Molecular and Functional Characterization of Histone Derived Antimicrobial Peptides from Marine Organisms

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By

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To My Mother…

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Certificate

*This is to certify that the thesis entitled "**Molecular and Functional Characterization of Histone Derived Antimicrobial Peptides from Marine Organisms"** is an authentic record of the research work carried out by **Mr. Naveen Sathyan** under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements of the degree of **Doctor of Philosophy Under the Faculty Of Marine Sciences** of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

Dr. Rosamma Philip

Kochi – 16

Date: 2/5/2015

Declaration

*I hereby declare that the thesis entitled "**Molecular and Functional Characterization of Histone Derived Antimicrobial Peptides from Marine Organisms"** is a genuine record of research work done by me under the supervision and guidance of **Dr. Rosamma Philip, Associate Professor,** in the Department of the Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.*

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Certificate

This is to certify that this thesis entitled ***"Molecular and Functional Characterization of Histone Derived Antimicrobial Peptides from Marine Organisms"*** *is a bonafide record of research work carried out by* ***Mr. Naveen Sathyan*** *under my supervision and guidance. I also certify that all relevant corrections and modifications suggested by the audience during the presynopsis seminar and recommended by the doctoral committee has been incorporated in this thesis.*

Dr. Rosamma Philip

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Naveen Sathyan

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General Introduction

1.1 Introduction

Antimicrobial peptides (AMPs) are gene encoded, small sized, generally cationic, amphiphathic peptides characterized by antimicrobial activity against bacteria, fungi, viruses and other pathogens. They are a major component of the innate immune defense system of almost all living organisms, ranging from bacteria to humans and represent the first line of defense against the invading microbial pathogens (Boman, 1995; Zasloff, 2002). Antimicrobial peptides represent a heterogeneous group displaying multiple modes of action that are determined by the sequence and concentration of peptides. Their remarkable specificity for prokaryotes with low toxicity for eukaryotic cells has favored their investigation and exploitation as new antibiotics (Zasloff, 1992).

The number of bacteria gaining resistance to conventional antibiotics is on increase recently and therefore there is a great demand for novel therapeutants. The Infectious Diseases Society of America launched its 10 × 20 initiative in the year 2010, calling for a worldwide participation for developing new antibacterial drugs, with the aim of developing ten new

antibiotic agents by the year 2020 (Boucher et al., 2009). Endogenous antimicrobial peptides appear to be exciting candidates to be developed into new antibacterial agents. Antimicrobial peptides have retained potency, in the face of exceedingly mutable pathogens over an evolutionary time span (Tossi, 2005). Their case is further strengthened by their broad spectrum antimicrobial activity and high selectivity. Much of the research over the past decade was focused on bioprospecting for novel antimicrobial compounds capable of exhibiting a broad spectrum activity against a wide range of microorganisms. Antimicrobial peptides were found to be exciting molecules as they display direct microbicidal activities towards bacteria, fungi, parasites and viruses (Jenssen et al., 2006). New advancements in bioinformatics tools have revolutionized AMP research by facilitating the structural modification of AMPs aimed at synthesizing more selective and effective drugs. The increasing data on AMPs is a positive indication that antimicrobial peptides have a great potential to be the next breakthrough in therapeutics.

1.2 Source of Antimicrobial Peptides

Antimicrobial peptides were first isolated and characterized from bacteria, that are produced to kill other microbes, and thereby reducing the competition for space, food and other nutrients (Willey & Donk, 2007). However, the wide recognition of AMPs started in the 1960s with the discovery of peptides with antimicrobial properties in polymorphonuclear leukocytes (Zeya & Spitznagel, 1963; Zeya & Spitznagel, 1966). These peptides were later named as defensins (Selsted et al., 1984; Ganz et al., 1985). Bioprospecting for AMPs in invertebrates were initiated in 1980s (Steiner, 1982) and since then thousands of AMPs have been isolated and

reported from a wide range of organisms. These peptides can be synthesized at a low metabolic cost and easily stored in large amounts. The ability to recognize common characteristics of pathogens instead of identifying unique and specific tags particular to a specific pathogen make AMPs safe and efficient (Danilova, 2006). It is predicted that each species is capable of producing more than two dozen AMPs (Hancock & Rozek, 2002). Though AMPs demonstrate similarity in their biophysical properties, there are differences in amino acid sequence, even in the same class of AMPs isolated from two different species of animals, even if they are closely related.

The Antimicrobial Peptide database (second version) (http://aps.unmc.edu/AP/statistic/statistic_structure.php) abbreviated as the APD2 (Wang & Wang, 2004; Wang et al., 2009a) as on April, 2015 contain 2531 different antimicrobial peptides having an average length of 32.47 residues and average net charge of +3.19. Majority of the total 2531 reported AMPs are from animals (75.73%) and the rest has been reported from plants (13.55%), bacteria (8.43%), fungi (0.43%) and protozoan (0.23%) along with synthetic peptides (1.58%).

1.3 Structural Diversity of Antimicrobial Peptides

Antimicrobial Peptides exhibit remarkable diversity and can be categorized into many subtypes based on different criteria: origin, size, charge, length, structure, amino acid sequence, biological action and mechanism of action but it has been shown that secondary structure is the only meaningful criterion to sort and classify them (Epand & Vogel, 1999; van't Hof et al., 2001). Based on structure, Hancock & Lehrer (1998) proposed four classes of AMPs, which are β-sheet, α-helical, loop and extended peptides (Fig. 1.1). The

α-helical form the largest and the most common family of AMPs in nature. They are highly positively charged peptides with a notable amphipathic behavior, and have a tertiary structure with a hinge in the middle (Gennaro & Zanetti, 2000; Tossi et al., 2000). β-sheet antibacterial peptides form a family of AMPs composed of β-sheet peptides that are conformationally constrained and stabilized by one to five disulfide bridges (Brogden, 2005). Looped antibacterial peptides are characterized by their looped structure imparted by the presence of a single bond (disulfide or amide or isopeptide bond). Linear extended antibacterial peptides are characterized by unusual amino acid composition due to overexpression of one or more amino acid. They are linear in shape and do not show any secondary structure either in α-helix or in β-sheet.

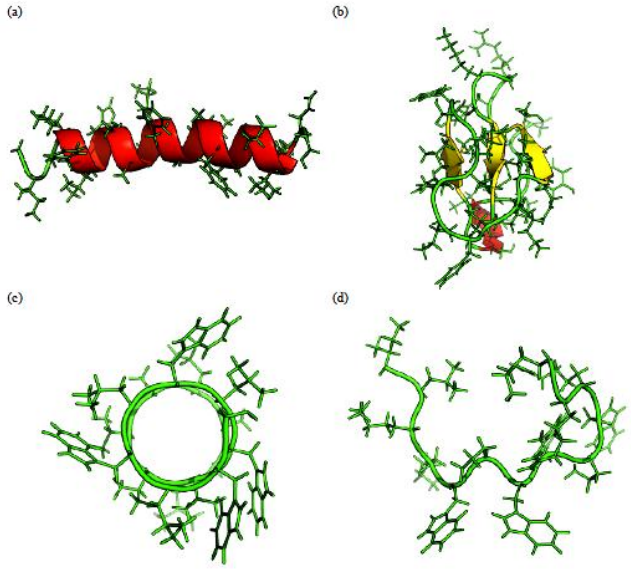


Fig. 1.1. Three dimensional model structures representing the differences between the four classes of cationic peptides; (a) α-helical peptide, (b) β-defensin, (c) Loop structure and (d) Extended peptide. (Adopted from Cézard et al., 2011).

1.4 Biophysical Parameters of Antimicrobial Peptides

Antimicrobial peptides possess some common characteristic features which are important for their antimicrobial activity. One of the striking characteristic features of AMPs is preservation of function without any conservation in size and sequence. The reason for common antimicrobial function in spite of the difference in sequence of AMPs can be attributed to the necessity of the host immune system to successfully adapt to different environments by retaining its efficiency against specific microbial pathogens (Tossi et al., 2000). Antimicrobial peptides are small sized having a length of less than 50 amino acids, although a small group of longer AMPs have been reported. Majority of AMPs deposited in APD2 database fall in the length range of 20 to 40 amino acid residues.

Majority of AMPs reported to date are characterized to have a net positive charge in the range of +2 to +9 (Yeaman and Yount, 2003). Net positive charge is important for the initial electrostatic attraction of AMPs to negatively charged phospholipid membranes of bacteria and other microorganisms. The APD2 database contains antimicrobial peptides with charge ranging from -4 to +10, and the majority lies in the charge range of +2 to +4.

Upon interaction with the target membranes almost all AMPs form an amphipathic structure in order to accommodate the inherently amphipathic nature of the membrane lipid matrix. The proportion of hydrophobic and hydrophilic surface varies among peptides (Segrest et al., 1994). Hydrophobicity of a peptide is defined as the proportion of hydrophobic residues within a peptide and is normally around 50 % for most AMPs. Hydrophobicity is an important physico-chemical characteristic which is

considered to be independent of other structural parameters (Yount & Yeaman, 2005). Hydrophobicity is an essential feature for antimicrobial peptide membrane interactions, as it determines the extent to which a peptide can insert into the lipid bilayer. Polar angle is a measurement of the relative proportion of polar versus nonpolar facets of a peptide conformed to an amphipathic helix (Yount & Yeaman, 2005). The polar angle has also been shown to correlate with the overall stability and half-life of peptide-induced membrane pores (Yount & Yeaman, 2005).

It can be said that the activity of AMPs is not determined by a single factor but by a combination of factors including sequence, hydrophobicity and position of cationic residues. Amphipathicity, hydrophobicity, polar angle and conformation of a peptide also play a role in the antimicrobial activity; however there is no strict rule regarding the optimal number of charged and hydrophobic residues for maximum antimicrobial activity and minimum cytotoxicity as it varies widely among different peptides and within a given structural group (Khandelia et al., 2008).

1.5 Mode of Action of Antimicrobial Peptides

1.5.1 Antibacterial Activity

Majority of AMPs kill bacteria by membrane disruption leading to pore formation and membrane depolarization. The bacterial cell membranes are made up of negatively charged phospholipids such as phosphatidylglycerol, cardiolipin, or phosphatidylserine (Cronan, 2003). Unlike bacterial membranes, mammalian membranes are rich in sterols and zwitterionic phospholipids with neutral net charge including phosphatidylethanolamine, phosphatidylcholine, or sphingomyelin. This difference is between bacterial

cell and mammalian cell is the basis of AMP specificity. Moreover, presence of significant amounts of cholesterol in mammalian membranes reduce the activity of AMPs by affecting the fluidity and dipole potential of phospholipids, in addition to stabilizing the lipid bilayers and delaying the binding of peptides to the membranes (Tytler et al., 1995; Matsuzaki, 1999).

A comprehensive account of the mode of action of AMPs on bacterial cells has been explained by Giuliani et al. (2007). Electrostatic interaction between the cationic AMPs and negatively charged membrane of the target cell is the driving force behind the binding and accumulation of the peptides on the surface of the target cell membrane. Thereafter bound peptide remains on the membrane with its long helical axis parallel to the membrane surface until a threshold concentration is reached. Once the threshold concentration is reached, peptide-peptide and peptide–lipid interactions will create a complex structure, which is associated with the specific antimicrobial action. AMPs interact with membranes and tend to divide peptides into two mechanistic classes: membrane disruptive and non-membrane disruptive (Powers and Hancock, 2003).

The barrel-stave model was the first mechanism proposed to explain bacteria killing by AMPs (Ehrenstein et al., 1977). According to this model, AMPs accumulate in circular patterns on the bacterial surface as monomers and assume an orientation parallel to the lipid bilayer. This is followed by perpendicular orientation and insertion into the lipid core of the membrane resulting in a shape resembling a barrel whose staves are the α-helical AMPs (Yang et al., 2001). According to Carpet model (Pouny et al., 1992), the AMPs aggregate onto the bilayer surface but, unlike barrel stave model, keep a parallel alignment to the membrane surface during the process (Bechinger, 1999). The peptides accumulated coat the bacterial surface in a carpet-like way

and eventually the membrane is disrupted due to unfavourable energetics. The toroidal pore model (Hancock and Chapple, 1999) combines the actions of the barrel-stave and carpet models. The AMPs aggregate on the membrane similarly to the carpet model and then insert themselves perpendicularly into it, causing its deformation (Matsuzaki et al., 1996). (Yamaguchi et al., 2002). The aggregate channel model (Hancock and Chapple, 1999) proposes that after binding to the phospholipid head groups, the peptides insert into the membrane and then cluster into unstructured aggregates allowing the dynamic formation of pores that span the membrane for short periods.

