig. 4.3: Caudal part of fish sample collected from Vizhinjam

4.3.1.2 Case study 2: Ullal (Mangalore, Karnataka)

93 individuals from Ullal (Mangalore) were intoxicated after consuming head portions of red snapper (fish weight unknown) and

hospitalized. The fishes were purchased from an exporting company near Ullal and the effected individuals are the nearby residents of the same area. Intoxicated patients include 32 male and 61 females, at an age category of 18-56. The symptoms started after consuming the fish 2 hr to 3 days. 90% of the affected individuals recovered within one to three days and 5% recovered within 10 days. In 5% of the individuals, the symptoms persist for almost one month. No mortality were observed during the intoxication. The major symptoms observed during the toxicity include abdominal pain, leg pain, weakness and body pain.

Species authentication of the samples collected as head portions, caudal part and filleted fish parts on partial sequencing yielded an average length of 641 bp for COI gene (Accn. No. MF383185) and identified as *Lutjanus bohar*.

Clinical diagnoses of CFP are reliable when a detailed and comprehensive history of the food source, onset of the illness and description of symptoms are accounted (Stewart et al., 2010). Table 4.2 shows the comparison of clinical symptoms observed during the intoxication and frequency of symptoms.

Table 4.2: Comparison of Clinical symptoms observed during ciguatera intoxication

		Outbreak 1: Vizhinjam, Trivandrum		Outbreak 2: Ullal, Mangalore	
	Number of individuals affected	**6**		**93**	
1	Male	**1**		**32**	
2	Female	**2**		**61**	
3	Children	**3**		**0**	
4	Area	**Vizhinjam, Kerala**		**Ullal, Mangalore, Karnataka**	
5	Age	**-**		**18 – 56**	
6	Date and time	**15 Jan 2016**		**30 Sept 2016**	
I	**Gastrointestinal Symptoms**	**Report (Present/Absent)**	**Frequency of Symptoms**	**Report (Present/Absent)**	**Frequency of Symptoms**
1	Diarrhea	Yes	10%	Yes	Below 5%
2	Vomiting	Yes	10-20%	Yes	10-20%
3	Nausea	Yes	10-20	Yes	10-20%
4	Abdominal pains	Yes	40-50%	Yes	40-50%
5	Weakness	Yes	40-50%	Yes	40-60%
6	Body pain	Yes	30-40%	Yes	40-60%
7	Joint pain	ND	-	Yes	40-60%
8	Knee pain	ND	-	Yes	40-60%
9	Leg pain	ND	-	Yes	40-60%
10	Chest burning	Yes	Below 5%	Yes	Below 5%
II	**Cardiovascular Symptoms**				
1	Bradycardia	Yes	50-70%	Yes	10-20%
2	Hypotension	Yes	20%	Yes	40-50%
3	Hypertension	Yes	20%	Yes	40-50%
4	Breathing problem	Yes	5-10%	Yes	5-10%
III	**Neurological Symptoms**				
A	**Peripheral Nervous System Symptoms**				
1	Paresthesia- Extremity	Yes	50-60%	Yes	NA
2	Circumoral Paresthesia	Yes	40-50%	Yes	NA
3	Temperature Dysesthesia	Yes	NA	ND	-
4	Dental pain/ feeling like teeth are loose or falling out	Yes	NA	ND	-
5	Myalgia	ND	-	ND	-
6	Arthralgia	ND	-	ND	-
7	Pruritis	Yes	NA	ND	-
8	Dysuria	ND	-	ND	-

B	**Central Nervous System Symptoms**				
1	Vertigo	Yes	40-60%	Yes	40-60%
2	Dizzy	Yes	40-60%	Yes	40-60%
3	Lipothymy	ND	-	ND	-
4	Loss of consciousness	ND	-	Yes	Below 5%
5	Cerebellar Syndrome	ND	-	ND	-
6	Balance Disturbance	Yes	30-40%	Yes	40-60%
7	Hallucinations				
8	Depression	Yes	40-60%	Yes	40-60%
9	Memory/Concentration	Yes	NA	ND	-
10	Behavioral disturbance	ND	-	ND	-
11	Visual Disturbance	Yes	NA	Yes	Below 5%
13	Giddiness	Yes	NA	Yes	40-60%
IV	**Other Symptoms:**				
1	Headache	Yes	40-60%	Yes	40-60%
2	Weakness	Yes	20-40%	Yes	40-60%
3	Fatigue	Yes	10-20%	Yes	40-60%
4	Respiratory Disturbance	Yes	5%	Yes	Below 5%
5	Chills/Sweating	Yes	10%	Yes	20-30%
	Duration ofsymptoms				
1	Time of Onset	**After consuming 6 hours**		**After consuming 2 hours to 3 days**	
2	Time taken for recovery	**Below 1 to 4 days (5 persons) 6 months (1 person)**		**Below 1 day to 3 days 90% 5% within 10 days 5% 15-30 days**	
3	Most prominent symptoms	**Bradycardia (Low heart rate) Giddiness Paresthesia of upper and lower limbs**		**Abdominal pain Leg pain Weakness Body pain**	
4	Age of most effected individual (Mention whether Male or Female)	**45 year male (one person)**		**20 years above major**	
5	Age category of Male Patients	**45 year (one male)**		**30 years above**	
6	Age category of Female Patients	**8 years above**		**18 years above**	
7	Death reports (If any)	**No**		**No**	

ND - Not Detected; NA- Not Available

Most prominent symptom in Vizhinjam intoxication is Bradycardia, which is considered as an evidence of severe ciguatera along with hypotension. Reports are there from Hawaii (Villareal et al., 2007) and South

Pacific (Bagnis et al., 1979) for the association of this symptom during intoxication. The other prominent symptoms are abdominal pain, leg pain, body pain, giddiness and paresthesia of upper and lower limbs from the outbreak investigations, which was similar to the previous reports on clinical diagnosis of ciguatera toxicity in humans (Caillaud et al., 2010; Farrell et al., 2016; Friedman et al., 2017).

4.3.2 Results of Mouse bioassay

The intra-peritoneal injection of fish extract into mice induced symptoms as indicated in case of CTX toxicity (Figure 4.4). A detailed description of signs of profile in mice up to a 24-hour period observation is given in Table 4.3. The evaluation of Mouse bioassay toxicity test of samples has interpreted as shown in Table 4.4. The prominent symptoms include pilo-erection, diarrhea, lachrymation, dyspnea, gasping, progressive hind limb paralysis, wobbly upright gait, terminal convulsions with tail arching, breathing difficulties, slow loco motor activity, hypothermia etc. However these symptoms were, absent in control and negative (non-toxic) fish samples. The relationship between dose and time to death is used to quantify toxicity of the extract which is ranged from 30 minutes to >10 hr.

Fig. 4.4: Mouse bioassay observation and ip injection of fish ether extract

4.3.2.1 Case study 1:

The lethal dose is estimated to be 3.25 MU/20mg of ether extract and the amount of CTX toxicity in fish sample is equivalent to 16.25ng of CTX -1, which is significantly higher than the reported levels of CTX intoxication in humans. Weight loss observed in the positive sample is calculated as 13%.

4.3.2.2 Case Study 2:

The lethal dose is estimated to be 2.17 MU/20mg of ether extract and the amount of CTX toxicity in fish sample is equivalent to 10.84ng of CTX toxicity and also 13% weight loss is observed in mice.

4.3.3 Toxicity in reef fish samples:

The red snapper species, *L.bohar* (8 fish samples) were collected from Kollam (8°56′19″N, 76°32′25″E), Thoppumpady (9°56'7"N, 76°15'33''E) and Mangalore (12°50′23″N, 74°47′24″E) along south west coast of India, confirmed as positive samples for ciguatoxicity using Mouse bioassay. The samples are weighed more than 4kg with an average weight of 6.13±1.17 kg. Symptoms observed as per the previous records are shown in Table 4.3. The lethal dose estimated to be 2.08 MU/20mg of ether extract and the amount of CTX toxicity in fish sample is equivalent to 10.4 ng of CTX toxicity and 10% weight loss is observed in intoxicated mice.

Table 4.3: Mouse Bioassay: Symptoms observed after administration of fish extract

Sign	Evaluation	Animal Responses	
		Control	Toxin treated
Hypothermia	Thermometer	35^0C - 38^0 C	Below 33^0C
Piloerection	Observation	None	Mild to marked
Lachrymation	Observation	Normal	Mild to severe
Hyper salivation	Observation	Absent	Mild to severe
Dyspnea	Observation	Absent	Mild to severe
Wobbly upright gait	Observation	Absent	Present
Gasping	Observation	Absent	Mild to marked
Withdrawal reflex	Grasp hind leg	Withdrawal	Reduced to absent
Mild gasping	Observation	Absent	Present
Diarrhea	Observation	Absent	Mild
Breathing difficulties	Observation	Absent	Rapid shallow to intermittent gasping
Loco motor activity	Observation	Normal	Slow to absent
Hind limb paralysis	Observation		Progressive paralysis from hind limb extending to fore limbs
Convulsions	Observation	Absent	Tonic and/or Jumping

Table 4.4: Interpretation of Mouse Bioassay

Test Sample	No. of dead mouse(s) in 24 hr	Weight loss >5% after 24 hr injection	Conclusion
Suspected Sample 1	1/2	Yes	Positive, Inedible
Suspected Sample 2	0/2	Yes	Positive, Limited edibility
Control	0/2	No	Negative, Edible

Source: EU-NRL, (ANSES, 2016)

The death of 1 or 2 mice within 24 hours is interpreted as a positive result and fish samples were accepted as not edible. In the absence of death, weight loss >5% after 24 hours of injection of toxin of at least one mouse is considered as a positive result. When no mortality or weight loss occurs, then the sample is edible without doubt. Yasumoto et al. (1984) suggested that any fish containing above 2.5 MU/100g should be avoided as food. Therefore the result of identified toxic specimens from the study showed an increased level of Mouse Unit for the fish extracts.

MBA (Mouse Bioassay) provides a measure of total toxicity based on the biological response of the animal to the toxins but there is no specific information is provided on individual toxins. Banner et al. (1960) introduced the MBA for CTXs and this is the most widely used mammalian *in vivo* model for toxicity screening of CTXs (Caillaud et al., 2010).

4.3.4 Dose v/s Time Death Relationship

Based on the 12 toxic samples analyzed, the toxicity was determined as 3.15, 2.17 and 2.08 MU/20mg of ether extract. LD_{50} observed for the toxic samples analyzed during the study were calculated as 14 µg/kg (Figure 4.5).

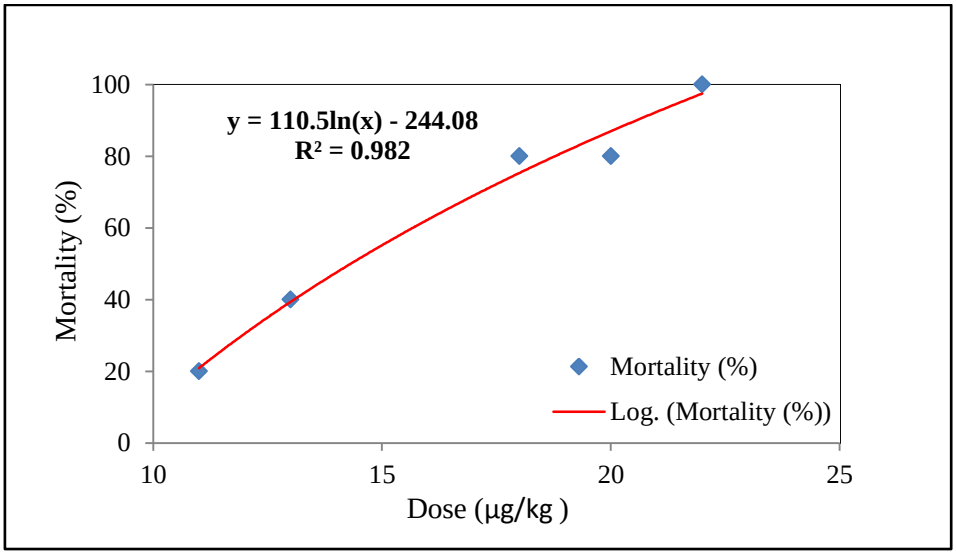


Fig. 4.5: Mouse Ciguatoxin Bioassay Dose-Response Curve

y = 110.5ln(x) - 244.0

For LD_{50} we have to find out the 'x' corresponding to the 'y =50'

50 = 110.5ln(x) - 244.0

294=110.5ln(x)

ln(x) =294/110.5

ln(x) =2.66

x=14.305

Finally LD_{50}= 14.305ug/kg

The MBA allows a reasonable and sensitive detection of CTXs, whenever signs of intoxication of mice are consistent with CTXs (Lehane & Lewis, 2000), but its utility is limited by the requirement of a dose-response curve (Hoffman et al., 1983; Lewis, 2003) with purified CTXs for accurate quantification (since the dose-response curve is not linear) (Lewis, 2003)

4.3.5 Changes in Histological and Biochemical parameters

4.3.5.1 Changes in blood serum parameters

In CTX positive samples, total protein and albumin values decreased dramatically within 2-3 hr of IP injection of extract. All mice's are weighed around 20± 2g. Albumin and Globulin are important blood serum proteins and 0could be utilized as a measure of response to injury, inflammation and stress in the animal (Zaias et al., 2009). The reference level for A:G ratio should be above 1. A value less than one is clinically significant. Here the albumin level in blood serum decreased significantly ($P<0.05$) due to toxicity. Hence it is assumed that, there is change in liver sections, since it is involved in the synthesis of albumin. Difference in A: G ratio is an indication of toxin affect in kidney. Changes in the blood serum parameters indicate that the CTX toxin affects the organ system and metabolism of affected mice. The data regarding the changes in different blood serum parameters are given in Table 4.5. In case of total protein, albumin and A: G ratio there is no significant difference

(P<0.05) between control mice and CTX negative ones. An analysis of variance showed significant reduction in total protein, albumin and A: G ratio in CTX positive samples compared with control and CTX negative samples. CTX positive samples showed a significant increase (P< 0.05) in AST and ALT when compared to control and negative samples.

Table 4.5: Changes in different blood serum parameters in toxin injected mice

Parameter	Control Mice	CTX positive	CTX negative
Total Protein	5.21 ± 0.02^{a}	3.45 ± 0.44^{b}	5.28 ± 0.49^{a}
Albumin	2.85 ± 0.19^{a}	1.51 ± 0.08^{b}	2.68 ± 0.10^{a}
A:G Ratio	1.41 ± 0.17^{a}	1.06 ± 0.21^{b}	1.44 ± 0.07^{a}
AST (SGOT) Serum	9.00 ± 0.96^{a}	226.00 ± 63.51^{b}	8.88 ± 0.99^{a}
ALT (SGPT) Serum	9.03 ± 1.32^{a}	380.50 ± 113.74^{b}	6.90 ± 0.57^{a}

Results of multiple comparison of means in biochemical parameters of Mice Blood Serum (Tukey Alpha, P<0.05)

4.3.5.2 Changes in Histopathological analysis

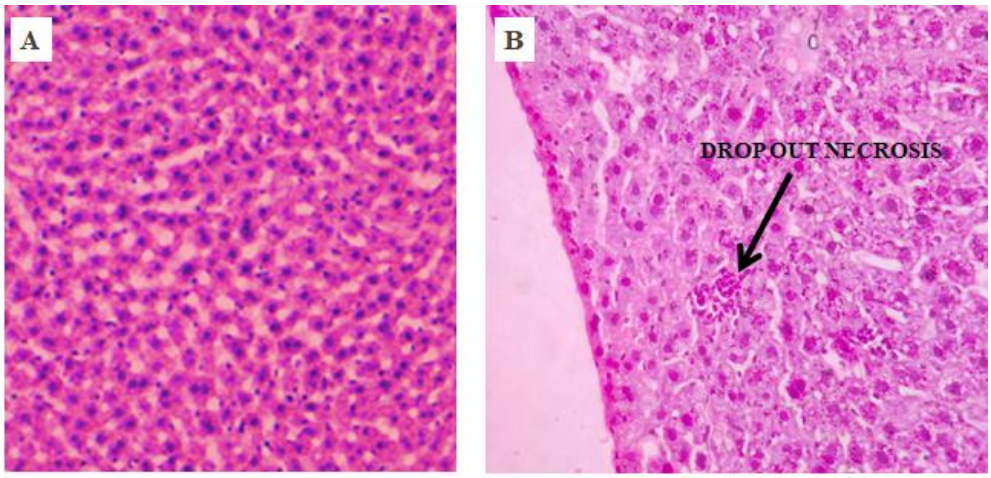
Mouse bioassay of toxic fish extract was found to induce toxic effects in mice when administered intraperitoneally. Histopathological analysis aimed to demonstrate that the ingestion of CTX extract can induce the toxicity symptoms or death in mammal and affect the histological aspects of vital organs. Microscopic examination of liver control sections show that normal liver parenchyma composed of congested central veins with hepatocytes radiating outwards from the central veins to the portal tract. The portal tract show hepatic artery, portal venules and bile ductules along with sparse

inflammatory infiltrate composed predominantly of lymphocytes along with plasma cells. Toxin effected liver section exhibit the major changes in the portal tract which show hepatic artery, portal venule and bile ductules along with dense perivascular inflammatory infiltrate and also focal areas of drop out necrosis identified (Figure 4.6). Microscopic examination of brain control sections show normal brain parenchyma. But the toxin effected brain sections show brain parenchyma with perivascular lymphocytic infiltrate in meninges (Figure 4.7).

Histopathological sections of heart from control and toxin treated has showed normal morphology. Sections from control and toxin treated kidney show normal renal parenchyma composed of cortex and medulla. Cortex shows mature glomerulli, and surrounding tubules of both proximal convoluted type and distal convoluted type. The cells of the tubules show normal morphology of cells. Medulla shows collecting tubules and ducts extending into renal pelvis. Interstitium of kidney is not expanded, and vessels show normal morphology.

Sections from control and toxin treated small intestine showed normal intestinal parenchyma with normal villi:crypt ratio and lymphocytic population noted in the villi core and in between epithelial cells (Figure 4.8). Acute and chronic mice intoxication symptoms and histopathological effects

of ciguatoxin and gambierol have been reported earlier (Terao et al., 1991; Ito et al., 2003).



A. Control Liver section showing nomal liver architecture and components of basic liver lobules. B. Ciguatoxin affected mice Liver section showing lobules with Necrosis (At 10X)

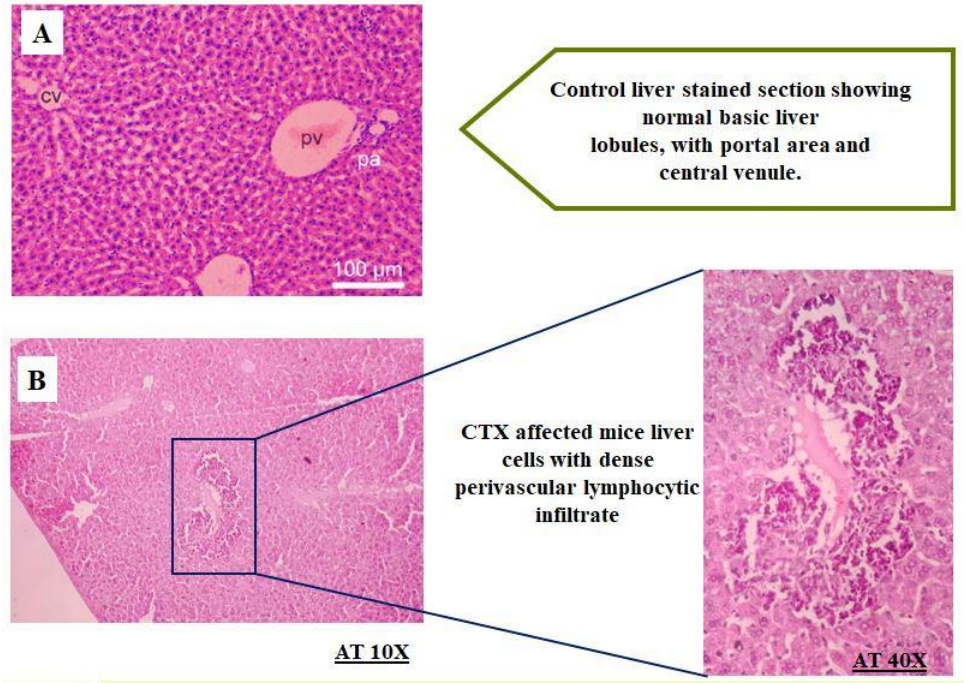


Fig. 4.6: Histopathological section- CTX effects in Liver

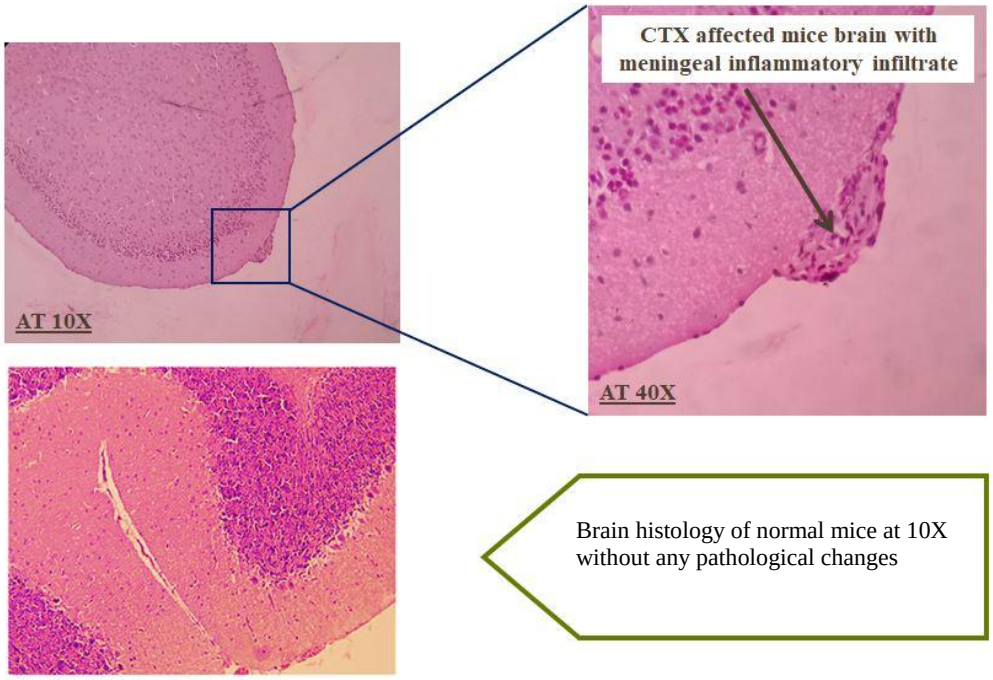


Fig. 4.7: Histopathological section – CTX effects in Brain section

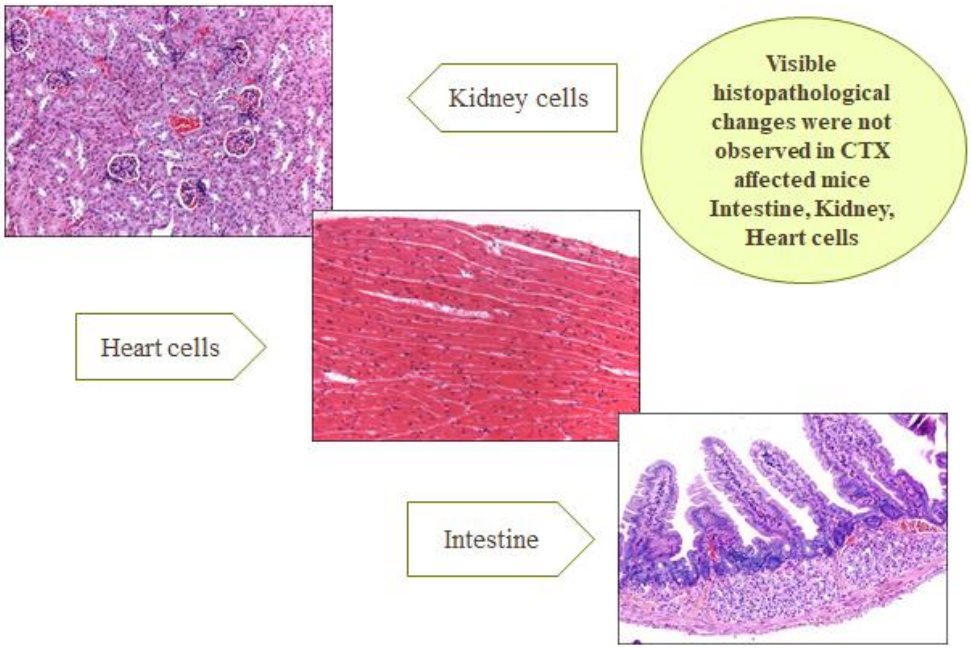


Fig. 4.8: Histopathological section – CTX effects in Kidney, Heart and Intestine (At 10X)

Along Indian coast, CFP incidents are rare. This study is the first report in incidence of ciguatoxin poisoning along Kerala coast and signifies the importance of seafood safety. In the absence of commercial testing, a precautionary approach is necessary for the surveillance of CTX intoxication along the Indian coast. MBA is considered as a toxicological tool accessible only to selected laboratories. Hence carried out mouse bioassay test to identify and quantify CTX toxins in order to provide further support for the clinical diagnosis of the CFP incident. Proficiency in the ability to identify the toxic fish and effective clinical recognition will definitely improve our understanding of the source of poisoning. The implementation of regulatory criteria for CTXs would be needed with respect to aspects like identification of ciguatoxic fish mainly reef associated fish, regulatory measures such as ban or size restrictions on high-risk species and misdiagnosis or under-reporting of CFP cases. A rapid and reliable instrumentation method through mass spectrometry, with the aim of routine monitoring and screening of CFP in reef fishes along the Indian coast is the need of the hour. The study also signifies the need for creating awareness regarding consumption of coral reef fishes and its consequences among the public.

Ciguatoxin sample analysis data based on mouse bioassay method using IOC manuals and guides (Lewis, 1995) has been submitted as an agenda

item 3 in Codex Committee on Contaminants in foods, eleventh session (CCCF, 2017). In present scenario, where food safety is becoming prime concerns of all people, it is felt that the ciguatera poisoning will assume and will be a major concern for the marketing of reef associated finfishes. The amount of toxins is directly correlated to the size of the fish and results indicated that large sized fishes had more ciguatoxin in comparison to small fishes (Pottier et al., 2001). Hence it is advisable for the consumers to take only *L. bohar* of small size. Ban or size restrictions on certain reef fish species can be taken as an initial safety measure to protect the consumers from the lethal effects of this toxicity.

4.4 Conclusion

Lutjanus bohar commonly known "*Chempalli* or Red snapper" has identified as toxic sample from four different locations, viz., Vizhinjam, Thoppumpady, Kollam (Kerala Coast) and Mangalore (Karnataka coast). All the samples tested are weighed more than 5 kg. Out of the 262 samples investigated for Mouse Bioassay, 12 samples were detected as positive and the lethal dose estimated in Mouse Unit as 3.15, 2.17 and 2.08/20 mg of fish ether extract. LD_{50} value based on all the 12 toxic samples assayed for *in vivo* Mouse bioassay along Indian Coast was estimated as 14.3µg/kg in mice. Clinical symptoms of data collected from affected individuals showed

similarity among the symptoms reported earlier from Indian Coast (Rajeish et al., 2016). The number of individuals in initial intoxication is less compared to the later. The symptomatology in the hospitalized patients verified with earlier reports of Ciguatera Fish Poisoning (Rajeish et al., 2016) with typical clinical signs like gastrointestinal, neurological and cardiovascular symptoms. Organ specific toxicity were observed in liver and brain sections of intoxicated mice, with alterations in liver enzymes (ALT & AST) and also in total protein level in blood. The development and verification of additional chemical methods for CTX will depend upon the Mouse Bioassay result.

Fish carrying ciguatoxin do not exhibit any symptoms and it is practically difficult to ascertain whether the fish is toxic or not. Mouse bioassay of fish extract is considered as a reliable approach to detect the presence of sub lethal doses of CTXs through intermittent observation of symptoms for up to 48 hr (Caillaud et al., 2010). Existence of CTXs along Indian coast calls for a need for good surveillance system and analytical confirmatory methods for the protection of consumers along with exporters.

Annexure -I

Questionnaire

Clinical symptoms of Ciguatera intoxication in effected individuals at Time of Diagnosis

The details given will be kept confidential and the data will be used only for research purpose

Sl No.	Questions	Report (Present/ Absent)	Frequency of Symptoms (%)	Other Details (If any)
I	**Number of individuals effected**			
1	Male			
2	Female			
3	Children			
4	Area			
5	Age			
6	Date and time			
II	**Gastrointestinal Symptoms**			
1	Diarrhea			
2	Vomiting			
3	Nausea			
4	Abdominal pains			
5	Any other symptoms			
III	**Cardiovascular Symptoms**			
1	Bradycardia			
2	Hypotension			
3	Hypertension			
4	Tachycardia			
5	Arrhythmia			
6	Any other symptoms			
IV	**Neurological Symptoms**			
A	**Peripheral Nervous System Symptoms**			
1	Paresthesia- Extremity			
2	Circumoral Paresthesia			
3	Temperature Dysesthesia			
4	Dental pain/ feeling like teeth are loose or falling out			

5	Myalgia			
6	Arthralgia			
7	Pruritis			
8	Dysuria			
9	Any other symptoms			
B	**Central Nervous System Symptoms**			
1	Vertigo			
2	Dizzy			
3	Lipothymy			
4	Loss of consciousness			
5	Cerebellar Syndrome			
6	Balance Disturbance			
7	Hallucinations			
8	Depression			
9	Memory/Concentration			
10	Behavioral disturbance			
11	Visual Disturbance			
12	Multi-tasking Problems			
13	Giddiness			
14	**Any other Symptoms**			
V	**Other:**			
1	Headache			
2	Weakness			
3	Fatigue			
4	Asthenia			
5	Respiratory Disturbance			
6	Chills/Sweating			
VI	**Duration of symptoms**			
1	Time of Onset			
2	Time taken for recovery			
3	Most prominent symptoms			
4	Age of most effected individual (Mention whether Male or Female)			
5	Age category of Male Patients			
6	Age category of Female Patients			
7	Death reports (If any)			
Name of the Doctor / Hospital				

Annexure -II

IOC Mouse bioassay for Ciguatoxin (Check list)

Sl No.	Symptoms	1st hour	2nd hour	3rd hour	4th hour	5th hour	6th hour	24th hour
1.	Hypothermia	+	+	+	ND	ND	ND	ND
2.	Hypothermia below 33^0 C	+	+	+	ND	ND	ND	ND
3.	Piloerection	+	+	+	ND	ND	ND	ND
4.	Diarrhoea	+	+	+	ND	ND	ND	ND
5.	Lachrymation	+	+	+	ND	ND	ND	ND
6.	Hyper salivation	+	+	+	ND	ND	ND	ND
7.	Dyspnoea	+	+	+	ND	ND	ND	ND
8.	Wobbly upright gait	+	+	+	ND	ND	ND	ND
9.	Gasping	+	+	+	ND	ND	ND	ND
10.	Mild gasping	+	+	+	ND	ND	ND	ND
11.	Terminal Convulsion with tail arching	+	+	+	ND	ND	ND	ND
12.	Hind limb paralysis	+	+	+	ND	ND	ND	ND
13.	Progressive hind limb paralysis	+	+	+	ND	ND	ND	ND
14.	Progressive paralysis from hind extending to fore limbs	+	+	+	ND	ND	ND	ND
15.	Convulsions	+	+	+	ND	ND	ND	ND
16.	Mild Convulsions preceding death>30 sec	+	+	+	ND	ND	ND	ND
17.	Respiratory problems	+	+	+	ND	ND	ND	ND
18.	Respiratory failure	+	+	+	ND	ND	ND	ND
19.	Death from respiratory failure	+	+	+	ND	ND	ND	ND
20.	Slow Movements	+	+	+	ND	ND	ND	ND
21.	Slow locomotor activity	+	+	+	ND	ND	ND	ND
22.	Breathing Difficulties	+	+	+	ND	ND	ND	ND
23.	Sluggish	+	+	+	ND	ND	ND	ND

Note: ND – Not Detected as mice death occurs within 3-4 hours from the time of injection.

Chapter 5

Characterization of Indian Ocean Ciguatoxin and its Congeners in Individual Specimens of Red Snapper (*Lutjanus bohar*) by Liquid Chromatography Tandem Mass Spectrometry

Contents

5.1 Introduction

Identification and characterization of ciguatoxin implicated in reef fishes along Indian EEZ has bought a new challenge to our fisheries sector in terms of food and consumer safety. Due to the complex structure and existence of different congeners present in individual toxin, bioassay directed physico-chemical methods are normally used for the determination of these toxins (Holland, 2008). Bioassay depends on the response of symptoms observed in tested animals and practiced as a screening toxicological tool for many laboratories. Further, the ethical concerns over animal studies also pose difficulties in the regular monitoring of food safety issues related with toxins. In this perspective, physico-chemical methods based on chromatographic and

spectroscopic techniques play a vital role in the quantitative analysis of known seafood toxins. Different instrumentation methods are available in the field of marine algal toxin research viz., Gas Chromatography (GC), Thin Layer Chromatography (TLC), HPLC-UVD/FLD, LC-MS, Capillary Electrophoresis (CE) etc. (Quilliam, 2003; Shi, 2012). Mass spectrometry with Electron Spray Ionization (ESI) or Atmospheric pressure Ionization mode (API) and Multiple Reaction Monitoring (MRM) transitions is preferred in the identification of molecular ions of individual toxins and its congeners in a mixture or complex form of compound (Quilliam, 2003; Núñez et al., 2005). Tandem Mass Spectrometry allows universal detection capability, high sensitivity, selectivity and specificity in detection of complex molecules. Through the precise and accurate quantitation of molecular ions, identification of novel toxins from a complex mixture is also made possible in addition to routine monitoring of food safety.

LC-MS/MS is a combination of LC system for separation of analyte, an ionization interface to ionize the molecules, and the MS in which the ions are separated according to their mass-to-charge ratio (*m/z*) and detected in a high vacuum environment (McMaster, 2005). ESI, APCI, APPI, FAB and MALDI are the key ionization interfaces used for detection and separation of analytes, in which ESI is the most common ionization interface for more polar

compounds and it acquires both positive and negative ion spectra (Quilliam, 2003; McNabb et al., 2005). The mass analyzer known as the heart of MS are of three types viz., Quadrupole, Ion Trap (IT), and Time of Flight (TOF). MALDI-TOF-MS has been mostly used in the proteomic analysis or in the analysis of large biomolecules (Moyer et al., 2002; Cai et al., 2005; McMaster, 2005; Reyzer & Caprioli, 2007) and it cannot be combined with LC and provides relatively poor quantitative reproducibility (Cai et al., 2005). In ion trap mass spectrometry (ITMS) low dynamic range could be easily used for quantitation of mass range lower than 4000 Da (March, 1997; Aebersold & Mann, 2003). TOF-MS can also obtain high mass resolutions to enable accurate mass measurements, which aid in developing chemical formulae of novel toxins (Moyer et al., 2002). Triple quadrupole Ion Trap MS comprises of Source (Q0), Quadrupole (Q1), Collision cell (Q2) Quadrupole (Q3) and Detector (internal structure given in Chapter 2). Triple Quad Mass Spectrometry works in combination with ESI Ionization mode and MRM transitions are widely used in marine and fresh water algal toxin detection and delivers molecular mass and structural information in full scan and/or daughter ion scan mode; hence it is considered as a highly sensitive and selective detector for quantitative analysis of complex mixtures.

Ion spray mass detection was first introduced by Lewis et al. (1994) and a CTX Rapid Extraction Method known as CREM was also developed by Lewis et al. (2009). 20 different congeners were identified for CTX from the Pacific, Caribbean and Indian Ocean regions (Caillaud et al., 2010). Potent polyether ciguatoxin responsible for foodborne disease accumulates the toxin at risk levels above 0.1ppb (Lewis et al., 2009). CTX also exhibits multiple classes of charged ions which are difficult to monitor by bioassays (Table 5.1).

Table 5.1 Group of CTXs isolated from Pacific, Caribbean and Indian Ocean regions

Origin	Examples of CTX	Molecular weight (Da)	Source	References
Pacific Type I & Type II (P-CTX)	CTX (CTX1B, CTX-1) CTX2A2 (CTX-2, 52-epi-54-deoxyCTX) CTX2B2 (CTX-3, 54-deoxyCTX) CTX4A CTX4B (GTX-4B, Gt 4b)	1110.6 1094.5 1060.8 1022.8 1056.0	Both Carnivorous and Herbivorous fishes and Dinoflagellate *G. toxicus*	Murata et al., 1990; Lewis et al., 1991; Lewis et al., 1993; Satake et al., 1993a
Caribbean (C-CTX)	CTX-1 & CTX-2	1140.7	Carnivorous fishes	Lewis et al., 1998
Indian Ocean (I-CTX)	CTX-1, CTX-2, CTX-3 & CTX-4	1140.6 1157.6	Carnivorous fishes	Hamilton et al., 2002b

All known ciguatoxin (CTXs) and brevetoxin (PbTxs) normally comes within the mass range of 700 to 1400 Daltons (Hamilton et al., 2002a & 2002b). All CTXs identified till date is heat stable polyether toxins and P-CTX-1 remains the most potent ciguatoxin (Lewis et al., 1991). CTX-1 contributes approximately 90% of the total lethality of carnivorous ciguateric fish capture in the western Pacific Ocean (Lewis & Sellin, 1992). CTX isolation and characterization from Caribbean and Pacific were confirmed by

various researchers (Murata et al., 1989 & 1990; Lewis et al., 1991; Vernoux & Lewis, 1997; Lewis et al., 1998; Yasumoto et al., 2000). P-CTX and C-CTX were well characterized which showed the comparison with brevetoxin (PbTxs) reported by Lombet et al. (1987) and Poli et al. (1997). Indian Ocean Ciguatoxin was isolated and characterized from Red bass (*L. bohar*) and red emperor (*L. sebae*) fish species collected from the bank fishery to the North of the Republic of Mauritius and the molecular masses were identified as *m/z* 1141.6 and 1157.6 Da (Hamilton et al., 2002b). Ciguatera Fish Poisoning was confirmed from *Lutjanus bohar* through Mouse Bioassay method (Rajeish et al., 2016; Rajisha et al., 2017a and 2017b). Trace quantities of CTX in fish samples always make the extraction methods and detection of ciguatoxin a very challenging task. In this chapter an attempt was made in to the optimization of CTX using size exclusion chromatography and Solid Phase Extraction methods using HPLC Tandem Mass Spectrometry. LC-MS/MS methods were used to compare novel compounds produced by ciguatoxin implicated reef fish *L. bohar* along Indian Coast. Two validated extraction methods were followed for the isolation of toxic eluant and compared the extracted ion chromatograms obtained for the previous reports of CTX molecular mass range were compared.

5.2 Materials and methods

5.2.1 Sample preparation

Red snapper (*L. bohar*), species implicated in ciguatera intoxication in Vizhinjam and Mangalore, with mouse toxicity 3.25MU and 2.17MU were taken for the analysis. Fish sample including head, tail and muscle portions were pooled (7.496 kg) and cooked for 30-40 minutes at 120^0C in an autoclave and allowed to cool. The flesh was then removed and minced. This sample was subjected to liquid-liquid partitioning using size exclusion method and solid phase extraction for the detection of CTXs. All chemicals used were LC-MS/MS grade solvents (JT baker™, Fisher Scientific).

5.2.2 Extraction of CTXs using Size Exclusion chromatography

This extraction procedure is based on the chromatographic methods described by Hamilton et al. (2002b) and Vernoux and Lewis (1997) with some minor modifications. The steps involved in the chromatographic purification method comprised of Florisil adsorption chromatography, Sephadex LH 20 Chromatography and HPLC separation of toxic fractions.

Approximately 4.98kg, minced sample were taken for the preparation of crude extract. Samples were initially identified as toxic by Mouse bioassay method. Lipid extraction was performed as per Lewis (1995), given in IOC

manuals and Guides (Detailed description is given in Chapter 4). Crude lipophilic extract collected in the form of diethyl ether fractions was then dissolved in 80% methanol and washed again with n-hexane. Then methanol phase is collected and evaporated using flash evaporator.

5.2.2.1 Purification using chromatography methods

5.2.2.1a. Florisil Adsorption Chromatography

The lipid soluble residue (4.98 kg flesh yielded 9.72g lipid soluble extract) was dissolved in hexane acetone (3:1) and kept as five partitions for one sample extraction. Florisil embedded column was prepared by 100g Florisil with 60-100 mesh size (Sigma-Aldrich) (Figure 5.1). Conditioning of the column was done by passing 3 bed volumes (V_b) of acetone: methanol (9:1) and five bed volumes of hexane: acetone (3:1). After conditioning the column, sample was loaded and eluted with acetone: methanol (9:1). The toxic fractions were collected in the acetone: methanol phase. These fractions were tested using Mouse bioassay method and symptoms observed in mice were compared with previous data. Figure 5.3 indicates schematic representation of Florisil Adsorption Chromatography

5.2.2.1b. Sephadex LH20 size exclusion chromatography

Sephadex column was prepared by Sephadex®LH20, 25 to 100µm bead size (Sigma-Aldrich) gel filtration media (3cm diameter X 120cm height; V_b =

80ml) (Figure 5.2). One gram LH 20 swells for at least three hours in 4ml methanol. Conditioning of the column was done with $3V_b$ of methanol. After loading the toxic Eluent, column is eluted with two V_b of dichloromethane: methanol (1:1) and the resulting toxic zone was again applied to a Sephadex LH20 (3.2cm diameter X 94cm height; V_b= 80ml) column. Again loaded the sample and eluted with methanol (~120ml). Evaporated and dried weight of extract was taken.

Fig. 5.1: Florisil (60-100 mesh size) Column Chromatography

Fig. 5.2: Sephadex LH-20 Gel Filtration Chromatography

5.2.2.1c. HPLC column separation of Toxic eluant

Lachrom Merck-Hitachi Interface D7000 HPLC was used for the analysis. HPLC column Lichrocart® 100 RP-18, 250x4cm (5µm) was used and eluted with methanol: water (9:1) at a flow rate 0.5mL/min and gradient programme is given in Table 5.2. Evaporated and toxic fractions collected were reconstituted in 1.5 ml methanol. Toxic fractions were confirmed by mouse bioassay and dried under nitrogen and stored at -20^{0}C for LC-MS/MS analysis. Figure 5.4 depicts schematic representation of Sephadex LH-20 and HPLC separation of toxic fractions.

Table 5.2: Gradient Programme for separation in HPLC

Time (min.)	MeOH (A)	H_2O (B)
0	10	90
5	25	75
10	0	50
15	75	25
20	90	10
25	75	25
27	50	50
30	10	90

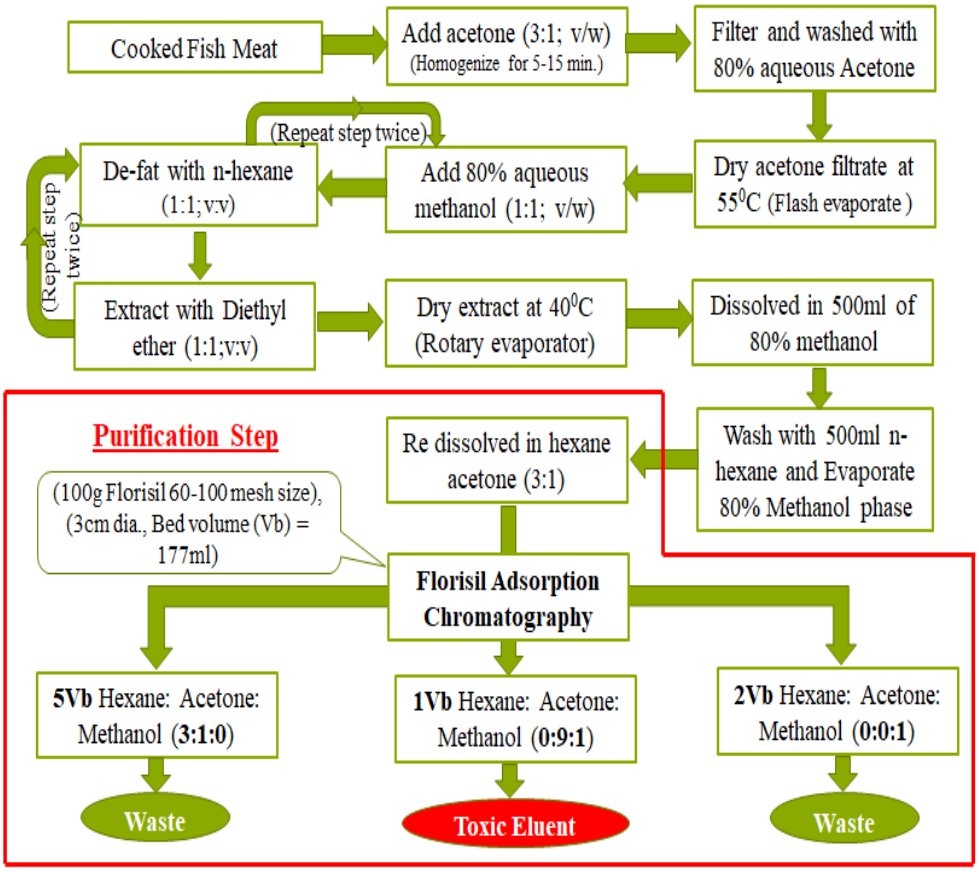


Fig. 5.3: Schematic representation of Florisil Adsorption Chromatography. Toxicity in eluted fractions was confirmed in parallel using mouse bioassay (Lewis, 1995; Hamilton et al., 2002b)

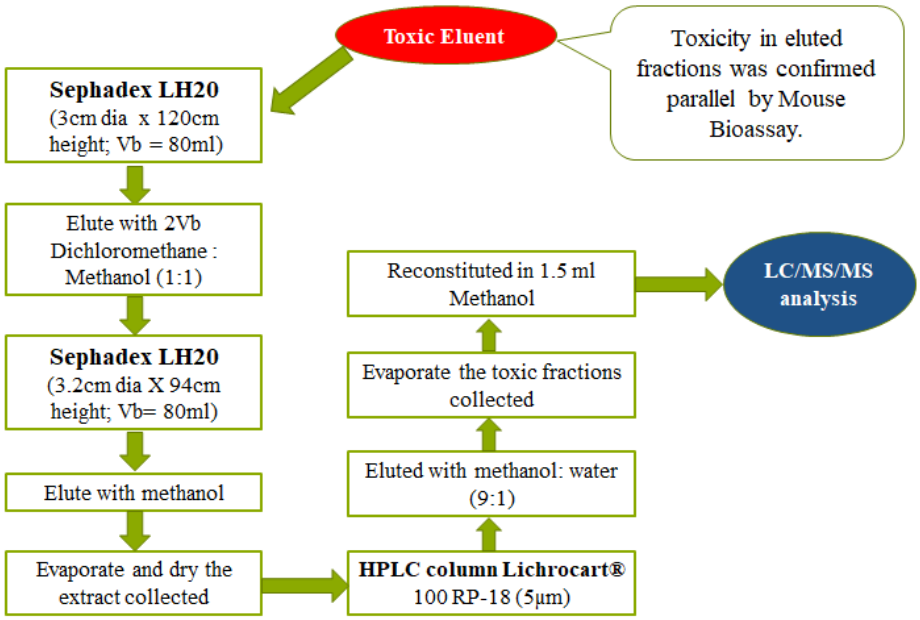


Fig. 5.4: Schematic representation of Sephadex LH-20 and HPLC separation of toxic fractions eluted from Florisil adsorption method. Toxicity in eluted fractions was confirmed in parallel using mouse bioassay (Lewis, 1995; Hamilton et al., 2002b)

5.2.3 Solid Phase Extraction (SPE) of the fish extracts

During the size exclusion chromatography method the final concentration of dried toxic eluent is comparatively small; hence it is difficult to concentrate the complete elution of toxic fractions. Hence Solid phase Extraction (SPE) method was also followed using the fish sample. Solid phase extraction is an efficient method for sample preparation when the sample is less in a complicated matrix (Lewis et al., 2009).

Solid phase extraction was carried out as per the Ciguatoxin Rapid Extraction Method (CREM) developed by Lewis et al. (2009) with minor modifications. Initially different types of reversed phase Sep Pak® C18 and

normal phase Silica cartridges and elution conditions were conducted. The optimized procedure used for a 500mg Sep Pac® Vac 6cc C18 and Silica (Waters) cartridges. 4g minced fish cooked in a 50 ml centrifuge tube, homogenized with 16 ml methanol: hexane (3:1). Then centrifuged and discarded the upper hexane layer. Filtered 12 ml MeOH layer with 0.45 PTFE membrane filter.

5.2.3.1 C18 SPE Clean up (Sep Pac® Vac 6cc, 500mg, Waters)

Initially the SPE cartridge column was conditioned with 4 ml H_2O and then sample was applied, followed by washing with 6.5 ml 65% methanol and eluted with 8 ml 80% methanol. Approximately 7.5 ml of sample collected and added 0.5 ml 65% methanol. To the collected sample added 5 ml 1M NaCl and 7.5 ml Chloroform with vigorous shaking and centrifuge 4 min at 2000rpm. Then discarded upper aqueous layer and evaporated lower layer reconstituted in 4 ml chloroform.

5.2.3.2 Silica SPE Clean up (Sep Pac® Vac 6cc, 500mg, Waters)

Conditioned the column with 4ml Chloroform and applied sample to SPE column. Then washed with 5 ml chloroform and eluted with 10 ml 90% chloroform. All samples were evaporated under Nitrogen and dissolved in 400µl 50% aqueous methanol for LC-MS/MS Analysis. Figure 5.5 showed the flow diagram of CTX Rapid Extraction Method

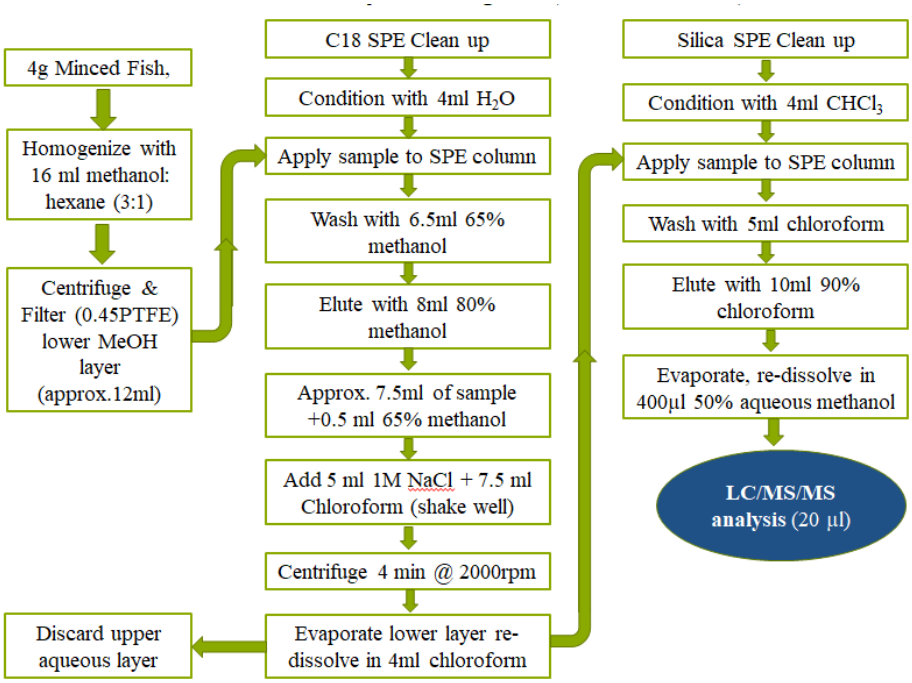


Fig. 5.5: Flow Diagram of CTX Rapid Extraction Method (Lewis et al., 2009)

5.2.4 Mouse Bioassay

All the eluted toxic fractions from size exclusion, HPLC and SPE chromatography methods were collected and dried under nitrogen and completely removed all the solvents. Female albino mice were injected with these extracts prepared in Tween 60/0.9% saline and observed the symptoms of toxicity to identify the toxic eluted fractions.

5.2.5 LC/MS/MS analysis

API 4000 QTRAP LC/MS/MS (AB Sciex, figure 5.6) were used for the detection. The toxic eluent analyzed in QTRAP scanned over 700-1400 m/z under electrospray ionization using the following conditions given in Table 5.3.

Table 5.3: MS operating parameters

1	Entrance Potential (eV)	10
2	Curtain gas (CUR)	10
3	Collision gas	Medium
4	Ion Spray voltage (IS)	5500
5	Temperature from Ion Source (TEM)	350°C
6	Nebulizing gas (GS1)	20
7	Dwell time(ms)	150

Fig. 5.6: AB Sciex 4000 Triple Quadrupole Mass Spectrometer

5.2.5.1. Column used for HPLC Eluted fractions:

LC comprised a Hibar® HR Purospher STAR RP-18 end capped (3µm) column.

5.2.5.2. Column used for Solid phase extraction Eluant:

The LC system comprised a C18 column (Phenomenex Luna 3µ C_{18} (2) 100A, 150x 2.00 mm).

LC mobile phase is aqueous 2 mM ammonium formate and 0.1% formic acid (Solvent A) and 95% acetonitrile with 2 mM ammonium formate and 0.1% formic acid (Solvent B). Linear gradient programme given in table 5.4.

Table 5.4: Linear Gradient programme (flow rate of 500µl/min)

Time (min.)	Solvent A (%)	Solvent B (%)	B Curve
0	65	35	6
12	5	95	6
15	65	35	6

5.3 Results and Discussion

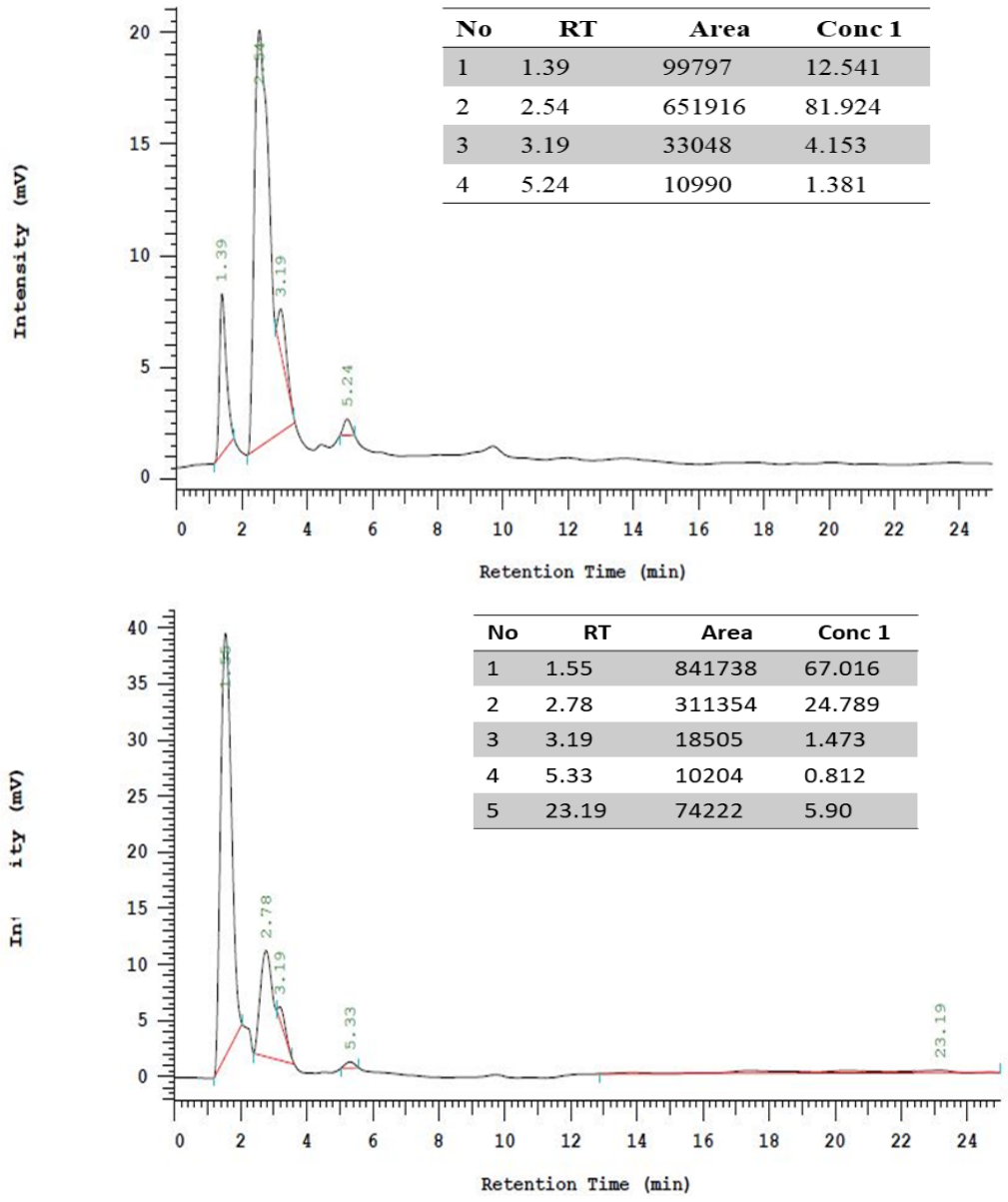
5.3.1 Separation of crude lipophilic toxic ether extract

The extracted samples were separated in to 5 liquid partitions during evaporation and the toxic diethyl ether phase collected as 1.37g, 1.99g, 1.61g, 2.33g and 2.42g respectively in each fraction. These fraction quantities were further subjected to elute through the chromatographic separation and SPE method. Through Sephadex LH-20 and HPLC eluant programme, the maximum quantity of pooled dried extract recovery is 1.38µg only, which shows considerable loss of toxicity during extraction steps. Hence it is understood that the sample is in a complex matrix and SPE is a better option. CREM method (Lewis et al., 2009) using C18 and Silica cartridge is a random solid phase extraction method in which the extract is finally eluted in 50% aqueous methanol (400µl) and the initial sample quantity (4g) is also very less, hence dried weight of extract cannot be taken for toxin recovery. Hence the dried form of extract could not be quantified using this method.

5.3.2 HPLC separation of toxic fractions

Figure 5.7 shows the reversed phase HPLC chromatogram of I-CTX extract partially purified using Florisil and Sephadex LH-20 chromatography methods.

These fractions were collected and tuned for the molecular masses of I-CTX using Mass Spectrometry under ESI conditions. The toxic fractions collected were considerably at low intensity when compared to SPE fraction analysis.



No	RT	Area	Conc 1
1	1.39	99797	12.541
2	2.54	651916	81.924
3	3.19	33048	4.153
4	5.24	10990	1.381

No	RT	Area	Conc 1
1	1.55	841738	67.016
2	2.78	311354	24.789
3	3.19	18505	1.473
4	5.33	10204	0.812
5	23.19	74222	5.90

Fig. 5.7: HPLC separation of CTXs containing a mixture of 5.24µg of CTX extract by Reversed phase C18 HPLC column monitored at 215nm (Eluant MeOH: H_2O; 9:1)

5.3.3 Ciguatoxin Rapid Extraction Method (CREM) for mass spectrometry

Lewis et al. (2009) developed the simplified solid phase extraction method for P-CTX detection and quantification. The major modifications made in the CREM method are the change in sample quantity which is taken as 4g instead of 2g fish flesh. SPE cartridges were selected as 500mg 6cc (Waters) whereas in CREM method it is 900mg. For LC-MS/MS analysis of SPE toxic fractions, LC column was selected as Phenomenex Luna 3µ C_{18} (2) 100A, 150x 2.00mm, which also differ from original CREM method. The initial tuning of crude extract was done and MRM method developed for analysis. During SPE step, eluted toxic fractions were collected in chloroform, which is further evaporated and reconstituted in aqueous methanol. Complete removal of trace quantities of all the solvents is mandatory for the elimination of impurities during the extraction. Otherwise the presence of solvents will affect the peak intensity.

5.3.4 Toxicity analysis using Mouse assay

Intraperitoneal injection of toxic fractions collected from Sephadex and SPE chromatography methods were induced symptoms in mice. The observed symptoms were similar to the earlier reported data given in chapter 4. Death of mice is absent since the quantity of toxin in sample matrix is very low, but 10% weight loss observed in CTX affected mice. Symptoms showed during MBA help to identify the toxic fractions during each chromatographic step.

5.3.5 Compound optimization using Manual tuning method in QTRAP LC-MS/MS

The major aim was to determine the optimized instrument settings and ion transitions for each CTX congeners using AB Sciex Q TRAP MS/MS with maximum detection sensitivity. Initially performed a survey scan based on detailed review data of existing molecular masses of ciguatoxin reported from various geographical regions. Survey scan conducted within the mass range m/z 700 – 1400 (a mass range that covers all known CTXs and PbTXs, lipophilic polyether toxins) in positive and negative mode using TRAP function to identify different molecular weight (Lewis et al., 1991). Therefore each extracted toxic eluent was infused directly in to the MS at a rate of 10µl per minute and at a concentration of 1µg/mL, although some extracted factions were found considerably less concentration. The instrument was set to allow passage of the molecular ions through the Q1 detector, and then Q3 was scanned for ion fragments. The software ramps up various instrument parameters in order to identify optimum settings for each of the most three abundant product ions. Prominent molecular masses were tuned and identified as m/z 1117.303, 1125.576, 1245.67 and 1291.695 Da (Figures 5.8 to 5.11). Each molecular mass obtained were subjected to further tuning including product ion scan and Multiple Reaction Mode (MRM) scan and developed methods for these molecular weights using Compound optimization Manual tuning programme in QTRAP (Table 5.5).

Table 5.5: MRM method developed for the analysis of I-CTX based on different MS operating parameters

Precursor Ion (m/z) Q1	Product Ion (m/z) Q2	DP (eV)	EP (eV)	CE (eV)	CXP (eV)	CUR	Collision gas	IS	TEM (°C)	GS1	Dwell time (ms)
1117.303	436.8	61	10	37	12	10	Medium	5500	350	20	150
	188.7	61		41	18						
	408.7	61		43	12						
1125.576	859.1	81	10	19	26	10	Medium	5500	350	20	150
	592.7	81		37	18						
	440.9	81		45	12						
1245.668	424.8	81	10	45	12	10	Medium	5500	350	20	150
	979.1	81		19	16						
	440.9	81		45	14						
1291.695	1025.2	81	10	19		10	Medium	5500	350	20	150
	889.2	81		23							
	425.0	81		49							

Mass spectra of HPLC and SPE toxic fractions were obtained using QTRAP MS based on the developed MRM method. Three most intense product ion spectra tuned for each molecular masses and selected for MRM method. Three toxic HPLC fractions and two SPE fractions were analysed for the tuned molecular masses. Figure 5.12 depicts the extracted ion chromatogram (XIC) obtained for 1.37g of ether extract eluted through HPLC method with total ion chromatogram (TIC) intensity (Figure 5.13) 4500cps. Peak list obtained for the XIC of 1.37g of ether extract is given in table 5.6. Figure 5.14 & 5.15 gives the XIC and TIC of 1.99g of ether extract obtained with a peak intensity at 5300cps. Peak list for the XIC of 1.99g of ether extract is given in table 5.7. Figure 5.16 &5.17 gives the XIC and TIC for 2.42g of ether extract with maximum intensity at $1.00e^4$ cpc. Peak list obtained for figure 5.16 is givn in table 5.8. Figure 5.18 & 5.19 gives the XIC and TIC of 1.61g SPE eluted ether toxic fractions with peak intensity $1.18e^4$ cps. Peak list obtained for the eluted fractions were given in table 5.9. XIC and TIC of 2.33g of SPE eluted ether fractons were given in figure 5.20 & 5.21. Peak list of data obtained for the XIC is given in table 5.10.

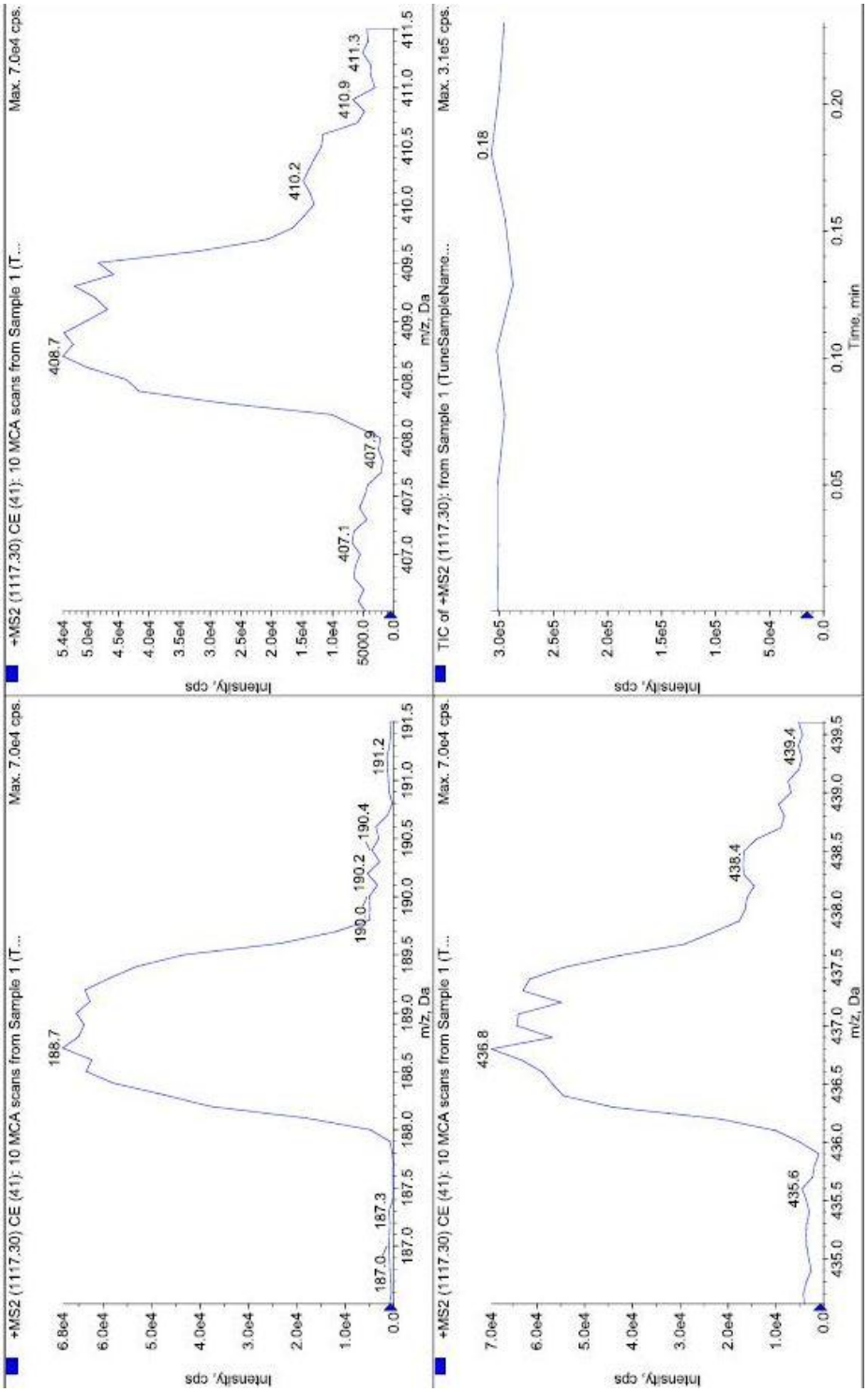


Fig. 5.8: Infusion compound optimization in positive mode with Q1 mass 1117.303 and Q3 masses 188.7, 408.7 & 436.8

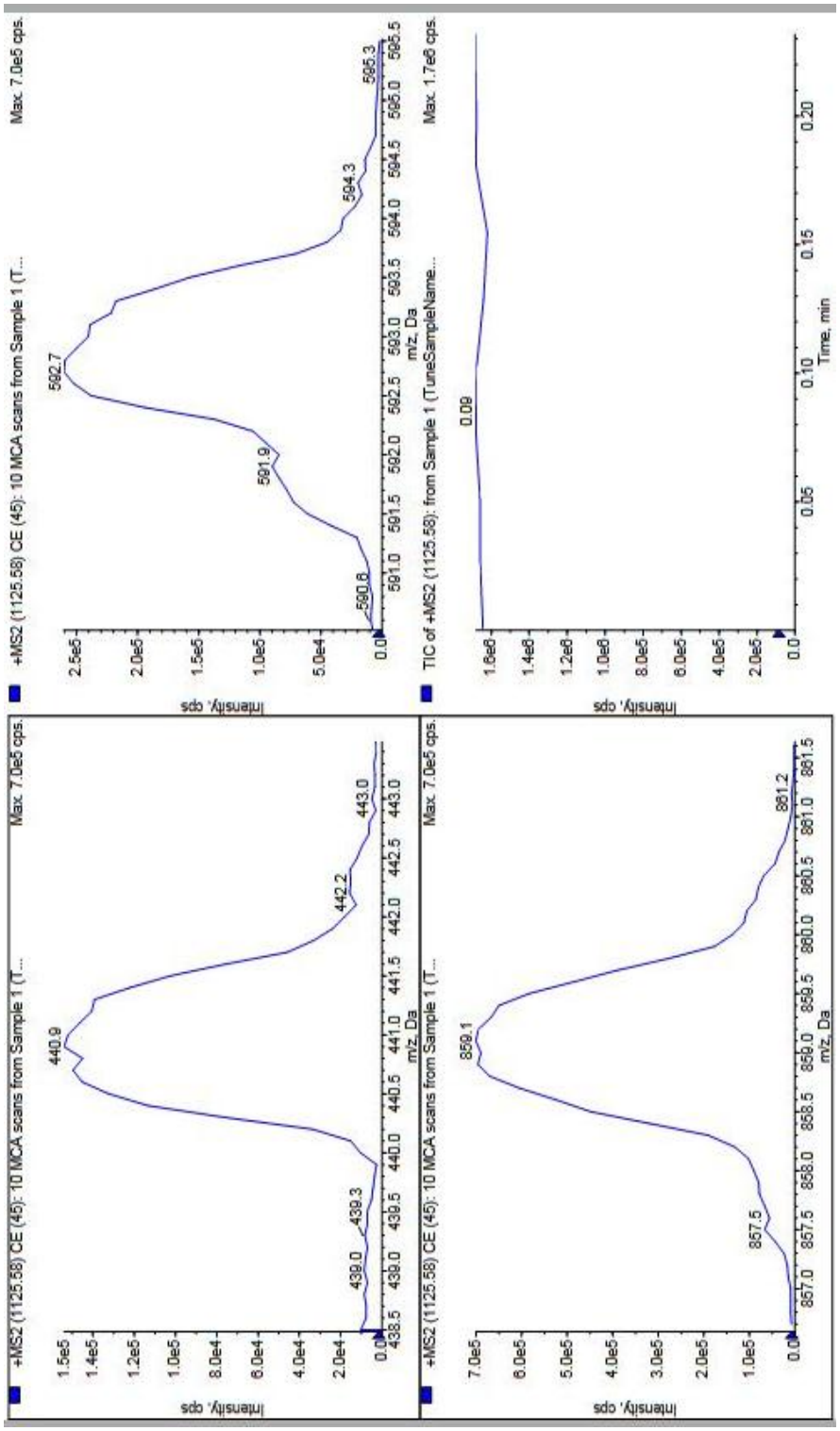


Fig. 5.9: Infusion compound optimization in positive mode with Q1 mass 1125.576 and Q3 masses 440.9, 592.7 & 859.1