There are evidences indicating that AMPs have non-membrane disruptive mechanism (intracellular targets) as well (Yeaman & Yount, 2003). Antimicrobial peptides, like the 21 amino acid Buforin II and arginine rich AMPs, do not permeabilize the bacterial outer membrane, but penetrate it to accumulate in the cytoplasm, exerting its cytotoxic activity (Park et al., 2000; Futaki et al., 2001). Once translocated in the cytoplasm, AMPs are able to act on many levels: inhibition of cell-wall synthesis, inhibition of enzymatic activity, inhibition of DNA, RNA and protein synthesis, binding to DNA, altering cytoplasmic membrane (inhibition of septum formation) and/or activation of autolysin.

1.5.2 Antitumor Activity

A number of studies have shown tumor cells to be susceptible to antimicrobial peptides. AMPs can differentiate cancerous cells from their non-transformed counterparts. The basis for this difference is not fully clarified; changes in membrane potential due to higher metabolism, higher exposure of acidic phospholipids in the outer leaflet of membrane, or cytoskeleton

alteration and possible alterations in the extracellular matrix have been proposed as potentially implied in the process (Andreu & Rivas, 1998).

1.5.3 Bacterial Resistance to Antimicrobial Peptides

Antimicrobial peptides are generally not known to trigger resistance in bacteria, however, exceptions are there. Both Gram positive and Gram negative bacteria have been reported to develop mechanisms to reduce the electrostatic forces, thereby reducing the AMP uptake and thus blocking the first step of the bacterial killing process (Peschel & Sahl, 2006). A classic example is of *Staphylococcus aureus*, in which reduction of net negative charge of bacterial membrane is achieved by the transportation of positively charged D-alanines from the cytoplasm to the anionic teichoic acids of the bacterial wall by dltA-D gene products (Peschel et al., 2006). Production of proteases to digest highly positively charged peptides (Devine et al., 1999), increasing the hydrophobic interactions within their outer membrane (Groisman et al., 1992), overexpression of efflux pumps to export AMPs (Nikaido, 2006), formation of biofilms (Nikaido, 1996) and synthesis of molecules to bind AMPs in order to neutralize them (Otto, 2006). Nevertheless, there are many studies which conclude that developing resistance against AMPs will be difficult for bacteria (Steinberg et al., 1997; Ge et al., 1999; Mosca et al., 2000; Zhang et al., 2005). One has to bear in mind that to grow a resistance against AMP, the bacteria would have to redesign its whole membrane. The fact that bacteria are still sensitive to AMPs, makes it very unlikely for a resistance phenomenon to suddenly arise.

1.5.4 Therapeutic Potentials

Presently, the clinical trials of AMPs are mainly focussed on the development of medications with topical application. An α-helical magainin variant peptide MSI-78 has been developed by Magainin Pharmaceuticals which is in phase-III clinical trials for its efficacy against polymicrobic foot-ulcer infections in diabetes. Bacterial lantibiotic peptide nisin is undergoing clinical trials against *Helicobacter pylori* stomach ulcers. Iseganan, a protegrin derivative (Mosca et al., 2000), is undergoing phase III clinical study (Intrabiotics, Mountain View, CA, USA), to investigate iseganan HCl (Giles et al., 2002) in the prevention of ventilator-associated pneumonia (VAP). A histatin-derived peptide is undergoing phase I clinical trials (Periodontix Inc., USA) for the application against oral candidiasis and Trimeris. Durham (NC, USA) has successfully completed a phase II clinical trial, in which peptide T-20 (Su et al., 1999) was found to reduce viral load in HIV-infected patients by up to 97 %. Demegen (Pittsburgh, USA) has successfully tested peptide D2A21 (Robertson et al., 1998) in animal studies and is in process of developing it as therapeutic for several types of cancer and are also working on its gel formulation as a wound healing product to treat infected burns and wounds. Demegen's P113D derived from histatins (Sajjan et al., 2001), had been granted orphan drug status for the treatment of cystic fibrosis infections. Entomed's (Illkirch, France) product, heliomicin (Lamberty et al., 2001) for systemic antifungal treatment is under preclinical stage. Human lactoferricin (AM Pharma, Netherland) and bactericidal/permeability-increasing protein (Xoma, USA; Gray et al., 1989) have also proved their potential to be developed into drugs with systemic applications. Neuprex™, a BPI-derived peptide rBPI 21 (Xoma Corp., USA; Horwitz et al., 1996), has proved its potential against meningococcal sepsis in phase II/III clinical trials and more

than 1000 patients have received NEUPREX in clinical studies without any safety concerns. Several AMPs are also in preclinical development stage (Zhang et al., 2004; Kristensen, 2004; Scott et al., 2004). The pharmacology and pharmacokinetics of AMPs are still unknown and further research is required before the feasibility of peptide therapeutics will be generally accepted by the pharmaceutical industry.

1.6 Antimicrobial Peptides of Marine Origin

Invertebrates and fishes of marine origin mainly depend on innate immune system to combat microbial pathogens. Their survival over millions of years in pathogen loaded marine environment suggests that they might have evolved a large number of chemical tools including AMPs to tackle pathogens. Therefore bioprospecting for AMPs from marine organisms is expected to return rich dividends. Marine antimicrobial peptides are reported from poriferans, cnidarians, crustaceans, molluscs, tunicates and teleost fishes (Tincu et al., 2004; Patrzykat and Douglas, 2003; Smith et al., 2010; Otero-González et al., 2010; Sperstad et al., 2011).

The bioactive peptides isolated from sponges include Discodermin A (Matsunaga et al., 1985), jaspamide (Scott et al., 1988), theonellamide F (Fusetani and Matsunaga, 1993), theonegramide (Bewley and Faulkner, 1994), Callipeltin A (Zampella et al., 1996), hymenamides (Kobayashi et al., 1993) and Phoriospongin (Capon et al., 2002). From coelentratres aurelin was isolated from scyphoid jellyfish, *Aurelia aurita* (Ovchinnikova et al., 2006). Polychaetes are the most investigated annelids with respect to AMPs. Arenicin (Ovchinnikova et al., 2004), perinerin (Pan et al., 2004) and hedistin (Tasiemski et al., 2007) have been reported from polychaetes.

Molluscs rely predominantly on cellular responses mediated by haemocytes and humoral immune responses that employ AMPs to tackle invading microorganisms (Hooper et al., 2007). A number of antimicrobial peptides belonging to different families have been isolated and cloned from molluscs. The main classes of AMPs isolated from marine molluscs include defensins (Hubert et al., 1996; Charlet et al., 1996; Mitta et al., 1999a; Boulanger et al., 2004; Seo et al., 2005; Schmitt et al., 2010; Adhya et al., 2012; Zhang et al., 2015), mytilins (Charlet et al., 1996; Mitta et al., 2000a; Mitta et al., 2000b; Gestal et al., 2007; Parisi et al., 2009), myticin (Mitta et al., 1999b) and big defensin (Wei et al., 2003; Gerdol et al., 2012). In addition to the above classes of molluscan AMPs, a few other AMPs have been reported from marine molluscs and these include dolabellin B2 (Iijima et al., 2003), littorein (Defer et al., 2009), proline-rich peptides (Dolashka et al., 2011), myticusin-1 (Liao et al., 2013), Myticusin-1 (Liao et al., 2013), cgMolluscidin (Seo et al., 2013) and mytichitin-CB (Qin et al., 2014).

Among marine crustaceans, AMP have been isolated from crabs (Schnapp et al., 1996), lobsters (Hauton et al., 2006), crayfish (Jiravanichpaisal et al., 2007) and several shrimp species (Destoumieux et al., 2000). The important AMPs isolated includes penaeidins (Destoumieux et al., 1997; Destoumieux et al., 2000; Cuthbertson et al., 2004; Chen et al., 2004; Kang et al., 2004; Shanthi and Vaseeharan, 2012), crustin (Relf et al., 1999; Bartlett et al.,2002; Rattanachai et al., 2004; Hauton et al., 2006; Zhang et al., 2007; Afsal et al., 2011; Amparyup et al., 2008; Krusong et al., 2012; Kim et al., 2012; Cui et al., 2012), anti-lipopolysaccharide factors or ALFs (Tanaka et al., 1982; Liu, et al., 2005; Somboonwiwat et al., 2005; Nagoshi, et al., 2006; de la Vega et al., 2008; Afsal et al., 2011; Afsal et al., 2012a; Afsal et al., 2012b), arasin (Stensvag et al., 1998; Imjongjirak et al., 2011), carcinin

(Brockton et al., 2007), scygonadin (Wang et al., 2006) and defensins (Bateman et al., 2002; Pisuttharachai et al., 2009; Montero-alejo et al., 2012). Other Amps include proline/arginine-rich (or PRP-rich) (Schnapp et al., 1996), callinectin (Khoo et al., 1999), scygonadin (Huang et al., 2006), homarin (Battison et al., 2008), aracin (Stensvag et al., 1998), hyastatin (Sperstad et al., 2009) and tachyplesin (Shigenata et al., 1993).

Antimicrobial peptides isolated from Echinoderms include strongylocins (Li et al., 2008a; Li et al., 2010a; Li et al., 2010b) and centrocins (Li et al., 2010b) reported from sea urchins. Halocyamines (Azumi et al., 1990), clavanins (Lee et al., 1997a), styelins (Lee et al., 1997b) and clavaspirin (Lee et al., 2001) are antimicrobial peptides isolated from Ascidians.

Many AMPs have been isolated and identified from marine teleost fishes and these include pardaxin (Oren and Shai, 1996), misgurin (Park et al., 1997), pleurocidin (Cole et al., 1997; Cole et al., 2000), epinecidin-1(Yin et al., 2006), cathelicidin (Uzzel et al., 2003; Chang et al., 2006; Maier et al., 2008), β-defensin (Zhou et al., 2007; Casadei et al., 2009; Nam et al., 2010), piscidins (Siphadoung and Noga, 2001; Chinchar et al., 2004; Silphaduang et al, 2006; Buonocore et al., 2012; Bae et al., 2014), hepcidin (Shike et al., 2002; Xu et al., 2008a; Wang et al., 2009b; Chaithanya et al., 2013a; Chaithanya et al., 2013b) and Liver-expressed antimicrobial peptides (Shinner et al., 1996; Li et al., 2014; Liu et al., 2014; Seo et al., 2014;Li et al., 2015).

Several histone derived AMPs have been reported from marine organisms. Histone proteins are involved in the packaging of DNA, essentially serving to wind up the long DNA strands in a spool-like manner. Various types of histone proteins are found in eukaryotic nuclei and these include H1, H2A, H2B, H3 and H4. The potentials of histone proteins as antimicrobial

agents was known since 1958 (Hirsch, 1958), but at that time little attention was paid to the discovery as there was no theoretical concept as to how they might interact with bacteria for the host's benefit. Since then not much research was carried out on the antimicrobial potentials of histone proteins until the mid 1990s, when Frohm et al., (1996) reported the contribution of histone proteins to the antibacterial activity of wound blister fluid. Then reports began to emerge that histones account for a large proportion of the antibacterial activity of skin exudates from amphibians (Park et al., 1998) and teleost fish (Robinette et al., 1998; Patrzykat et al., 2001; Richards et al., 2001). Noga et al. (2001) reported the activity of histone proteins against fish-parasitic dinoflagellates. Histones have been reported to be present in the skin mucus of several fish taxa, including Salmoniformes, Siluriformes and Pleuronectiformes (Smith & Fernandes, 2009).