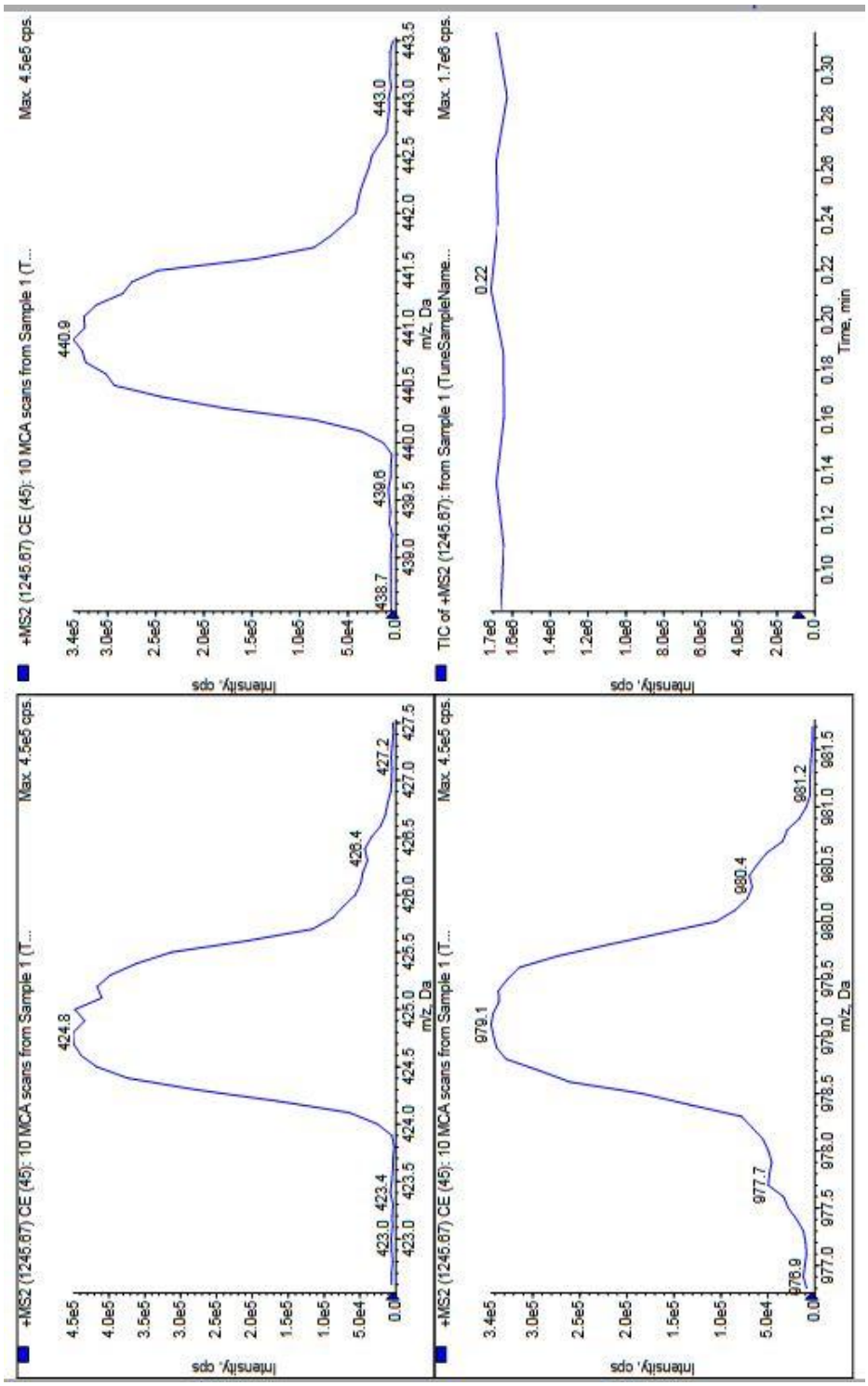


Fig. 5.10: Infusion compound optimization in positive mode with Q1 mass 1245.668 and Q3 masses 424.8, 440.9 & 979.1

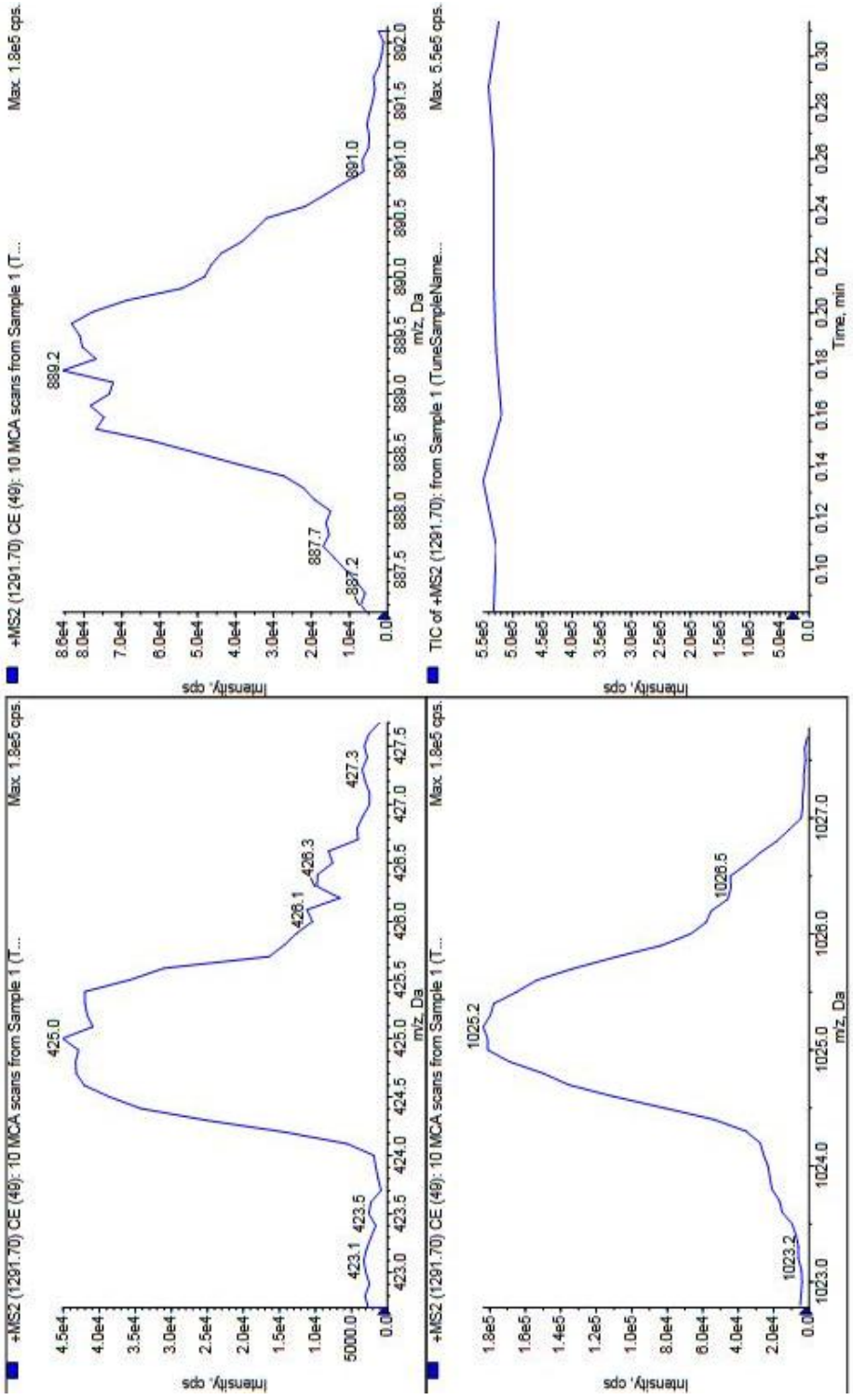


Fig. 5.11: Infusion compound optimization in positive mode with Q1mass 1291.695 and Q3 masses 425, 889.2 &1025.2

5.3.6 Mass Spectral Data for HPLC eluted fractions

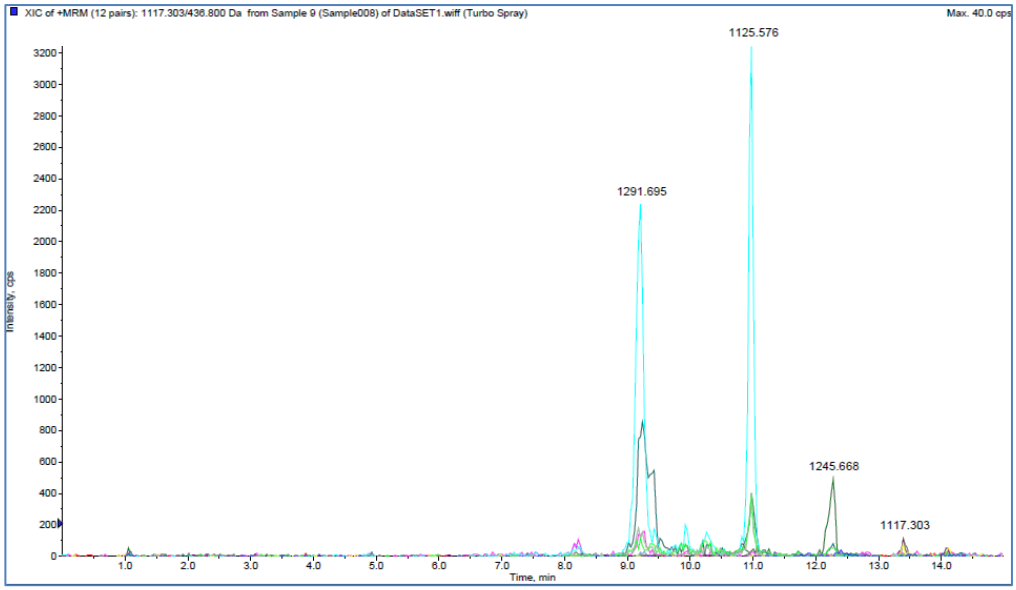


Fig. 5.12: XIC of 1.37g of ether extract with molecular ions m/z 1117, 1125, 1245 and 1291 Da

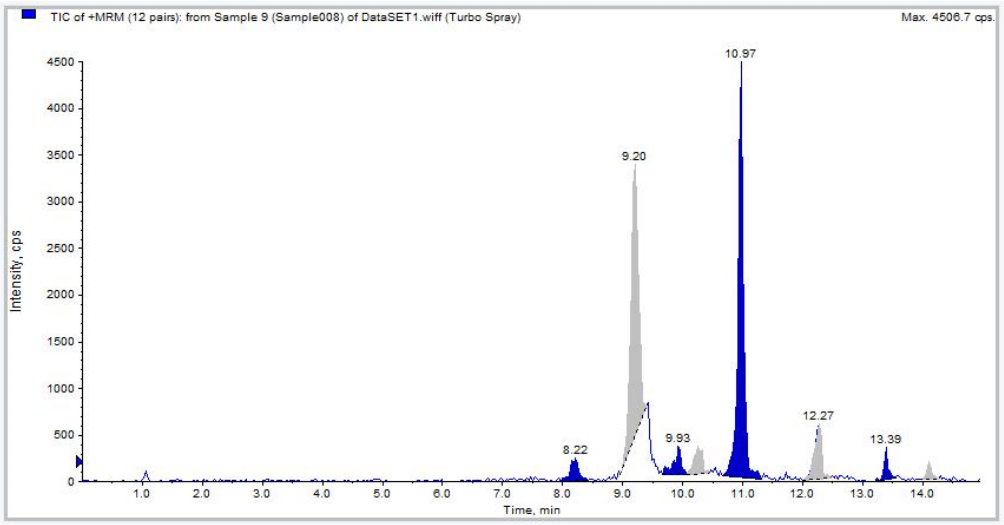


Fig. 5.13: TIC of 1.37g of ether extract with peak intensity 4500cps

Table 5.6: Peak list obtained for the Extracted Ion Chromatograms given in figure 5.9

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	8.2152	2467.7549	3.6160	249.8316	2.6565	0.4650
2	9.2034	2.5237e4	36.9798	2924.6893	31.0992	0.4341
3	9.9312	2579.4533	3.7797	320.0242	3.4029	0.4030
4	10.2631	2827.6123	4.1433	293.3513	3.1193	0.3720
5	10.9708	27569e4	40.3965	4491.9784	47.7648	0.6511
6	12.2721	4910.9194	7.1960	594.7841	6.3245	0.4651
7	13.3929	1568.7278	2.2987	339.6564	3.6117	0.3720
8	14.1060	1085.1339	1.5900	190.0589	2.021	0.3100

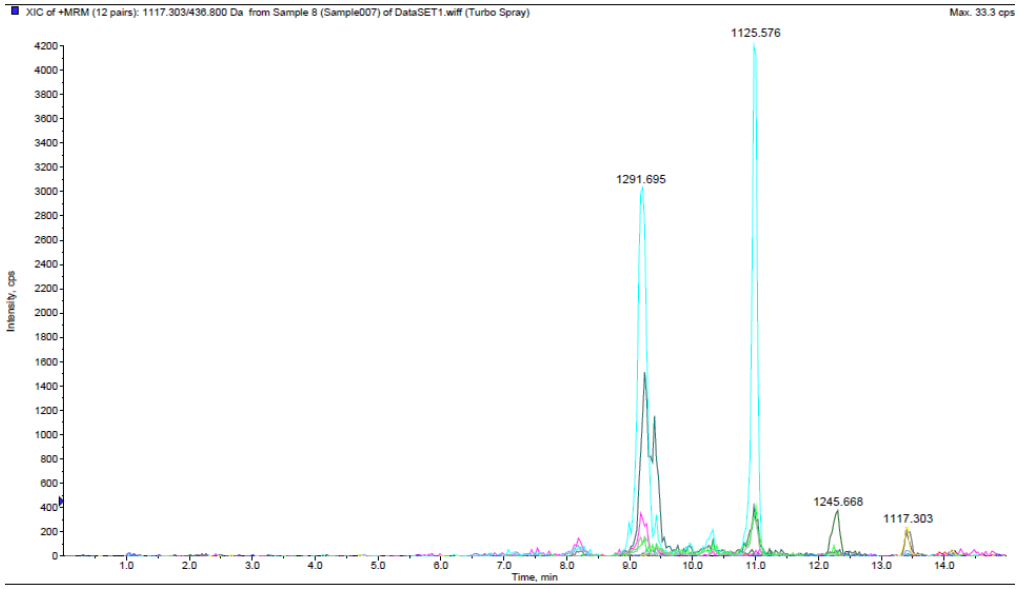


Fig. 5.14: XIC of 1.99g of ether extract with molecular ions m/z 1117, 1125, 1245 and 1291 Da

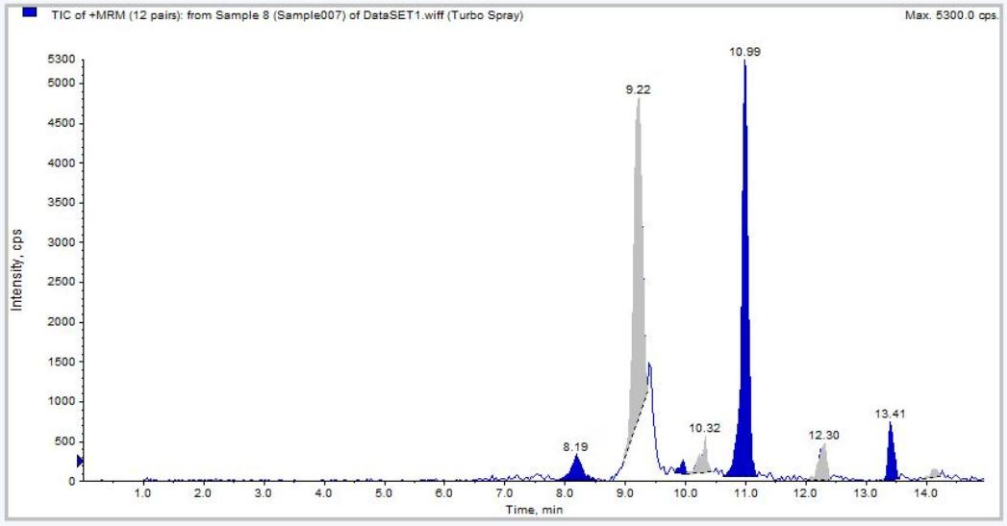


Fig. 5.15: TIC of 1.99 g of ether extract with peak intensity 5300cps

Table 5.7: Peak list obtained for the Extracted Ion Chromatogram given in figure 5.11

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	8.1881	4129.6951	4.3980	327.3321	2.7484	0.6201
2	9.2241	3.8494e4	40.9956	4148.7990	34.8355	0.4031
3	9.9561	936.2793	0.9971	167.9137	1.4099	0.1860
4	10.3218	3218.1932	3.4273	458.0746	3.8462	0.4341
5	10.9890	3.7478e4	39.9134	5460.8408	45.8520	0.5581
6	12.2990	4123.3636	4.3913	466.3744	3.9159	0.3720
7	13.4053	4583.7604	4.8603	764.9922	6.4233	0.2790
8	14.1222	954.9306	1.0170	115.3718	0.9687	0.2790

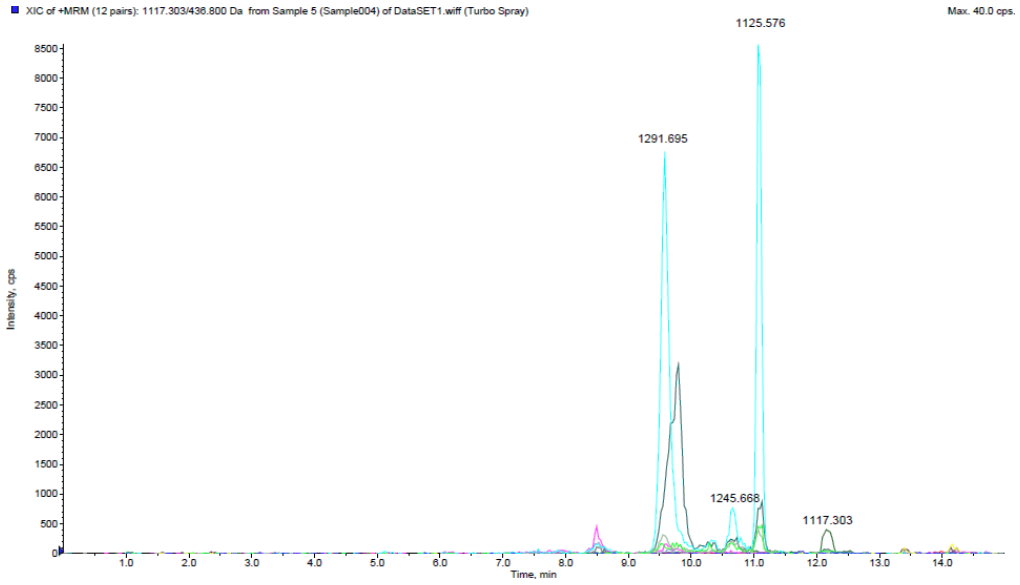


Fig. 5.16: XIC of 2.42g of ether extract with molecular ions m/z 1117, 1125, 1245 & 1291 Da

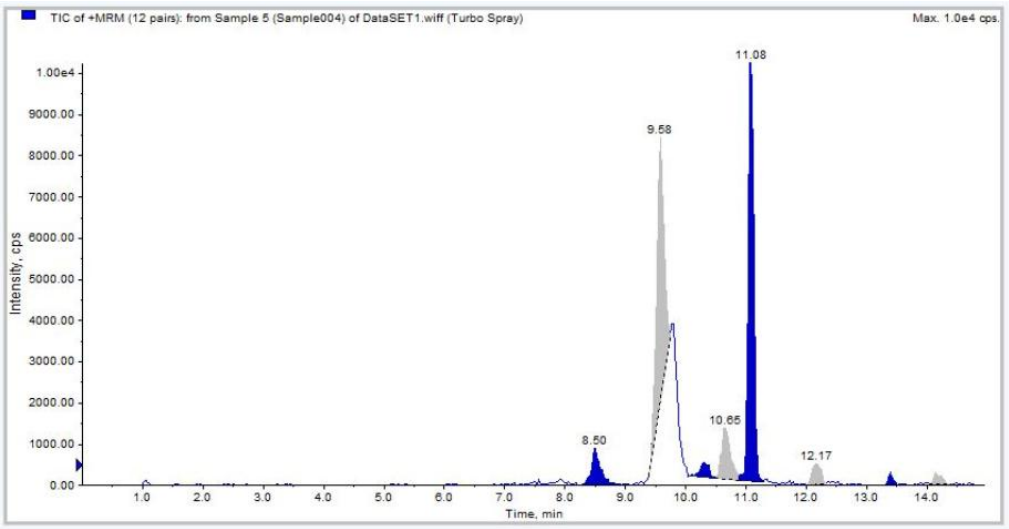


Fig. 5.17: TIC of 2.42g of ether extract with peak intensity1.00e4 cps

Table 5.8: Peak list obtained for the Extracted Ion chromatogram given in figure 5.13

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	8.4961	9890.4428	6.5351	888.6950	4.2987	0.8061
2	9.5824	5.2497e4	34.6872	6351.1295	30.7209	0.3410
3	10.3007	3536.3731	2.3367	365.7820	1.7693	0.4030
4	10.6498	1.2761e4	8.4319	1301.2355	6.2942	0.4030
5	11.0809	6.2452e4	41.2651	1.0644e4	51.4863	0.4030
6	12.1655	5407.2824	3.5729	510.6876	2.4702	0.3101
7	13.3965	2306.6565	1.5241	321.1056	1.5532	0.5891
8	14.1438	2492.6493	1.6470	290.9329	1.4073	0.3720

5.3.7 Mass Spectral Data of SPE Fractions

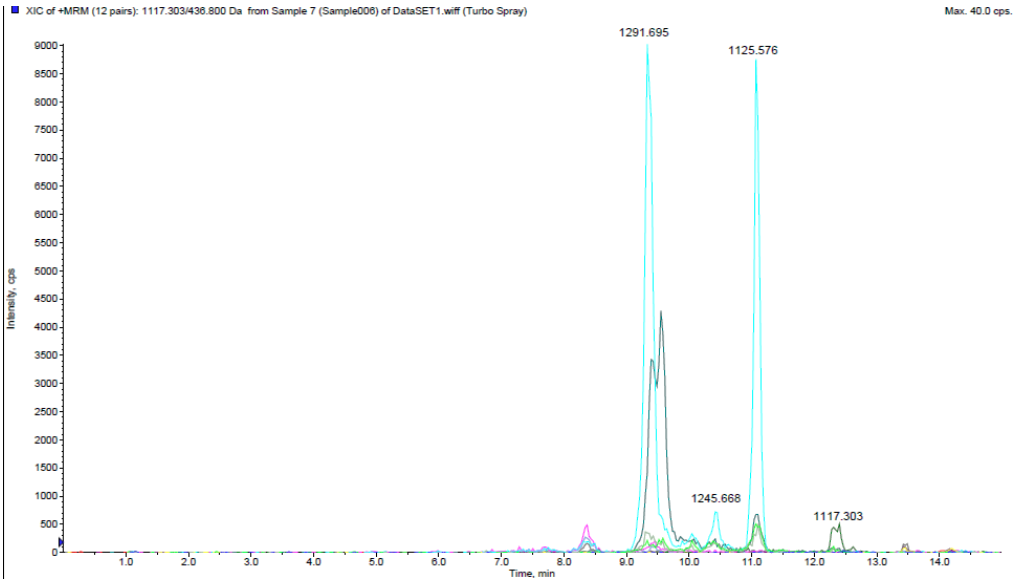


Fig. 5.18: XIC of 1.61g of ether extract with molecular ions m/z 1117, 1125, 1245 & 1291 Da

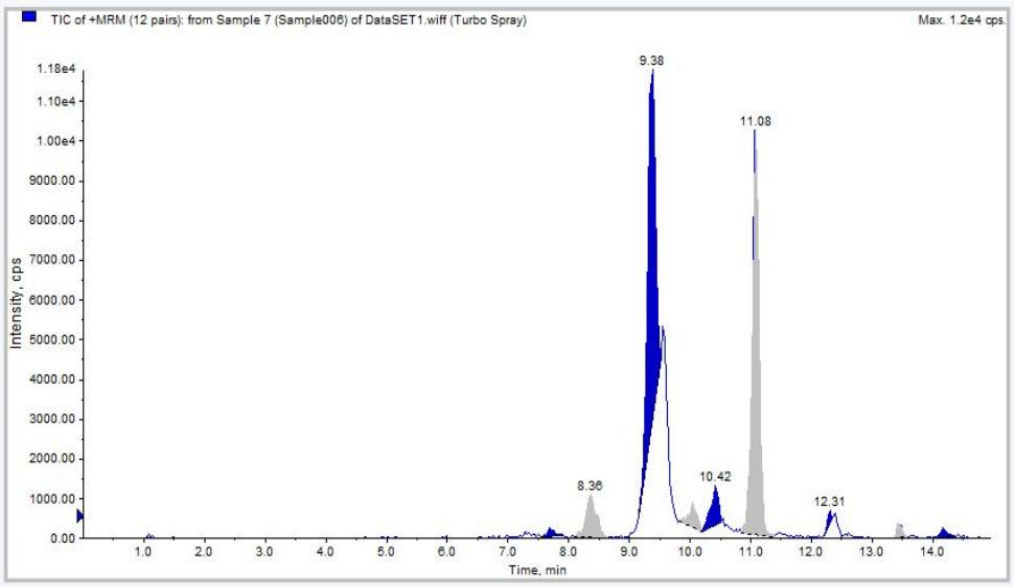


Fig. 5.19: TIC of 1.61g of ether extract with peak intensity 1.18e4 cps

Table 5.9: Peak list obtained for the Extracted Ion chromatogram given in figure 5.15.

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	7.9073	3174.8617	0.8905	459.6755	0.8943	0.4030
2	8.5775	$1.5992e^4$	4.4853	1985.0045	3.8620	0.4341
3	9.5562	$1.4699e^5$	41.2288	$2.2879e^4$	44.5126	0.3100
4	9.7194	$7.8859e^4$	22.1183	9691.2042	18.8552	0.3410
5	10.1650	8133.8730	2.2814	1233.3741	2.3996	0.3100
6	10.4681	$1.1292e^4$	3.1672	1474.8733	2.8695	0.3101
7	11.0514	$8.0220e^4$	22.4999	$1.2205e^4$	23.7466	0.4961
8	12.2537	6783.3865	1.9026	768.6299	1.4954	0.6201
9	13.3621	5084.3760	1.4261	701.3938	1.3646	0.4651

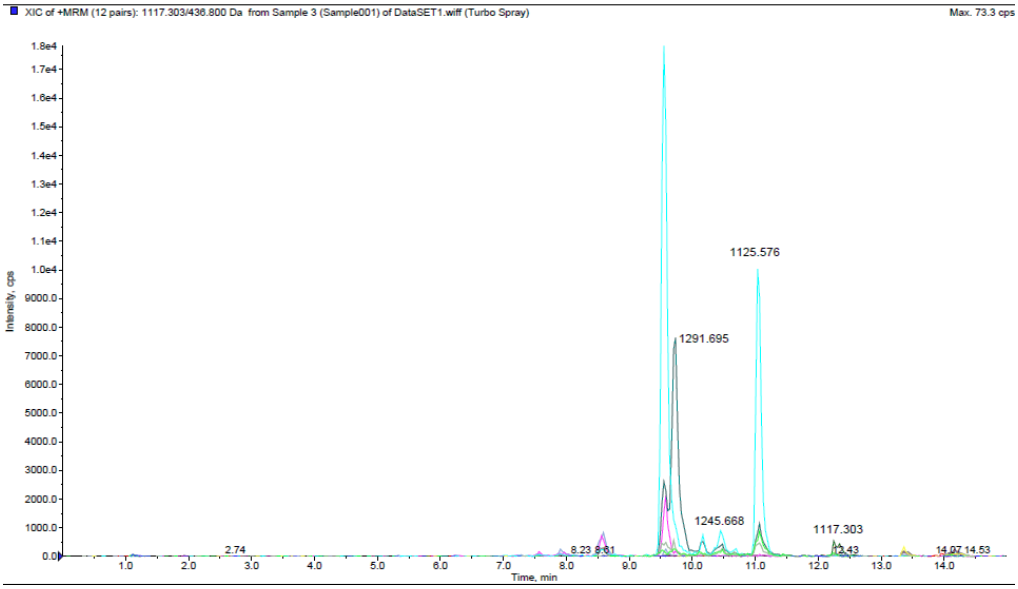


Fig. 5.20: XIC 2.33g of ether extract with molecular ions m/z 1117, 1125, 1245 and 1291 Da

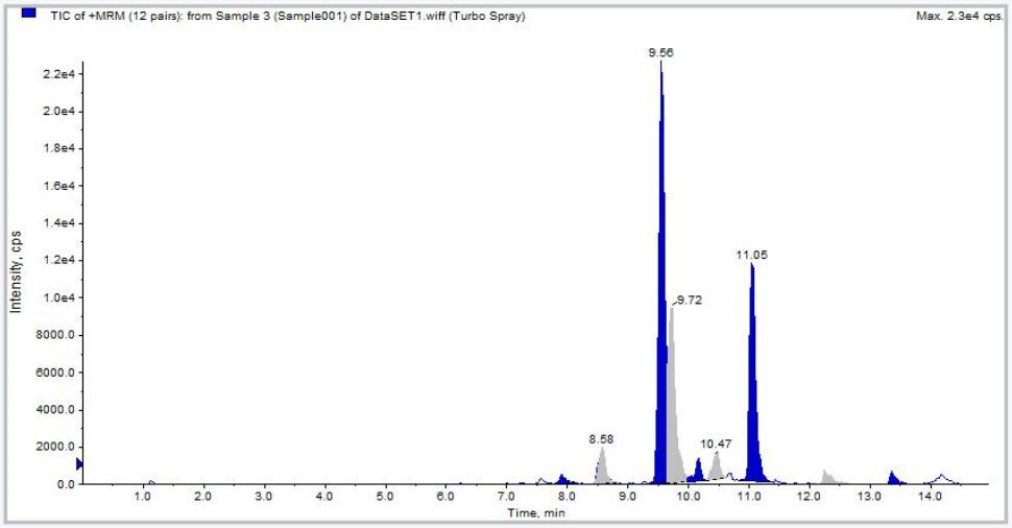


Fig. 5.21: TIC of 2.33g of ether extract with peak intensity 2.2e4 cps

Table 5.10: Peak list obtained for the Extracted Ion chromatogram given in figure 5.17

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	7.6947	1754.9175	0.249	206.8455	0.8901	0.3720
2	8.3609	1.2420e4	6.5459	1084.2109	4.6657	0.5581
3	9.3811	7.9910e4	42.1160	9002.5467	38.7410	0.3720
4	10.0453	5580.4758	2.9411	621.1047	2.6728	0.3720
5	10.4203	8228.2433	4.3366	987.2087	4.2483	0.3100
6	11.0784	7.6865e4	40.5111	1.0422e4	44.8492	0.5271
7	12.3068	1109.9482	0.5850	314.9080	1.3552	0.1240
8	13.4386	2071.0620	1.0915	366.3264	1.5764	0.2170
9	14.1730	1798.1461	0.9477	232.6628	1.0012	0.4030

This study, reports the isolation and characterization of I-CTX from reef fish *L. bohar* samples collected from the Indian Coast. For this purpose, two extraction procedures for ciguatoxin using Size Exclusion method and solid phase extraction method were selected from Hamilton et al. (2002b) and Lewis et al. (2009). The extracted ion chromatogram shows the same molecular ion spectrum for each extracted fractions. Lewis et al. (1991) conducted purification and characterization of ciguatoxins from Moray eel viscera, in which mass spectra of CTX-1 showed m/z 1111.6 ± 0.1, which was earlier reported by Murata et al. (1990) with m/z 1111.5843± 0.0053. It is comparable with the molecular mass m/z 1117.303 Da identified in this study from Indian Coast, which is named as I-CTX-1117. Lewis et al. (1991) also suggested glycerol: thioglycerol (1:1) run for CTX-1 and CTX-2 for m/z 1219&1327 and 1203&1311, which is confirmed as high and low energy CID spectra of 1111 ion. The molecular masses identified in this study also compared with the m/z 1245 and 1291 ions as high and low energy spectra of

1117 ions. Lewis & King, (1996) identified fourteen Pacific CTX congeners with m/z 1111.6, 1095.7 and 1127.7 Da. Hence it is felt that molecular mass m/z 1125.668 is comparable with the identified 1127.7 Da.

Mass spectral data showed and confirmed the presence of CTX molecular ions in the collected fish specimens of *L. bohar* from Indian coast. An MRM method obtained by tuning the mass range 700- 1300 Daltons and the prominent ion spectra obtained for the molecular masses 1117, 1125, 1245 and 1291 were identified from the extracted samples. But the ether extract obtained from the samples are very less in quantity and further purification using HPLC and SPE yielded only microgram quantities of toxic fractions. It is also observed that there is considerably low intensity in peaks obtained from HPLC eluted toxic fractions when compared to SPE. CTX dried extract is obtained in the form white amorphous solids for further structural analysis (Lewis et al., 1991).

Table 5.11: Comparison of peak intensity obtained from HPLC and SPE fractions

Toxic Fractions	Dried ether extract (g)				
	HPLC			SPE	
	1.37	1.99	2.42	1.61	2.33
Peak intensity (cps)	4500	5300	1.00e4	1.18e4	2.2e4

Peak intensity is higher in the SPE fractions (Table 5.11) proved considerable losses in the toxin level while doing the HPLC separation of ether extract. The SPE collected fractions were not able to evaporate to microgram quantity

level, since the initial sample quantity taken is only 4g. Hence there is a need for the method development to exclude the limitations of loss toxicity while doing extraction. Since the followed extraction methods are developed for P-CTX and C-CTX extraction and purification based on the chemical nature of toxins, the major aim the doctoral work focused on the development of new extraction and purification of CTX under Indian conditions.

5.4 Conclusion

Conditions for CTXs detection along Indian Coast using an API 4000 QTRAP MS were optimized. The ultimate isolation of CTXs from pre-purified extracts obtained after liquid-liquid extraction, solid-phase extraction, and size-exclusion chromatography were achieved by reversed-phase C18, normal phase silica and HPLC columns. Survey scan method tuned for all cyclic polyether toxin s (m/z 700 to 1400) and MRM based tuning method is followed in the selected mass range reported for ciguatoxin. CTX toxic fractions collected as five toxic zones through the chromatographic methods. Splitting the output to a mass spectrometer, under ESI conditions and collected fractions identified the CTXs with m/z I-CTX-1117.303, I-CTX-1125.576, I-CTX-1245.668 and I-CTX-1291.695 Da. Based on the peak intensity of eluted ions, SPE method has found more effective than Size Exclusion extraction methods.

Chapter 6

Development of a New Extraction Method for Purification of Indian Ocean Ciguatoxin

Contents

6.1 Introduction

Gymnodimine (GYM), Spirolides (SPXs) and Ciguatoxins (CTXs) are not coming under the legislation of European Union, hence Mouse bioassay is still considered as a reference method for lipophilic toxins (Otero et al., 2011; Antelo et al., 2014). Functional and chemical assays based determination of biotoxins requires a certified reference standard which is not available for CTX group of toxins. Therefore, LC-MS/MS had turned into an essential research tool for the determination of CTX; however this method should be validated. Based on an inter laboratory validation exercise from National Reference Laboratories Network it is agreed as a suitable reference method (These et al., 2011; Veilleri et al., 2011; Antelo et al., 2014). Electron Spray Ionization Mass Spectrometry is a powerful analytical technique which offers speed, accuracy and high sensitivity (Quilliam, 2003). The LC-MS

combination include both the HPLC separation and detection and characterization by MS. ESI is a soft ionization technique, which generates a mass spectrum in which the protonated $[M+H]^+$ or cationized ($[M+Na]^+$ and $[M+K]^+$ etc.) molecules typically correspond to the base peak and can be easily interpreted. The degree of fragmentation in an ESI-MS is controlled by Collision-Induced Dissociation (CID) in tandem mass spectrometry or by varying the orifice (cone) voltage.