Histone H2A is the most potent antibacterial agent among the core histone proteins. The whole histone H2A protein has a molecular mass of 13.6 kDa and exhibit bactericidal activity against Gram positive bacteria (Fernandes et al., 2002). Histone H2A protein was reported to exhibit a weak antifungal activity against the yeast, *Saccharomyces cerevisiae*, but did not show any haemolytic activity to trout erythrocytes (Fernandes et al., 2002). In addition to histone H2A whole protein, fragments of histone H2A derived from the N-terminus region take part in defence against the microbial pathogens. Histone H2A, thus participate in host defense response through producing novel antimicrobial peptides (AMPs) from its N-terminus in vertebrates and invertebrates. In response to epidermal injury, *Parasilurus asotus*, a catfish, secreted a strong antimicrobial peptide into the epithelial mucosal layer. The 2 kDa antimicrobial peptide was parasin I (Park et al., 1998). Parasin I showed high similarity to buforin, an antimicrobial peptide

derived from N terminal region of toad histone H2A which implies that Parasin I is cleaved from the catfish histone H2A. Cho et al. (2002) studied the mechanism of Parasin I production from *Parasilurus asotus* histone H2A and concluded that cathepsin D cleaves the Ser19-Arg20 bond of histone H2A to produce parasin I. Hipposin is a polypeptide of 51 amino acids isolated from the skin mucus of Atlantic halibut *Hippoglossus hippoglossus* (Birkemo et al., 2003). Hipposin has been shown to be derived from histone H2A and shows sequence similarity with the buforin 1 of Asian toad and the parasin-1 of catfish. Hipposin was found to be active against several Gram positive and Gram negative bacteria (Birkemo et al., 2004). Fractions of histone H2A, actin and filamin A, each around 2 kDa and exhibiting antimicrobial activity were identified from celomocyte extract of the starfish *Asterias rubens* (Maltseva et al., 2004; Maltseva et al., 2007). Using biochemical approaches histone H2A fragments with antimicrobial activity was reported in the shrimp *Litopenaeus vannamei* (Patat et al., 2004). Li et al. (2006) was the first to report the presence of a H2A derived antimicrobial peptide from scallop *Chlamys farreri*. DeZoysa et al. (2009) identified a 40-amino acid AMP designated as abhisin from the N-terminus histone H2A sequence of the abalone *Haliotis discus*. A 52 amino acid histone H2A derived AMP, teleostin was identified from the histone H2A sequence amplified from two teleost fishes, *Tachysurus jella* and *Cynoglossus semifasciatus* was reported by Chaithanya et al. (2013c).

Antimicrobial activity was also reported for core histone H2B, first reported from mouse macrophages (Hiemstra et al., 1993). Antimicrobial activity of histone H2B was reported from skin exudates of channel catfish, *Ictalurus punctatus* in which the protein was found to be active against the fish pathogens (Robinette et al., 1998). From the acidic extract of the surface

mucus of Atlantic cod (*Gadus morhua*), histone H2B protein was purified which exhibited antimicrobial property (Bergsson et al., 2005). Among crustaceans, histone H2B derived antimicrobial peptides was reported in the shrimp *Litopenaeus vannamei* (Patat et al., 2004). First report of HPLC based purification of a histone H2B derived AMP cv-H2B-1 in mollusc was that of*Crassostrea virginica* by Seo et al. (2010). Later, from *Crassostrea virginica* three more histone H2B derived AMPs (cv-H2B-2, -3 & -4) were reported (Seo et al., 2011).

In case of histone H3, synthetic H3–like peptides were reported to exhibit antibacterial activity (Tsao et al., 2009) and H3 is present in normal epidermal mucus extruded from the hagfish *Myxine glutinous* (Subramanian et al., 2007). The epidermal mucus has a significant role in innate immunity of hag fish against infectious pathogens and histone H3 forms an important component of mucus aiding in immunity. Antimicrobial property has also been reported in Histone H4 protein. Histone H4 has been found to be one of the active factors in human sebocyte secretions (Lee et al., 2009). Histone H4 from shrimp (*Litopenaeus vannamei*) haemocytes has antibacterial properties (Patat et al., 2004), but reports for its antimicrobial role in other marine or aquatic organisms are scanty.

Anti-infective properties of linker histone proteins are also well known. Hiatone H1 exhibits antimicrobial properties and has been isolated from several species, including humans (Kashima, 1991), mice (Hiemstra et al., 1993), fish (Fernandes et al., 2002; Richards et al., 2001; Lüders et al., 2005) and shrimp (Patat et al., 2004). Histone H1, when isolated from Coho salmon, is active against *E. coli* with a MIC of 31 μg mL^{-1} (Richards et al., 2001). In winter flounder, the expression of this protein is up-regulated following immune stimulation, which coincides with an increase in the antibacterial

activities of serum and mucus (Patrzykat et al., 2003), indicative of its role in systemic as well as mucosal response to non-self-challenge. A 7.2 kDa antimicrobial peptide termed as oncorhyncin-2 has been isolated from rainbow trout, *Oncorhynchus mykiss* skin secretions (Fernandez et al, 2004). Oncorhyncin-2, a histone H1 fragment, has activity in the submicromolar range against Gram positive and Gram negative bacteria and shows negligible hemolytic activity towards trout enzyme. A 6.7 kDa antimicrobial peptide oncorhyncin-III was isolated from *Oncorhynchus mykiss* (Fernandez et al, 2003). The peptide is a cleavage product of the non-histone chromosomal protein H6 (residues 1-66). Histones have a conserved sequence exhibiting similarity across divergent phyla and hence, it is highly likely that histones from most eukaryotic species will show similar antimicrobial activity. Major studies on histone derived AMPs from marine organisms are given in Table 1.3.

Table. 1.3 Histone derived AMPs reported from marine organisms

Protein	Active Factor	Source	Reference
Histone H1	Whole protein (20.7 kDa) N-terminus (26 aa) (HSDF-1) C-terminus (69 aa) (oncorhyncin II) Fragment (not specified)	Salmon Salmon Rainbow trout Shrimp	Richards et al. (2001) Patrzykat et al. (2001) Fernandes et al. (2004) Patat et al. (2004)
Histone H2A	Whole protein (13.5 kDa) Whole protein (13.5 kDa) Whole protein (13.5 kDa) N-terminus (51 aa) (hipposin) N-terminus (19 aa) (parasin) N-terminus (40 aa) (abhisin) N-terminus (52 aa) (Teleostin)	Channel catfish Rainbow trout Shrimp Halibut Catfish Abalone Teleost Fishes	Robinette et al. (1998) Fernandes et al. (2002 Patat et al. (2004) Birkemo et al. (2003) Park et al. (1998) De Zoysa et al. (2009) Chaitanya et al (2013c)
Histone H2B	Whole protein (13.8 kDa) Whole protein (15.5 kDa) Whole protein (13.5 kDa) Whole Protein Isoforms (13.6kDa)	Cod Catfish Shrimp Oyster	Bergsson et al. (2005) Robinette et al. (1998) Patat et al. (2004) Seo et al. (2011)

Histone H3	Whole protein (15.3 kDa)	Shrimp	Patat et al. (2004)
Histone H4	Whole protein (11.3 kDa)	Shrimp	Patat et al. (2004)
Histone H6	Whole protein (6.7 kDa) (oncorhyncin III)	Fish	Fernandes et al. (2003)

1.7 Recombinant Expression Vectors of AMPs

Recombinant expression of AMPs provides a means to produce sufficient quantities of peptides for carrying out biological assays and structural studies (Ingham and Moore, 2007). An expression system for AMPs has to be carefully chosen as several peptide features such as size, folding etc. should be taken into consideration (Desai et al., 2010). Bacteria and yeasts are the most commonly used expression hosts (almost 97.4% of heterologous-expressed AMPs) for recombinant production of AMPs (Li et al., 2008b). Plants are also emerging as a promising host for AMP production. Transgenic plants with heterologous AMP genes can be directly used as microbial resistant varieties by simply expressing the peptide in the desired crop without the need for purifying the peptides (Giddings et al., 2000; Desai et al., 2010).

Escherichia coli is the most commonly used bacterial expression system for AMP production (Ingham and Moore, 2007; Parachin et al., 2012). Recombinant protein production in *E. coli* is cost effective owing to its rapid growth, availability of a wide range of commercial expression vectors, well-established DNA manipulation protocols and extensive knowledge regarding its genetics, biochemistry and physiology (Sorensen & Mortensen, 2005). Other than *E. coli*, *Propionibacterium freudenreichii* (Brede et al., 2005) and *Bacillus subtilis* (Chen et al., 2009) have also been successfully used as a

heterologous host for recombinant expression of AMPs. The widely used expression vector for AMP production in bacteria is the Novagen developed pET series The most frequently used *E. coli* expression strains include BL21 (DE3), Origami, pLysS, and Rosetta (Novagen) and C41 (DE3) (Parachin et al., 2012). During recombinant expression, AMP is often fused to a carrier protein having anionic properties to prevent its activity on host, to protect it from proteases to provide stability to AMP (Parachin et al., 2012). Carrier proteins have also been reported to facilitate the solubility of AMPs (Esposito and Chatterjee, 2006; Chatterjee and Esposito, 2006). Several fusion proteins that have been used to express and purify antimicrobial peptides include small ubiquitin- like modifier (SUMO), glutathione S-transferase (GST), F4 fragment of PurF and green fluorescent protein (GFP). (Parachin et al., 2012). Studies carried out to develop efficient techniques for recombinant production of AMPs strongly endorse bacteria as a host organism. The rate of expression of active peptides might be hindered by its size and cysteine content in cases where AMPs require certain types of post-translational modifications such as formation of disulfide bonds (Puertas et al., 2011). In case of AMPs requiring complex post- translational modifications that are not possible in a prokaryote host, different hosts have to be considered for AMP production. Cathelicidins (Chang et al., 2005), defensins (Gueguen et al., 2006), crustins (Supungul et al., 2008; Amparyup et al; 2008) etc. are main classes of marine derived AMPs expressed in an *E. coli* expression system.

Second to bacteria, yeasts are widely preferred as hosts for recombinant expression of AMPs. *Saccharomyces cerevisiae* and *Pichia pastoris* are the commonly utilized yeast expression systems. In case of AMPs requiring post-transcriptional and post-translational modifications, such as glycosylation, yeast hosts have an advantage over their bacterial counterparts (Cregg et al.,

2009). Yeasts grow faster and are more cost-effective in comparison to mammalian cell cultures and yeast hosts generally results in higher yields and productivity of the desired protein. Yeast expressed recombinant protein require less purification steps, have increased protein yield and facilitated scale-up process (Romanos et al., 1992; Byrne et al., 2005). Though *S. cerevisiae* is the most preffered host for the majority of recombinant proteins (Mattanovich et al., 2012), the bulk of heterologous AMPs has been expressed in *P. pastoris* (Puertas et al., 2011). In case of scygnodin, an AMP from the gonads of mud crab, expression in *P. pastoris* was found to achieve a 1.3-fold increase in production of recombinant AMPs when compared to *E. coli* (Peng et al., 2012). *P. pastoris* is definitely a host expression system, but, it still requires many modifications to achieve the desired yield of recombinant AMPs. Ch-penaeidin (Li et al., 2005), epinecidin-1 (Yin et al., 2006), big defensin (Zhao et al., 2007), Scallop histone H2A derived AMP (Li et al., 2007), tachyplesin II (Xu et al., 2008b) and Sp-ALFs (Liu et al., 2012) are few example of AMPs of marine origin, cloned and expressed in *P. pastoris.*

Genetically modified plants have also been used as expression hosts for proteins (Desai et al., 2010). The expression of AMPs in microbial hosts is often hindered due to their antimicrobial nature. Plant expression systems can be a suitable alternative for AMP production as it will not be affected by the high level expression of AMPs and will also facilitate complex folding and processing pathways. Therefore, though not very popular, plant systems can be an interesting choice for the expression of several families of AMPs with specific expression requirements (Jennings et al., 2005; Ireland et al., 2006).

Antimicrobial peptides find application in numerous industries such as biotechnology, pharmaceutical, cosmetic and food. But structural variation and difficulties in large scale production of AMPs restrict their utility on a

commercial basis. Production costs in case of AMPs with high molecular masses and complex folding, impair their large-scale chemical synthesis. Another major limitation in handling AMPs is that they are easily degraded by endopeptidases. Despite enormous challenges that need to be overcome in order to obtain stable, pure, and functional recombinant AMPs, tools in genetic engineering techniques have been under constant development and optimization. Efforts for the development of new production systems and designs of fermentation processes are essential for the establishment of a cost effective methodology for large scale AMP production.

1.8 Significance of the Study

Antibiotic resistance has become a global public-health problem, and thereby there is a dire necessity for novel antibiotics. Antimicrobial peptides have shown great potential to be developed into a new class of antibiotics. Although, a number of antimicrobial peptides from different organisms have been reported throughout the world; there is a very limited report of antimicrobial peptides from marine organisms inhabiting Indian seas. Marine antimicrobial peptides represent a largely unexploited resource that can follow a route from the ocean to the clinics. The main aim of the present study was to bioprospect for novel histone derived antimicrobial peptides from marine organisms inhabiting the Indian seas with an intention to develop new therapeutic agents for the benefit of human kind. Moreover, better understanding of the role of histone derived antimicrobial peptides in marine organisms would help better understand their immune system which in turn would aid in better health management of marine organisms in aquaculture.

1.9 Objectives of the Study

The specific objectives of the study are:

1) Detection and molecular characterization of histone derived Antimicrobial Peptides from marine organisms.

2) Recombinant Expression of histone based AMP genes and to test its bioactivity.

3) Characterization and testing the Bioactivity of histone derived synthetic antimicrobial peptides.

1.10 Outline of the Thesis

The thesis is presented in seven chapters. Chapter 1 is the general introduction. Chapter 2 and 3 deals with the molecular characterization and phylogenetic analysis of histone H2A derived antimicrobial peptides from marine molluscs (Sunettin and Molluskin) and Elasmobranch, the Round whip ray, *Himantura pastinacoides* (Himanturin) respectively. Chapter 4 deals with Molecular characterization of histone H2A derived antimicrobial peptides designated as Harriottin (1-3), from Sicklefin Chimaera *Neoharriotta pinnata* and its phylogenetic analysis based on Cytochrome Oxidase-I (CO1) gene and Histone genes. Chapter 5 deals with the recombinant expression of Harriottin-1 and Harriottin-2 using pET32a+ expression vector system and *E. coli* expression host system (Rosetta-gami^{TM} B (DE3) pLysS) and testing the antibacterial activity of recombinant Harriottin-1 and -2. Chapter 6 deals with the antibacterial, anticancer and cytotoxicity testing of synthetic Harriottin-3 (a histone H2A derived AMP identified from Sicklefin Chimaera). The present

study is summarized in Chapter 7 with special emphasis on salient findings of the study. This is followed by a list of References, GenBank accessions and Publications.

2

Molecular Characterization and Phylogenetic Analysis of Histone Derived Antimicrobial Peptides from Marine Molluscs.

2.1 Introduction

Marine molluscs comprises of one of the largest and enigmatic groups of marine organisms adapted to almost every kind of habitat ranging from deep sea hydrothermal vents to intertidal regions. They exhibit high diversity not only in habitat but also in size, anatomical structure, morphology and behavior. Molluscs have more varied forms than any other animal phyla. The cephalopod molluscs comprising of squid, cuttlefish and octopus are the most neurologically advanced of all invertebrates and hence regarded as the most intelligent invertebrates. Molluscan resources have been exploited by humans for ages. Many marine molluscan species form important food source for man. Squids, cuttle fishes, mussels and oysters are highly valued commercial food species. The graceful shells of gastropods and the pearls from oysters are also of high commercial value.

Being marine organisms, these molluscs thrive in a microbe rich environment and are continuously at war with diverse microbial pathogens of varying virulence. Generally marine invertebrates including molluscs lack a specific adaptive immunity and therefore depend entirely on innate immunity including cellular and humoral immunity for mounting an efficient host defense (Tincu and Taylor, 2004). Among the innate immune defenses, antimicrobial peptides (AMPs) comprising small sized, cationic and amphipathic peptides with broad spectrum antimicrobial activity form a significant component.

Antimicrobial Peptides have received increasing attention in recent years as their contribution to host defense mechanisms and their potential as new pharmaceutical substances is becoming increasingly appreciated. This is mainly because of the broad spectrum activity of AMPs and the rapid development of microbial resistance to conventional antibiotics. The evolution of antibiotic-resistant bacteria has stirred the search for new potential bactericidal agents for which antimicrobial peptides have attained great attention as promising candidates with huge potentials as future antibiotic agents (Van't Hof et al., 2001). Antimicrobial peptides are important components of the innate immune system of almost all multicellular organisms (Hancock, 1997; Boman, 2003; Patrzykat and Douglas, 2003; Tincu and Taylor, 2004; Jenssen et al., 2006; Lai and Gallo, 2009; Guani et al., 2010). Marine molluscs depend entirely on innate immune system to protect themselves against pathogenic organisms and thus form a promising source for novel AMPs.

Antimicrobial peptides (AMPs) from a wide variety of phylogenetically diverse invertebrates have been reported, ever since the discovery of cecropin from the insect *Hyaophora cecropia* by Steiner et al. (1981). Initial reports on

the AMPs from molluscs started to emerge in the mid-1990s and were based on biological activities of biochemically purified fractions (Li et al., 2011). Up to date a number of antimicrobial peptides categorized as defensins, big-defensins, mytilins, myticins, mytimycins and histone derived AMPs have been reported from various marine molluscs including oysters, clams, abalones, mussels, scallops and gastropods (Tincu and Taylor, 2004; Cheng-Hua et al., 2008; Li et al., 2011).

Numerous AMPs have been purified from marine molluscs utilizing analytical techniques. Charlet et al. (1996) initiated the HPLC based purification of AMPs from molluscs by isolating six AMPs which include two defensins (peptide A, B), two myticins (A, B) and one mytimycin from *Mytilus edulis.* In the very same year Hubert et al. (1996) isolated defensin (MGD-1) from *Mytilus galloprovincialis*. HPLC based purification of two myticins (A, B) and three mytilins (C, D, G-1) was reported from *Mytilus galloprovincialis* (Mitta et al., 1999; Mitta et al., 2000). Wei et al. (2003) reported a new family of AMPs in molluscs i.e., big-defensin (RPD-1) from *Ruditapes philippinarum.* Defensin (AOD) from *Crassostrea virginica* (Seo et al., 2005) and littorein from gastropod, *Littorina littorea* was reported by Defer et al. (2009).

Several AMPs and isoforms of previously known AMPs have been discovered by employing molecular techniques. Mitta et al. (1999) was the first to report the gene based identification of AMPs from molluscs. They discovered defensin (MGD-2) from mussel *Mytilus galloprovincialis*. Numerous AMPs have then been reported from molluscs and these include two defensins from *Argopecten irradians* (Song et al., 2006), defensin (Cg-Def) from *Crassostrea gigas* (Gueguen et al., 2006), two defensins from *Crassostrea virginica* (Cunningham et al., 2006), big-defensin (AiBD) from

Argopecten irradians (Zhao et al., 2007) and two mytimycins (P, V) from mussel *Mytilus galloprovincialis* (Sonthi et al., 2011). Many isoforms of these AMPs from molluscs have been isolated and studied using molecular techniques (Patrzykat and Douglas, 2003; Yount et al., 2006; Li et al., 2011).

AMPs derived from histone proteins represent an important category of peptide antibiotics. Histone derived antimicrobial peptides with broad spectrum activity has been reported from a number of organisms (Kawasaki and Iwamuro, 2008). Histone derived AMPs reported from molluscs are mainly derived from histone proteins belonging to H2A and H2B family. Seo et al. (2010) was the first to purify and report a histone H2B derived AMP, cv-H2B-1 in molluscs from *Crassostrea virginica*. Later, from *Crassostrea virginica* three more histone H2B derived AMPs (cv-H2B-2, -3 & -4) were reported (Seo et al., 2011). Histone H2A derived AMPs are the most studied ones among the class of histone derived AMPs. Molluscan histone H2A derived AMPs have been reported from Scallop *Chlamys farreri* (Li et al., 2007) and Abalone *Haliotis discus discus* (De Zoysa et al., 2009). Pacific White Shrimp *Litopenaeus vannamei* is another marine invertebrate from which histone H2A derived AMP has been identified and reported (Patat et al., 2004). Among marine vertebrates histone H2A derived antimicrobial peptides have been reported from Catfish *Parasilurus asotus* (Park et al., 1998), Atlantic Halibut *Hippoglossus hippoglossus* (Birkemo et al., 2003) and Rainbow Trout *Oncorhynchus mykiss* (Fernandes et al., 2004).

In terms of number of species, molluscs constitute the second largest animal phyla and are represented copiously in marine environment. They are important from economic point of view as many of their representatives constitute highly valuable commercial fisheries and also form an integral part of aquaculture industry. Inspite of the rich diversity of marine molluscs, it has

to be said that only negligible number of species has been investigated with respect to AMPs. Present study was carried out to identify histone H2A derived antimicrobial peptides from marine molluscs so as to understand the role of histones in the innate immune defense of molluscs. The study is focused on identification of histone H2A derived antimicrobial peptide sequence from marine molluscs. The molluscs considered for the study include Marine clam (*Sunetta scripta*), Back Water Oyster (*Crassostrea madrasensis*), Rock Oyster (*Saccostrea cucullata*), Grey Clam (*Meretrix casta*), Fig Shell (*Ficus gracilis*) and Ribbon Bullia (*Bullia vittata*). The bivalves *S. scripta, C. madrasensis, S. cucullata* and *M. casta* are relished as delicacies, whereas, the gastropods *F. gracilis* and *B. vittata* are highly valued for their graceful shells. Since these bivalves are important from economic point of view, understanding their innate immunity will go a long way in better exploitation and management of these molluscan resources. The molluscan species considered for the study has never been investigated for AMPs and hence the study provides a baseline for extending the search for AMPs to marine molluscs.

2.2 Materials and Methods

2.2.1 Sample Collection

Live animal samples were collected from the intertidal and pelagic regions of Kerala coast. Marine Clam, *Sunetta scripta* was obtained from sandy intertidal region of Calicut Sea. Rock Oyster, *Saccostrea cucullata* was collected from rocky shores of Calicut Sea. Fig Shell, *Ficus gracilis* was obtained from coastal waters of Calicut Sea. Back Water Oyster, *Crassostrea madrasensis* and Grey Clam, *Meretrix casta* were collected from Vembanad

estuary, Kochi. Ribbon Bullia, *Bullia vittata* was sampled from the intertidal region of Vypeen, Kochi. Photographs of the live animal samples collected for the study is shown in Fig. 2.1.

Out of the six collected species of Phylum Mollusca, 4 belongs to Class: Bivalvia and two to Class: Gastropoda. The clams, *S. scripta* and *M. casta* belongs to Class: Bivalvia, Order: Veneroida and Family: Veneridae. The oysters *C. madrasensis* and *S. cucullata* belong to Class: Bivalvia, Order: Ostreoida and Family: Ostreidae. The Fig shell, *F. gracilis* belongs to Class: Gastropoda, Order: Littorinimorpha and Family: Ficidae. The Ribbon bullia, *B. vittata* belongs to Class: Gastropoda, Order: Neogastrapoda and Family: Nassariidae.

2.2.2 RNA Extraction

2.2.2.1 RNase Free Anticoagulant and Water

The anticoagulants, water and other required materials (including glass wares, homogenizers, gloves, scissors and forceps), used for RNA isolation were made free of RNase by treating with 0.1 % Diethyl pyro carbonate (DEPC). For the purpose the required materials and solutions were incubated with 0.1 % DEPC overnight followed by autoclaving at 15 lbs pressure for 1 hour. Autoclaving for an hour ensures complete evaporation of DEPC as ethanol and CO_2 leaving behind RNase free utensils, anticoagulant solution and water.

2.2.2.2 Haemolymph Collection

Samples were transported to laboratory in live condition. Haemolymph was collected from byssus muscles of bivalves and foot region of gastropods

using 1 ml syringe rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7).