Chemical nature of CTX showed that it is lipophilic polycyclic ether compound (Caillaud et al., 2010). Hence the toxic fractions can be accumulated in the lipid content. These lipid fractions were lost in quantity while doing the CTX extraction. Hence it was observed that for the maximum recovery of CTX, complete extraction of the lipid content in the form of ether fractions is needed. Based on this basic idea, an extraction method is developed which differ completely from the reported methods. This chapter discuss about a new extraction protocol developed for the detection of CTX using mass spectrometry. The main focus of the extraction method is the purification of maximum weight of toxic extract for structural analysis using NMR and IR spectral data.

6.2 Materials and methods

6.2.1 Sample Collection

The red snapper species, *L. bohar* (8 fish samples) collected from Kollam (8°56′19″N, 76°32′25″E), Thoppumpady (9°56’7”N, 76°15’33’’E) and

Mangalore (12°50′23″N, 74°47′24″E) along south west coast of India, confirmed as positive samples for ciguatoxicity using Mouse bioassay taken for the analysis.

6.2.2 Extraction Procedure

The extraction method includes a combination of two extraction procedures. Lipid extraction by Folch Method (Folch et al., 1957) and Solid phase extraction of crude lipid extract using Sep- Pak Florisil and Primary Secondary Amine cartridges (Waters) (Yogi et al., 2014). Chloroform: methanol (2:1) was added to 100g fish tissue and homogenized (v/w 1:15; extract in 3 steps). Pooled the three extract and measured the volume. Added 20% water mixed and kept for overnight separation and then transferred the chloroform layer through anhydrous Na_2SO_4. Collected layer concentrated and added 5 ml Methanol. Evaporated the solvent using nitrogen and calculated the lipid content. When the lipid content is less than 2g fat, then added 30ml Methanol and 1.5 ml 150% potassium hydroxide (Increased the volume of extraction solvent according to the lipid content). Non Saponifiable Matter (NSM) from the extracted lipid content was refluxed for 30 min in boiling water bath under N_2, cool slightly and added 20 ml distilled water. Extract three times with 20ml petroleum ether and pooled the extract. Washed three times with distilled water, filtered through Na_2SO_4 and evaporated under Nitrogen gas. Crude NSM is collected and reconstituted in 5 ml methanol. Then Solid Phase Extraction method is followed using Sep Pak® Vac 6cc

(500mg) Florisil Cartridge and Sep Pak® Vac 6cc (500mg) PSA Cartridge (Waters). During the Florisil SPE cartridge eluted with 4ml ethyl acetate and Methanol (9:1 ratio) and dried under nitrogen stream. PSA is eluted with 4 ml acetonitrile and 3ml methanol and dried using nitrogen. The dried extract is reconstituted in 1 ml methanol for LC-MS/MS analysis. A detailed description of extraction method is given in results (Figure 6.1)

6.2.3 LC MS MS

AB Sciex 4000 Q TRAP MS/MS is used for the analysis. The chromatogram for ciguatoxin obtained with a BEH C18 Column coupled with a Water Acquity UPLC (1.0 × 50 mm id, 1.7 µm particle size). The flow rate was 500µl/min with a gradient starting at 75% B, increased to 90% B in 11 min, and held for 4 min (Table 6.1). Solvent A is 5 mM ammonium formate and 0.1% formic acid in water and solvent B is methanol (MeOH)]. LC-MS/MS grade (J T Baker® Brand and Merck) solvents and chemicals were used in analysis.

Table 6.1 Linear Gradient Programme (Flow rate 500µl/min)

Time (min.)	Solvent A (%)	Solvent B (%)
0	25	75
2	25	75
11	10	90
15	10	90
20	65	35

6.3 Results and Discussion

6.3.1 Isolation and Purification of CTXs using newly developed extraction procedure

CTXs were isolated from eight suspected *L. bohar* fish samples weighed more than 4kg with an average weight of 6.13±1.17 kg. The fish specimens were pooled and taken for isolation and purification as depicted in figure 6.1. The lethal dose for reef fish samples estimated to be 2.08 MU/100g of ether extract. The amount of toxicity in fish sample is equivalent to 10.4ng and 10% weight loss observed in intoxicated mice. Initial Mouse bioassay toxicity and observed symptoms were detailed in Chapter 4.

6.3.1.1 Separation of Non Saponifiable Matter (NSM)

The new extraction method is optimized and finalized after a number of trials and errors. The major difficulty during the extraction of CTX involves the trace quantity of toxic extract in dried form. This problem was solved up to an extent by this method. Various SPE columns and cartridges were trialed and Florisil and PSA SPE method was found to be more suitable for CTX analysis. The CTXs eluted as a single toxic fraction and dried extract obtained as white amorphous solids as reported by Lewis et al. (1991). The dried weight samples eluted approximately 53µg, 104µg, 81.9µg and 68µg in four separate extractions using the new protocol.

Ciguatoxin is a polyether compound, lipid containing quaternary nitrogen atom, one or more hydroxyl groups and a cyclopentanone moiety,

hence it is not considered as a phosphatidic ester (Scheuer, 1994). Since it is a lipophilic compound, the separation of crude lipid using Folch extraction method is followed. The NSM content in the fish flesh separated. Care should be taken while doing the methanol and KOH extraction step. Because the quantity of lipid always remain less than 2 g for the given quantity of solvents. When the lipid content is high, the quantity of solvents should be increased.

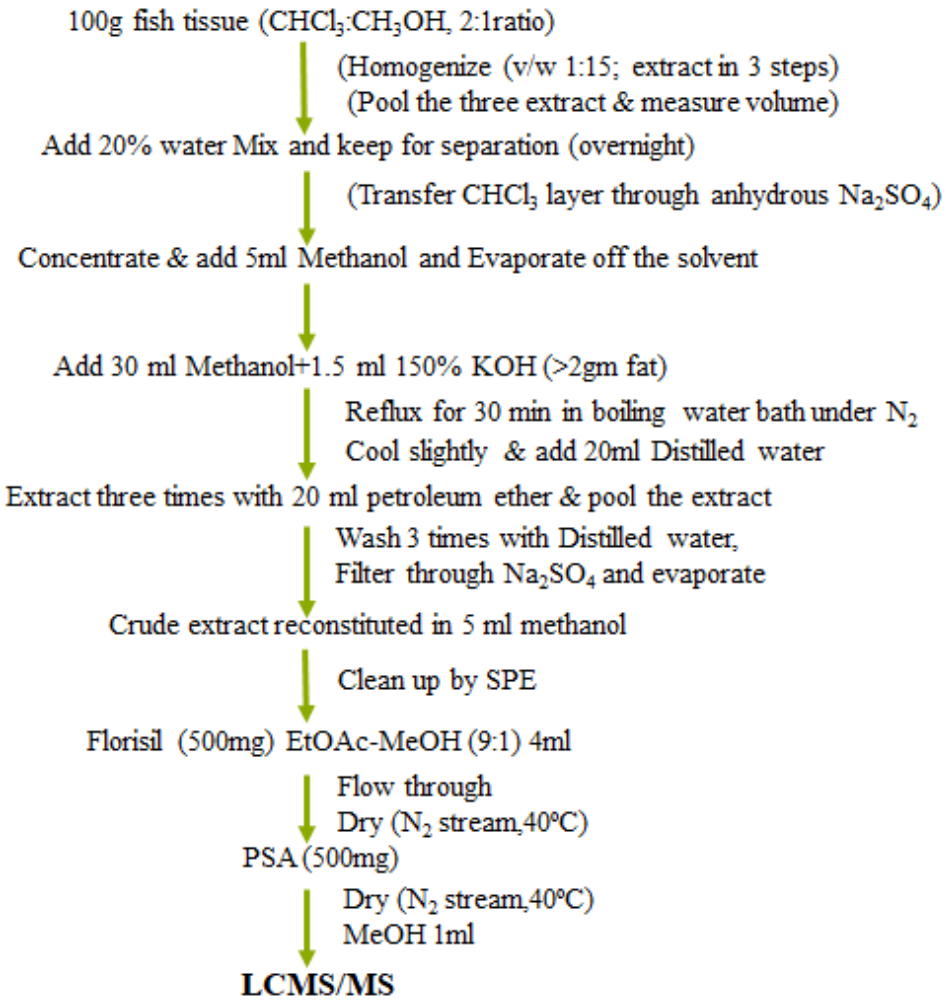


Fig. 6.1: Schematic representation of the novel extraction protocol for CTX Purification

6.3.2 Mass Spectra

CTX ions showed as single charged ions and tuned in positive mode using infusion compound optimization method with search ranges from m/z 1111.0 to 1113.0 Da, 1141.0 to 1143.0 Da, 1158.0 to 1159.5 Da and build final method using three most intense peaks. In all search range of molecular ions base peak ions selected as the precursor ion and the product ions are in auto select mode. For the mass range m/z 1111.0 to 1113.0 Da, the base peak ion mass (precursor ion) obtained as 1112.606 Da. The final Q1 scan mass is 1112.801 with an intensity of 536980 cps (5 MCA average) and the final product ion masses were m/z 782.641, 86.232 and 104.227 Da with an intensity of 10480, 7360 and 37280 cps respectively. For m/z 1141.0 to 1143.0 Da, precursor ion was 1142.606 Da and the final mass obtained as 1142.605 Da with an intensity of 484720 cps. The most intense final product ion masses were m/z 86.2, 147.1 and 104.1 Da with an intensity of 8400, 7480 and 6330 cps respectively. For the search range m/z 1158.0 to 1159.5 Da, the base peak ion mass is 1158.794Da and the final Q1 scan gives the m/z as 1158.803 Da. The final product ion masses include m/z 828.6, 86.2 and 104.1 with an intensity of 16860, 9340 and 7800 cps respectively. The other molecular masses m/z 1117.303, 1125.576, 1245.576 and 1245.668 Da tuned earlier in Chapter 5 was also included in the MRM method (Table 6.2) for detection of

CTX using Tandem Mass Spectrometry. Figure 6.2 to 6.4 gives the manual tuning spectra for identified molecular masses with three most intense precursor ions

Table 6.2: MRM method developed for the analysis of I-CTX using all the tuned molecular ions

Precursor Ion (m/z) Q1	Product Ion (m/z) Q2	DP (eV)	EP (eV)	CE (eV)	CXP (eV)	CUR	Collision gas	IS	TEM (0C)	GS1	Dwell time (ms)
1112.900	782.6 104.1 86.3	81	10	19 77 119	14 14 8	10	Medium	5500	350	20	150
1141.998	147.1 104.1 86.3	71	10	23 79 129	12 14 8	10	Medium	5500	350	20	150
1158.803	828.6 104.1 86.3	71	10	19 83 129	14 12 8	10	Medium	5500	350	20	150
1117.303	436.8 188.7 408.7	61	10	37 41 43	12 18 12	10	Medium	5500	350	20	150
1125.576	859.1 592.7 440.9	81	10	19 37 45	26 18 12	10	Medium	5500	350	20	150
1245.668	424.8 979.1 440.9	81	10	45 19 45	12 16 14	10	Medium	5500	350	20	150
1291.695	1025.2 889.2 425.0	81	10	19 23 49	18 14 12	10	Medium	5500	350	20	150

Five Indian Ocean CTX congeners were identified using the newly developed extraction method with molecular masses m/z 1117, 1112, 1141, 1158 and 1125, named as I-CTX-1117, I-CTX-1112, I-CTX- 1141, I-CTX-1158 and I-CTX- 1125 respectively. Pottier et al. (2002a) identified similar five new C-CTX congeners from *Caranx latus* with pseudo molecular ions at m/z 1141.58, 1143.60, 1157.57, 1159.58 and 1127.57 Da. These five congeners are closely related and existed as multiple forms of ions. The molecular masses detected from Indian Ocean regions were also closely related as multiple ions. The mass range for CTX ions are 900 – 1200 Da obtained using ammonium formate and methanol as eluant. Vernoux and Lewis (1997) detected m/z 1140.58 for C-CTX; hence it is considered as I-CTX has chemical and structural similarity with Caribbean CTX (Hamilton et al., 2002b). Molecular masses m/z 1141.58 and 1158.62 Da was identified for Indian Ocean CTX by Hamilton et al. (2002b). Abraham et al. (2012) generated product ion spectra for C-CTX with m/z 1159, 1139, 1143 and 1123. All the mass spectra obtained during triple Quadrupole mass spectrometry method which exhibit characteristic similarity with previous reports of C-CTX and I-CTX (Vernoux & Lewis, 1997; Lewis et al., 1998; Hamilton et al., 2002b; Pottier et al., 2002a; Abraham et al., 2012).

Mass Spectral Chromatogram for all the molecular masses identified from CTX Extract was given in figure 6.5. Extracted ion chromatogram (XIC) for molecular masses m/z 1141.998 & 1158.980 are shown in figure 6.6 with total ion chromatogram (figure 6.7) intensity $1.3e^4$cps. Table 6.3 gives the peak list of XIC and TIC obtained for the respective figures. XIC and TIC of m/z 1117.303Da showed in figure 6.8 & 6.9 respectively with peak intensity 967cps and peak list details are given in table 6.4. Figure 6.10 and 6.11 gives the XIC and TIC of molecular masses identified as m/z 1112.900 Da with peak intensity $1.20e^6$ cps and peak list data are given in table 6.5. Figure 6.12 & 6.13 gives the XIC and TIC of molecular masses identified as m/z 1141.998 and 1112.9 Da with peak intensity 8000 cps and peak list details are given in table 6.6. XIC and TIC of molecular masses identified as m/z 1117, 1125, 1158 & 1112 Da were given in figure 6.14 and 6.15 with peak intensity 2200cps and peak list data given in table 6.7. XIC ad TIC of molecular masses identified as m/z 1117, 1125, 1158, 1141 & 1112 Da were given in figure 6.16 & 6.17 with peak intensity 1800 cps and table 6.8 gives the details of peak list data.

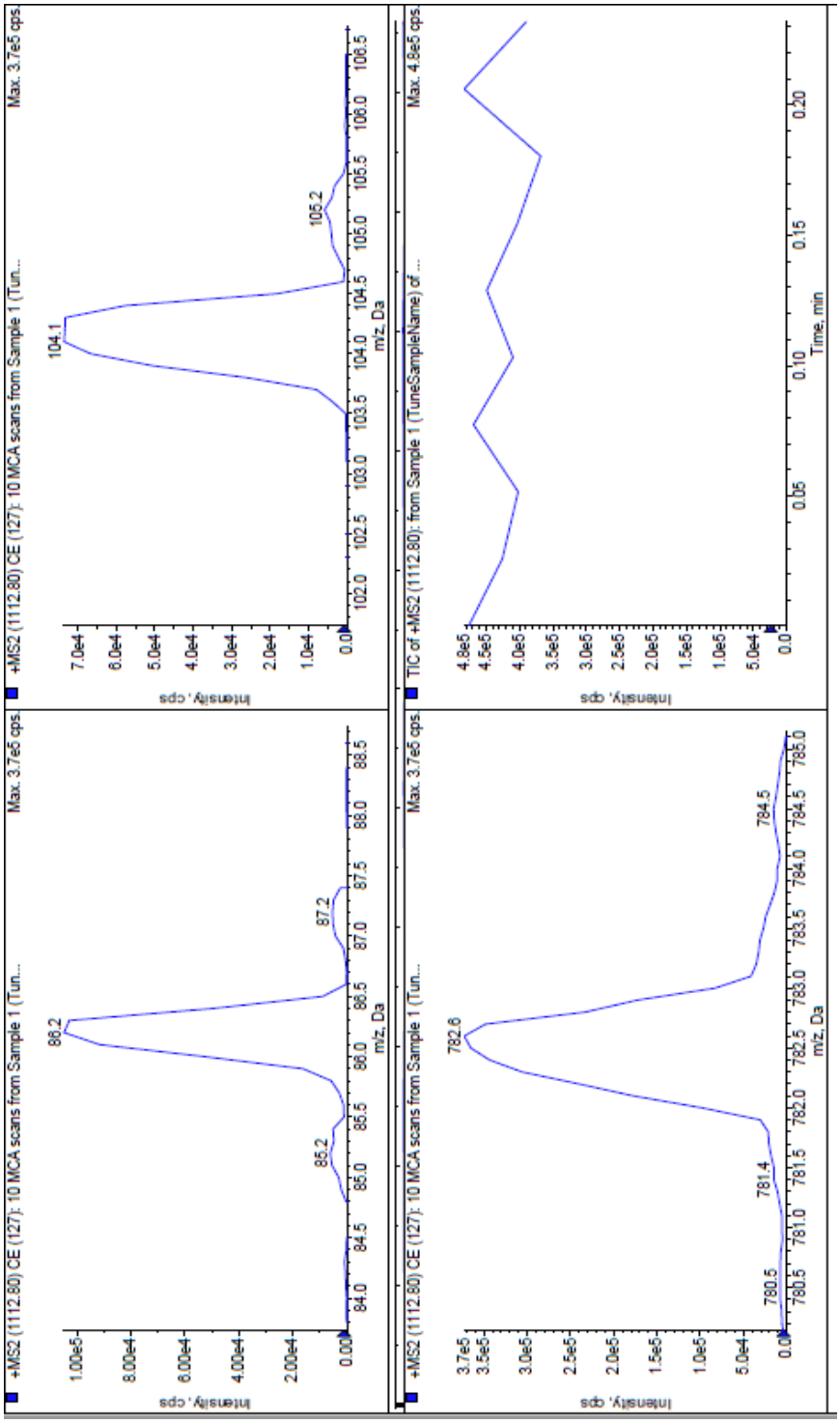


Fig. 6.2: Tuned spectra for m/z 1112.801 with three product ions as 86.2, 104.1 and 782.6

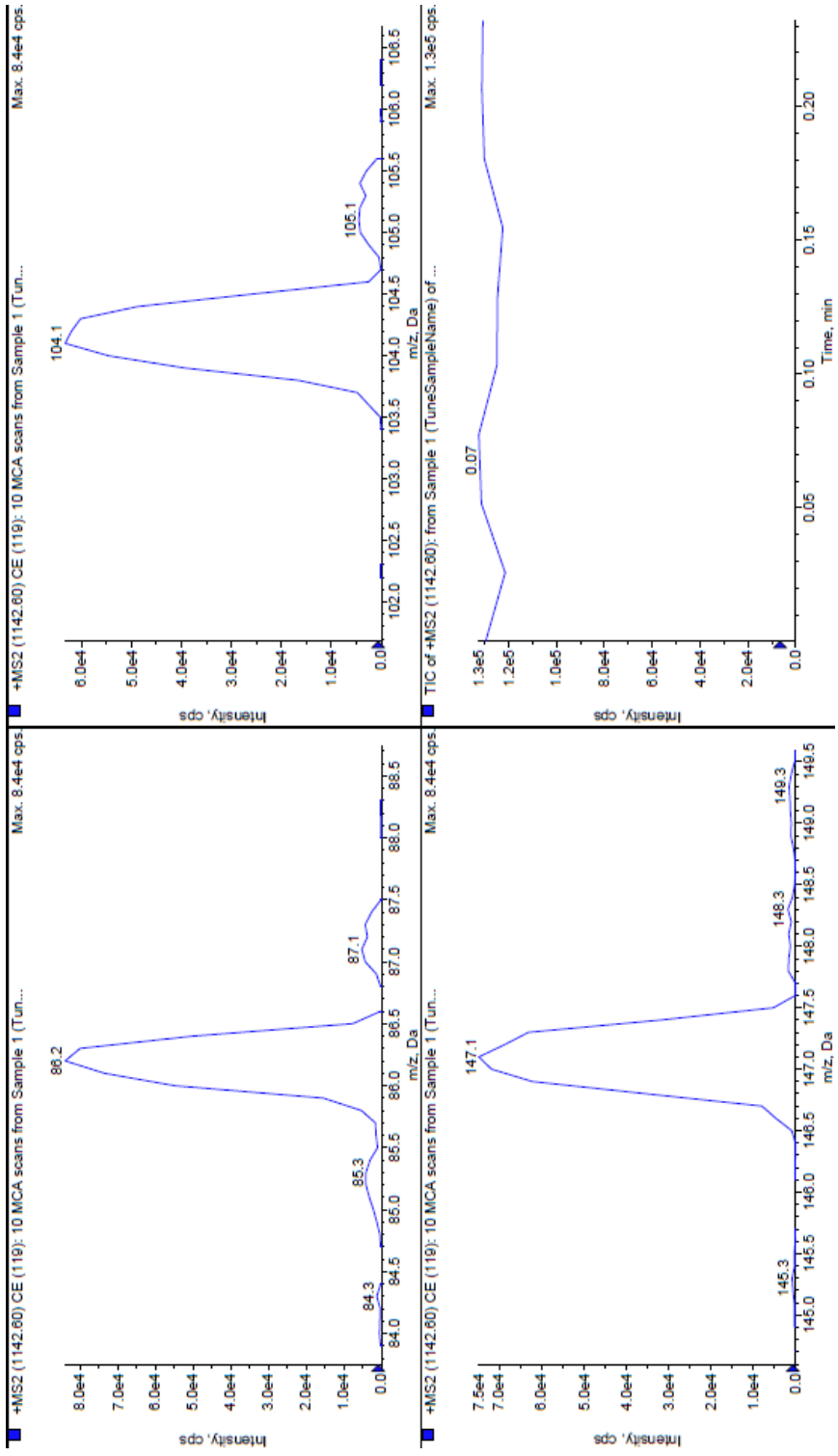


Fig. 6.3: Tuned spectra for m/z 1142.605 with three product ions as 86.2, 104.1 and 147.1

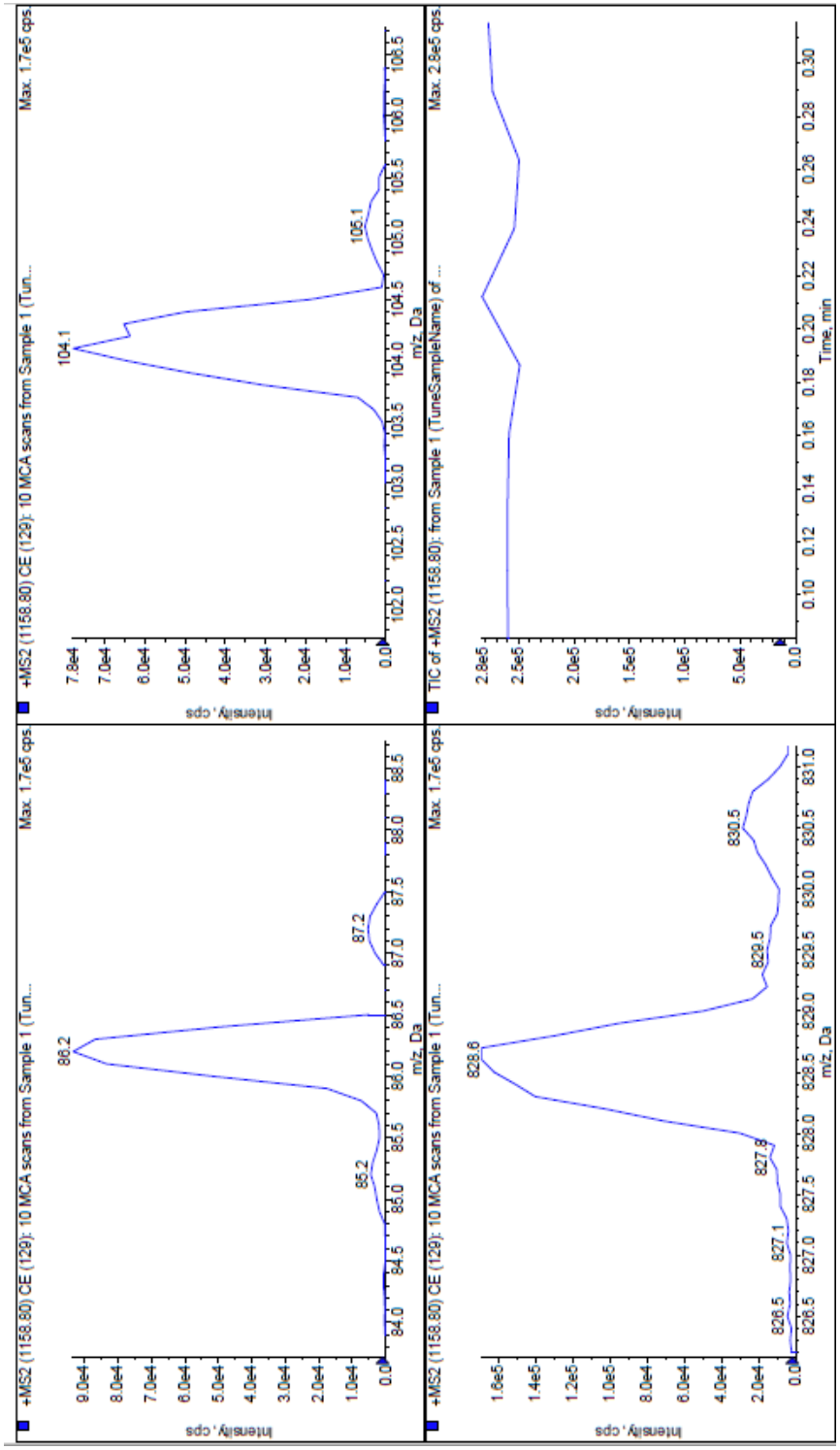


Fig. 6.4: Tuned spectra for m/z 1158.803 with three product ions as 86.2, 104.1 and 828.6

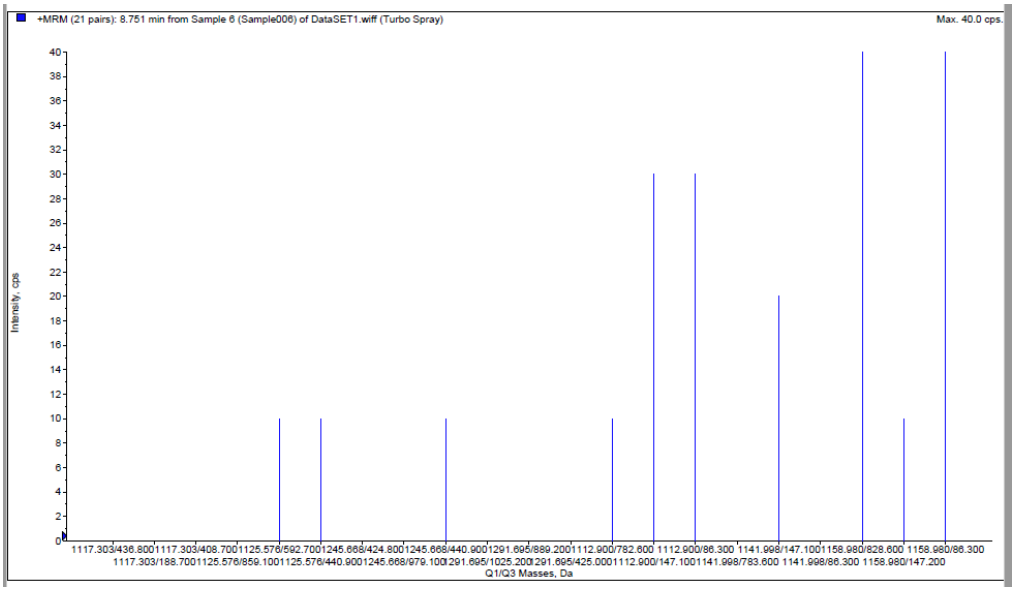


Fig. 6.5: Mass Spectral Chromatogram for CTX Extract

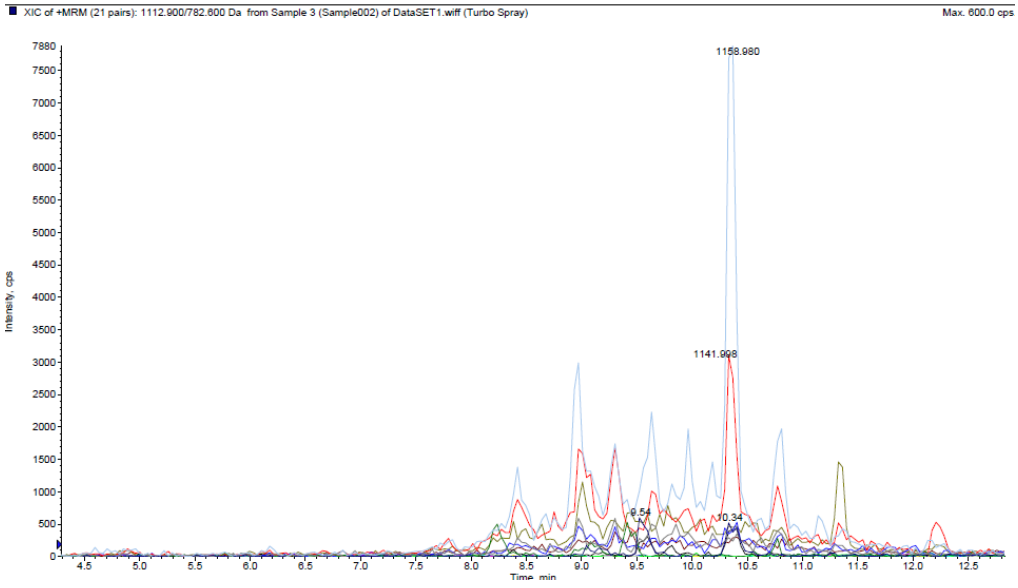


Fig. 6.6: XIC of molecular masses identified as m/z 1158.980 and 1141.998 Da

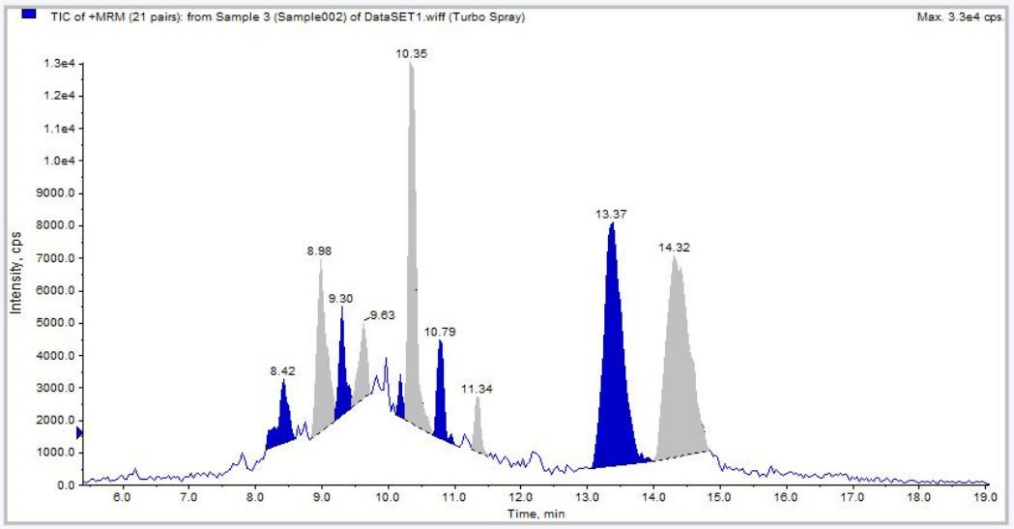


Fig. 6.7: TIC of molecular masses identified as m/z 1158.980 and 1141.998 Da

Table 6.3: Peak list of XIC and TIC shown in Figure 6.6 & 6.7

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	8.4152	20319e4	2.1147	1966.0084	2.4759	0.4412
2	9.3013	2.0480e4	2.1315	3351.1555	4.2203	0.2574
3	9.6320	1.6921e4	1.7610	2311.8133	2.9114	0.2574
4	10.3496	7.8768e4	8.1978	1.2068e4	15.1978	0.4044
5	11.3414	1.0502e4	1.0930	1803.2441	2.2709	0.2574
6	13.3682	1.4548e5	15.1407	7604.0441	9.5762	0.9193
7	14.3152	1.4979e5	15.5896	6248.6618	7.8693	0.8825

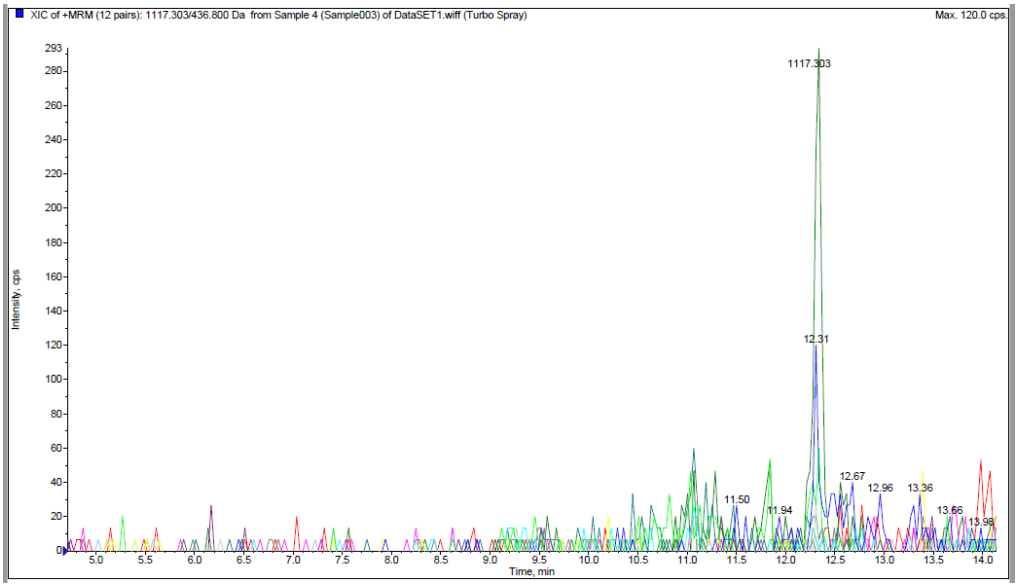


Fig. 6.8: XIC of molecular masses identified as m/z 1117.303 Da

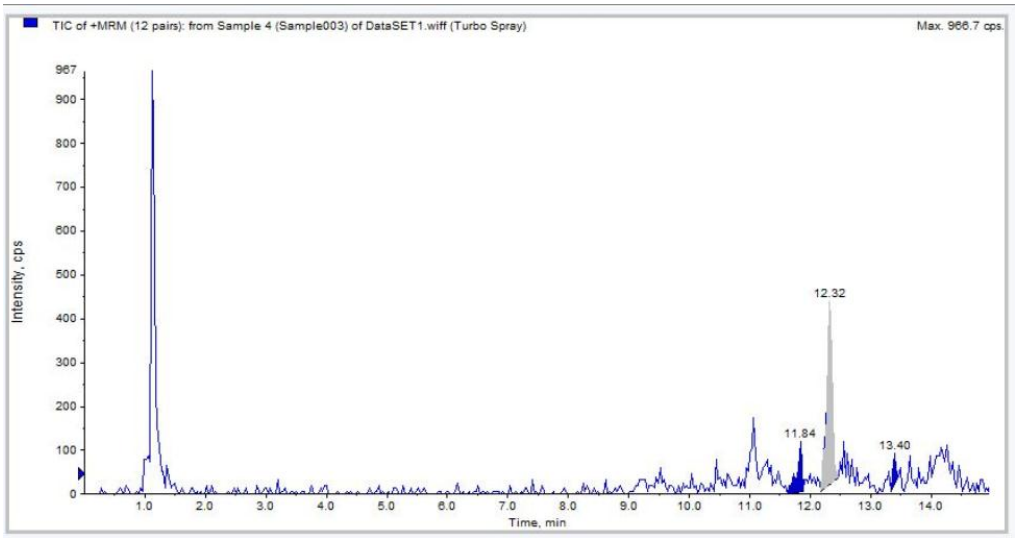


Fig. 6.9: TIC of molecular masses identified as m/z 1117.303 Da

Table 6.4: Peak list of XIC and TIC shown in Figure 6.8 & 6.9

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	11.8386	589.0778	17.1484	116.3458	18.7811	0.2480
2	12.3193	2604.3085	75.8128	433.5551	69.9864	0.2790
3	13.3962	241.7987	7.0389	69.5836	11.2325	0.1240