2.2.2.3 Total RNA Extraction

Total RNA was extracted from the haemolymph collected from the live Molluscs using TRI® Reagent (Sigma), following the manufacturer's instructions. Briefly, one ml of haemolymph was homogenized with TRI Reagent (1 ml) in a tissue homogenizer. The homogenate was transferred to 1.5 ml micro centrifuge tubes (MCTs) and allowed to stand for 5 mins at room temperature. To the homogenate was added 0.2 ml chloroform, shaken vigorously for 15 seconds, allowed to stand at room temperature for 15 mins and centrifuged at 12,000 x g for 15 mins at 4^{o} C. Centrifugation separated the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was transferred to a fresh MCT and 0.5 ml isopropanol was added. The samples were allowed to stand for 5-10 min at 4^{o} C and then centrifuged at 12000 x g for 10 min at 4^{o} C. The RNA pellet obtained was washed by adding 1 ml 75 % ice cold ethanol. The samples were vortexed and centrifuged at 7500 x g for 5 min at 4^{o} C. RNA pellets were dried for 5-10 min and dissolved in RNase free water by repeated pipetting with a micropipette at 55-60^{o} C for 10-15 min.

2.2.2.4 Determining Quality and Quantity of RNA

The purity and quality of the RNA was visualized using electrophoresis on 0.8% agarose gel (Fig. 2.2). RNA was quantified and qualified by measuring optical density (O.D) at 260 and 280 nm in a UV Spectrophotometer (U-2900, Hitachi). The ratio of absorbance at 260 to 280 nm is an indication of RNA quality. Only RNAs with absorbance ratio (A260:

A280) ≥1.8 were used for cDNA synthesis. Optical density when measured at 260 nm, 1 O.D of RNA = 40 µg/ml, and hence, RNA concentration was calculated as:

RNA concentration (µg/ml) = O.D at 260 nm x Dilution factor x 40

2.2.3 cDNA Synthesis

Single stranded cDNA was synthesised from good quality RNA (RNAs having an absorbance ratio of ≥1.8 at A260 and A280) using reverse transcription in a 20 µl reaction mixture containing 5 µg total RNA, 1x RT buffer, 2 mM dNTP, 2 mM oligo d(T20), 20 U of RNase inhibitor and 100 U of MMLV Reverse transcriptase (Fermentas, Inc.). The reaction was carried out at 42^{o} C for 1 h followed by an inactivation step at 85^{o} C for 15 min. The synthesized cDNA was stored at -20^{o} C for further use.

2.2.4 PCR Amplification

The PCR amplification of cDNA with gene specific primers were performed, first, to amplify a constitutively expressed gene, the beta-actin as an internal control to verify reverse transcription reaction and thereafter to amplify histone H_2A gene sequences. The sequences of the primers used to amplify beta-actin and histone H2A genes is given in Table 1.

The PCR amplification of AMP genes with specific primers were performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The PCR condition involved an initial denaturation of 94^{o} C for 2 minutes followed by 35 cycles of 94^{o} C for 15 seconds at 60^{o} C (annealing temperature) for 30

seconds and 68^{o} C for 30 seconds and a final extension at 68^{o} C for 10 minutes. PCR products were analysed by electrophoresis in 1.5% agarose gel.

Table 1. Primer sequences used to amplify β-actin and histone H2A gene sequences.

Primer	Primer Sequence (5'-3')	Product size	Reference
β-actin	F: agcaagagaggtatcctcac R: gcatttcctgtggacaatgg	945 bp	Hosoi et al., 2007
HH2A-1	F: atgtccggrmgmggsaarac R: gggatgatgcgmgtcttcttgtt	249 bp	Birkemo et al., 2003
HH2A-2	F: gaattcatgtctggacgaggaaaggg R: gcggccgcatagtttcccttacggagcaga	117 bp	Li et al., 2007

2.2.5 Agarose Gel Electrophoresis

For analysis, 0.8 % Agarose gel was prepared for RNA and 1.5 % gel for PCR products. Agarose gel was prepared in 1 x TBE buffer (Tris-base -10.8 g, 0.5 M EDTA- 4 ml, Boric acid- 5.5 g, double distilled water- 100 ml, pH- 8.0) and SYBR® safe (1 µl / ml of agarose gel) was added. After cooling to 45^{o} C, the agarose was poured on to gel tray and was allowed to solidify. The gel tray was submerged in 1 x TBE buffer filled in a buffer tank. Ten micro litre of PCR product was mixed with 2 µl of 6 x gel loading buffer (1 % Bromophenol blue- 250 µl, 1 % xylene cyanol-250 µl, glycerol–300 µl, double distilled water–200 µl) and loaded into the well. Electrophoresis was carried out at a voltage of 3-5 volt/cm. The gel was visualized on a UV transilluminator using the Gel Doc XR system and the quantity one programme (Bio-Rad Hercules, Ca).

2.2.6 Sequencing

Amplicons obtained were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 377 DNA sequencer at M/s SciGenom Sequencing Facility, Kakkanad, Kochi, Kerala.

2.2.7 Sequence Analysis and Molecular Characterization

The nucleotide sequences of AMP genes were analysed and assembled using GeneTool software. Translation of the obtained nucleotide sequence was performed using the translation tool of Expert Protein Analysis System (http://web.expasy.org/translate/). Homology searches of the nucleotide sequence and deduced amino acid sequence were performed using BLASTn and BLASTp suite of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment of the peptide with previously reported histone derived AMPs from other animals were performed with ClustalW tool of BioEdit software. Enzyme cleavage site in the amino acid sequence was identified using PeptideCutter tool (http://web.expasy.org/peptide_cutter/). Physicochemical parameters of the deduced peptide were calculated using the ProtParam tool (http://cn. expasy.org/tools/protparam.html), Protein Calculator v 3.3 (http://protcalc. sourceforge.net/), PepDraw (http://www.tulane.edu/~biochem/) and Protean module of the DNASTAR Lasergene sequence analysis software suite. Data for secondary structure and spatial organisation of the obtained amino acid sequence was generated using SWISS-MODEL, a homology based protein modelling server (Guex and Peitsch, 1997; Schwede et al., 2003 and Arnold et al., 2006). Using the data generated by SWISS-MODEL, 3-Dimensional structure of amino acid sequence was predicted using PyMOL software. The secondary structural analysis of the peptides was carried out using secondary

structure assignment program, STRIDE (http://webclu.bio.wzw.tum.de/cgi-bin/stride/stridecgi.py). Antimicrobial activity of the peptide was predicted using Antimicrobial Peptide Predictor Program of Antimicrobial peptide database 2 (APD2; http://aps.unmc.edu/AP/main.php).

2.2.8 Phylogenetic Analysis

Nucleic acid sequences of histone H2A genes reported from various organisms were retrieved from GenBank database and multi aligned using ClustalW and GeneDoc computer programmes. Phylogenetic analysis was carried out using MEGA software version 5.05 and the phylogenetic tree was constructed by Neighbour Joining (NJ) method with complete deletion of gaps and subjected to 1000 repetitions of bootstrap. Kimura 2 parameter (K2P) model was used to construct NJ and ML tree for CO1 genes.

2.3 Results

2.3.1 Histone Sequence detected in *Sunetta scripta*

2.3.1.1 Sequence Analysis

A 246 bp fragment cDNA encoding 82 amino acids from the mRNA of *Sunetta scripta* was obtained by Reverse Transcription PCR using HH2A-1 primers (Fig. 2.3). The nucleotide and deduced amino acid sequence of the obtained amplicon is shown in Fig 2.4. BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptide belonged to histone H2A family. The obtained nucleotide and deduced amino acid sequences were deposited in GenBank database (GenBank ID: **HQ720149**).

Multiple-sequence alignment of the amino acid sequence of the peptide with previously reported histone H2A derived AMPs revealed that the first 51 amino acid sequence at the N-terminal of the deduced peptide showed similarity to previously reported histone H2A derived AMPs i.e., hipposin, buforin I, buforin II, abhisin and those reported from *Litopenaeus vannamei* and *Chlamys farreri* (Fig. 2.5). This 51 amino acid H2A derived peptide sequence from *Sunetta scripta* was termed as 'Sunettin'.

The 51 amino acid Sunettin was found to have a predicted molecular weight of 5.43 kDa and a theoretical isoelectric point (p*I*) of 11.9 as predicted by PROTPARAM software. Sunettin was found to be rich in arginine (13.7%), glycine (19.6 %), alanine (9.8 %), leucine (11.3 %), valine (10.3 %) and lysine (11.8 %) as reported in all other histone H2A derived AMPs. Protein Calculator v 3.3 predicted Sunettin to have a net charge of +13.8, +12.1 and +6.9 at pH 4, 7 and 10 respectively. Hydrophobicity of peptide was found to be +50.02 kcal/mol (29 %) as predicted by PepDraw.

Analysis of Sunettin using Protean module of the DNASTAR Lasergene sequence analysis software suite revealed that the peptide will have a concentration of 1.82 mg/ml for an absorbance of 1 OD measured at 280 nm and 1 µg of the peptide would contain 184.29 pMoles. Hydrophobic amino acids constituted 24.17% while polar amino acids represented 18.76% of the total weight of Sunettin.

Schiffer-Edmundson helical wheel analysis of Sunettin clearly showed a perfect amphipathic nature (Fig. 2.6). Majority of hydrophilic residues such as serine (S), lysine (K), histidine (H), arginine (R), asparagine (N), glutamine (Q) and majority of hydrophobic residues such as valine (V), alanine (A), tyrosine (Y), phenylalanine (F), leucine (L), isoleucine (I), methionine (M)

occupied positions opposite to each other in the helical wheel. This type of arrangement for hydrophobic and hydrophilic amino acid also confirms the helical nature of the peptide. This result suggests an amphipathic nature and an α-helical structure for the Sunettin.

The secondary structure analysis of Sunettin showed that the peptide contains 44 % α-helical region (22 amino acid residues), 30 % turns (15 amino acid residues) and 26 % coils (13 amino acid residues). No β-sheet region has been noticed in the secondary structure of Sunettin (Fig. 2.7; a-b). The calculations were made omitting the initial amino acid, Met, which was excluded by the STRIDE programme. The α-helical region in Sunettin is from Arg 17 to Ala 21, Val 27 to Gly 37 and Ala 45 to Tyr 50. Turns can be observed in Sunettin from Ser 2 to Gly 5, Lys 9 to Lys 15 and Ala 40 to Val 43. Coils can be observed in Sunettin from Lys 6 to Gly 8 and Gly 22 to Pro 26. Coils can also be seen at Ser 16, Asn 38, Tyr 39, Gly 44 and Leu 51. Tertiary structure of Sunettin was created using PyMOL software using data generated in pdb format by SWISS-MODEL. SWISS-MODEL also predicted an α-helical structure for Sunettin (Fig. 2.8).

Analysis of Sunettin for its antimicrobial activity was carried out with Antimicrobial Peptide Database which predicts Sunettin to be an Antimicrobial peptide with a protein binding potential of 2.3 kcal/mol.

2.3.1.2 Phylogenetic Analysis

Phylogenetic tree based on nucleotide sequence was constructed by Neighbour Joining (NJ) method with complete deletion of gaps and subjected to 1000 repetitions of bootstrap. The phylogenetic tree branched into three main clusters, a cluster of higher vertebrates comprising of mammals and birds, a cluster formed by fishes and frogs and a cluster of invertebrates.

Histone H2A sequence from *Sunetta scripta* was found to align along with the invertebrate cluster (Fig. 2.9).