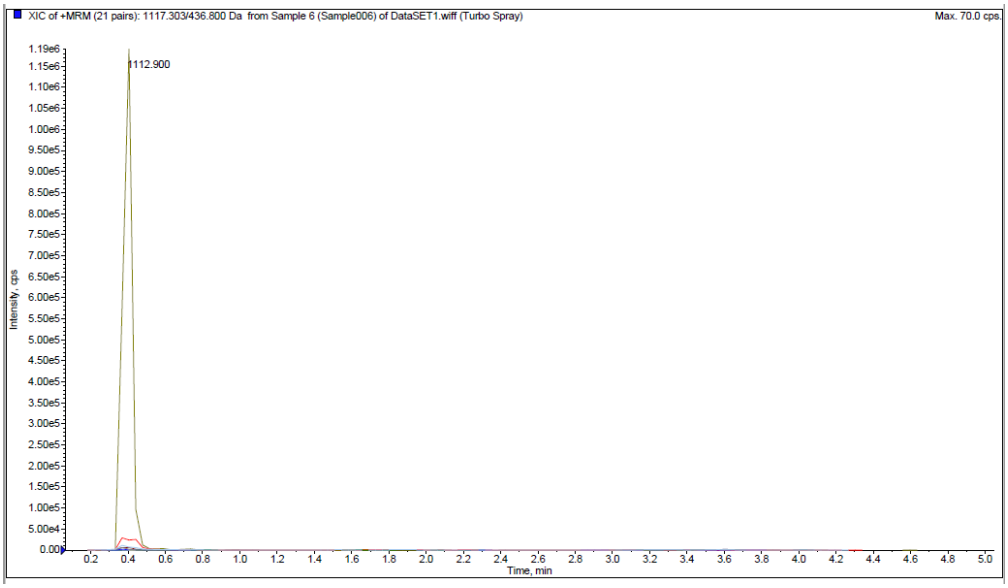


Fig. 6.10: XIC of molecular masses identified as m/z 1112.900 Da

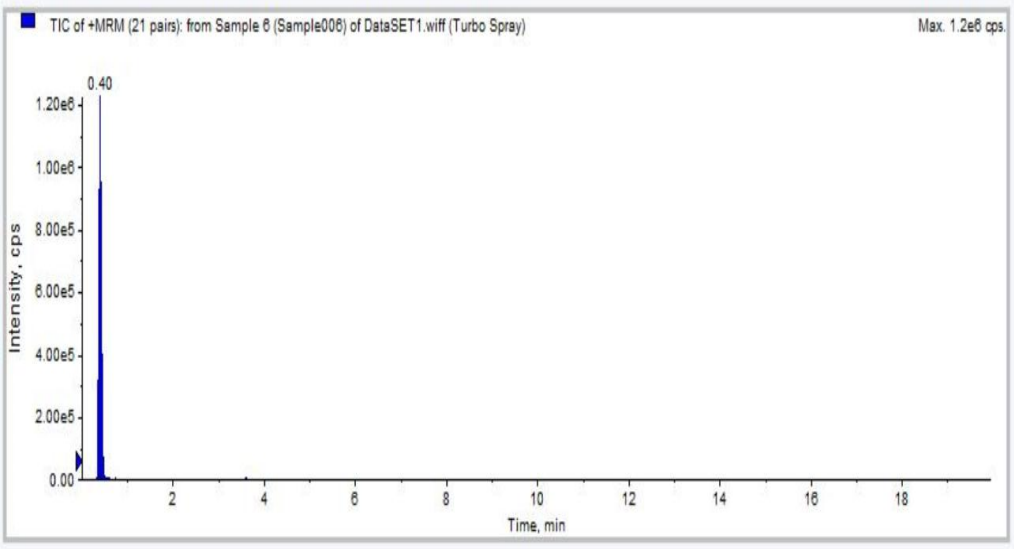


Fig. 6.11: TIC of molecular masses identified as m/z 1112.900 Da

Table 6.5: Peak list of XIC and TIC shown in Figure 6.10 & 6.11

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	0.3391	4.4856e6	100.00	1.2476e6	100.00	0.4780

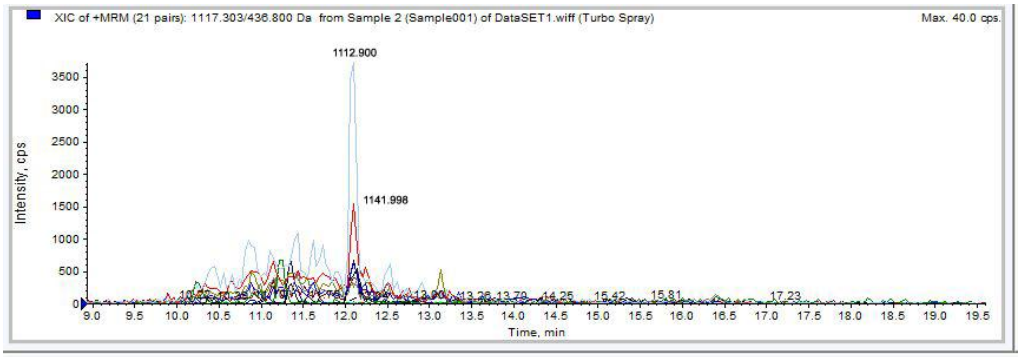


Fig. 6.12: XIC of molecular masses identified as m/z 1141.998 and 1112.9 Da

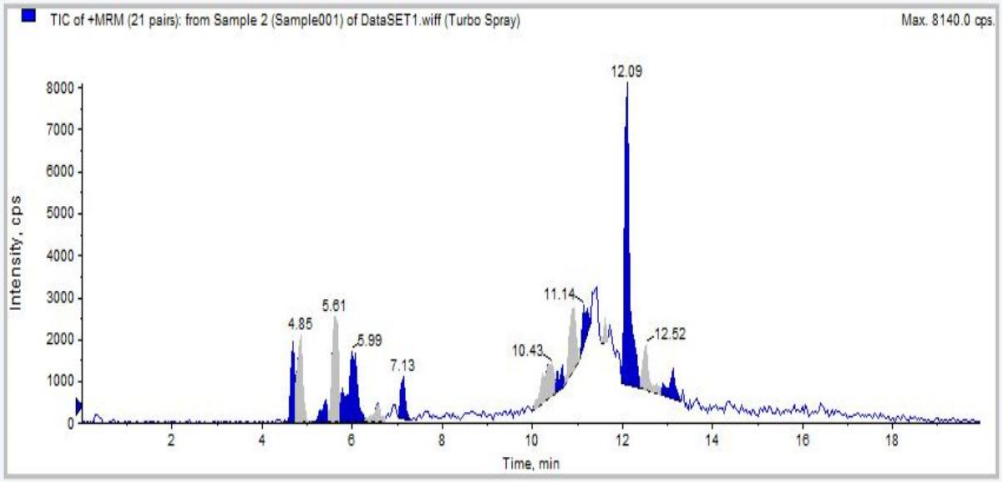


Fig. 6.13: TIC of molecular masses identified as m/z 1141.998 and 1112.9 Da

Table 6.6: Peak list of XIC and TIC shown in Figure 6.12 & 6.13

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	7.1260	6949.3310	3.3529	1022.0005	4.2884	0.2574
2	10.8989	1.5311e^4	7.3874	1547.0065	6.4914	0.3309
3	11.1413	6695.6954	3.2306	949.977	3.9863	0.2206
4	11.6154	1654.7377	0.7984	543.1211	2.2790	0.1103
5	12.0910	5.5983e^4	27.0110	7258.3926	30.4570	0.4045
6	12.5226	1.0932e^4	5.2743	1176.4091	4.9363	0.4780

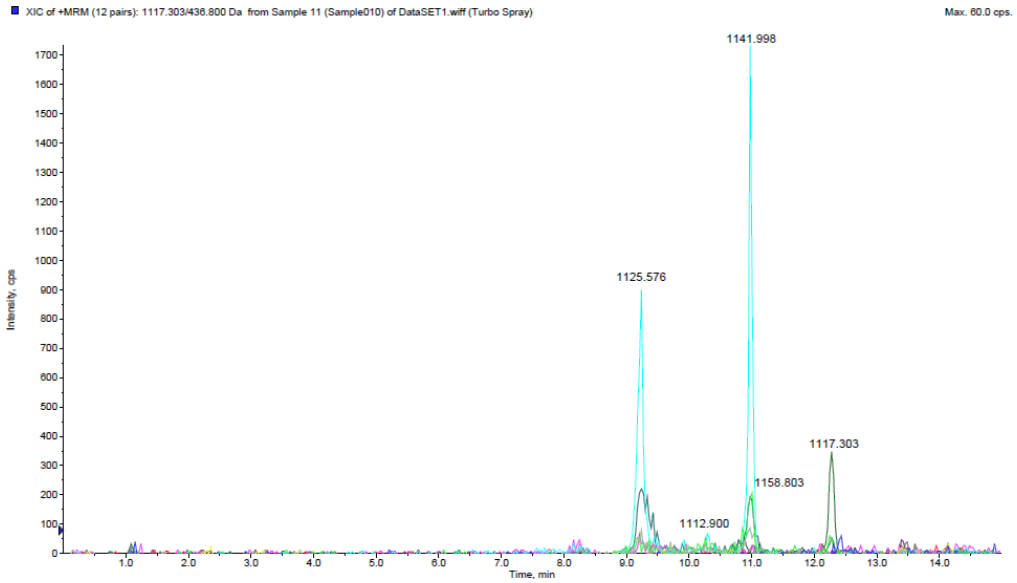


Fig. 6.14: XIC of molecular masses identified as m/z 1117, 1125, 1158 & 1112 Da

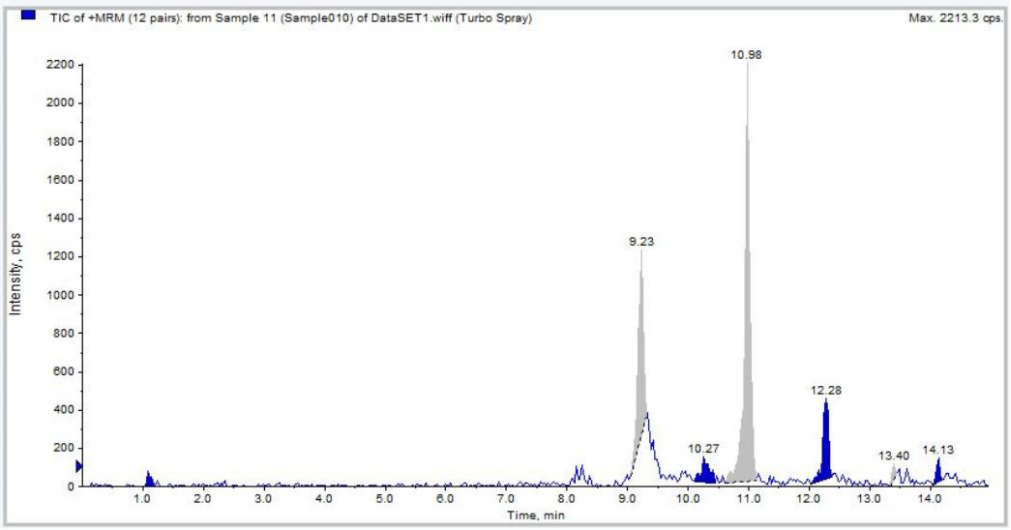


Fig. 6.15: TIC of molecular masses identified as m/z 1117, 1125, 1158 & 1112 Da

Table 6.7: Peak list of XIC and TIC shown in Figure 6.14 & 6.15

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	1.0896	396.8334	1.7414	80.9533	2.0126	0.1860
2	9.2324	5419.5348	23.7825	977.0829	24.2911	0.2480
3	10.2663	1252.5338	5.4965	137.5399	3.4194	0.3410
4	10.9791	$1.2302e^4$	53.9869	2199.3952	54.6787	0.4961
5	12.2786	2591.8580	11.3738	417.5640	10.3810	0.3410
6	13.3995	303.7952	1.3331	81.4100	2.0239	0.1240
7	14.1340	520.8573	2.2857	128.4527	3.1934	0.1550

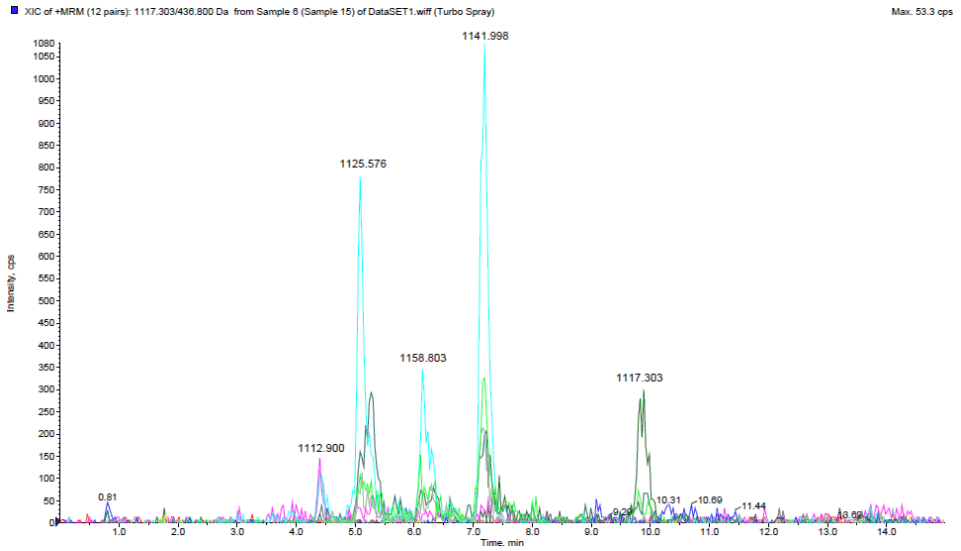


Fig. 6.16: XIC of molecular masses identified as m/z 1117, 1125, 1158, 1141 & 1112 Da

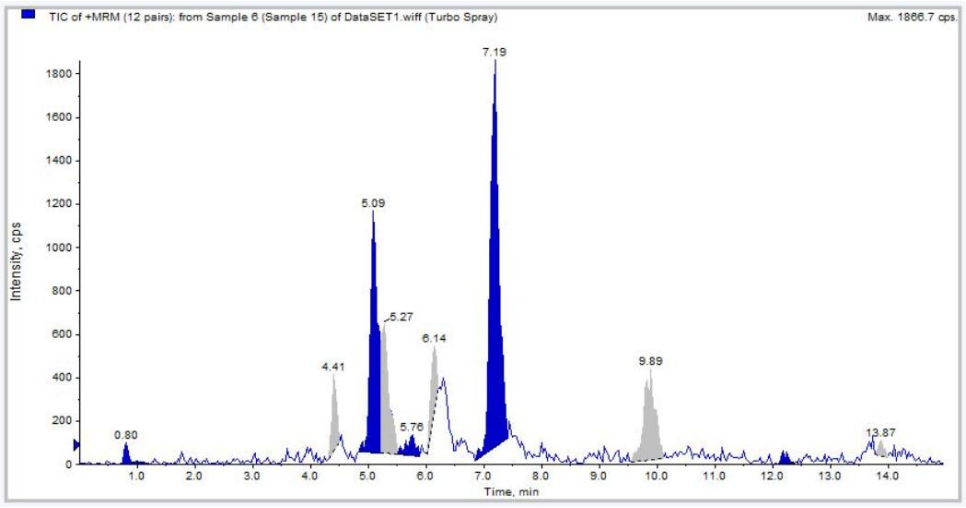


Fig. 6.17: TIC of molecular masses identified as m/z 1117, 1125, 1158, 1141 & 1112 Da

Table 6.8: Peak list of XIC and TIC shown in Figure 6.16 & 6.17

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)
1	0.8049	706.8845	1.6029	100.0633	2.0382	0.5271
2	4.4082	1767.3209	4.0076	352.3383	7.1767	0.1860
3	5.0870	9311.8158	21.1156	1112.6793	22.6639	0.3720
4	5.2666	5518.2785	12.5133	595.9214	12.1382	0.3100
5	5.7574	975.3412	2.2117	100.4697	2.0464	0.3720
6	6.1431	1829.0016	4.1475	294.6334	6.0013	0.1860
7	7.1887	$1.7914e^4$	40.6218	1792.9958	36.5211	0.5581
8	9.8910	5196.2061	11.7830	418.6337	8.5270	0.5581
9	12.1774	421.6310	0.9561	66.5065	1.3547	0.2790
10	13.8690	458.8704	1.0405	75.2379	1.5325	0.2170

As per the new extraction method, the molecular ions m/z 1141 and 1158 has been reported from Indian Ocean by different researchers (Hamilton et al., 2002a; 2002b; Pottier et al., 2002a). The other molecular masses identified m/z 1112, 1117, 1125 had shown the mass spectra reported for C-CTX 1 and C-CTX 2 (Lewis et al., 1991; Satake et al., 1996; Vernoux & Lewis, 1997). The new molecular ions identified using the new extraction method showed similarity to Indian Ocean CTX reported. Initially different extraction methods were included as size exclusion chromatography and C_{18} & silica SPE methods to optimize the CTX determination methods in MS using the positive samples collected from South West Coast of India. These samples were very low in quantity as it is procured in the form of fish remnants and only few molecular ions were determined using CREM and preparatory HPLC extraction methods. The molecular ions obtained through the previous methods include 1117, 1125, 1245 and 1291 Da. Two molecular ions 1117 and 1125 Da are repeatedly getting the newly isolated methods. There is

considerable loss of quantity of toxic extract while doing the previous extraction methods, from the lipid fractions. Since CTX is a lipophilic compound, the toxin is accumulated in the unsaponifiable part of the lipid and through the Folch extraction method NSM is completely separated. NSM is again passed through the SPE for the purification of toxin content. Through the NSM separation, it was concluded that the interference of other fatty acid esters were omitted and the separation of crude toxin content is comparatively much easier. MS detected the presence of 1245 and 1291 as an interference of other lipid fractions with CTX congeners which alter the tuning of ions. Hence NSM separation method showed that m/z 1117.303, 1112.9, 1141.998, 1158.980 and 1125.576 Da as the molecular masses for I-CTX from *L. bohar* samples along Indian coast.

6.4 Conclusion

As per the new extraction protocol, the molecular masses for Indian Ocean CTX were identified as m/z 1112.900, 1117.303, 1141.998, 1158.980 and 1125.576 Da. The lethal dose value obtained for extracted *L. bohar* specimens were comparatively low compared to the other human intoxicated fish samples collected from Ullal, Mangalore and Vizhinjam, Trivandrum. But the toxin recovery of eluted samples showed good peaks with maximum intensity in less toxic samples. Hence it was proved that the developed

extraction method is suitable for the determination of trace quantities of CTX from fish samples. The characterized ions are related with the molecular mass reported for I-CTX and C-CTX from different coasts. CTX characterization based on their molecular masses has been reported for the first time from Indian Coast. The new congeners of CTX with molecular masses 1112.900, 1117.303 and 1125.576 Da were isolated from *L. bohar* samples. Molecular masses 1141.998 and 1158.98 were identical to the masses reported by Hamilton et al. (2002b). Now there is a need for the identification of structural relationship of I-CTX ions for the confirmation of the isolated molecular masses. The molecular masses were validated by using NMR study and structural comparison of CTX ions.

Chapter 7

Molecular Structural Characterization and Comparison of Indian Ocean Ciguatoxin (I-CTX) Isolated from *Lutjanus bohar*

Contents

7.1 Introduction

Families of structurally related cyclic polyether toxins resembling the brevetoxin (PbTx) class of toxins are involved in ciguatoxin food borne disease (Lewis et al., 1991; Lewis & Jones, 1997; Lewis et al., 1998). Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) methods are used to characterize CTXs. For Pacific CTX, two types are reported, type-1 having 13 numbers of ether rings and 60 numbers of carbons and type-2 having 13 numbers of ether rings and 57 numbers of carbons (Murata et al., 1990; Lewis et al., 1991; Lewis et al., 1993; Satake et al., 1993b). Caribbean ciguatoxins C-CTX 1 and C-CTX 2 had 14 number of ether rings and 62 number of carbons (Lewis et al., 1998). Details of CTX structural elucidation based on number of ether rings and number of carbons were given in table 7.1. Murata et al. (1990)

reported structure of CTX from Moray eel and its precursor from *G. toxicus* on the basis of NMR & MS measurements. In this chapter an attempt has been made to elucidate and compare the structure of Indian Ocean Ciguatoxin (I-CTX) isolated from *Lutjanus bohar*.

Table 7.1: Details of CTX structures elucidated based on ether rings and carbons

Origin		No. of rings	No. of carbons	Molecular weigh	References
P-CTX	Type 1	**13**	**60**	1110.6 1094.5 1060.8	Murata et al., 1990 Lewis et al., 1991 Lewis et al., 1993 Satake et al., 1993b
	Type 2	**13**	**57**	1022.8 1056.0	
C-CTX		**14**	**62**	1141.6	Lewis et al., 1998
I-CTX		-	-	1140.6 1157.6	Hamilton et al., 2002b

7.2 Materials and methods

I-CTX extracted from *L. bohar* samples using the newly developed extraction protocol were subjected to UV visible spectrometric and IR analysis. NMR spectra of samples were combined for the identified molecular ions.

7.2.1 UV-Visible NIR Spectrophotometer

An absorbance maximum of ciguatoxin was determined by recording UV–Vis absorption spectra using a Varian, Carry 5000 spectrophotometer over a spectral range of 175 to 3300 nm.

7.2.2 Fourier Transform Infra-Red spectrometer (FTIR)

FTIR spectra was recorded using a Thermo Nicolet, Avatar 370 spectrometer (Waltham, USA) using Potassium bromide beam splitter and detector over a spectral range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1}. IR spectrum is useful in identifying the functional groups like –OH, -CN, -CO, -CH, $-NH_2$ etc.

7.2.3 Nuclear Magnetic Resonance (NMR)

All NMR spectra were recorded on Bruker Avance III 400 MHz spectrometer. 1D and 2D NMR spectra of Ciguatoxin purified extract were measured using 5 mm PABBO BB probe. Methanol-d_4 was used as solvent. The proton connectivity including hydroxyl protons was mainly established by ^1H -^1H COSY data.

7.3 Results and Discussion

Mass Spectra for new ciguatoxin congeners I-CTX 1141.998, 1158.98, 1117.303 1112.9 and 1125.576 Da were isolated from toxic *L. bohar* samples (Figure 7.1). A highly toxic fish is needed for attaining the completely purified CTX recovery in appropriate level from a sample matrix. Earlier reports on C-CTX and P-CTX structural elucidation data showed that, these were isolated from fishes weigh around more than 50kg or from the cultures of

dinoflagellate *G. toxicus* (Murata et al., 1990; Lewis et al., 1991; Satake et al., 1996; Vernoux & Lewis., 1997; Lewis et al., 1998). Whereas in this study the toxic samples weighed around five kilograms only and the quantity of purified toxin were present only in trace amount. The complete structural elucidation of CTX is a tiresome work and the trace amount of pure sample made it unattainable. Occurrence of CTX reported only two times from Indian waters and in west coast it is the first time. During the study period highly toxic samples were not traced out from Indian coast and forced to limit our studies to the possible end.

From the isolated samples, the purified extract eluted in trace levels as white amorphous solids which showed the presence of CTX in *L. bohar* samples. These fish specimens showed a 2.08 MU of observed mouse lethality, which is comparatively smaller than 2.5MU/100g fish suggested by Yasumoto et al. (1984). The NMR spectra of extracted and purified samples showed a comparative structural similarity and chemical shifts related to Caribbean CTX (Lewis et al., 1998). The even mass obtained for I-CTX with m/z 1141.9 is taken for the authentication of structural similarity with C-CTX. It indicated that, it contain even number of nitrogen, same as in the case of polyether toxins (Lewis et al., 1991; Lewis et al., 1998). Two dimensional ^{1}H-^{1}H NMR and one dimensional proton NMR, FTIR and Tandem Mass Spectrometry measurements

indicated that I-CTX was comparable to C-CTX1 and C-CTX 2 isolated previously (Murata et al., 1990; Lewis et al., 1991; Lewis et al., 1998).

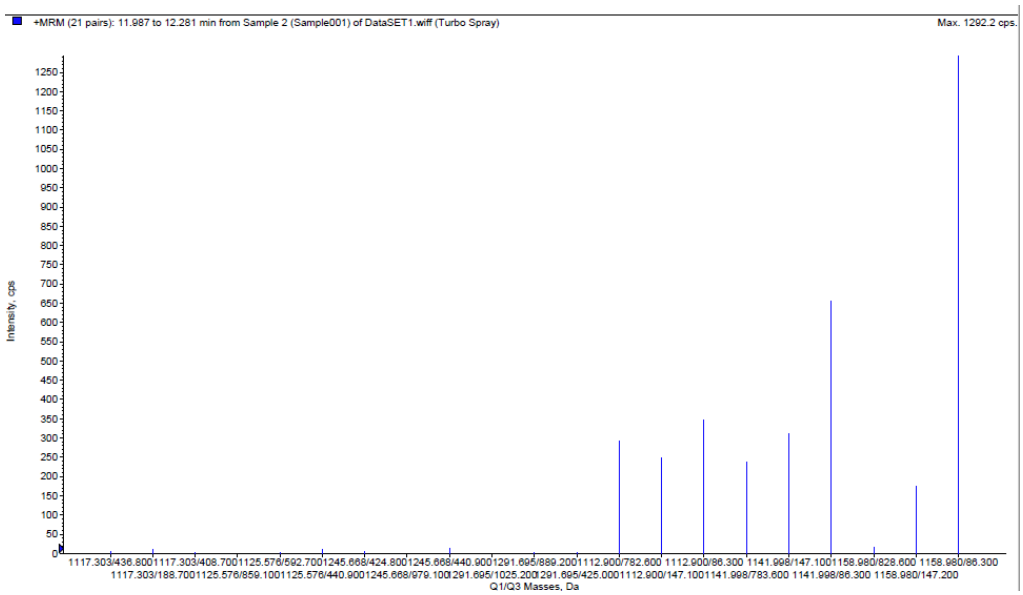


Fig. 7.1: Q1 and Q3 Mass Spectra of m/z 1117, 1112, 1141, 1158 and 1125 molecular ions

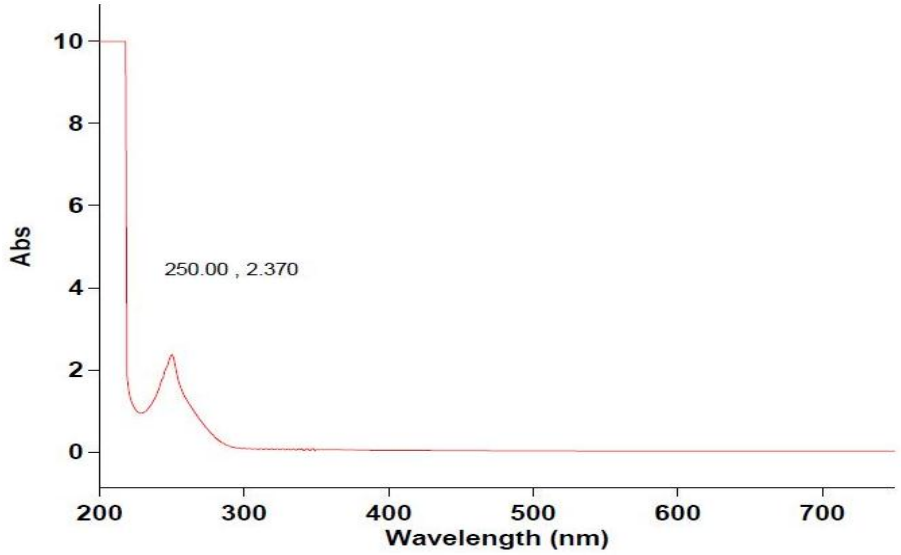


Fig. 7.2: UV Visible NIR Spectrum of CTX extract at 750 to 200nm (at wave length 250nm and absorbance at 2.37)

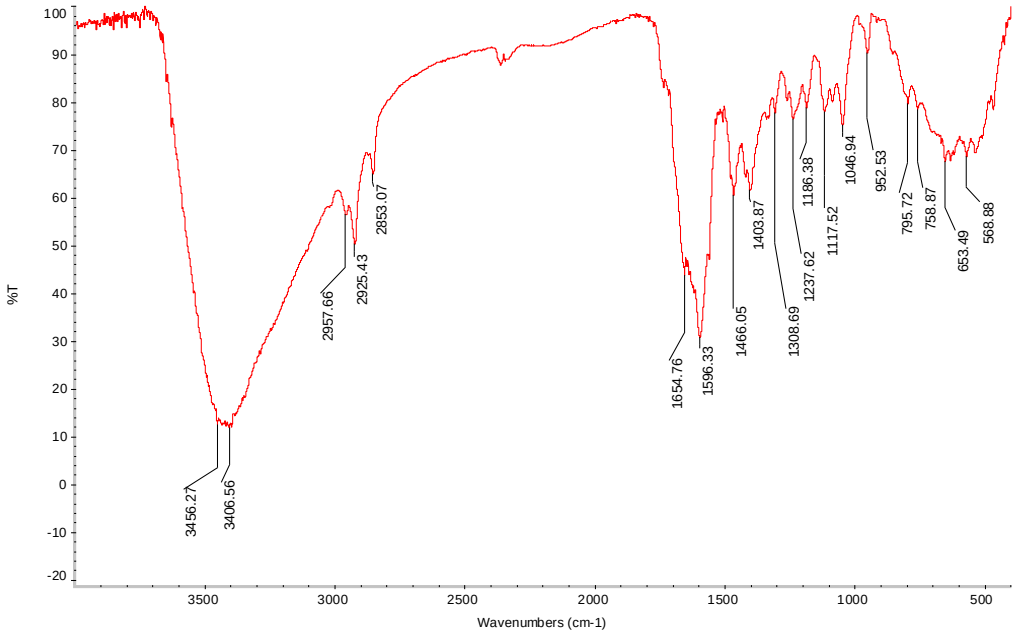


Fig. 7.3: Infrared Spectrum of CTX extract exhibited major characteristic bands at around the region 3200-2500 cm^{-1} and 2000- 500 cm^{-1}

CTX displays a single UV absorption peak at 250nm and absorbance at 2.37 (Figure 7.2). The most prominent features of Infrared spectrum (FT, Solid film) (Figure 7.3) of ciguatoxin are hydroxyl (3456 cm^{-1}) and ether (1046 - 1117 cm^{-1}) bands. C-H stretching vibration occurred around 3000 cm-1 region. Figure 7.4 gives the two dimensional 400 MHz ^{1}H- ^{1}H COSY Spectra of Indian Ocean CTX. The spectrum was obtained in deutero-methanol at 670K using a 400 MHz instrument. Figure 7.5 and 7.6 indicates ^{1}H NMR Spectrum in which I-CTX peaks are labelled. Table 7.2 gives ^{1}H Chemical shifts and couplings of I-CTX isolated from *L. bohar* compared with C-CTX -1.

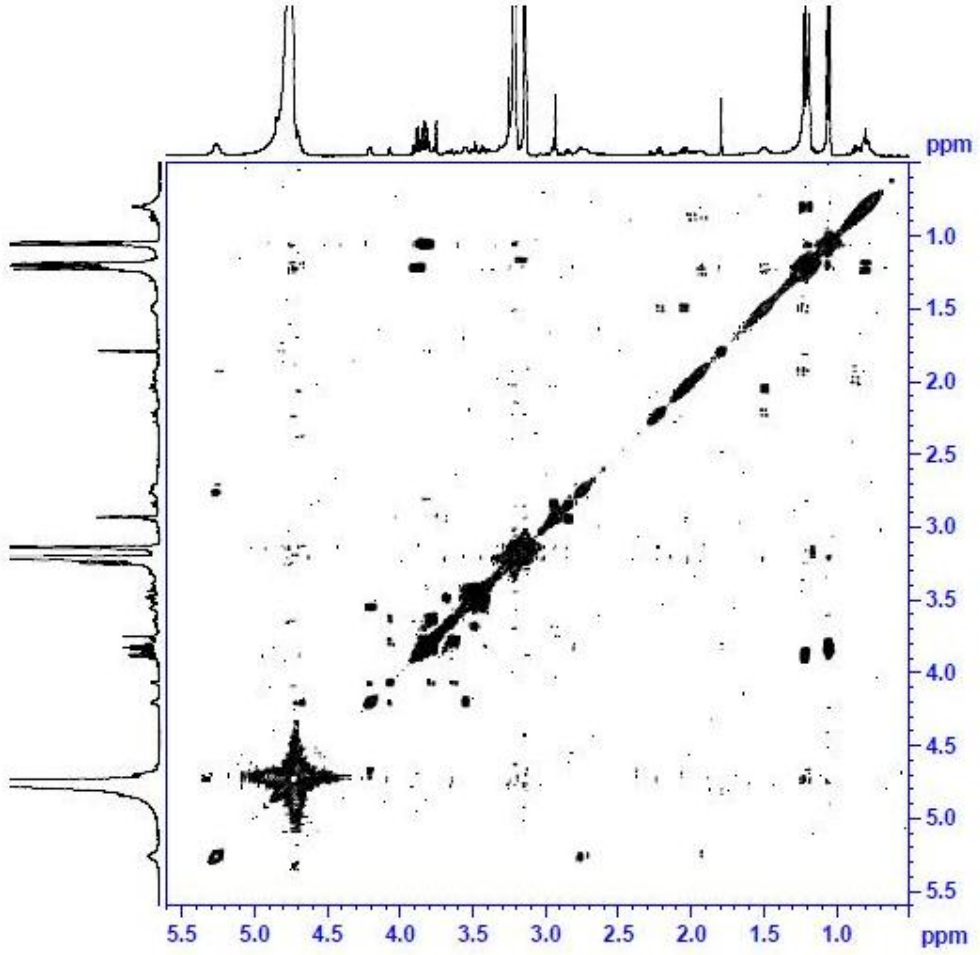


Fig. 7.4: Two dimensional 400 MHz ^{1}H- ^{1}H COSY Spectra of Indian Ocean CTX. The spectrum was obtained in deutero-methanol at 670K using a 400 MHz instrument.

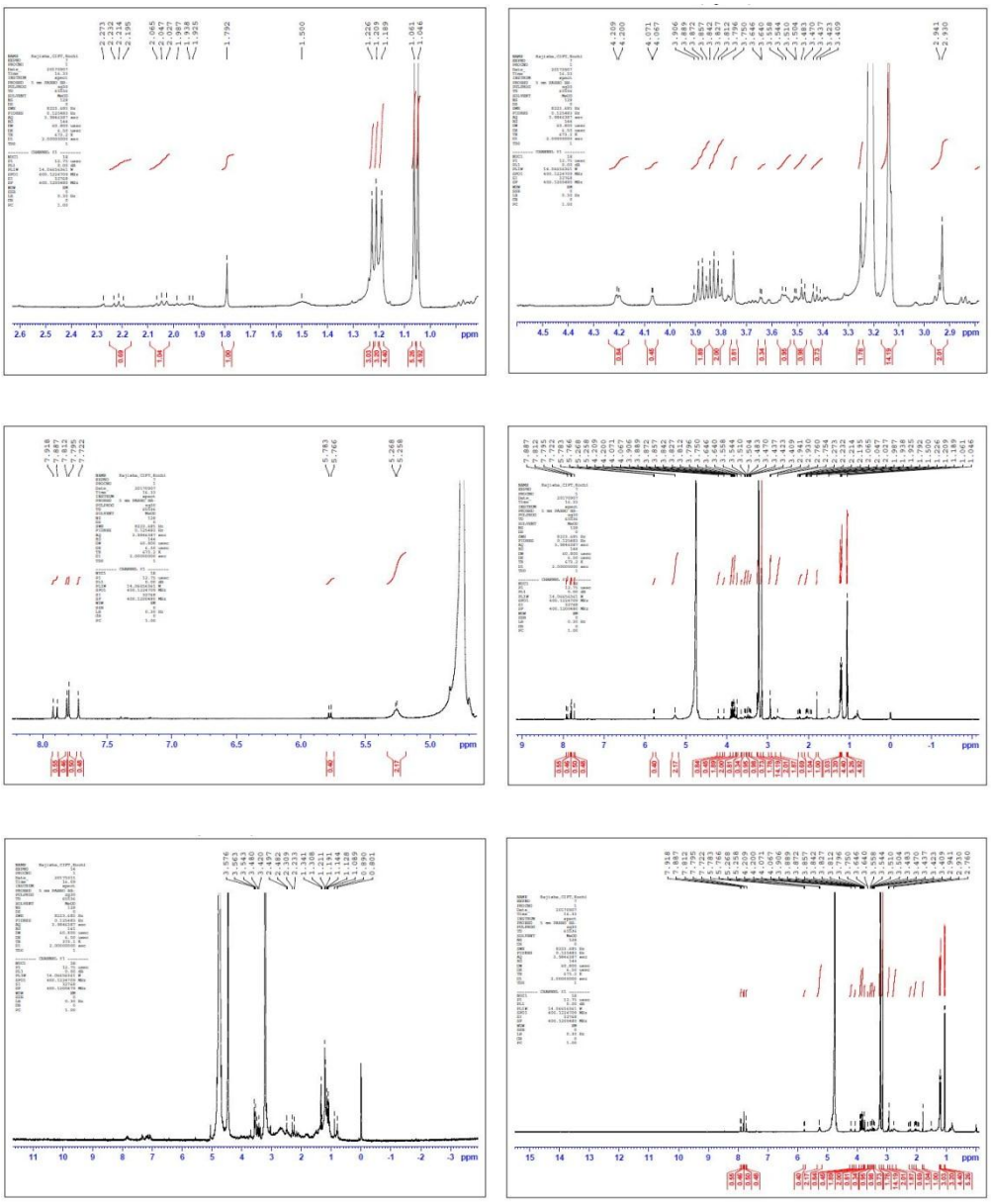


Fig. 7.5: ^{1}H NMR Spectrum of I-CTX

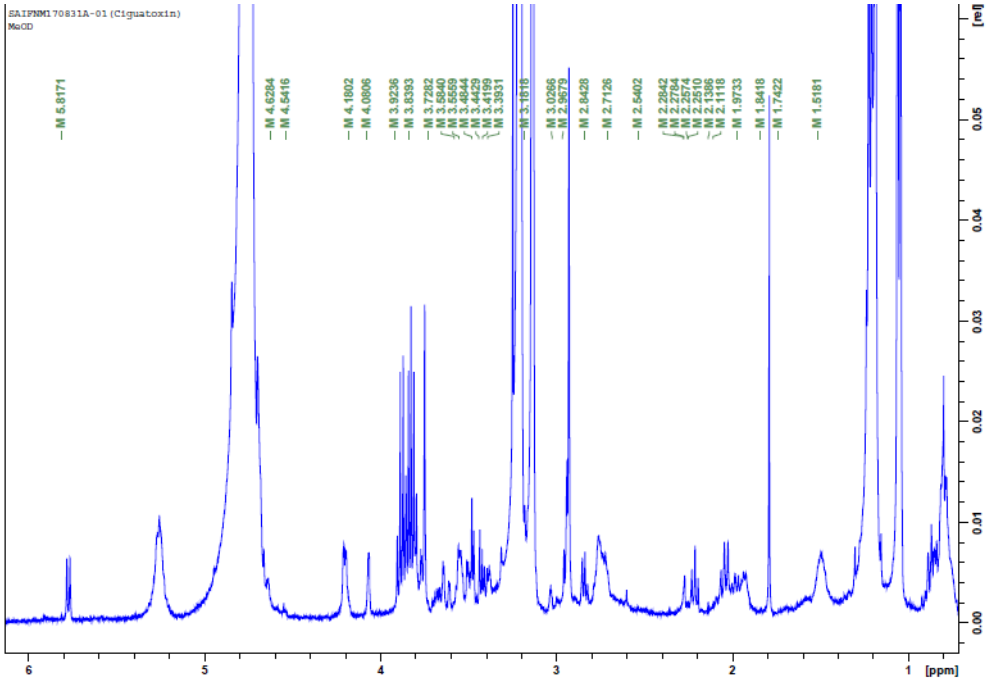


Fig. 7.6: The ^{1}H NMR spectrum in which I-CTX peaks are labeled

Table 7.2: ^{1}H Chemical shifts and couplings of I-CTX isolated from *L. bohar compared with C-CTX -1*

Position	I-CTX	C-CTX-1		Position	I-CTX	C-CTX-1
1	3.9236	3.94		22	2.2842	2.28
1	3.8393	3.83		25	2.9679	2.96
2	2.1118	2.12		25	2.2784	2.27
4	2.7126	2.71		26	3.7282	3.72
4	2.1118	2.11		27	3.584	3.58
5	3.3931	3.39		28	2.5402	2.53
6	3.4844	3.48		32	2.2574	2.25
7	1.7422	1.71		34	3.4429	3.44
8	3.1818	3.18		35	1.5181	1.51
10	1.8418	1.79		41	4.0806	4.08
11	3.4199	3.42		42	3.5559	3.55
14	5.8171	5.82		46	4.5416	4.54
15	4.1802	4.19		47	2.251	2.3
17	2.8428	2.84		50	1.9733	1.95
17	2.251	2.28		52	4.6284	4.62
22	3.0266	3.02		55	2.1386	2.14

I-CTX structure was compared with the C-CTX based on NMR experiments and Mass Spectrometry measurements. I-CTX is also considered as an exclusively trans-fused polyether compound (Lewis et al., 1998). This compound contains most of the ether rings. Proton NMR showed peaks according to the spectrum of C-CTX reported by Lewis et al. (1991 & 1998). Some peaks are merged together with peaks of other compounds in the sample, which is considered as small impurities. '*H NMR* studies have shown that it is a polar and highly oxygenated molecule belonging to the class of polyether compounds. Tachibana et al. (1987) proposed a molecular formula $C_{53}H_{77}NO_{24}$ for CTX with molecular mass 1112.2 Da. Legrand et al. (1998) suggested a probable molecular formula of $C_{60}H_{86}O_{19}$ for m/z 1111.5840. Presence of primary hydroxyl group is identified from the '*H NMR* spectra and it also showed the presence of 86 protons in the molecular structure of I-CTX. But in the absence of C-13 spectrum it is impossible to count the number of carbons and the degree of overlap. A satisfactory C-13 spectrum of I-CTX was not able to determine because of lack of toxin and attempts to isolate the larger crystals of toxin were failed. Researchers considered CTX as a single entity compound (Tachibana et al., 1987; Murata et al., 1990). In Pacific Type I and Type II, side chain carbons were present, which is absent in the structure of Caribbean CTX. In case of I-CTX, proton NMR spectrum showed the absence of side chain carbons. Figure 7.7 indicates probable structure of CTX comparable with I- CTX.

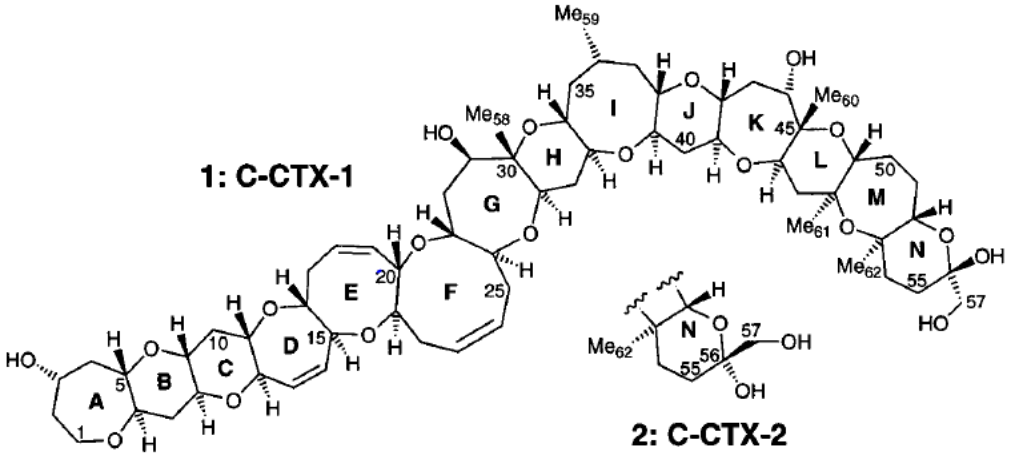


Fig. 7.7: Probable structure of CTX comparable with I- CTX

7.4 Conclusion

Structural similarity of I-CTX is almost closely related with C-CTX. The new molecular ions identified for Indian Ocean CTX showed similarity with Caribbean CTX. There is no similarity with Pacific Type - I and Type - II CTX. The NMR studies proved that the Caribbean and Indian ocean CTX are structurally and chemically existed as multiple ions in the form of lipid containing quaternary nitrogen, hydroxyl and carbonyl groups. Structural comparison of I-CTX is correlated with the C-CTX, but a complete structural elucidation was not possible from the data. This is because of the trace amount of toxin present in the specimens. Peaks are completely eluted with a good spectral data based on the quantity of toxin present in purified form. Hence the presence of a highly toxic fish is needed for the increased amount of purified CTX from samples. Therefore it was assumed that the presence of I-CTX in

the west coast Indian waters and it may be due to climate change and other environmental interruptions by which our coral ecosystem also started to inhabit the CTX accumulated toxic fishes or proliferation of the causative dinoflagellate *G. toxicus*.

Chapter 8

Summary and Recommendations

Incidents of CFP from Indian coast are an early warning to our fishery sector with great concern over seafood safety and risk assessment. Three incidents were reported from our coast during the study period (Rajeish et al., 2016; Rajisha et al., 2017a; 2017b) hence research in the area of emerging CTX toxicity is a matter of concern. The presence or existence of CTX from reef fishes also indicates a gradual proliferation of *G. toxicus* in our coral waters, which has not been reported earlier and this may be attributed to climate change and associated disruption of ocean beds and reefs, causes damage to animals and humans via dispersal of toxin. Symptoms related to CFP are quiet distinctive in nature and varies according to different geographical region, therefore prevention is the key method. A mandatory strategy taken at the distributor/marketing and government level to understand the details of fishing grounds, where the fish is coming from and size restrictions for carnivorous fishes will definitely block the extent of toxin into consumer level. Research in the area of CTX toxicity will be considered as an initial step to ensure the safety of highly priced and tasted reef fishes along with the export economy.

Under the doctoral programme, studies were conducted on the screening and investigation of ciguatoxin implicated in different finfishes inhabiting coral ecosystem along Indian Coast. Proper identification of fishes were ensured by morphological and DNA sequencing method. Primary investigation and screening of all the collected specimens were done by Mouse Bioassay and other biochemical parameters are tested for confirmation of toxicity in mice. Clinical manifestations of CTX associated in humans were also studied. Optimization of ciguatoxin using mass spectrometry was conducted by chromatographic extraction methods. A novel extraction method from Indian Ocean CTX were developed using toxic *L. bohar* fish samples. Structural comparisons of detected molecular masses were done using NMR, FTIR and UV visible NIR data.

The details of the study can be summarized as follows:

- A total of 262 samples were collected and investigated for screening of ciguatoxicity along Indian Coast from January 2015 to December 2017.
- The fish specimens (20 different species) screened for toxicity comprised of *Lutjanus argentimaculatus, L. fulvus, L. bohar, L. gibbus, L. johnii, Pristipomoides filamentosus, Pinjal pinjalo, Aprion virescens, Variola louti, Epinephelus bleekeri, E. coioides, E.*

diacanthus, E. chlorostigma, E. polylepis, E. merra, Otolithoides biauritus, Caranx ignobilis, Sphyraena putnamae, S. jello and *Lethrinus nebulosus.*

- Data based on previous reports of ciguatoxicity showed that among the collected specimens *L. bohar* was reported to be the most common fish implicated in ciguatoxicity.
- DNA barcoding helps to confirm the species level identification of unknown samples collected in degutted, filleted forms and fish body parts (head, tail, viscera etc.) among the 20 different finfish species.
- 30 numbers of fish samples were sequenced and deposited in GenBank NCBI with accession numbers.
- Phylogenetic tree of species based on COI gene showed its similarity to other respective sequences.
- *Lutjanus bohar* commonly known "*Chempalli* or Red snapper" has identified as toxic sample from four different locations, viz., Vizhinjam, Thoppumpady, Kollam (Kerala Coast) and Mangalore (Karnataka coast). All the samples tested weighed more than 5 kg.

- Out of the 262 samples investigated for Mouse Bioassay, 12 samples were detected as positive and the lethal dose estimated in Mouse Unit were 3.15, 2.17 and 2.08/100 of fish ether extract.
- LD_{50} value based on all the 12 toxic samples assayed for *in vivo* Mouse bioassay along Indian Coast was estimated as 14.3µg/kg in mice.
- Clinical symptoms of data collected from affected individuals showed similarity among the symptoms reported earlier from Indian Coast.
- The symptomatology in the hospitalized patients verified with earlier reports of Ciguatera Fish Poisoning include typical clinical signs like gastrointestinal, neurological and cardiovascular symptoms.
- Organ specific toxicity were observed in liver and brain sections of intoxicated mice, with alterations in liver enzymes (ALT & AST) and also in total protein level in blood.
- The development and verification of additional chemical methods for CTX will depend upon the Mouse Bioassay result.
- Determination of CTX detection was standardized using an API 4000 QTRAP MS/MS system.
- The ultimate isolation of CTXs from pre-purified extracts obtained after liquid-liquid extraction, solid-phase extraction, and size-exclusion

chromatography were achieved by reversed-phase C_{18}, normal phase silica and HPLC columns. Peak intensity is higher in the SPE fractions, proved considerable losses in the toxin level while doing the HPLC separation of ether extract.

- Survey scan method tuned for all cyclic polyether toxins (m/z 700 to 1400) and MRM based tuning method is followed in the mass range m/z 900 to 1300Da as per the reported CTX molecular fractions.
- CTX toxic fractions collected through Sephadex LH-20 size exclusion chromatography eluted as four toxic zones at each chromatographic step. Splitting the output to a mass spectrometer, under ESI conditions and collected fractions identified the CTXs with m/z I-CTX-1117.303, I-CTX-1125.576, I-CTX-1245.668 and I-CTX-1291.695 Da.
- Developed a new extraction method for purification of Indian Ocean Ciguatoxin using the separation of non Saponifiable matter and Florisil and PSA solid phase extraction methods
- MRM method is developed for the analysis of I-CTX using all the tuned molecular ions. Detected the presence of molecular masses m/z 1245.668 and 1291.695 Da identified using the size exclusion chromatography and CREM method, as an interference of other lipid fractions with CTX congeners which alter the tuning of ions.

- From Indian Ocean five CTX congeners were identified using the newly developed extraction method having molecular masses m/z 1117.303, 1112.900, 1141.998, 1158.803 and 1125.576, named as I-CTX-1117, I-CTX-1112, I-CTX- 1141, I-CTX-1158 and I-CTX- 1125 respectively.
- The new extraction method was developed with high toxin recovery and peak intensity from less toxic fish samples.
- The new molecular ions identified for Indian Ocean CTX showed similarity with Caribbean CTX. Two dimensional 400 MHz ^{1}H- ^{1}H COSY and ^{1}H NMR spectra of Indian Ocean CTX was obtained in deutero-methanol at 670K using a 400 MHz instrument. Chemical shifts from proton NMR and FTIR data showed similarity with Caribbean ciguatoxin. Indian ocean CTX is structurally and chemically existed as epimers or as multiple ions in the form of lipid containing quaternary nitrogen, hydroxyl and carbonyl groups.

Recommendations

Ciguatoxin has not been reported from Indian coastal regions before 2016. Now there is a prevalence of ciguatoxin from Indian Coast. That indicates the shift in the habitat of causative organisms producing CTX known as G. *toxicus*, may be due to climate change. Hence management is needed for protection of our aquatic habitats so that *G. toxicus* does not scatter and get bio accumulated in to our fishes. Now there is an urgent need to periodically survey all the reef fishery specimens which is part of our exporting and local marketing fisheries sector. A complete data base based on the analytical observations of fish specimens is needed for regular monitoring of CFP from Indian Coast. There is a possibility of bioaccumulation of ciguatoxin in our eco system and most of the fishes are coming under the category of ciguatoxic fishes as mentioned by USFDA hazards and controls guidance 2011 (FDA, 2011). Hence CTX is considered as a potential emerging hazard category and periodically risk analysis study is needed for regular monitoring of ciguatera fish poisoning.

References

Abraham, A., Jester, E.L.E., Granade, H.R., Plakas, S.M. and Dickey, R.W. (2012) Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. Food Chem. 131: pp 192-198.

Aebersold, R. and Mann, M. (2003) Mass spectrometry-based proteomics. Nature, 422 (6928), pp 198-207.

Aiyar, R. G. (1936) Mortality of fish of the Madras coast in June 1935. Curr Sci 4(7): pp488–489.

Allen, G. R. (1985) FAO Species Catalogue- 6.Snappers of the world.An annotated and illustrated catalogue of lutjanid species known to date. FAO, Rome. FAO Fish.Synop. 125(6):208 p.

ANSES, (2016) Méthoded'analyse en sécurité sanitaire des aliments: Détection des ciguatoxinesdans la chair de poisson par bioessaisursouris [Method of analysis for food safety: Detection of ciguatoxins in the Flesh by bio-assay on mouse:], ANSES / LSAliments / LSA-INS-0150 - Version 01, 06/04/2016, French Agency for Food, Environmental and Occupational Health & Safety: Maisons-Alfort, France, 16p.

Antelo, A., Alfonso, C. and Álvarez, M. (2014) Standards for freshwater and marine toxins. In Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, Third Edition, Botana, L. M. (Eds.), CRC Press, Taylor and Francis Group: pp 429-470.

AOAC. (2012) Association of Analytical Communities (AOAC) official methods of analysis, Natural Toxins, Chapter-49, pp 86-88

Arcila-Herrera, H., Castello-Navarrete, A., Mendoza-Ayora, J., Montero-Cervantes, L., González-Franco, M. F., Brito-Villanueva, W. O. (1998) Diez casos de ciguatera en Yucatán. Rev. Invest. Clin. 50 (2): 149-152.

Arena, P., Levin, B., Fleming, L.E., Friedman, M.A. and Blythe, D. (2004) A pilot study of the cognitive and psychological correlates of chronic ciguatera poisoning. Harmful Algae 3: pp 51-60.

Aune, T., Larsen, S., Aasen, J. A.,Rehmann, N., Satake, M. and Hess, P. (2007). Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. Toxicon 49(1), pp 1-7.

Azziz, B. E., Luber, G., Conklin, L., Tosteson, T. R., Granade, H. R., Dickey, R.W. and Backer, L. C. (2012) Assessing the incidence of ciguatera fish poisoning with two surveys conducted in Culebra, Puerto Rico, during 2005 and 2006. Environ. Health Perspect. 120: pp 526–529.

Baden, D., Fleming, L. E., Bean, J. (1995) Marine Toxins in handbook of clinical Neurology: Intoxications of the Nervous System Part-II, National Toxins and Drugs. (deWolff, F.A., ed.) Elsevier Press: Amsterdam, The Netherlands: 21: pp 141-175

Bagnis, R. and Legrand, A. M. (1987) Clinical features on 12,890 cases of ciguatera (fish poisoning) in French Polynesia. In: Progress in Venom and Toxin Research, (Gopalakrishnakone, P. and Tan, C.K., Eds). National University of Singapore and International Society of Toxicology, Asia-Pacific Section, Singapore. pp 372-377.

Bagnis, R., Chanteau, S., Chungue, E., Drollet, J., Lechat, I., Legrand, A., Pompon, A., Prieur, C., Roux, J. and Tetaria, C. (1985) Comparison of the cat bioassay, The mouse bioassay and the mosquito bioassay to detect ciguatoxicity in fish. In Proceedings of the fifth international coral reef congress, Tahiti, French Polynesia, pp. 491- 496.

Bagnis, R., Kuberski, T. and Laugier, S. (1979) Clinical Observations on 3,009 Cases of Ciguatera (Fish Poisoning) in the South Pacific. Am. J. Trop. Med. Hyg., 28: pp 1067-1073.

Banner, A. H., Helfrich, P., Scheuer, P. J. and Yoshida, T. (1963) Research on ciguatera in the tropical Pacific. In Proceedings of the 16th ann. Session Gulf, Caribbean Fisheries Institute, pp. 84-98.

Banner, A., Scheuer, P., Sasaki, S., Helfrich, P. and Alender, C. (1960) Observations on ciguatera type toxin in fish. Ann. N.Y. Acad. Sci., 90: pp770–787.

Baumann, F., Bourrat, M. B. and Pauillac, S. (2010) Prevalence, symptoms and chronicity of ciguatera in New Caledonia: Results from an adult population survey conducted in Noumea during 2005, Toxicon, 56 (5): pp 662–667.

Bhat, R.V. (1981) A report on an outbreak of mussel poisoning in coastal Tamil Nadu, India.National Institute of Nutrition, Hyderabad.

Bhimachar, B. S. and George, P. C. (1950) Abrupt set–backs in the fisheries of the Malabar and Kanara Coasts and "Red Water" phenomenon as their probable cause. Proc Indian Acad Sci 31(B): pp339–350

Bienfang, P., Oben, B., DeFelice, S., Moeller, P., Huncik, K., Oben, P., Toonen, R., Daly-Engel, T. and Bowen, B. (2008) Ciguatera: the detection of neurotoxins in carnivorous reef fish from the coast of Cameroon, West Africa. Afr. J. Mar. Sci., 30: pp 533-540.

Blythe, D. G. and de Sylva, D. P. (1990) Mother's milk turns toxic following fish feast. J. Am. Med. Assoc. 264 (16): pp 2074.

Blythe, D. G., De Sylva, D. P., Fleming, L. E., Ayyar, R. A., Baden, D. G. and Shrank, K. (1992) Clinical experience with I.V. Mannitol in the treatment of ciguatera. Bull. Soc. Pathol. Exot., 85: pp 425-426.

Botana, L. M. (2008) Seafood and Freshwater Toxins. Pharmacology, Physiology, and Detection; Ed.; CRC Press: Boca Raton, FL, USA, 1215 p.

Bradford, D. G. and Joseph, B. M. (2008) Epidemiological Impact of Toxic Episodes: Neurotoxic Toxins, in Seafood and Freshwater Toxins. Pharmacology, Physiology, and Detection; Edited by Botana, L.M., Ch., 4, CRC Press: Boca Raton, FL, USA, 2008.

Bruneau, A., Mahanty, S., al-Azraqui, T., MacLean, J., Bourque, M. and Desroches, F. (1997) Ciguatera fish poisoning linked to the ingestion of barracuda in a Montreal restaurant-Quebec. Can. Commun. Dis. Rep. 23(20): pp153-156.

Cai, Y., Sun, M. and Corke, H. (2005) HPLC characterization of betalains from plants in the amaranthaceae. J. Chromatogr. Sci. 43(9): pp 454-60.

Caillaud, A., de la Iglesia, P., Darius, H. T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain M. and Diogène, J. (2010). Update on Methodologies Available for Ciguatoxin Determination: Perspectives to Confront the Onset of Ciguatera Fish Poisoning in Europe, Marine Drugs, 8 (6): pp 1838-1907. doi:10.3390/md8061838.

CCCF (2017) Codex Committee on Contaminants in Foods, Joint FAO/WHO Food Standards Programme, Eleventh Session, Rio de Janeiro, Brazil, 3 – 7 April 2017 Agenda item 3 CF11/CRD37. Ciguatoxin Sample Analysis Data – India

CDCP, (2016) Centers for Disease Control and Prevention. Chapter 2: The pre-travel consultation; counseling & advice for travelers: Food poisoning from marine toxins. In CDC Health Information for International Travel; Oxford University Press: New York, NY, USA, 2016.

Chan, T. Y. K. (2013) Ciguatera caused by consumption of humphead wrasse. Toxicon, 76: pp 255–259.

Chan, T. Y. K. (2014). Large Outbreaks of Ciguatera after Consumption of Brown Marbled Grouper. Toxins, 6(7), 2041–2049. http://doi.org/10.3390/toxins6072041

Chan, T. Y. K. (2016) Characteristic features and contributory factors in fatal ciguatera fish poisoning-implications for prevention and public education. Am. J. Trop. Med. Hyg. 94: 704-709.

Chateau-Degat, M. L., Huin-Blondey, M. O., Chinain, M., Darius, T., Legrand, A. M., Ngoc, L. N., Laudon, F., Chansin, R. and Dewailly, E. (2007a) Prevalence of chronic symptoms of ciguatera disease in French Polynesian adults. Am. J. Trop. Med. Hyg. 77, pp 842–846.

Chateau-Degat, M., Beuter, A., Vauterin, G., Nguyen, N. L., Chinain, M., Darius, T., Legrand A., Chansin, R. and Dewailly, E. (2007b). Neurologic signs of ciguatera disease: Evidence of their persistence. Am. J. Trop. Med. Hyg. 77: pp 1170-1175.

Cheng, C. C. and Chung, C. H. (2004) Ciguatera fish poisoning: a challenge to emergency physicians Hong Kong J. Emerg. Med., 11(3): pp 173-177

Chinain, M., Darius, H. T., Ung, A., Fouc, M. T., Revel, T., Cruchet, P., Pauillac, S. and Laurent, D. (2010a) Ciguatera risk management in French Polynesia: The case study of Raivavae Island (*Australes archipelago*). Toxicon, 56 (5), pp 674–690.doi:10.1016/j.toxicon.2009. 05.032.

Chinain, M., Darius, H., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D. and Pauillac, S. (2010b) Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. Toxicon,56(5): pp739-750 doi: 10.1016/ j.toxicon.2009.06.013.

Chinain, M., Germain, M., Deparis, X., Pauillac, S. and Legrand, A.M. (1999) Seasonal abundance and toxicity of the dinoflagellate *Gambierdiscus* spp. (Dinophyceae), the causative agent of ciguatera in Tahiti, French Polynesia. Mar. Biol. 135: pp 259–267.

Coleman, A. M. E. (1990) Ciguatoxin-induced food poisoning in a community - implications for disease surveillance and medical-practice in Jamaica. West Indian Med. J. 39: 233-238.

Crump, J. A., McLay, C. L. and Chambers, S. T. (1999) Ciguatera fish poisoning. Postgrad. Med. J. Nov. 75: 678-679.

D' Silva, M., Anil, A., Naik, R. and D'Costa, P. (2012) Algal blooms: a perspective from the coasts of India, Natural Hazards: Journal of the International Society for the Prevention and Mitigation of Natural Hazards, 63 (2): pp 1225-1253

Dalzell, P. (1991) Secretariat of the Pacific Community Ciguatera Database. Secretariat of the Pacific Community, Noumea, New Caledonia.

Daranas, A. H., Norte, M. and Fernández, J. J. (2001) Toxic marine microalgae. Toxicon, 39(8): pp1101-1132.

Darius, H. T., Ponton, D., Revel, T., Cruchet, P., Ung, A., Fouc, M. T. and Chinain, M. (2007) Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. Toxicon, 50 (5): pp 612–626.

Dechraoui, B. M.-Y., Wang, Z. and Ramsdell, J. S. (2007) Optimization of ciguatoxin extraction method from blood for Pacific ciguatoxin (P-CTX-1). Toxicon, 49 (1): pp 100–105.

DeFouw, J., Egmond, H. and Speijers, G. (2001). Ciguatera fish poisoning: a review. National Institute of Public Health and Environment.RIVM, P.O. Box 1, 3720 BA, Bilthoven, RIVM report 388802021, 66p.

DeHaro, L., Pommier, P. and Valli, M. (2003) Emergence of imported ciguatera in Europe: report of 18 cases at the Poison Control Centre of Marseille. J. Toxicol. Clin.Toxicol. 41(7): pp 927-930.

DeMotta, E. G. and Noceda, D. G. (1985) Epidemiological, clinical and experimental aspects of ciguatera in Puerto Rico. In Proceedings of the 5th International Coral Reef Congress, Tahiti, French Polynesia, 27 May–1 June 1985: pp. 415-416.

DeSchrijver, K., Maes, I., De Man, L. and Michelet, J. 2002. An outbreak of diarrhoeic shellfish poisoning in Antwerp, Belgium. Eurosurveillance Monthly Archives 7(10): 138-141.

Devassy, V. P. and Nair, S. R. S. (1987) Discolouration of water and its effect on fisheries along the Goa coast. Mahasagar 20: pp 121–128.

Devassy, V. P., Bhattathiri, P. M.A, Qasim, S. Z. (1978) Trichodesmium–Phenomenon. Indian J Mar Sci 7: pp 168–186.

Dickey, R. W. (2008) Ciguatera Toxins: Chemistry, Toxicology and Detection. In Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd ed.; Botana, L. M., Ed.; CRC Press-Taylor & Francis: Boca Raton, FL, USA, 2008: pp. 479–500.

Dickey, R. W. and Plakas, S. M. (2010) Ciguatera: A public health perspective. Toxicon, 56 (2): pp123–136.

Dickey, R., Jester, E., Granade, R., Mowdy, D., Moncrief, C., Rebarchik, D., Robl, M., Musser, S. and Poli, M. (1999) Monitoring brevetoxins during a Gymnodinium breve red tide: comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. Natural Toxins 7, 157–165.

EFSA (2006) European Food Safety Authority, Risk characterization of ciguatera food poisoning in Europe: Framework partnership agreement. In Proceedings of the Meeting of the EFSA Advisory Forum, Utrecht, The Netherlands, 8–9 June 2006; Available online: https://www.efsa.europa.eu/sites/default/files/AF/160608a/160608-p10.pdf (accessed on 13 March 2017).

EFSA (2008a) Marine biotoxins in shellfish – Azaspiracid group - Scientific Opinion of the Panel on Contaminants in the Food chain. European Food Safety Authority Journal (723), 6(10): 52p.

EFSA (2008b) Marine biotoxins in shellfish - okadaic acid and analogues - Scientific Opinion of the Panel on Contaminants in the Food chain. European Food Safety Authority Journal (589), 6(1): 62p.

EFSA (2009a) Marine biotoxins in shellfish - Domoic acid. European Food Safety Authority Journal (1181), 7(7): 61p.

EFSA (2009b) Marine biotoxins in shellfish – Pectenotoxin group. European Food Safety Authority Journal (1109), 7(6): 47p.

EFSA (2009c) Marine biotoxins in shellfish - Yessotoxin group - Scientific Opinion of the Panel on Contaminants in the Food chain. European Food Safety Authority Journal (907), 7(2): 62p.

EFSA (2009d) Panel on Contaminants in the Food Chain. Marine biotoxins in shellfish - saxitoxingroup. European Food Safety Authority Journal (1019), 7(2): 76p.

EFSA (2009e) Scientific Opinion on marine biotoxins in shellfish - Palytoxin group. European Food Safety Authority Journal (1393), 7(12): 38p.

EFSA (2010a) Panel on Contaminants in the Food Chain. Scientific opinion on marine biotoxins in shellfish - emerging toxins: brevetoxin group. European Food Safety Authority Journal (1677), 8(7): 29 p.

EFSA (2010b) Scientific Opinion on marine biotoxins in shellfish – Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). European Food Safety Authority Journal (1628), 8(6): 39 p.

Falkowski, P. G. (1984) Physiological response of phytoplankton to natural light regimes. J. Plankton Res. 6(2): 295–307

FAO (2004) Marine biotoxins. FAO food and nutrition paper, Food and Agriculture Organisation of the United Nations, Rome, 80(7): 192 p.

FAO (2017) The State of World Fisheries and Aquaculture, Food and Agriculture Organisation of the United Nations, Rome, Italy, 2014:223p.

Farrell, H., Zammit, A., Manning, J., Shadbolt, C., Szabo, L., Harwood, D.T., McNabb, P., Turahui, J.A. and van den Berg D. J. (2016) Clinical diagnosis and chemical confirmation of ciguatera fish poisoning in New South Wales, Australia, Communicable Diseases Intelligence, 40 (1): pp 1-6.

FDA (2011) Food and Drug Administration, Fish and Fishery Products Hazards and Controls Guidance Fourth Edition, CHAPTER 6: Natural Toxins, 468p. https://www.fda.gov/downloads/Food/ Guidance Regulation / UCM251970.pdf

Folch, J., Lee, M. and Stanley, S.G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226(1), pp 497-509.

Frenette, C., MacLean, J. D. and Gyorkos, T. W. (1988) A Large Common-Source Outbreak of Ciguatera Fish Poisoning. J. Infect. Dis. 158 (5): pp 1128-1131.

Friedman, M.A., Arena, P., Levin, B., Fleming, L., Fernandez, M., Weisman, R., Bernstein, J., Schrank, K., Blythe, D., Backer, L. and Reich, A. (2007). Neuropsychological study of ciguatera fish poisoning: A longitudinal case-control study. Archives of Clinical Neuropsychology 22: pp 545-553.

Friedman, M.A., Fernandez, M., Backer, L.C., Dickey, R.W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Flores Quintana, H.A., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T. and Fleming, L.E. (2017). An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. Mar. Drugs, 15 (3), 72p.

Friedman, M.A., Fleming, L,E., Fernandez, M., Bienfang, P., Schrank, K., Dickey, R., Bottein, M.Y., Backer, L., Ayyar, R., Weisman, R., Watkins, S., Granade, R. and Reich, A. (2008) Ciguatera fish poisoning: Treatment, prevention and management. Mar Drugs 6: pp 456-479.

Froese, R. and Pauly, D. (2017) [Internet] FishBase.World Wide Web electronic publication.www.fishbase.org, (10/2017)

Garthwaite, I. (2000) Keeping shellfish safe to eat: a brief review of shellfish toxins, and methods for their detection. Trends in Food Science & Technology, 11(7), pp 235-244.

Gillespie, N. C., Lewis, R. J. and Pearn, J. H. (1986) Ciguatera in Australia. Occurrence, clinical features, pathophysiology and management. Med. J. Aust.,145 (11-12), pp 584–590.

Glaziou, P. and Martin, P. M. (1993) Study of factors that influence the clinical response to ciguatera fish poisoning. Toxicon. 31(9), pp 1151–1154

Glaziou, P. and Legrand, A. M. (1994) The Epidemiology of Ciguatera fish Poisoning. Toxicon 32(8): pp 863-73.

Godhe, A., Karunasagar, I. and Karunasagar, I, (1996) Gymnodinium catenatum on west coast of India. Harmful Algae News, An IOC Newsletter on toxic alage and algal blooms 15:1

Gopinathan, C. P., Rajagopalan, M., Kaladharan, P. and Prema, D. (2007) Training manual on phytoplankton identification/taxonomy, Fishery Environment Management Division, CMFRI, Kochi, 16p.

Granade, H. R., Cheng, P. C. and Doorenbos, N. J. (1976) Ciguatera I: brine shrimp (Artemia salina L.) larval assay for ciguatera toxins. J. Pharm. Sci., 65 (9), pp 1414–1415.

Guder, W.G.and Zawta B. (2001) the Quality of Diagnostic Samples. 1st ed. Darmstadt: GIT Verlag: pp 42-43

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids.Symp. Ser. 41: pp 95- 98.

Hallegraeff, G. M. (1995) Harmful algal blooms: A global overview. In Hallegraeff, G. M. et al. (Eds.), Manual on harmful marine microalgae, Paris, UNESCO. pp 1-22.

Hallegraeff, G.M. (1993) A review of harmful algal blooms and their apparent global rise. Phycologia 32: pp 79–99.

Halstead, B. (1978) Poisonous and Venomous Marine Animals of the World; Darwin Press: Princeton, NJ, USA, 1043p.

Halstead, B. W., Auerbach, P. S. and Campbell, D. R. (1990) A colour atlas of dangerous marine animals, Wolfe Medical Publications Ltd, W.S. Cowell Ltd, Ipswich, England, 192 p.

Hamilton, B., Hurbungs, M., Jones, A. and Lewis, R. J. (2002a) Multiple ciguatoxins present in Indian Ocean reef fish. Toxicon, 40 (9): pp 1347–1353.

Hamilton, B., Hurbungs, M., Vernoux, J. P., Jones, A. and Lewis, R. J. (2002b) Isolation and characterization of Indian Ocean ciguatoxin. Toxicon, 40 (6): pp 685–693.

Hoffman, P., Granade, H. and McMillan, J. (1983) The mouse ciguatoxin bioassay: a dose-response curve and symptomatology analysis. Toxicon 21(3), pp 363–369.