2.3.2 Histone H2A Sequence detected in *C. madrasensis, S. cucullata, M. casta, F. gracilis* and *B. vittata.*

2.3.2.1 Sequence Analysis

A 117 bp amplicon was obtained from the mRNA of *Crassostrea madrasensis*, *Saccostrea cucullata*, *Meretrix casta*, *Ficus gracilis* and *Bullia vittata* by Reverse Transcription PCR using HH2A-2 primers (Fig. 2.10). A 86 bp readable sequence encoding 28 amino acids was detected in *Crassostrea madrasensis*, a 89 bp readable sequence encoding 29 amino acids in *Saccostrea cucullata*, a 77 bp readable sequence encoding 25 amino acids in *Meretrix* casta, a 84 bp readable sequence encoding 28 amino acids in *Ficus gracilis* and a 82 bp readable sequence encoding 27 amino acids in *Bullia vittata*. The nucleotide and deduced amino acid sequences obtained from all five molluscs are shown in Fig. 2.11 (a to e). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptides belonged to histone H2A family. The obtained nucleotide and deduced amino acid sequences were deposited in GenBank database; *B. vittata* (GenBank ID: **HQ720143**), *C. madrasensis* (GenBank ID: **HQ720145**), *F. gracilis*(GenBank ID: **HQ720146**), *S. cucullata* (GenBank ID: **HQ720147**) and *M. casta* (GenBank ID: **HQ720148**).

Since similar sequences were obtained for all five molluscs, a 25 amino acid sequence (*Meretrix casta*) which was common to all sequences was considered for further analysis. Multiple-sequence alignment of the amino acid sequence with previously reported histone H2A derived AMPs revealed that

the 25 amino acid sequence of the deduced peptide showed similarity to previously reported histone H2A derived AMPs like Buforin I, Buforin II, Hipposin, Abhisin, Rainbow trout AMP and those reported from *Litopenaeus vannamei* and *Chlamys farreri* (Fig. 2.12). This 25 amino acid histone H2A derived peptide sequence was termed as 'Molluskin'.

Sequence analysis of the peptide was carried out using ProtParam software which predicted Molluskin to have a molecular weight of 2.84 kDa and a theoretical isoelectric point (p*I*) of 12.18. Molluskin was found to be rich in arginine (20 %), leucine (12 %), serine (12 %), glycine (12 %) and alanine (8 %) as reported in all other histone H2A derived AMPs. The 25 amino acid peptide was found to have a net charge of +7.1, +6.1 and +4 at pH 4, 7 and 10 respectively. Hydrophobicity of Molluskin was found to be +21.92 kcal/mol (32 %) as predicted by PepDraw.

Analysis of Molluskin using Protean module of the DNASTAR Lasergene sequence analysis software suite revealed that the peptide will have a concentration of 1.91 mg/ml for an absorbance of 1 OD measured at 280 nm and 1 µg of the peptide would contain 352.08 pMoles. Hydrophobic amino acids constituted 29.61 % while polar amino acids represented 23.47 % of the total weight of Molluskin.

Amphipathic nature of Molluskin was revealed by Schiffer-Edmundson helical wheel analysis. Majority of hydrophilic residues such as serine (S), lysine (K), histidine (H), arginine (R), asparagine (N), glutamine (Q) and majority of hydrophobic residues such as valine (V), glycine (G), alanine (A), tyrosine (Y), leucine (L), isoleucine (I), proline (P) occupied positions opposite to each other in the helical wheel, an arrangement which confirms the

helical nature of the peptide (Fig. 2.13). This result suggests an amphipathic nature and an α-helical structure for the Molluskin.

The secondary structure analysis of Molluskin showed that the peptide contains 64 % α-helical region (16 amino acid residues) and 36 % coils (9 amino acid residues). No β-sheet region has been noticed in the secondary structure of Molluskin (Fig. 2.14; a to b). Arg 2 to Ala 6 and Val 12 to Gly 22 constitute the α-helical region in Molluskin. Ser 1, Gly 7 to Pro 11 and Asn 23 to Ala 25 form the coiled regions of Molluskin. Three dimensional structure of Molluskin generated in PyMOL software is presented in Fig. 2.15.

Analysis of Molluskin for antimicrobial activity was carried out with Antimicrobial Peptide Database which predicts Molluskin to be an antimicrobial peptide with a protein binding potential of 2.96 kcal/mol.

2.3.2.2 Phylogenetic Analysis

Bootstrapped (1000 replications) phylogenetic tree constructed by NJ method confirmed the similarity of nucleotide sequences to the previously reported histone H2A nucleotide sequences (Fig. 2.16). The phylogenetic tree branched into clusters of mammals, birds, fishes, frogs and invertebrates. Sequences obtained from all five molluscs clustered along with the invertebrate groups.

2.4 Discussion

In invertebrates, humoral immunity mainly consists of antimicrobial agents present in the circulating body fluid (Tincu and Taylor 2004). Therefore identifying novel antimicrobial peptides provide information crucial

for elucidating invertebrate innate immunity. The 51 amino acid Sunettin and 25 amino acid Molluskin exhibited substantial similarity with previously reported histone H2A derived antimicrobial peptides. All of these reported AMPs are derived from the N-terminal region of histone H2A protein suggesting its participation in the innate immune defenses of an organism. Invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses to defend themselves against pathogens. In invertebrates, humoral immunity mainly consists of antimicrobial agents present in the circulating body fluid (Tincu and Taylor, 2004). Therefore identifying novel antimicrobial peptides provide information crucial for elucidating invertebrate innate immunity. AMPs derived from precursors are less understood in case of marine invertebrates and therefore the study would provide a better understanding of innate immune responses in invertebrates.

Histone H2A derived antimicrobial peptides are cleaved from their precursors mainly by the action of proteolytic enzymes. In Asian toad, *Bufo bufo gargarizans*, the intact histone H2A protein is secreted into the stomach and buforin I is produced by the action of pepsin isozymes cleaving the Try 39 – Ala 40 bond of intact protein (Kim et al., 2000). Similarly in cat fish (*Parasilurus asotus*), parasin I is produced by cleavage of Ser19-Arg20 bond of histone H2A by cathepsin D found in skin mucus of the fish (Cho et al., 2002). An inducible trypsin-like serine proteinase, the tryptase which has a preferential cleavage site in histone H2A molecule, 20 residues downstream N-terminus was reported from mast cell granules (Fiorucci et al., 1997). These findings suggest that the action of proteolytic enzymes on histone H2A of molluscs could result in the formation of active antimicrobial peptides like Sunettin and Molluskin assisting in their innate immunity.

Histone H2A derived antimicrobial peptides are reported to exhibit broad spectrum activity against both Gram-positive and Gram-negative bacteria and fungi. Hipposin, Parasin I and Buforin I are the most studied Histone H2A derived antimicrobial peptides. Hipposin showed strong antibacterial activity against several Gram positive and Gram negative bacteria and activity could be detected down to concentrations of 1.6 µg/ml (Birkemo et al., 2003). Parasin I was found to exhibit a minimum inhibitory concentration of 1 to 4 µg/ml against an array of Gram positive and Gram negative bacteria without any haemolytic activity (Park et al., 1998). Minimum inhibitory concentration in case of Buforin I was found to be 4 to 8 µg/ml against both categories of bacteria (Minn et al., 1998). Both Parasin I and Buforin I were found to exhibit antifungal activity against *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Candida albicans* with minimum inhibitory concentration of 2 µg/ml and 4 µg/ml respectively (Park et al., 1998; Minn et al., 1998). Abhisin, an antimicrobial peptide derived from histone H2A of disk abalone *Haliotis discus* was found to be active against *Listeria monocytogenes* (G+), *Vibrio ichthyoenteri* (G-) bacteria, and fungi (yeast) *Pityrosporum ovale*. Abhisin treatment (50 µg/ml) decreased the viability of THP-1 leukemia cancer cells by about 25 % without any effect on vero cells, suggesting that abhisin has cytotoxicity against cancer cells but not normal cells (Zoysa et al., 2009). Histone H2A derived AMPs exhibit strong activity against aquatic and human pathogens. Sunettin and Molluskin which exhibit sequence similarity to these potent AMPs are expected to demonstrate potent bioactivity.

Some differences in the amino acid sequences can be noted in the histone H2A derived AMPs reported from vertebrates and invertebrates (Fig. 2.5). Sunettin has a Ser at position 16 and same is the case with Molluskin at

corresponding position (N-terminus region). Histone H2A derived antimicrobial peptides reported from other invertebrates also possess Ser at the corresponding position, but in case of vertebrates, Thr is present in position of Ser. Amino acid Ile is present at position 30 of Sunettin and at position 15 (corresponding to 30 of Sunettin) of Molluskin. This is same for all invertebrates as they possess Ile at the corresponding position, whereas, in vertebrates Val occupies the position instead of Ile. Ser and Thr are hydrophilic and Ile and Val are hydrophobic and they do not contribute to the charge of the peptide, therefore their interchange will have no or very little effect on the activity of the peptides.

Histone proteins are required for the package of newly synthesized DNA into chromatin. Almost 85 to 90% of histone proteins are cell cycle regulated and are expressed in accordance with DNA replication. These proteins are mainly synthesized during G1 to S phase with maximum at S phase of the cell cycle. These histone proteins are termed as replication-dependent histones. The mRNA of replication-dependent histones lack a poly (A) tail. The mRNAs of these histones are accumulated and degraded rapidly in concert with DNA replication. However, 10 to 15 % of histones (a few members of Histone H2A and H3 family) are replication-independent and are not under the regulation of cell cycle. The mRNAs of these histones differ from replication-dependent histones in having a poly (A) tail. Replication-dependent histones and replication-independent histones are similar in sequence at N-terminal region but differ from each other at C-terminal region (Bonner et al., 1993). Since oligo dT primers were used for cDNA synthesis, it has to be understood that in the present work histone mRNAs with poly (A) tail was amplified with gene specific primers.

2.4.1 Sunettin

Sunettin exhibit strong similarity to these highly potent antimicrobial peptides. Antimicrobial Peptide Database predicts Sunettin to be an antimicrobial peptide since it form alpha helices and can have at least 7 residues on the same hydrophobic surface which allows the peptide to interact with membranes. Sunettin shows the characteristic features of antimicrobial peptides including high cationicity, higher hydrophobic residue and 2.3 kcal/mol protein binding potential.

As discussed earlier, it is evident from previous reports that histone derived antimicrobial peptides are formed by the action of proteolytic enzymes on histone proteins. The histone H2A sequence of *Sunetta scripta* was analysed using PeptideCutter tool of Expasy which reveals that proteolytic enzymes like Proteinase K, Thermolysin, Pepsin and Chymotrypsin are capable of cleaving the protein between position number 51 (Lys) and 52 (Arg) of amino acid sequence from the N-terminal and thereby releasing a 51 amino acid Sunettin. Hence it can be assumed that, when required, the cleavage activity of proteolytic enzymes on the histone H2A protein of *S. scripta* could release Sunettin which would assist the clam in its defense against pathogens.

Though Sunettin exhibit sequence similarity with previously reported histone H2A derived AMPs, it is more similar to Hipposin with regard to length. Owing to its similarity with Hipposin in terms of sequence and length, Sunettin is expected to have activity and mode of action comparable to Hipposin. Hipposin was found to kill bacterial cells by inducing membrane permeabilization, and this membrane permeabilization is promoted by the presence of the N-terminal Parasin domain (Bustillo et al., 2014). Being

similar to Hipposin, Sunettin is also anticipated to bring about the lysis of bacterial cells by membrane permeabilization mechanism. Schiffer-Edmundson helical wheel modeling of the peptide using Protean module revealed clustering of hydrophobic and hydrophilic/basic residues on opposing sides of the helical wheel and thereby confirmed the amphipathic nature of Sunettin. The cationic and amphipathic nature suggests that Sunettin, just like Hipposin and most AMPs would readily interact with target cell membranes and possibly would result in its permeabilization. The cationic charge would allow Sunettin to bind to the anionic surface of target bacterial cells and the amphipathic nature of the peptide would then allow it to interact with the cell membrane and bring about its permeabilization.

A molecular bootstrapped phylogenetic tree showing the relationship between histone H2A nucleotide sequence obtained from *S. scripta* and previously reported histone H2A sequences from other organisms was constructed. The tree demonstrates that the members of the family are derived from a common ancestor by a series of evolutionary changes. The phylogenetic tree branched into three main clusters or clades. Mammals and birds clustered into a single clade while lower invertebrates including fishes and amphibians formed another clade. Histone H2A sequences reported from invertebrates clustered into a single major clade. The bootstrap distance tree calculated based on histone H2A nucleotide sequences showed the clustering of *S. scripta* along with the invertebrate clade.

2.4.2 Molluskin

Molluskin also exhibited high sequence and structural similarity to previously reported histone H2A derived antimicrobial peptides. Based on its ability to form alpha helices and tendency to have at least 5 residues on the

same hydrophobic surface (which allows the peptide to interact with membranes), the Antimicrobial Peptide Database predicts Molluskin to be a peptide with antimicrobial activity. Molluskin exhibits all the characteristic features of antimicrobial peptides including high cationicity and higher content of hydrophobic residues along with a protein binding potential of 2.96 kcal/mol.

In order to understand the proteolytic cleavage involved in the formation of Molluskin we analyzed the histone H2A sequence obtained from *S. scripta* using PeptideCutter tool. PeptideCutter tool predicts extracellular digestive enzyme, trypsin to have potential cleavage site at amino acid position 15 and the enzymes, proteinase K and Asp-N endopeptidase to have a potential cleavage site at amino position 40 from the N-terminus. Proteolytic activity of these enzymes will result in the formation of a 25 amino acid Molluskin. These findings further suggest that the activity of proteolytic enzymes could transform the N-terminus of histone H2A in molluscs into an active antimicrobial peptide assisting in the innate immunity of the organisms. AMPs derived from precursors are less understood in case of marine invertebrates and therefore the study would provide a better understanding of their innate immune responses.

Buforins are one of the most potent antimicrobial peptides. In addition to their broad spectrum activity against bacteria and fungi (Park et al., 1996), they also possess antiendotoxic and anticancer activities (Cho et al., 2009). General mode of action of antimicrobial peptides is to kill cells through membrane disruption. Buforin II differs from this generalization as it does not cause significant membrane permeabilization (Kobayashi et al., 2000). Instead, Buforin II appears to readily enter bacterial cells in vivo (Park et al., 2000) and once inside, it is believed to cause bacterial cell lysis by interacting

with intracellular nucleic acids especially the major groove of DNA (Uyterhoeven et al., 2008). No cytotoxic activity against normal mammalian cells was observed for Buforin II (Takeshima et al., 2003). NMR structural studies showed that proline at position 11 serves as a hinge between a C-terminus helix and N-terminal region extended helical structure (Yi et al., 1996). This sole proline residue (Pro11) of Buforin II is necessary for effective translocation across cell membrane (Kobayashi et al., 2000; Park et al., 2000). Replacement or removal of Pro11 would readily hinder the effective membrane translocation of Buforin II (Park et al., 2000). Presence of a proline hinge as in Buforin II was also found to be a characteristic feature of Molluskin (Fig. 2.15). Presence of proline hinge clearly indicates that the antimicrobial activity of Molluskin lie in its ability to interact with nucleic acid rather than membrane permeabilization.

Antimicrobial peptides are also viewed as agents with therapeutic potential against cancer cells (Hoskin and Ramamoorthy, 2008). Buforin II exhibit selective cytotoxicity against cancer cells through interaction with cell surface gangliosides and once inside the cell they induced mitochondria-dependent apoptosis (Lee et al., 2008). Structural similarity to Buforin II would confer potential anticancer activity.

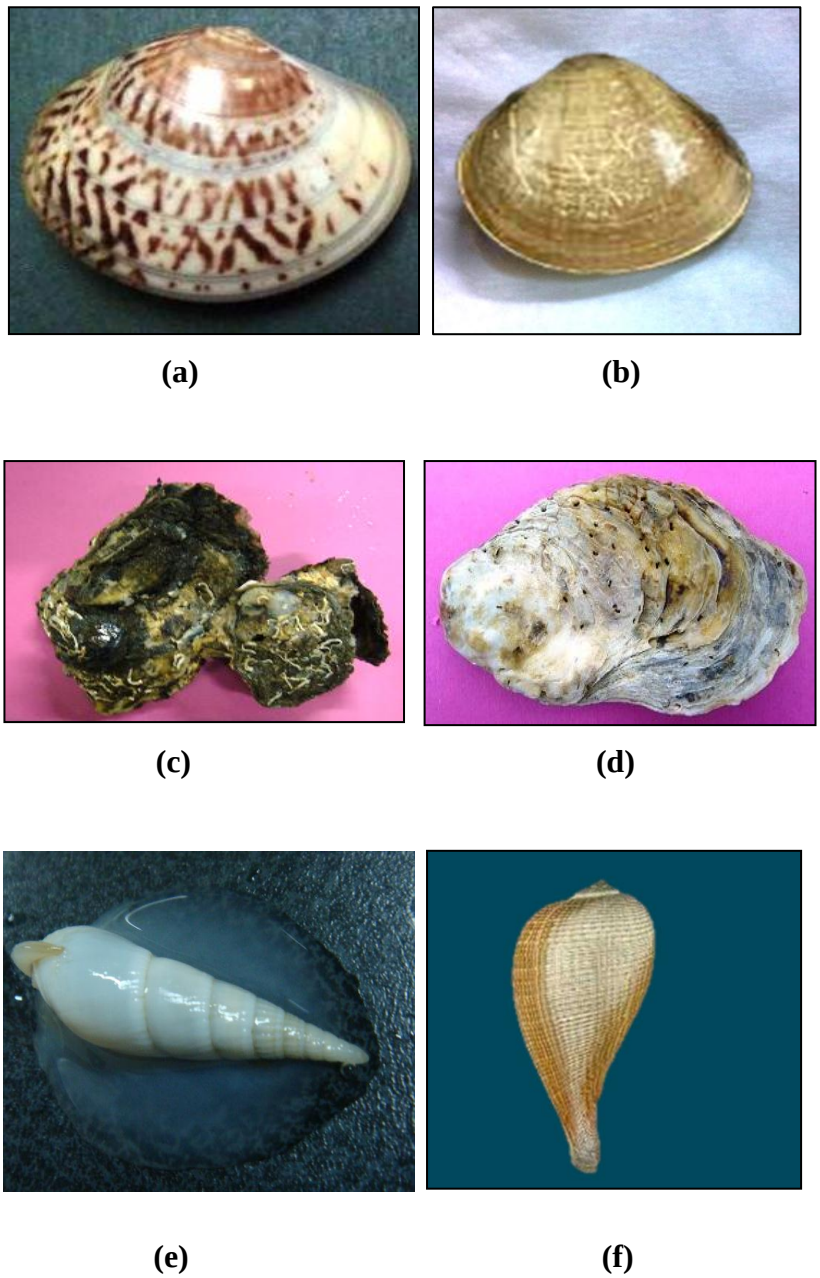
The molecular phylogenetic tree based on nucleic acid sequences of previously reported histone H2A derived AMPs demonstrates that the members of the family are derived from a common ancestor by a series of evolutionary changes (Fig. 2.16). Mammals and birds formed separate clades in the phylogenetic tree while fishes and frogs clustered into a single clade and The invertebrates formed a separate group. The nucleotide sequences of histone H2A genes obtained from all the five molluscs was found to align with the invertebrate group. Histone genes evolve very slowly and therefore,

evolutionary analyses of histones should be informative with regard to the phylogenetic relationships of distantly related organisms (Thatcher and Gorovsky, 1994).

Conclusion

A 51 amino acid peptide with antimicrobial sequence motif was identified from the histone H2A sequence obtained from *S. scripta* and was named as Sunettin. Similarly, from the histone H2A of *C. madrasensis*, *S. cucullata*, *M. casta*, *F. gracilis* and *B. vittata* a 25 amino acid peptide containing antimicrobial sequence motif was identified and named as Molluskin. High sequence similarity of both Sunettin and Molluskin to other histone H2A derived AMPs with proven antimicrobial activity, their physicochemical properties and molecular structure that are in agreement with those of traditional antimicrobial peptides strongly endorse them to be antimicrobial peptides.

This work was undertaken to study the presence of histone derived AMPs in molluscs depicting its possible role in innate immunity. Synthesizing histone derived AMPs for commercial applications would be a highly promising endeavor as an alternative to the conventional antibiotics which elicit drug resistance in microbes and impose tremendous ecological damage to the environment both terrestrial and aquatic. An objective in antimicrobial peptide research has been to use synthetic peptides as antibiotics and thereby limit the use of conventional antibiotics. Since both Molluskin and Sunettin are short peptides with a broad range of activity, both of them have the potential to be developed into effective therapeutic agents for use in aquaculture and pharmacy.



(a) **(b)**

(c) **(d)**

(e) **(f)**

Fig. 2.1. Pictures of the live mollusc samples collected for the study: (a) *Sunetta scripta*, (b) *Meretrix casta*, (c) *Crassostrea madrasensis*, (d) *Saccostrea cucullata*, (e) *Bullia vittata* and (f) *Ficus gracilis.*

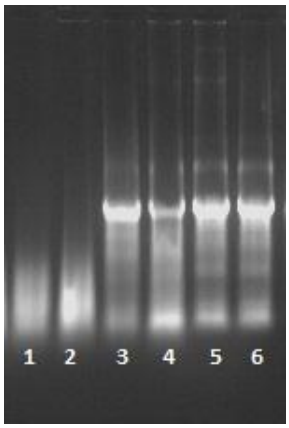


Fig. 2.1

Fig. 2.2. RNA electrophoresis in 0.8% Agarose Gel. Lane 1 and 2 show degraded RNA while lane 3 to 6 show good quality RNA.

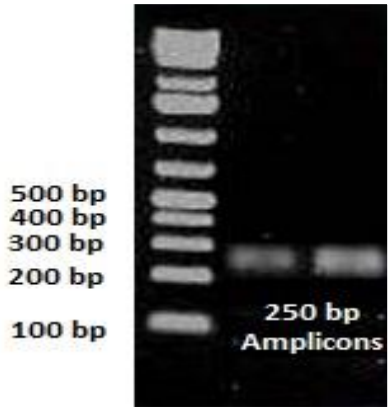


Fig. 2.2

Fig. 2.3. Amplicons of size 250 bp obtained from cDNA of *S. scripta* using HH2A-1 primer. A 100 bp ladder is loaded in first well for reference.

```
atgtctggacgaggaaagggtggaaagacaaagggaaaggcaaagagccgctcttctcgc
 M  S  G  R  G  K  G  G  K  T  K  G  K  A  K  S  R  S  S  R
gctggacttcagttccccgtgggtcgtatccaccgtctcctacgtaagggtaactacgcc
 A  G  L  Q  F  P  V  G  R  I  H  R  L  L  R  K  G  N  Y  A
gagagagttggagctggtgcacctgtgtatctcgccgccgtgctcgagtacttggccgcc
 E  R  V  G  A  G  A  P  V  Y  L  A  A  V  L  E  Y  L  A  A
gaagttttggaattggctggcaacgccgcacgagacaacaagaagacgcgcatcatcccc
 E  V  L  E  L  A  G  N  A  A  R  D  N  K  K  T  R  I  I  P
actcga

 T  R
```

Fig. 2.4. Necleotide sequence and deduced amino acid sequence of histone H2A amplified from *S. scripta*. The highlighted region denotes the active peptide region termed Sunettin.

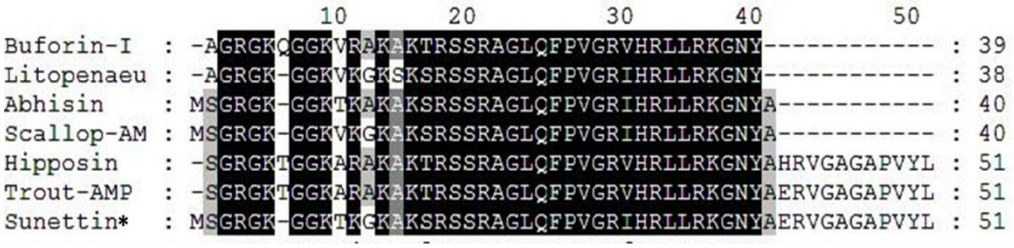


Fig. 2.5. ClustalW multiple alignment of Sunettin (*S. scripta*) with Hipposin (*Hippoglossus hippoglossus*), Buforin I (*Bufo bufo gargarizans*), Trout AMP (*Oncorhynchus mykiss*), Litopenaeus AMP (*Litopenaeus vannamei*), Scallop AMP, (*Chlamys farreri)* and Abhisin (*Haliotis discus*).