Hokama, Y., Honda, S., Kobayashi, M., Nakagawa, L., Asahina, A. and Miyahara, J. (1988) Monoclonal antibody (MAb) in detection of ciguatoxin (CTX) and related polyethers by the stick-enzyme immunoassay (S-EIA) in fish tissues associated with ciguatera poisoning. In Mycotoxins and Phycotoxins '88; Natori, S.; Hashimoto, K.; Ueno, Y. (Eds). Elsevier: Amsterdam, The Netherland: pp 303–310.

Hokama, Y., Takenaka,W. E., Nishimura, K. L., Ebesu, J. S. M., Bourke, R., Sullivan, P. K. (1998) A simple membrane immunobead assay for detecting ciguatoxin and related polyethers from human ciguatera intoxication and natural reef fishes. J. AOAC Int. 81: pp 727–735.

Holland, P. T. (2008). Analysis of marine toxins – techniques, method validation, calibration standards and screening methods. In Botana, L. M. (Ed.), Seafood and Freshwater Toxins: pharmacology, physiology and detection, Boca Raton, CRC Press, (2nd ed.) pp 21-50

Holmes, M. J., Lewis, R. J. and Gillespie, N. C. (1990) Toxicity of Australian and French polynesian strains of Gambierdiscustoxicus (Dinophyceae) grown in culture: characterization of a new type of maitotoxin. Toxicon 1990, 28 (10): pp 1159–1172.

Holmes, M. J., Lewis, R. J., Poli, M. A. and Gillespie, N. C. (1991) Strain dependent production of ciguatoxin precursors (gambiertoxins) by Gambierdiscus toxicus (Dinophyceae) in culture. Toxicon 29 (6): pp 761–775.

Hsieh, C. H., Hwang, K. L., Lee, M. M., Lan, C. H., Lin, W. F. and Hwang, D. F. (2009) Species identification of ciguatoxin-carrying grouper implicated in food poisoning. J. Food Prot. 72 (11): 2375–2379.

Hungerford, J. M. (1993) Seafood toxins and seafood products. J. Assoc. Off. Anal. Chem.76: pp 120–130.

ICES. (1984) International Council for the Exploration of the Sea, Report of the ICES special meeting on the causes, Dynamics and effects of exceptional marine blooms and related events. International council meeting paper. 42p.

Ito, E., Suzuki-Toyota, F., Toshimori, K., Fuwa, H., Tachibana, K., Satake, M. and Sasaki, M. (2003) Pathological effects on mice by gambierol, possibly one of the ciguatera toxins. Toxicon 42 (7): pp 733–740.

Johnson, A.M., Rohlfs, E.M. and Silverman L.M. (1999) Proteins. In: Burtis C.A., Ashwood E.R. (Eds.) Tietz Textbook of Clinical Chemistry, 3rd ed. Philadelphia: W.B. Saunders Company: pp 477-540

Jugnu, R. (2006) Studies on the prevalence of algal blooms along Kerala coast, India. PhD. Thesis, Cochin University of Science and Technology (CUSAT), Cochin. 320 p.

Juranovic, L. R. and Park, D. L. (1991) Food borne toxins of marine origin: ciguatera. Rev Environ Contam Toxicol 117: pp 51-94.

Karalis, T., Gupta, L., Chu, M., Campbell, B.A., Capra, M.A. and Maywood, P.A. (2000) Three clusters of ciguatera poisoning: clinical manifestations and public health implications. Med. J. Aust. 172 (4): pp 160–162.

Karunasagar, I., Gowda, H. S. V., Subburaj, M., Venugopal, M. N. and Karunasagar, I. (1984) Outbreak of Paralytic Shellfish Poisoning in Mangalore, west coast of India. Curr Sci 53(5): pp 247–249

Karunasagar, I., Joseph, B., Philipose, K. K. and Karunasagar, I. (1998) Another outbreak of PSP in India. Harmful Algae News, An IOC Newsletter on toxic algae and algal blooms 17:1

Karunasagar, I., Karunasagar, I., Oshima, Y. and Yasumoto, T. (1990) A toxin profile for shellfish involved in an outbreak of paralytic shellfish poisoning in India. Toxicon 28: pp 868–870

Katz, A. R., Terrell-Perica, S. and Sasaki, D. M. (1993) Ciguatera on Kauai: Investigation of factors associated with severity of illness. Am J Trop Med Hyg 49: pp 448-454.

Kosaki, T. I., Stephens, B. J., Anderson, H. H. (1968) Marine toxins from the pacific: III. Comparative bioassay of ciguatoxin (s) in the mouse and chicken. In Proceeding of the West Pharmacol Soc., 11 pp126–128.

Labrousse, H. and Matile, L. (1996) Toxicological biotest on Diptera larvae to detect ciguatoxins and various other toxic substances. Toxicon, 34 (8): pp881–891.

Lampel, K. A. (2012) Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins, 2nd ed. U.S. Food and Drug Administration: Silver Spring, MD, USA, 2012. Available online: www.fda.gov/Food/FoodborneIllness Contaminants/CausesOfIllnessBadBugBook/defaul.htm.

Lawrence, J., Loreal, H., Toyofuku, H., Hess, P., Iddya, K. and Ababouch, L. (2011) FAO Fisheries and Aquaculture Technical Paper-551, Assessment and Management of Biotoxin Risks in Bivalve Molluscs; Food and Agriculture Organisation of the United Nations: Rome, Italy, 337p.

Legrand, A. M., Teai, T., Cruchet, P., Satake, M., Murata, K. and Yasumoto, T. (1998) Two structural types of ciguatoxin involved in ciguatera fish poisoning in French Polynesia. In VIII International Conference on Harmful Algae; Reguera, B., Blanco, J., Fernandez, M.L, Wyatt, T., Eds.; Xunta de Galicia and International Oceanographic Commission of UNESCO: Paris, France, pp. 473–475.

Legrand, A., Fukui, M., Cruchet, P., Ishibashi, Y. and Yasumoto, T. (1992) Characterization of ciguatoxins from different fish species and wild Gambierdiscus toxicus.In Proceeding of the 3rd International Conference on Ciguatera, Puerto R. & Tosteson, T. R. (Ed.) Polysciences publications: Quebec, Canada, pp 25–32.

Lehane, L. and Lewis, R. J. (2000) Review Ciguatera: recent advances but the risk remains. Int. J. Food Microbiol. 61: pp 91-125.

Leung, K. P., Chan, T. Y. K. and Sze, F. K. H. (1992) Ciguatera poisoning in Hong Kong: A report of two cases. Southeast Asian J. Trop. Med. Public Health, 22: pp 543–544.

Lewis, N. D. (1986). Epidemiology and impact of ciguatera in the Pacific: A review. Mar. Fish. Rev. 48(4): pp6-13.

Lewis, R. J. (1993) An overview of ciguatera fish poisoning. SPC Fisheries Newsletter 65: pp 33- 36.

Lewis, R. J. (1995) Detection of Ciguatoxins and related Benthic Dinoflagellate Toxins: in vivo and in vitro Methods. In Manual on Harmful Marine Microalgae; Hallegraeff, G., Anderson, D. & Cembella, A., Eds.; IOC Manuals and Guides No.33, UNESCO: Paris, France; Volume 33, pp 135–161.

Lewis, R. J. (2000) Ciguatera management. SPC Live Reef Fish Inf. Bull. (7): pp11–13.

Lewis, R. J. (2001) The changing face of ciguatera. Toxicon, 39 (1): pp 97-106.

Lewis, R. J. (2003) Detection of toxins associated with ciguatera fish poisoning. In Manual on Harmful Marine Microalgae; Hallegraeff, G.M., Anderson, D.M., Cembella, A.D., Eds.; UNESCO: Paris France, pp 267–277.

Lewis, R. J. (2006) Ciguatera: Australian perspectives on a global problem. Toxicon, 48 (7): pp 799–809.

Lewis, R. J. and Jones, A. (1997) Characterization of ciguatoxins and ciguatoxin congeners present in ciguateric fish by gradient reverse-phase high-performance liquid chromatography/mass spectrometry. Toxicon, 35 (2), 159–168.

Lewis, R. J. and Sellin, M. (1992) Multiple ciguatoxins in the flesh of fish. Toxicon 30 (8): pp 915–919.

Lewis, R. J., Holmes, M. J, Alewood, P. F and Jones, A. (1994) Ionspray mass spectrometry of ciguatoxin-1, maitotoxin-2 and-3, and related marine polyether toxins. Nat. Toxins 1994, 2(2): pp 56-63.

Lewis, R. J., Molgó, J. and Adams, D. J. (2000) Ciguatera toxins: Pharmacology of toxins involved in ciguatera and related fish poisonings. In Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection; Botana, L.M., Ed.; Marcel Dekker: New York, NY, USA, pp 419-447.

Lewis, R. J., Norton, R. S., Brereton, I. M. and Eccles, C. D. (1993) Ciguatoxin-2 is a diastereomer of ciguatoxin-3. Toxicon, 31(5): pp 637-643.

Lewis, R. J., Sellin, M. (1993) Recovery of ciguatoxin from fish flesh. Toxicon 31(10): pp 1333–1336.

Lewis, R. J., Sellin, M., Poli, M. A., Norton, R. S., MacLeod, J. K. and Sheil, M. M. (1991) Purification and characterization of ciguatoxins from moray eel (Lycodontis javanicus, Muraenidae). Toxicon, 29 (9): pp 1115–1127.

Lewis, R. J., Vernoux, J. P. and Breretons, A. M. (1998) Structure of Caribbean ciguatoxin isolated from *Caranx latus*. J. Am. Chem. Soc. 120 (24), pp 5914–5920.

Lewis, R. J., Yang, A. J. and Jones, A. (2009) Rapid extraction combined with LC-tandem mass spectrometry (CREM-LC/MS/MS) for the determination of ciguatoxins in ciguateric fish flesh. Toxicon 54 (1), pp 62–66.

Lewis, R. Jones, A. and Vernouxs, J. (1999) HPLC/tandem electrospray mass spectrometry for the determination of Sub-ppb levels of Pacific and Caribbean ciguatoxins in crude extracts of fish.Anal. Chem. 71: pp 247–250.

Lewis, R.J. and King, G.K. (1996) Ciguatera (fish poisoning). In: Williamson, J.A., Fenner, P.J., Burnett, J.W., Rifkin, J.F. (Eds.), Venomous and Poisonous Marine Animals: A Medical and Biological Handbook. University of New South Wales Press, Sydney, 15: pp. 346–353,

Lewis, R.J., Wong Hoy, A.W. and McGriffin, D. C. (1992) Action of ciguatoxin on human atrial trabeculae. Toxicon 30 (8): pp 907-914.

Lieske, E. and Myers, R. (1994) Collins Pocket Guide. Coral reef fishes. Indo-Pacific & Caribbean including the Red Sea.Haper Collins Publishers, 400 p.

Lombet, A., Bidard, J. N. and Lazdunski, M. (1987) Ciguatoxin and brevetoxins share a common recept or site on the neuron a voltage-dependent Naþ channel. FEBSLett.219, pp 355–359.

Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M. and Wekell, M. M. (1993) Tetrazolium-based cell bioassay for neurotoxins active on voltage- sensitive sodium channels: semiautomated assay for saxitoxin, bre- vetoxin, and ciguatoxin. Anal.Biochem. 214, pp 190–194.

Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M., Hokama, Y., Dickey, R. W., Granade, H. R., Lewis, R. J., Yasumoto, T. and Wekell, M. M. (1995) Detection of sodium channel effectors: directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxin and seafood extracts. J. AOAC Int. 78, pp 521–527.

Maralit, B. A., Aguila, R. D., Ventolero, M. F. H., Perez, S. K. L., Willette, D. A. and Santos, M. D. (2013) Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety. Food Control 33: pp 119-125.

March, R. E. (1997) An introduction to quadrupole ion trap mass spectrometry. Journal of Mass Spectrometry, 32(4): pp 351-369.

McMahon, T. and Silke, J. 1998.Re-occurrence of winter toxicity. Harmful Algae News, 17: 12-16.

McMaster, M. C. (2005) LC/MS: A practical user's guide. Hoboken, NJ: John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: 10.1002/0471736589.ch1

McNabb, P., Selwood, A. I., and Holland, P. T. (2005) Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and inter laboratory study. Journal of the Association of Official Analytical Chemists, 88(3): pp 761-772.

Meyer, L., Carter, S. and Capper, A. (2015) An updated ciguatoxin extraction method and silica cleanup for use with HPLC – MS/MS for the analysis of P-CTX-1, PCT-X-2 and P-CTX-3, Toxicon 108, 249-256.

Morris, J. G., Lewin, P., Hargrett, N.T., Smith, C.W., Blake, P.A. and Schneider, R. (1982) Clinical-features of ciguatera fish poisoning - a study of the disease in the United-States Virgin Islands. Arch Intern Med 142: pp 1090-1092.

Morris, P. D., Campbell, D. S. and Freeman, J. I. (1990) Ciguatera fish poisoning: an outbreak associated with fish caught from North Carolina coastal waters South. Med. J. 83 (4), 379–382.

Morton, S. L., Norris, D. R. and Bomber, J. W. (1992) Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. J. Exp. Mar. Biol. Ecol. 157(1): pp 79-90

Moulignier, A., Binet,D. and Frottier, J. (1995) Ciguatera fish poisoning: also in Europe.J Neurol. Neurosurg.Psychiatry. 59(2): pp 192.

Moyer, S. C., Cotter, R. J. and Woods, A. S. (2002) Fragmentation of phosphopeptides by atmospheric pressure MALDI and ESI/ion trap mass spectrometry. Journal of the American Society for Mass Spectrometry, 13(3): pp 274-283.

MPEDA, (2017) The Marine Products Export Development Authority (MPEDA)[Internet] Ministry of Commerce & Industry, Government of India, -[cited 2017 Dec 29] Available from: http://mpeda.gov.in/MPEDA/product_exports.php

MTNN, (2016) Many hospitalized, consuming fish head curry in Ullal. [online news paper] Mangalore Today News Network, Oct -01, 2016. http://www.mangaloretoday.com/main/Many-hospitalized-consuming-fish-head-curry-in-Ullal.html?fromNewsdog=1

Munday, R. and Reeve, J. (2013) Risk Assessment of Shellfish Toxins. Toxins 5(11): pp 2109-2137.

Murata, M., Legrand, A. M., Ishibashi, Y., Yasumoto, T. (1990) Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate Gambierdiscus toxicus. J. Am. Chem. Soc. 112 (11): pp 4380-4386.

Murata, M., Legrand, A.M., Ishibashi, Y. and Yasumoto, T. (1989) Structures of ciguatoxin and its congener. J. Am. Chem. Soc., 111 (24): pp 8929-8931.

Myers, R. F. (1999) Micronesian reef fishes: a comprehensive guide to the coral reef fishes of Micronesia, 3rd revised and expanded edition. Coral Graphics, Barrigada, Guam. 330 p.

Nagabhushanam, A. K. (1967) On an unusually dense phytoplankton 'bloom' around Minicoy Island (Arabian Sea) and its effect on the local tuna fisheries. Curr Sci 36: pp 611–612.

Nagai, H., Mikami, Y., Yazawa, K., Gonoi, T. and Yasumoto, T. (1993) Biological activities of novel polyether antifungals, gambieric acids A and B from a marine dinoflagellate *Gambierdiscus toxicu*s. J. Antibiot. 46: 520–522.

Nagai, H., Murata, M., Torigoe, K., Satake, M. and Yasumoto, T. (1992) Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus toxicus*. J. Org. Chem. 57: pp 5448–5453.

NCBI, (2017) National Center for Biotechnology Information (NCBI) [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [cited 2017 Dec 29]. Available from: https://www.ncbi.nlm.nih.gov/

Nixon, S.W. (1995) Coastal marine eutrophication: a definition, social consequences and future concerns. Ophelia 41: pp 199-220.

Núñez, O., Moyano, E. and Galceran, M. T. (2005) LC–MS/MS analysis of organic toxics in food. Trends in Analytical Chemistry, 24(7): pp 683-703.

Ojeda, M. C. L., Otero, E. C., Yamashita M.Y. and Sasaki, M. (2008) Polycavernosides and Gambierol: Chemistry, Pharmacology, Toxicology and detection. In Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd ed.; Botana, L.M., Ed.; CRC Press-Taylor & Francis: Boca Raton, FL, USA, pp 597-628.

Olsen, D. A., Nellis, D. W. and Wood, R. S. (1984) Ciguatera in the Eastern Caribbean. Mar. Fish Rev. 46 (1): pp 13–18.

Oshiro, N., Yogi, K., Asato, S., Sasaki, T., Tamanaha, K., Hirama, M., Yasumoto, T. and Inafuku, Y. (2010) Ciguatera incidence and fish toxicity in Okinawa, Japan. Toxicon, 56(5):656-661

Otero, P., Alfonso, A., Alfonso, C., Rodríguez, P., Vieytes, M. R. and Botana, L.M. (2011) Effect of uncontrolled factors in a validated liquid chromatography–tandem mass spectrometry method question its use as a reference method for marine toxins: Major causes for concern. Anal. Chem. 83: pp 5903–5911.

Padmakumar, K. B., Menon N. R., and Sanjeevan, V. N. (2012) Is Occurrence of Harmful Algal Blooms in the Exclusive Economic Zone of India on the Rise?. International Journal of Oceanography, Article ID 263946: pp 1- 7 doi:10.1155/2012/263946.

Paerl, H. W. (1988) Nuisance phytoplankton blooms in coastal, estuarine and inland waters. Limnol.Oceanogr. 33: pp 823-847.

Pearn, J. (1995) Ciguatera-a potent cause of the chronic fatigue syndrome. Journal of Immunology and Immuno Pharmacology, 15: pp 63–65.

Pearn, J. (2001) Neurology of ciguatera. *Br. Med. J. 70(1)*: pp 4-8.

Pearn, J. H., Lewis, R. J., Ruff, T., Tait, M., Quinn, J., Murtha, W., King, G., Mallett, A. and Gillespie, N. C. (1989) Ciguatera and mannitol: Experience with a new treatment regimen. Med. J. Aust. 151: pp 77–80.

Perl, T. M., Bedard, L., Kosatsky, T., Hockin, C. J., Todd, E. C. D. and Remis, R. S. (1990) An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. N. Engl. J. Med. 322: pp1775-1780.

Poli, M. A., Lewis, R. J., Dickey, R. W., Musser, S. M., Buckner, C. A. and Carpenter, L. G. (1997) Identification of Caribbean ciguatoxins as the cause of an outbreak of fish poisoning among U.S. soldiers in Haiti. Toxicon 35 (5): pp 733–741.

Pottier, I., Hamilton, B., Jones, A., Lewis, R. J. and Vernoux, J. P (2003) Identification of slow and fast-acting toxins in a highly ciguatoxic barracuda (Sphyraena barracuda) by HPLC/MS and radiolabeled ligand binding. Toxicon 42 (6): pp 663–672.

Pottier, I., Vernoux, J. P, Jones, A. and Lewis, R. J. (2001) Ciguatera fish poisoning in the Caribbean islands and Western Atlantic. Rev. Environ. Contam.Toxicol. 168: pp 99-141.

Pottier, I., Vernoux, J. P, Jones, A. and Lewis, R. J. (2002b) Analysis of toxin profiles in three different fish species causing ciguatera fish poisoning in Guadeloupe, French West Indies. Food Addit. Contam. 19 (11): pp 1034–1042.

Pottier, I., Vernoux, J. P, Jones, A., Lewis, R. J. (2002a) Characterization of multiple Caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (Caranxlatus) by high-performance liquid chromatography/mass spectrometry. Toxicon 40 (7): pp 929–939.

Prabhu, M. S., Ramamurthy, S., Kuthalingam, M. D. K., Dhulkhed, M. H. (1965) On an unusual swarming of the planktonic blue–green algae *Trichodesmium* spp., off Mangalore. Curr Sci 34:95

Prakash, A., Sharma, V. (1964) On the occurrence of 'red water' phenomenon on the west coast of India. Curr Sci 33: pp168–170

Prasad, R. R., Jayaraman, R. (1954) Preliminary studies on certain changes in the plankton and hydrological conditions associated with the swarming of Noctiluca. Proc Indian Acad Sci 49 (2B): pp 49–57

Quilliam, M. A. (2003) The role of chromatography in the hunt for red tide toxins. Journal of Chromatography A, 1000(1-2): pp 527-548.

Ragelis, E. P. (1984) Ciguatera seafood poisoning: overview. In: Ragelis, E.P. (Ed.), Seafood Toxins. American Chemical Society, Washington, DC, pp. 22–36.

Rajeish, M., Shekar, M., Madhushree, H. N. and Venugopal, M. N. (2016) Presumptive case of ciguatera fish poisoning in Mangalore, India. Research Communications, Curr. Sci., 111 (9): pp 1543-1547.

Rajisha, R., Kishore P., Panda, S. K., Ravishankar, C. N. and Kumar, A. K. (2017a). Confirmation of Ciguatoxin Fish Poisoning in Red Snapper, Lutjanus bohar (Forsskål,1775) by Mouse Bioassay. Fishery Technology, 54: pp1-4.

Rajisha, R., Kishore, P., Panda, S. K., Harikrishnan, G., Ajitha, K.C., Suresh, M. K., Chowdhury, L. M., Ravishankar, C. N. and Kumar, A. K. (2017b). Incidence of Ciguatoxin Fish Poisoning in Trivandrum, India. Indian Journal of Fisheries 64(4): pp 129-133, DOI: 10.21077/ ijf.2017.64.4.71558-20

Ramaiah, N., Paul, J. T., Fernandes, V., Raveendran, T., Raveendran, O., Sundar, D., Ravichandran, C., Shenoy, D. M., Mangesh, G., Kurian, S., Gerson, V. J., Shoji, D. T., Madhu, N. V., Kumar, S. S., Lokabharathi, P. A. and Shetye, S. R. (2005) The September 2004 stench off the southern Malabar coast - A consequence of holococcolithophore bloom. Curr. Sci. 88: pp551–554

Randall, J. E. (1958) A Review of Ciguatera, Tropical Fish Poisoning, with a Tentative Explanation of its Cause. Bull. Mar. Sci. 8 (3): pp 236-267.

Reyzer, M. L. and Caprioli, R. M. (2007) MALDI-MS-based imaging of small molecules and proteins in tissues. Current Opinion in Chemical Biology, 11(1): pp 29-35.

Richardson, K. and Jorgensen, B. B. (1996) Eutrophication, definition, history and effects. In: Jorgensen, B.B., Richardson, K. (Eds.), Eutrophication in Coastal Marine Ecosystems. American Geophysical Union, Washington, DC, pp. 1–20.

Roberts, D., Davies, C., Mitchell, A., Moore, H., Picton, B. and Portig, A. (2004) Strangford Lough Ecological Change Investigation (SLECI). Report to Environment and Heritage Service by the Queen's University, Belfast. Available online at http://www.ni-environment.gov.uk/ print/0execsum. pdf.

Rodrigue, D. C., Etzel, R. A., Hall, S., de Porras, E., Velasquez, O. H. and Tauxe, R.V., Kilbourne, E. M. and Blake, P. A. (1990) Lethal paralytic shellfish poisoning in Guatemala. Am. J. Trop. Med. Hyg. 42: pp 267–271.

Roeder, K., Erler, K., Kibler, S., Tester, P., Van The, H., Nguyen-Ngoc, L., Gerdts, G. and Luckas, B. (2010) Characteristic profiles of Ciguatera toxins in different strains of *Gambierdiscus* spp. Toxicon 56(5): pp 731-738.

Ruff, T. A. (1989) Ciguatera in the Pacific: a link with military activities. Lancet 1(8631): pp 201–205.

Ruff, T. A. and Lewis, R. J. (1994). Clinical aspects of ciguatera: an overview. Mem. Queensl. Mus. 34: pp 609–619.

Sahayak, S., Jyothibabu, R., Jayalakshmi, K. J., Habeebrehman, H., Sabu, P., Prabhakaran, M. P., Jasmine, P., Shaiju, P., Rejomon, G., Thresiamma, J. and Nair, K. K. C. (2005) Red tide of Noctiluca miliaris off south of Thiruvananthapuram subsequent to the 'stench event' at the southern Kerala coast. Curr Sci 89: pp 1472– 1473

Satake, M., Fukui, M., Legrand, A., Cruchet, P. and Yasumoto, T. (1998) Isolation and structures of new ciguatoxin analogs, 2, 3-dihydroxyCTX3C and 51-hydroxyCTX3C, accumulated in tropical reef fish. Tetrahedron Lett. 39 (10): pp 1197–1198.

Satake, M., Ishibashi, Y., Legrand, A. and Yasumoto, T. (1996) Isolation and structure of ciguatoxin-4 A, a new ciguatoxin precursor, from cultures of dinoflagellate *Gambierdiscus toxicus* and parrot fish *Scarus gibbus*. Biosci.Biotechnol.Biochem. 60 (12): pp 2103–2105.

Satake, M., Murata, M. and Yasumoto, T. (1993a) The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. Tetrahedron Lett. 34 (12): pp 1975-1978.

Satake, M., Murata, M. and Yasumoto, T. (1993b) Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. J. Am. Chem. Soc. 115 (1): pp 361–362.

Scheuer, P. J. (1994) Tetrahedron perspective number 2: Ciguatera and its off-shoots-chance encounters en route to a molecular structures. Tetrahedon 50 (1): pp 3-18.

Schumann, G., Bonora, R., Ceriotti, F. and Ferard, G. (2002) IFCC Primary reference procedure for the measurement of catalytic activity concentrations of enzymes at 37°C. Part 5: reference procedure for the measurement of catalytic concentration of aspartate aminotransferase. Clin. Chem. Lab. Med.,40: pp 725-33

Segar, K., Karunasagar, I. and Karunasagar, I. (1989) Dinoflagellate toxins in shellfishes along the coast of Karnataka. In: Joseph MM (ed) The First Indian Fisheries Forum Proceedings, Asian Fisheries Society, Indian Branch, Mangalore, pp 389–390

Sheppard, C. and Rioja-Nieto, R. (2005) Sea surface temperature 1871-2099 in 38 cells in the Caribbean region.Mar. Environ. Res. 60(3): pp 389-396.

Shi, F. (2012) Chemical and toxicological investigation of the toxic dinoflagellate, Karenia brevisulcata.Ph.D. Thesis, Lincoln University, New Zealand, 216p.

Shi, F., McNabb, P., Rhodes, L., Holland, P., Webb, S., Adamson, J., Immers, A., Gooneratne, R. and Holland, J. (2012) The toxic effects of three dinoflagellate species from the genus Karenia on invertebrate larvae and finfish. New Zealand Journal of Marine and Freshwater Research, 46 (2): pp 149-165.

Silas, E. G., Alagarswami, K., Narasimham, K. A., Appukuttan, K. K. and Muthiah, P. (1982) Country Report – India. In: Davy FB, Graham M (eds) Bivalve culture in Asia and the Pacific. Proc. Workshop held in Singapore, 16–19 Feb, 1982. International Development Centre, Ottawa, pp 34–431

Sims, J. K., (1987) Theoretical Discourse on the Pharmacology of Toxic Marine Ingestions. Ann. Emer. Med. 16: pp 1006-1015.

Skinner, M. P., Brewer, T. D., Johnstone, R. Fleming, L. E. and Lewis, R. J. (2011) Ciguatera fish poisoning in the Pacific islands (1998 to 2008). PLoS Negl. Trop. Dis. 5 (12): e1416.

Smayda, T. J. (1989) Primary production and the global epidemic of phytoplankton blooms in the sea: a linkage? In: Cosper, E.M., Bricelj, V.M., Carpenter, E.J. (Eds.), Novel Phytoplankton Blooms. Springer-Verlag, New York, NY, pp 449–484.

Smayda, T. J. (1992) Global epidemics of noxious phytoplankton blooms and food chain consequences in large ecosystems. In: Sherman, K., Alexander, L.M., Gold, B.D. (Eds.), Food Chains, Models and Management of Large Marine Ecosystems. Westview Press, San Francisco, pp 275–307.

Smith, M. M. and Heemstra, P. C. (1986) Smiths' sea fishes. Springer Verlag, Berlin. 1047 p.

Smith, P., Chang, F. H. and Mackenzie, L. (1993) Toxic phytoplankton and algal blooms, summer 1992/93. Paper presented at the Marine toxins and New Zealand shellfish, proceedings of a workshop on research issues, 10-11 June 1993.

Sona, A., Kumar, K. K., Mukundan, M. K., Jugnu, R., Kripa, V. and Gopinathan, C. P. (2003) On occurrence of certain biotoxins along the Kerala Coast. In: Symposium on Seafood Safety Status and Strategies, 28 - 30 March 2002, Cochin.

Stewart, I., Eaglesham, G. K., Poole, S., Graham, G., Paulo, C., Wickramasinghe, W., Sadler, R. and Shaw, G. R. (2010) Establishing a public health analytical service based upon chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples.Toxicon, 56 (5): pp 804-812

Sulochanan, B., Dineshbabu, A. P., Saravanan, R., Bhat, S. G. and Lavanya, S. (2014) Occurrence of Noctiluca scintillans bloom off Mangalore in the Arabian Sea. Indian J. Fish., 61(1): pp 42-48.

Tachibana, K., Nukina, M., Joh, Y. and Scheuer, P. J. (1987) Recent Developments in the Molecular Structure of Ciguatoxin, Biological Bulletin, 172 (1): pp 122-127

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: pp 1596–1599.

Terao, K., Ito, E., Oarada, M., Ishibashi, Y., Legrand, A. M. and Yasumoto, T. (1991) Light and electron microscopic studies of pathologic changes induced in mice by ciguatoxin poisoning. Toxicon 29 (6), pp 633–643.

Tester, P. A., Feldman, R. L., Nau, A.W., Kibler, S. R. and Litaker, R. W. (2010) Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. Toxicon 56 (5): pp 698–710.

The Hindu, (2004) Stench from sea: children hospitalized. The Hindu, Kerala Edition, 17 September 2004.

The Hindustan Times, (2004) Thousands of fishes killed by toxic algae in Kerala, 18 September 2004.

These, A., Klemm, C., Nausch, I. and Uhlig, S. (2011) Results of a European interlaboratory method validation study for the quantitative determination of lipophilic marine biotoxins in raw and cooked shellfish based on high-performance liquid chromatography-tandem mass spectrometry. Part I: collaborative study. Anal Bioanal Chem. 399(3): pp 1245-1256.

Thomas, L. (1998) Clinical Laboratory Diagnostics 1st ed. Frankfurt:TH-Books Verlagsgesellscchaft: pp 652-656

Times of India (2016) Hundreds taken ill after consuming fish heads in Ullal, [online news] Times of India, Oct 1, 2016. https://timesofindia. indiatimes.com/city/mangaluru/ Hundreds-taken-ill-after-consuming-fish-heads-in-Ullal/articleshow/54630403.cms

Ting, J. Y. and Brown, A. F. (2001) Ciguatera poisoning : a global issue with common management problems. Eur. J. Emerg. Med. (8): 295–300.

Ting, J., Brown, A. and Pearn, J. (1998) Ciguatera poisoning: an example of a public health challenge. Aust.N. Z. J. Publ. Health 22: pp 140–142.

Underdal, B., Yndestad, M., and Aune, T. (1985). DSP intoxication in Norway and Sweden, Autumn 1984 - Spring 1984. In D. M. Anderson, A. W. White, and D. G. Baden (eds.), Toxic Dinoflagellates. Amsterdam, The Netherlands: Elsevier.http://agris.fao.org/agris-search/search.do? recordID =US8644422

UNESCO (2016) United Nations Educational, Scientific and Cultural Organization, Climate Change Education and COP22, Tackling climate change through Education for Sustainable Development, http://unesdoc.unesco.org/images/0024/002464/246479E.pdf.

Van Dolah, F. M. (2000) Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 2000, 108 (Suppl. 1): pp 133–141.

Velieri, F., Bacchiocchi, S., Graziosi, T., Latini, M., Orletti, R. and Bacci, C. (2011) Valutation of official mouse bioassay for the detection of lipophilic marine toxin. Italian Journal of Food Safety, Vol. 1(2): pp 85-89

Vernoux, J. P. and Lewis, R. J. (1997) Isolation and characterization of Caribbean ciguatoxins from the horseeye jack (*Caranx latus*). Toxicon 35 (6): pp 889–900.

Vernoux, J. P., Lejeune, J. (1994) Ciguatera in the French West Indies. Mem. Qld. Mus.34: pp 631–638.

Villareal, T. A., Hanson, S., Qualia, S., Jester, E. L. E., Granade H. R. and Dickey, R. W. (2007) Petroleum production platforms as sites for the expansion of ciguatera in the northwestern Gulf of Mexico. Harmful Algae.6 (2): pp 253-259.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R. and Hebert, P. D. (2005) DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 360(1462): pp1847-1857.

Wells, M. L., Trainer, V. L., Smayda, T. J., Karlson B.S.O., Trick, C.G., Kudela, R. M., Ishikawa, A., Bernard, S., Wulff, A., Anderson, D.M. and Cochlan, W.P. (2015) Harmful algal blooms and climate change: Learning from the past and present to forecast the future. Harfmful algae. 49: pp 68-93

Wong, C. K., Hung, P., Lee, K. L. H. and Kam, K. M. (2005) Study of an outbreak of ciguatera fish poisoning in Hong Kong. Toxicon2005, 46 (5): pp 563–571.

Wong, C. K., Hung, P.and Lo, J. Y. (2014). Ciguatera fish poisoning in Hong Kong--a 10-year perspective on the class of ciguatoxins.Toxicon. 86: pp 96-106.

Wong, C., Hung, P., Lee, K. and Kam, K. (2009) Solid-phase extraction clean-up of ciguatoxin contaminated coral fish extracts for use in the mouse bioassay. Food Addit.Contam. 26: pp 236–247.

Wu, J. J., Mak, Y. L., Murphy, M. B., Lam, J. C., Chan, W. H., Wang, M., Chan, L. L. and Lam, P. K. (2011). Validation of an accelerated solvent extraction liquid chromatography-tandem mass spectrometry method for Pacific ciguatoxin-1 in fish flesh and comparison with the mouse neuroblastoma assay. Anal Bioanal Chem. 400 (9): pp 3165–3175.

Yasumoto, T. and Murata, M. (1993) Marine toxins. Chem. Rev. 93: pp 1897–1909.

Yasumoto, T., Igarashi, T., Legrand, A., Cruchet, P., Chinain, M., Fujita, T. and Naokis, H. (2000) Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. J. Am. Chem. Soc. 122: pp 4988–4989.

Yasumoto, T., Nakajima, I., Bagnis, R. and Adachi, R. (1977) Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43(8): pp 1021-1026.

Yasumoto, T., Oshima, Y. & Yamaguchi, M. (1978) Occurrence of a New Type of Shellfish Poisoning in the Tokohu District. Bull. Jap. Soc. Sci. Fish. 44(11): 1249-1255.

Yasumoto, T., Raj, U. and Bagnis, R. (1984) Seafood Poisonings in Tropical Regions, Laboratory of Food Hygiene, Faculty of Agriculture: Tohoku University, Japan, 74 p.

Yasumoto, T., Satake, M., Fukui, M., Nagai, H., Murata, K. and Legrand, A.M. (1993) A turning point in ciguatera study. In Toxic Phytoplankton Blooms in the Sea; Smayda, T.J., Shimizu, Y., Eds.; Elsevier Science Publishers: New York, NY, USA pp 455–460.

Yogi, K., Oshiro, N., Matsuda, S., Sakugawa, S., Matsuo, T. and Yasumoto, T. (2013) Toxin profiles in fish implicated in ciguatera fish poisoning in Amami and Kakeroma Islands, Kagoshima Prefecture, Japan. Food Hygiene and Safety Science, 54(6): pp 385–91.

Yogi, K., Sakugawa, S., Oshiro, N., Ikehara, T., Sugiyama, K. and Yasumoto, T. (2014) Determination of Toxins Involved in Ciguatera Fish Poisoning in the Pacific by LC/MS. J AOAC Int. 97(2): pp 398–402.

Zaias, J., Mineau, M., Cray, C., Yoon, D., and Altman, N. H. (2009) Reference Values for Serum Proteins of Common Laboratory Rodent Strains. J. Am. Assoc. Lab. Anim. Sci. 48(4): pp 387–390.

Zheng, L., He, J., Lin, Y., Cao, W., and Zhang , W. (2014) 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. Acta Oceanol. 33(4): pp 55–76

Zimmermann, K., Deuis, J. R., Inserra, M. C., Collins, L. S., Namer, B., Cabot, P. J., Reeh, P. W. Lewis, R. J. and Vetter, I. (2013) Analgesic treatment of ciguatoxin-induced cold allodynia. Pain, 154: 1999–2006.

PUBLICATIONS

Research Paper

Rajisha, R., Kishore P., Panda, S. K., Ravishankar, C. N. and Kumar, A. K. (2017). Confirmation of Ciguatoxin Fish Poisoning in Red Snapper, Lutjanus bohar (Forsskål, 1775) by Mouse Bioassay. Fishery Technology, 54: pp1-4.

Rajisha, R., Kishore, P., Panda, S. K., Harikrishnan, G., Ajitha, K.C., Suresh, M. K., Chowdhury, L. M., Ravishankar, C. N. and Kumar, A. K. (2017). Incidence of Ciguatoxin Fish Poisoning in Trivandrum, India. Indian Journal of Fisheries 64(4): pp 129-133, DOI: 10.21077/ijf.2017.64.4.71558-20

Seminar Proceedings

Rajisha, R., Kishore, P., Panda, S.K and Kumar, A.K. (2017) Determination of Ciguatoxin in Fish by Liquid Chromatography Tandem Mass Spectrometry and Comparison with Mouse Bioassay. In: (Thomas, S.N., Rao, B.M., Madhu, V.R., Asha, K.K., Binsi, P.K., Viji, P., Sajesh, V.K. and Jha, P.N., Eds) Fostering Innovations in Fisheries and Aquaculture: Focus on Sustainability and Safety –Book of Abstracts, 11th Indian Fisheries and Aquaculture Forum, ICAR – Central Institute of Fisheries Technology, Kochi and Asian Fisheries Society, Indian Branch, 21 – 24 November, 2017, Kochi, India, pp.429.

Popular Articles

Rajisha, R., Kishore,P., Panda,S.K., Kumar,A., Sankar,T.V., Ganesan,B., Harikrishnan,G. and Chowdhury,M.L (Submitted on 28/10/2016) Lutjanus bohar strain CG01 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial GenBank: KY057337.1. Ciguatoxin in fish from India.

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Research Note

Confirmation of Ciguatoxin Fish Poisoning in Red Snapper, *Lutjanus bohar* (Forsskål, 1775) by Mouse Bioassay

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Ciguatoxin is an important biotoxin resulting from the consumption of coral reef fishes. The biotransformation of Gambiertoxins in large fishes makes it more potent and significant in respect of human health (Friedman et al., 2017; Dickey et al., 2010; Lehane & Lewis 2000). A benthic dinoflagellate known as *Gambierdiscus toxicus*, is responsible for the production of Gambiertoxin. Ciguatoxin, a tasteless, colourless, odourless, heat and acid stable, lipophilic polyether compound which is stable at freezing temperature also (Abraham et al., 2011). CTXs are secondary metabolites with numerous congeners having different molecular structure have been reported from different geographical origins namely Pacific (P-CTX), Caribbean (C-CTX) and Indian (I-CTX) (Caillaud et al., 2010). This region-specific biotoxin has been reported very recently from Mangalore coast (Rajeish et al., 2016). Earlier in January 2016 the same fish species detected as ciguatoxic from Vizhinjam coast, Kerala and caused intoxication in local population were reported by Rajisha et al. (2017). The existence of ciguatoxicity has not been indicated by any highly visible surface phenomenon such as red tide as seen in the case of Paralytic Shellfish Poisoning (de Fouw et al., 2001), hence an early warning to the alarm of CFP incidence is not possible. The geographical distribution of ciguatoxic fish poisoning (CFP) affected globally in the tropical and subtropical regions and people suffered from this intoxication were reported to be ranging from 10 000 to 50 000 (Lewis, 2001) and 50 000 to 5 00 000 individuals annually (Caillaud et al., 2010), which shows the intensity of occurrence even though it is difficult to ascertain the under-reporting cases of CFP (Friedman et al., 2017). A wide variety of 400 finfish species are found to elaborate ciguatoxin (Lewis, 2006) and it is responsible for the substantial economic loss because of the chronic health impacts after fish consumption. Mouse bioassay (MBA) has been widely used for the selective determination of ciguatoxicity in fishes (Banner et al., 1960).

A total of 262 reef associated finfish samples were collected from different sources across Kerala, Tamil Nadu, Maharashtra, Gujarat and Karnataka for the determination of ciguatoxicity using MBA. Screened samples for ciguatoxicity comprised of four species of snappers *viz., Lutjanus argentimaculatus, L. fulvus, L. bohar and L. gibbus;* two species of Barracuda *viz., Sphyraena putnamae and S. jello;* four species of reef cod *viz., Epinephelus bleekeri, E. coioides, E. diacanthus and E. chlorostigma;* other species include *Pristipomoides filamentosus, Otolithoides biauritus, Caranx ignobilis, Lethrinus nebulosus, Variola louti, Aprion virescens etc.* DNA barcoding based on COI gene is employed for species-level fish identification using DNeasy Blood & Tissue Kit (Qiagen, Germany). The raw DNA sequences obtained by sequencing were edited and aligned using Bio Edit version 7.0.5.2 (Hall, 1999). Mouse bioassay of fish ether extracts used for toxin analysis (ANSES, 2016; Lewis. 1995). Fish sample extracted by acetone from 100 g of cooked (70°C) and minced tissue homogenate. After evaporation of acetone, residue dissolved in methanol-water mixture (9:1) and liquid-liquid partition with n-hexane allows the removal of non-polar lipids. The residue obtained after evaporation of methanol phase taken up in ethanol-water (1:3) and separated with diethyl ether. Ether phase evaporated and dissolved in chloroform: methanol (97:3) and residue collected after this phase is

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reconstituted in 1% 0.5 mL Tween 60/0.9% saline and filtered through 0.45 PTFE membrane filter prior to administering (intra peritoneal) into mice. Female albino mice weighed around 20±2 g was taken for analysis. Control mice injected 0.5 mL 1% Tween 60/0.9% saline alone.

Fish samples were partially sequenced which yielded an average length of 641bp for cytochrome oxidase subunit I (COI) gene (Accession No. MF383185). A BLAST analysis of COI sequences showed 100% similarity to *L. bohar*. Phylogenetic tree (Fig. 1) for the COI sequences with other Lutjanus sp. showed it to be closest to *L. bohar*. Species identification through molecular taxonomic techniques provides a better understanding of the species. Two fish samples of *Lutjanus bohar* (weighed 6.39 and 5.62 kg respectively) collected from Thoppumpady (Kerala) and Mangalore (Karnataka) in October 2016, were confirmed for ciguatoxicity using Mouse bioassay method (ANSES, 2016; Lewis, 1995). A check list (Table 1) of symptoms observed during experiment was prepared for up to 24 h after intra peritoneal injection in to mice. Symptoms started with reduced locomotor activity, diarrhea within 1 h of intra peritoneal (i.p) injection of fish extract. The death of one or two mice within 24 h interpreted as positive and it is confirmed that the fish were not fit for human consumption. Symptoms observed included progressive paralysis (hind limb), breathing difficulties, convulsion (body muscles contract and relax rapidly), stretching of hairs in an erected manner (piloerection) etc. within 4 h of administration of extract. This extraction method allows the quantification of ciguatoxin in fish flesh up to 20 mg of ether extract. The lethal

Table 1. IOC Mouse bioassay for Ciguatoxin (Check list)

Sl No.	Symptoms	1st h	2nd h	3rd h	4th h	24th h
1.	Hypothermia	+	+	+	ND	ND
2.	Hypothermia below 33°C	+	+	+	ND	ND
3.	Piloerection	+	+	+	ND	ND
4.	Diarrhoea	+	+	+	ND	ND
5.	Lachrymation	+	+	+	ND	ND
6.	Hyper salivation	+	+	+	ND	ND
7.	Dyspnoea	+	+	+	ND	ND
8.	Wobbly upright gait	+	+	+	ND	ND
9.	Gasping	+	+	+	ND	ND
10.	Mild gasping	+	+	+	ND	ND
11.	Terminal Convulsion with tail arching	+	+	+	ND	ND
12.	Hind limb paralysis	+	+	+	ND	ND
13.	Progressive hind limb paralysis	+	+	+	ND	ND
14.	Progressive paralysis from hind extending to fore limbs	+	+	+	ND	ND
15.	Convulsions	+	+	+	ND	ND
16.	Mild Convulsions preceding death>30 sec	+	+	+	ND	ND
17.	Respiratory problems	+	+	+	ND	ND
18.	Respiratory failure	+	+	+	ND	ND
19.	Death from respiratory failure	+	+	+	ND	ND
20.	Slow Movements	+	+	+	ND	ND
21.	Slow locomotor activity	+	+	+	ND	ND
22.	Breathing Difficulties	+	+	+	ND	ND
23.	Sluggish	+	+	+	ND	ND

Note: ND – Not Detected as mice death occurs within 3-4 h from the time of injection.

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dose i.e., LD_{50} dose for a 20 g mouse is equal to one Mouse Unit (MU) which is equivalent to five Nano gram CTX (Lewis, 1995). Dose and time to death relationship for a mix of ciguatoxin typically found in carnivorous fish are defined according to the equation: log MU = 2.3 log (1 + 1/T), where T is the time to death in hour (Lewis, 1995). The toxic fraction suspended in 0.5 ml 1% Tween 60/0.9% saline and assayed in duplicate. As the death time calculated during the experiment is 2.67 h, the lethal dose estimated to be 2.08 MU 20 mg^{-1} of ether according to the equation and the amount of CTX toxicity in fish sample is equivalent to 10.4 ng of CTX -1.

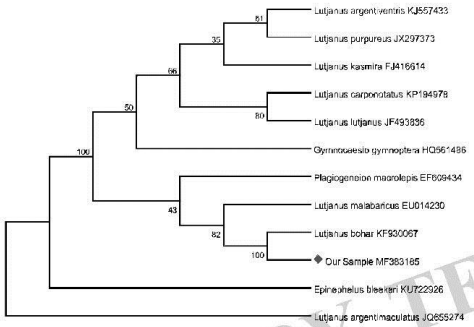

Fig. 1. Phylogenetic tree of Lutjanus bohar generated based on partial (641bp) COI gene sequences.

Ciguatoxin sample analysis data based on IOC manuals and guides mouse bioassay method (Lewis, 1995) has been submitted as an agenda item 3 in Codex Committee on Contaminants in foods, eleventh session (CCCF, 2017). In the absence of commercial testing, a reliable laboratory analytical method is needed to confirm the presence of ciguatoxicity among the reef fish samples meant for human consumption. Fish carrying ciguatoxin do not exhibit any symptoms and it is practically difficult to ascertain whether the fish is toxic or not. Existence of CTXs along Indian coast calls for a need for good surveillance system and analytical confirmatory methods for the protection of consumers along with exporters. Hence mouse bioassay of fish extract considered as a reliable approach to detect the presence of sub lethal doses of CTXs through intermittent observation of symptoms for up to 48 h (Caillaud et al., 2010). In present scenario, where food safety is becoming prime concerns of all people, it is felt that the ciguatera poisoning will assume and major concern for the marketing of reef associated fin fishes. The amount of toxins is directly correlated to the size of the fish and results indicated that large sized fishes had more ciguatoxin in comparison to small fishes (Pottier et al., 2001). Hence it is advisable for the consumers to take only *L. bohar* of small size. Ban or size restrictions on certain reef fish species can be taken as an initial safety measure to protect the consumers from the lethal effects of this toxicity.

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References

Abraham, A., Jester, E.L.E., Granade, H.R., Plakas, S.M. and Dickey, R.W. (2011) Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. Food Chem. 131: 192-198

Banner, A., Scheuer, P., Sasaki, S., Helfrich, P. and Alender, C. (1960) Observations on ciguatera type toxin in fish. Ann. N.Y. Acad. Sci. 90: 770-787

Caillaud, A., de la Iglesia, P., Darius, H. T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain M. and Diogène, J. (2010) Update on Methodologies Available for Ciguatoxin Determination: Perspectives to Confront the Onset of Ciguatera Fish Poisoning in Europe, *Marine Drugs*, 8 (6): 1838-1907. Doi:10.3390/md8061838

CCCF(2017) Codex Committee on Contaminants in Foods, Joint FAO/WHO Food Standards Programme, Eleventh Session, Rio de Janeiro, Brazil, 3 – 7 April 2017 Agenda item 3 CF11/CRD37. Ciguatoxin Sample Analysis Data - India

de Fouw, J., Egmond, H. and Speijers, G. (2001). Ciguatera fish poisoning: a review. RIVM report 388802021

Dickey, R.W. and Plakas, S.M. (2010) Ciguatera: A public health perspective. Toxicon, 56, 123-136

Friedman, M.A., Fernandez, M., Backer, L.C., Dickey, R.W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Flores Quintana, H.A., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T. and Fleming, L.E. (2017) An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. Mar. Drugs. 15: 72

FISHERY TECHNOLOGY

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-98

Lewis, R.J. (1995) Detection of Ciguatoxins and related Benthic Dinoflagellate Toxins: *in vivo* and *in vitro* Methods. In *Manual on Harmful Marine Microalgae*; Hallegraeff, G., Anderson, D., Cembella, A., Eds.; IOC Manuals and Guides No.33, UNESCO: Paris, France. 33: 135-161

Lehane, L. and Lewis, R.J. (2000) Review Ciguatera: recent advances but the risk remains. Int. J. Food Microbiol. 61: 91-125

Lewis, R. J. (2001) The changing face of ciguatera. Toxicon. 39: 97-106

Lewis, R.J. (2006)Ciguatera: Australian perspectives on a global problem. Toxicon. 48(7): 799-809

Pottier, I.,Vernoux, J. and Lewis, R. (2001) Ciguatera fish poisoning in the Caribbean islands and Western Atlantic. Rev. Environ. Contam. Toxicol. 168: 99-141

Rajeish, M., Shekar, M., Madhushree, H.N. and Venugopal, M.N. (2016) Presumptive case of ciguatera fish poisoning in Mangalore, India. Research Communications, Curr. Sci. 111 (9): 1543-1547

Rajisha, R., Pankaj Kishore., Satyen Kumar Panda., Harikrishnan, G., Ajitha, K. C., Suresh, M. K., Labrechai Mog Chowdhury., Ravishankar, C.N. and Ashok Kumar, K. (2017) Incidence of Ciguatoxin Fish Poisoning in Trivandrum, India. Indian J. Fish. (In Press)

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DOI: 10.21077/ijf.2017.64.4.71558-20

Note

Incidence of ciguatoxin fish poisoning in Trivandrum, India

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ABSTRACT

Ciguatoxin (CTX) is a visibly unidentifiable, colourless, odourless, heat stable and lipid soluble polyether marine biotoxin associated with human illness. Marine dinoflagellates under the genus *Gambierdiscus* are responsible for producing ciguatoxins (CTX). The ciguatoxin gets accumulated in herbivorous fishes, gets biotransformed in carnivorous fishes and finally reach fish consumers. In January 2016, individuals who consumed red snapper in Trivandrum, Kerala, India were suspected to be intoxicated with ciguatera based on characteristic symptoms as assessed by medical team from Trivandrum Medical College, Kerala, India. The red snapper species was identified and confirmed as *Lutjanus bohar* by DNA barcoding. Mouse bioassay was carried out to detect the presence of ciguatoxin and the tested mice showed symptoms related to suspected CTX toxicity. Significantly higher level of ciguatoxin lethal dose was estimated which was found equivalent to 16.25 ng of CTX-1 which led to 13% of weight loss in tested mice. Medical professionals also investigated clinical manifestations of suspected toxicity in hospitalised individuals. This study indicated that there is a need for regular surveillance of seafood landed across the coast and consumer's awareness for their safety.

Keywords: Ciguatera fish poisoning, Ciguatoxin, *Lutjanus bohar*, Mouse bioassay

Ciguatoxin (CTX) affects approximately 50,000 to 500,000 people per year (Meyer *et al.*, 2015). Ciguatera fish poisoning (CFP) causes acute gastrointestinal and neurological symptoms, including vomiting, diarrhoea, abdominal pain, severe localised itching, tingling of extremities and lips, dysesthesia, as well as other chronic symptoms (Lewis, 2001; 2006). CFP occurs due to consumption of reef fishes of larger size in tropical and subtropical regions. More than 400 fish species are reported to be causative agents for ciguatera poisoning. Larger fishes accumulate gambiertoxins and their biotransformation in the fish makes it more potent in comparison to those in small fishes (Lehane and Lewis, 2000; Farrell *et al.*, 2016). The dinoflagellate species *Gambierdiscus toxicus* is the main source of the production of this marine toxin and its accumulation in fishes (FAO, 2004). Ciguatoxin is a colourless, odourless, heat stable and lipid soluble polyether compound. This toxin remains unaffected by freezing, drying or cooking process (Lewis, 2006; Abraham *et al.*, 2011). CTXs are secondary metabolites with numerous congeners having different molecular structure reported from different geographical areas such as Pacific, Caribbean and Indian regions (Caillaud *et al.*, 2010).

Incidence of CTX toxicity from Kerala coast has not been reported so far, as it had escaped many a times the attention of medical practitioners. The present study investigated a recent incident of food poisoning suspected due to ciguatera toxin from red snapper in Trivandrum, which presented characteristic symptoms in those who consumed the fish.

Fish samples for the investigations were collected from Vizhinjam, Kerala, India in January 2016, where intoxication in local population were reported. The poisoning occurred due to the consumption of cooked "red snapper" fish coming under the genus *Lutjanus*, purchased from the local fish market. Medical team from Trivandrum Medical College observed that the intoxicated persons had consumed same fishes and developed neurological and gastrointestinal complications similar to that of ciguatera fish poisoning. The fish samples collected were stored at -20°C until analysis.

Caudal peduncle samples of the fishes were used for species authentication by sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene. The total genomic DNA from the samples was isolated using DNeasy Blood and Tissue Kit (Qiagen, Germany),

as per manufacturer's instructions and concentration as well as purity of the extracted DNA was estimated using a biospectrometer (Eppendorf, Germany). The COI gene was amplified using universal primer pair (Ward *et al.,* 2005) (Table 1).

Table 1. Primers used for identification of fish implicated in Ciguatera fish poisoning

Primer name	Sequence
Fish F1	5'- TCAACCAACCACAAAGACATTGGCAC -3'
Fish R1	5'- TAGACTTCTGGGTGGCCAAAGAATCA -3'

The reaction was carried out in 25µl volume, containing mixture of 1x taq buffer, 2.5 mM $MgCl_2$ and 50 µM of each primer, 200 µM of each dNTP, 0.5 U taq DNA polymerase, 75 ng of template DNA and autoclaved double distilled water to make up the volume to 25 µl. The reaction mixture was thermal cycled for 35 cycles of 30 s at 94°C, 30 s at 52°C, 45 s at 72°C and final extension of 10 min at 72°C, with an initial denaturation step at 95°C for 4 min. The PCR products were sequenced bidirectionally using ABI 3730 capillary sequencer in the sequencing facility. The raw DNA sequences obtained were edited and aligned using BioEdit version 7.0.5.2 (Hall, 1999). The edited partial sequences of COI gene were analysed for species identification using the NCBI BLAST search engine and the sequences were submitted to GenBank database (NCBI, USA).

Extraction of ciguatoxin from fish samples was carried out as per IOC manuals and Guides No. 33 (Hallegraeff *et al.*, 1995) and European Union Reference method (ANSES, 2016) for mouse bioassay of ciguatoxin. Fifty gram of fish sample was cooked at 70°C for 15 min, and cooled to room temperature. Tissue samples were then minced, diluted with acetone [3:1 V:W (ml g^{-1})] and homogenised for 5-15 min using a homogeniser (PRO Scientific Inc., USA) under iced condition. The homogenised samples were filtered using Whatman no.1 filter paper and the filtrate collected in a round bottom flask. Residual acetone and water were removed using a rotary evaporator (Heidolph, Germany) operated at 55°C. The dried extract was transferred to a separatory funnel, added methanol:water (9:1), shaken well followed by extraction with 1:1 (v/v) n hexane and the upper hexane layer was discarded. This extraction process was repeated twice. The residual methanol:water was removed using vacuum evaporator. Further, ethanol:water (1:3) was added and shaken with diethyl ether (1:1) to separate the layers and the ether layer was collected. Ether extraction was repeated twice and ether fractions were pooled at an elevated temperature of 40-55°C. The dried ether extract collected were assumed to contain the CTXs.

Ether extract was dissolved in chloroform:methanol (97:3) mixture and dried under N_2. The dried ether fraction was suspended in 1-5% tween 60/0.9% saline, sonicated for 180 s and filtered through 0.45 PTFE membrane filter prior to administering into mice.

Female albino mice weighing 20±2 g were used for the assay done in duplicate, by intraperitoneal injection with 0.5 ml of the prepared extract, whereas control mice were injected with only 0.5 ml tween 60/saline solution. Details such as time of injection, weight of mice, amount of extract (g) administered, time of onset and nature of signs and time of death were recorded for each injection. The post-injection behaviour was observed and recorded for at least 24 h. Weight loss in injected mice was also recorded at an interval of minimum 3 h duration.

The symptomatology in the hospitalised patients corroborated with earlier reports of ciguatera fish poisoning (Rajeish *et al.,* 2016) with typical clinical signs like gastrointestinal, neurological and cardiovascular symptoms. All six patients (one male and five females) were admitted with CFP symptoms of vomiting, diarrhoea, paraesthesia of upper limbs and lower limbs. Out of this, five patients belonged to one family comprising husband, wife and three daughters who live near Chakkipara Market, Trivandrum. Their symptoms started six hours after consumption of fish dish (chempalli curry). Symptoms like vomiting, diarrhoea, circumoral paraesthesia and paraesthesia of limbs were common to all family members. One of the three daughters also had paradoxical temperature reversal (cold objects sensed as hot and hot objects sensed as cold). All the patients were haemo-dynamically stable except the husband who had sinus bradycardia (low heart rate). The sixth patient was a female and her major symptom was giddiness. She also had abdominal pain and paraesthesia of limbs. In the affected individuals, the onset of ciguatera toxicity started within 24 h of consumption of fish curry and symptoms lasted for 1-4 days. However, in case of one individual, it persisted for six months as reported previously by Glaziou and Martin (1993) and Pearn (1995). Clinical diagnoses of CFP are reliable when a detailed and comprehensive history of the food source, onset of the illness and description of symptoms are accounted (Stewart *et al.,* 2009).

All the above said patients were treated by giving supportive measures like intravenous fluids and antiemetics. The husband's bradycardia improved and normal heart rate was restored after 2 days. Patients were in better condition at the time of discharge and the only symptom that persisted was paraesthesia of limbs.

The partial sequence of the mitochondrial CO1 gene from the tissue samples yielded an average length of 627 bp (Accession. No. KY057337). These sequences were used for identification of species based on the similarity search using the NCBI BLAST search engine and the species was identified as *Lutjanus bohar,* commonly known as two spot red snapper. *L. bohar* is a reef-associated tropical fish distributed along the Indo-Pacific region with earlier reports of ciguatera poisoning (Halstead *et al.,* 1990) and large fishes from oceanic areas in the western Pacific are often ciguatoxic (Dalzell, 1992). According to Oshiro *et al.* (2010), *L. bohar* weighing less than 4 kg to be non-toxic and 11.9% of the species exhibit CTX toxicity.

Intra-peritoneal injection of toxin extract from fish tissue induced symptoms in mice as indicated in case of CTX toxicity. A detailed description of symptoms of toxicity recorded in mice up to a 24 h period observation is given in Table 2. The prominent symptoms included piloerection, diarrhoea, lachrymation, dyspnoea, gasping, progressive hind limb paralysis, wobbly gait, terminal convulsions with tail arching, breathing difficulties, slow locomotor activity and hypothermia. However, these symptoms were absent when extract from control and negative (non-toxic) fish samples were administered in mice. The relationship between dose and time to death was used to quantify toxicity of the extract which ranged from 30 min to >10 h.

Traditional method of detecting the presence of ciguatoxin in fish involves testing lipid extracts by mouse bioassay (Lewis and Sellin,1993) and the most widely used mouse bioassay method was described by Yasumoto *et al.* (1984) which has been accepted worldwide. The lethal dose *i.e.,* LD_{50} dose for a 20 g mouse is equal to one Mouse Unit (MU) which is equivalent to 5 ng CTX-1. One MU is equivalent to 5 ng, 18 ng and 48 ng for Pacific CTXs, P-CTX-1, P-CTX-2 and P-CTX-3, respectively (Lewis *et al.,* 1991; Lewis and Sellin, 1993) and 72 ng for pure Caribbean CTX-1 (Pottier *et al.,* 2003). Dose and time to death relationship for a mix of ciguatoxins typically found in carnivorous fish is defined according to the equation:

$$\text{Log MU} = 2.3 \log (1 + 1/T)$$

where, T is the time to death in hours (Lewis and Sellin, 1992). The lethal dose was estimated to be 3.25 MU per 20 mg of ether extract and the amount of CTX toxicity in fish sample is equivalent to 16.25 ng of CTX -1, which is significantly higher than the reported levels of CTX intoxication in humans. It was formerly suggested that any fish containing above 2.5 MU 100 g^{-1} should be avoided as food (Yasumoto *et al.,* 1984) since ciguatoxins are potent neurotoxins that may have long-term neurological effects. The average weight loss observed in the positive sample was calculated as 13%. The evaluation of toxicity of the fish tissue samples based on mouse bioassay was interpreted as shown in Table 3. The death of 1 or 2 mice within 24 h is interpreted as positive for ciguatoxicity and the fish sample is rated as inedible. In the absence of death, weight loss >5% after 24 h of injection of atleast one mouse is considered as a positive result for ciguatoxicity and the fish sample is considered as edible to limited extent. When there is no mortality or weight loss, then the sample is edible without doubt. MBA (mouse bioassay) provides a measure of total toxicity based on the biological response of the animal to the toxins but no specific information is provided on individual toxins.

Table 2. Symptoms of ciguatera toxicity recorded during mouse bioassay

Symptoms	Evaluation	Animal responses	
		Control	Toxin treated
Hypothermia	Thermometer	35 - 38^{0}C	Below 33^{0}C
Piloerection	Observation	None	Mild to marked
Lachrymation	Observation	Normal	Mild to severe
Hyper salivation	Observation	Absent	Mild to severe
Dyspnoea	Observation	Absent	Mild to severe
Wobbly upright gait	Observation	Absent	Present
Gasping	Observation	Absent	Mild to marked
Withdrawal reflex	Grasp hind leg	Withdrawal	Reduced to absent
Mild gasping	Observation	Absent	Present
Diarrhea	Observation	Absent	Mild
Breathing difficulties	Observation	Absent	Rapid shallow to intermittent gasping
Locomotor activity	Observation	Normal	Slow to absent
Hind limb paralysis	Observation		Progressive paralysis from hind limb extending to fore limbs
Convulsions	Observation	Absent	Tonic and/or Jumping

Table 3. Interpretation of ciguatoxicity of fish tissue samples based on mouse bioassay

Test sample	No. of dead mouse (s) in 24 h	Weight loss >5% after 24 h injection	Conclusion
Suspected sample 1	One of two	Yes	Positive, Not edible
Suspected sample 2	0	Yes	Positive, limited edibility
Control	0	No	Negative, edible

Source: EU-NRL (ANSES, 2016)

Banner *et al.* introduced the MBA for CTXs in1960 and this is most widely used mammalian *in vivo* model for toxicity screening of CTXs (Caillaud *et al.,* 2010). Along Indian coast, CFP incidents are infrequent. Routine analysis of samples by mouse bioassay cannot be recommended since it is non-specific and ethically objectionable (Abraham *et al.,* 2011) and considered as a toxicological tool accessible only to selected laboratories. We carried out mouse bioassay test to identify and quantify CTX toxins in order to provide further support for the clinical diagnosis of the CFP incident. Proficiency in the ability to identify the toxic fish and effective clinical recognition will definitely improve our understanding of the source of poisoning.

This study is the first report in incidence of ciguatoxin poisoning along Kerala coast and signifies the importance of seafood safety. In the absence of commercial testing, a precautionary approach is necessary for the surveillance of CTX intoxication along the Indian coast. The implementation of regulatory criteria for CTXs would be needed with respect to aspects like identification of ciguatoxic fish mainly reef associated fish, regulatory measures such as ban or size restrictions on high-risk species and misdiagnosis or under-reporting of CFP cases. A rapid and reliable instrumentation method through mass spectrometry, with the aim of routine monitoring and screening of CFP in reef fishes along the Indian coast is the need of the hour. The study also signifies the need for creating awareness regarding consumption of coral reef fishes and its consequences among the public.

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References

Abraham, A., Jester, E. L. E., Granade, H. R., Plakas, S. M. and Dickey, R. W. 2011. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. *Food Chem.*, 131: 192-198.

ANSES 2016. *Methoded'analyse en securite sanitaire des aliments: Detection des ciguatoxinesdans la chair de poisson par bioessaisursouris [Method of analysis for food safety: Detection of ciguatoxins in the Flesh by bio-assay on mouse:],* ANSES / LSAliments / LSA-INS-0150 - Version 01, 06/04/2016 (French Agency for Food, Environmental and Occupational Health and Safety: Maisons-Alfort, France), 16 pp.

Banner, A., Scheuer, P., Sasaki, S., Helfrich, P. and Alender, C. 1960. Observations on ciguatera-type toxin in fish. *Annals. N. Y. Acad. Sci.*, 90: 770-787.

Caillaud, A., de la Iglesia, P., Darius, H. T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain M. and Diogene, J. 2010. Update on methodologies available for ciguatoxin determination: Perspectives to confront the onset of Ciguatera fish poisoning in Europe. *Marine Drugs*, 8(6): 1838-1907. doi:10.3390/md8061838.

Dalzell, P. 1992. Ciguatera fish poisoning and fisheries development in the South Pacific region. *Bull. Soc. Pathol. Exot.,* 85: 435-444.

FAO 2004. Marine biotoxins. *FAO food and nutrition paper*, Food and Agriculture Organisation of the United Nations, Rome, 80(7): 192 pp.

Farrell, H., Zammit, A., Manning, J., Shadbolt, C., Szabo, L., Harwood, D. T., McNabb, P., Turahui, J. A. and van den Berg D. J. 2016. Clinical diagnosis and chemical confirmation of ciguatera fish poisoning in New South Wales, Australia. *Communicable Diseases Intelligence*, 40(1): 1-6.

Glaziou, P. and Martin, P. M. 1993. Study of factors that influence the clinical response to ciguatera fish poisoning. *Toxicon.* 31(9): 1151-1154.

Hall, T. A. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucl. Acids. Symp. Ser.,* 41: 95-98.

Halstead, B. W., Auerbach, P. S. and Campbell, D. R. 1990. *A colour atlas of dangerous marine animals,* Wolfe Medical Publications Ltd, W. S. Cowell Ltd., Ipswich, England, 192 pp.

Hallegraeff, G. M., Anderson, D. M. and Cembella, A. D. 1995. Manual on harmful marine microalgae, *IOC Manuals and Guides No. 33,* UNESCO, p. 135-161.

Lehane, L. and Lewis, R. J. 2000. Review Ciguatera: recent advances but the risk remains. *Int. J. Food Microbiol.,* 61: 91-125.

Lewis, R. J. and Sellin, M. 1992. Multiple ciguatoxins in the flesh of fish. *Toxicon*, 30(8): 915-919.

Lewis, R. J. 2001. The changing face of ciguatera. *Toxicon,* 39(1): 97-106.

Lewis, R. J. 2006. Ciguatera: Australian perspectives on a global problem. *Toxicon*, 48(7): 799-809.

Lewis, R. J. and Sellin, M. 1993. Recovery of ciguatoxin from fish flesh. *Toxicon,* 31(10): 1333-1336.

Lewis, R .J., Sellin, M., Poli, M. A., Norton, R. S., MacLeod, J. K. and Sheil, M. M. 1991. Purification and characterisation of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). *Toxicon*, 29(9): 1115-1127.

Meyer, L., Carter, S. and Capper, A. 2015. An updated ciguatoxin extraction method and silica cleanup for use with HPLC - MS/MS for the analysis of P-CTX-1, PCT-X-2 and P-CTX-3. *Toxicon,* 108: 249-256.

Oshiro, N., Yogi, K., Asato, S., Sasaki, T., Tamanaha, K., Hirama, M., Yasumoto, T. and Inafuku, Y. 2010. Ciguatera incidence and fish toxicity in Okinawa, Japan. *Toxicon,* 56(5): 656-661.

Pearn, J. 1995. Ciguatera - a potent cause of the chronic fatigue syndrome. *Int. J. Immunopharmacol.*, 15: 63-65.

Pottier, I., Hamilton, B., Jones, A., Lewis, R. and Vernoux, J. 2003. Identification of slow and fast-acting toxins in a highly ciguatoxic barracuda (*Sphyraena barracuda*) by HPLC/MS and radiolabeled ligand binding. *Toxicon,* 42(6): 663-672.

Rajeish, M., Shekar, M., Madhushree, H. N. and Venugopal, M. N. 2016. Presumptive case of ciguatera fish poisoning in Mangalore, India. Research Communications. *Curr. Sci.*, 111(9): 1543-1547.

Stewart, I., Eaglesham, G., Poole, S., Graham, G., Paulo, C., Wickramasinghe, W., Sadler, R. and Shaw, G. 2009. Establishing a public health analytical service based upon chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples. *Toxicon,* 56(5): 804-812.

Yasumoto, T., Raj, U. and Bagnis, R. 1984. Seafood poisonings in tropical regions, Laboratory of Food Hygiene, Faculty of Agriculture, Tohoku University, Japan, 74 pp.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R. and Hebert, P. D. 2005. DNA barcoding Australia's fish species. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.,* 360(1462): 1847-1857.

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Determination of ciguatoxin in fish by liquid chromatography tandem mass spectrometry and comparison with mouse bioassay

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A visibly unidentifiable colourless, odourless, heat stable, lipid soluble polyether marine biotoxin known as Ciguatoxin (CTX) responsible for human illness has been identified from certain fish collected from Cochin, Trivandrum and Mangalore. Our study showed that among the 14 species collected, *Lutjanus bohar* (7.54 kg) has been identified for ciguatoxicity. Detection of the toxin was done using mouse bioassay which showed symptoms related to suspected CTX toxicity. Significantly, a higher level of 2.17 mouse unit Ciguatoxin was estimated which is equivalent to 10.84 ng of CTX toxicity and 13% of weight loss. Solid phase extraction (SPE) was employed for the separation and purification of the toxin from the fish extract, which was confirmed and quantified by gradient reverse phase liquid chromatography tandem mass spectrometry (LC- MS/MS). The Extracted Ion Chromatogram of CTX fish extract of *Lutjanus bohar* showed m/z 1117.303 Da precursor/product confirmatory ion transitions.

Nucleotide

GenBank

Lutjanus bohar strain CG01 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

GenBank: KY057337.1

FASTA Graphics

Go to:

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VERSION     KY057337.1
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  ORGANISM  Lutjanus bohar
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  AUTHORS   Rajisha,R., Kishore,P., Panda,S.K., Kumar,A., Sankar,T.V.,
            Ganesan,B., Harikrishnan,G. and Chowdhury,M.L.
  TITLE     Ciguatoxin in fish from India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 621)
  AUTHORS   Rajisha,R., Kishore,P., Panda,S.K., Kumar,A., Sankar,T.V.,
            Ganesan,B., Harikrishnan,G. and Chowdhury,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-OCT-2016) Quality Assurance and Management (QAM)
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            Matsyapuri P.O., CIFT Junction, Willingdon Island, Cochin, Kerala
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