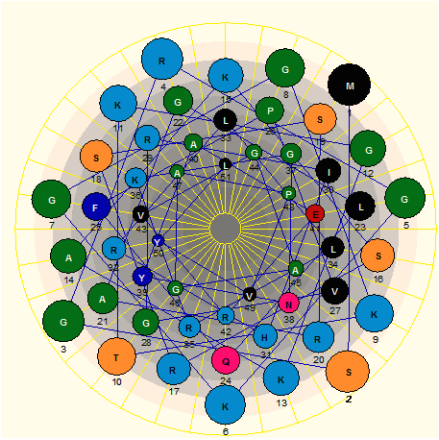
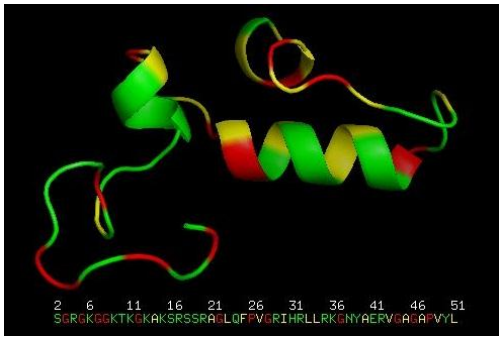


Fig 2.6. Schiffer-Edmundson Helical wheel representation of Sunettin generated using DNASTAR software. Hydrophobic and hydrophilic amino acids are seen occupying opposite positions.

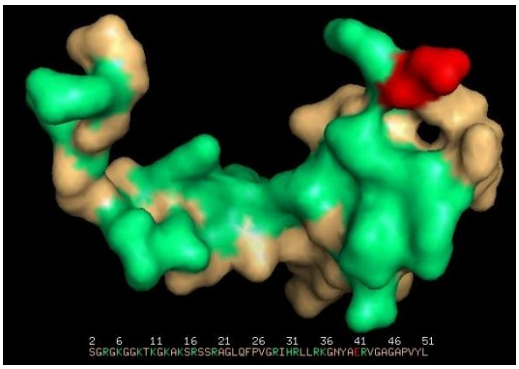


(a)

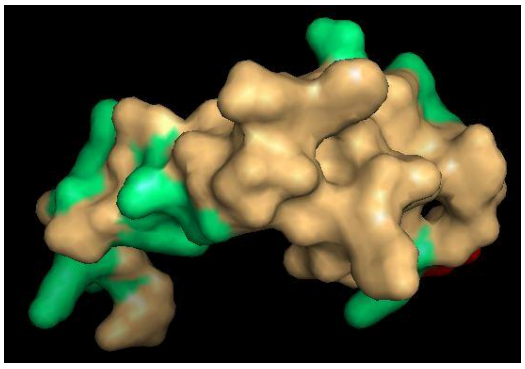
(b)

Fig. 2.7. Secondary structure of Sunettin. **(a)** Secondary structure of Sunettin created in PyMol software. Green colour denotes hydrophilic region, yellow colour denotes hydrophobic regions. Glycine and Proline which cannot be assigned a proper status is denoted by red colour. **(b)** STRIDE analysis of

secondary structure of Sunettin. The red regions represent α-helical arrangement while the yellow regions represent coils and turns.



View 1



View 2

Fig. 2.8. Tertiary Structure of Sunettin created in PyMol software. Green colour regions denote positively charged amino acids while brown coloured regions denote neutral ones. Negatively charged amino acids are denoted by red coloured region.

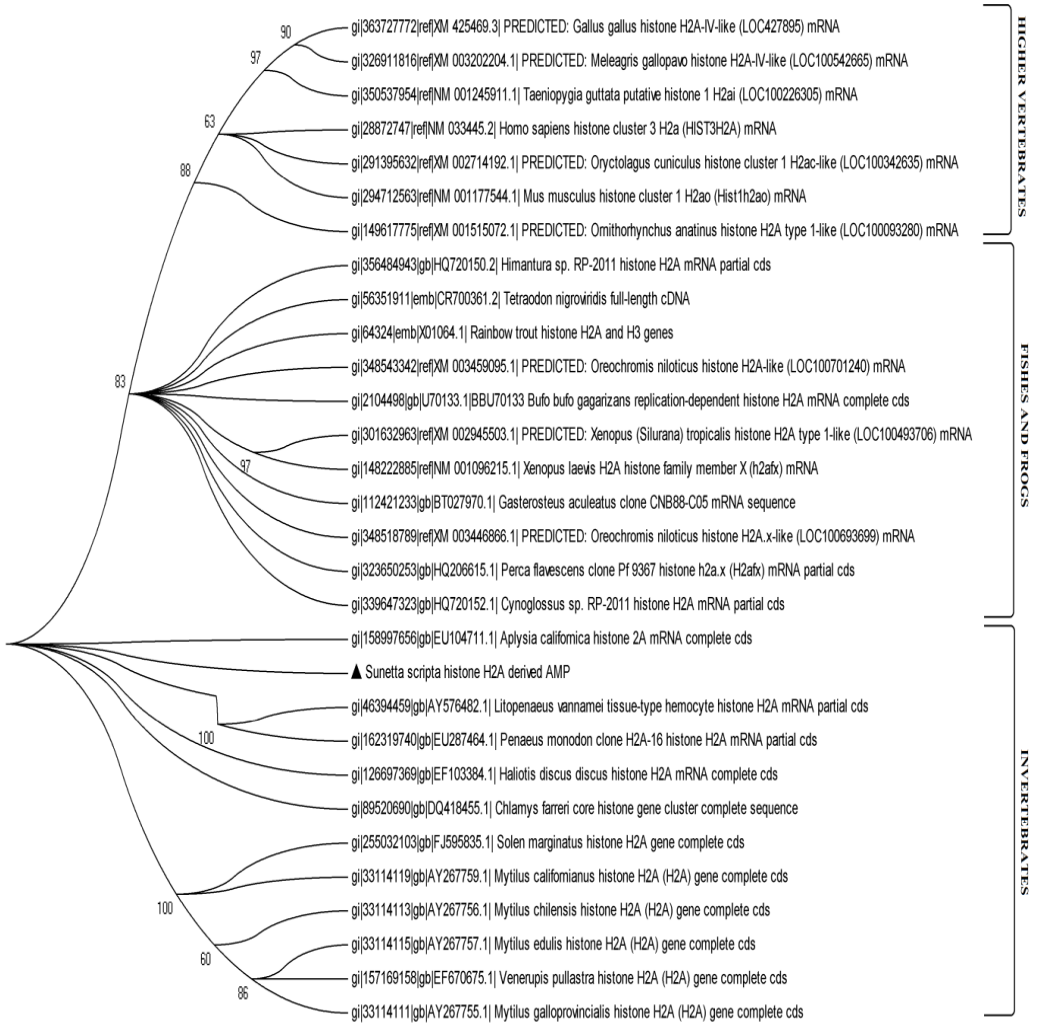


Fig 2.9. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the nucleotide sequence of *S. scripta* to the nucleotide sequences of previously reported histone H2A from various organisms downloaded from NCBI database.

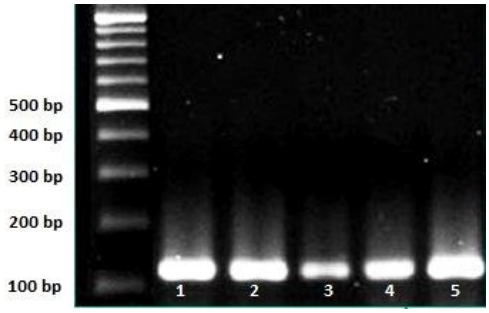


Fig. 2.10. Amplicons of size 117 bp obtained from (1) *Crassostrea madrasensis*, (2) *Saccostrea cucullata*, (3) *Meretrix casta*, (4) *Ficus gracilis* and (5) *Bullia vittata* by PCR amplification of respective cDNAs using primer HH2A-2. A 100 bp ladder is loaded in first well for reference.

```
agagcagtcccgctcttctcgcgctggacttcagttccccgtgggtcgtatccaccgtctg
  E  Q  S  R  S  S  R  A  G  L  Q  F  P  V  G  R  I  H  R  L
ctccgtaagggaaactatgcggccga
 L  R  K  G  N  Y  A  A
                                (a)

agagcagtcccgctcttctcgcgctggacttcagttccccgtgggtcgtatccaccgtctg
  E  Q  S  R  S  S  R  A  G  L  Q  F  P  V  G  R  I  H  R  L
ctccgtaagggaaactatgcggccgaag
 L  R  K  G  N  Y  A  A  E
                                (b)

agtcccgctcttctcgcgctggacttcagttccccgtgggtcgtatccaccgtctg
   S  R  S  S  R  A  G  L  Q  F  P  V  G  R  I  H  R  L
ctccgtaagggaaactatgcg
 L  R  K  G  N  Y  A
                                (c)

ccgctcttctcgcgctggacttcagttccccgtgggtcgtatccaccgtctg
  R  S  S  R  A  G  L  Q  F  P  V  G  R  I  H  R  L
ctccgtaagggaaactatgcggccg
 L  R  K  G  N  Y  A  A
                                (d)

cagtcccgctcttctcgcgctggacttcagttccccgtgggtcgtatccaccgtctg
 Q  S  R  S  S  R  A  G  L  Q  F  P  V  G  R  I  H  R  L
ctccgtaagggaaactatgcggcc
 L  R  K  G  N  Y  A  A
                                (e)
```

Fig. 2.11. Nucleotide and deduced amino acid sequences of histone H2A amplified from (a) *Crassostrea madrasensis*, (b) *Saccostrea cucullata*, (c) *Meretrix casta*, (d) *Bullia vittata* and (e) *Ficus gracilis.*

```
                    10        20        30        40        50
Molluskin*  : ----------------SRSSRAGLQFPVGRIHRLLRKGNYA---------- : 25
BuforinII   : ----------------TRSSRAGLQFPVGRVHRLLRK---------- : 21
BuforinI    : -AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY---------- : 39
Litopenaeu  : -AGRGK-GGKVKGKSKSRSSRAGLQFPVGRIHRLLRKGNY---------- : 38
Abhisin     : MSGRGK-GGKTKAKAKSRSSRAGLQFPVGRIHRLLRKGNYA---------- : 40
Scallop-AM  : MSGRGK-GGKVKGKAKSRSSRAGLQFPVGRIHRLLRKGNYA---------- : 40
Trout-AMP   : -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL : 51
Hipposin    : -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYL : 51
```

Fig. 2.12. ClustalW multiple alignment of Molluskin with Buforin I & 2 (*Bufo bufo gargarizans*), Litopenaeus AMP (*Litopenaeus vannamei*), and Abhisin (*Haliotis discus)*, Scallop AMP (*Chlamys farreri*), Hipposin (*Hippoglossus hippoglossus*) and Trout AMP (*Oncorhynchus mykiss*).

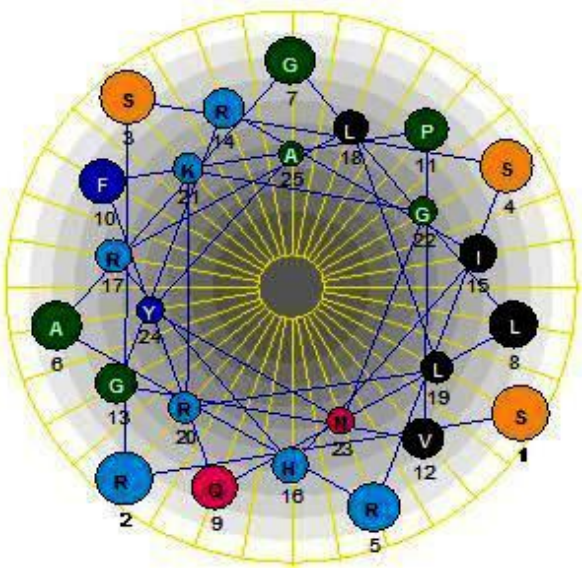
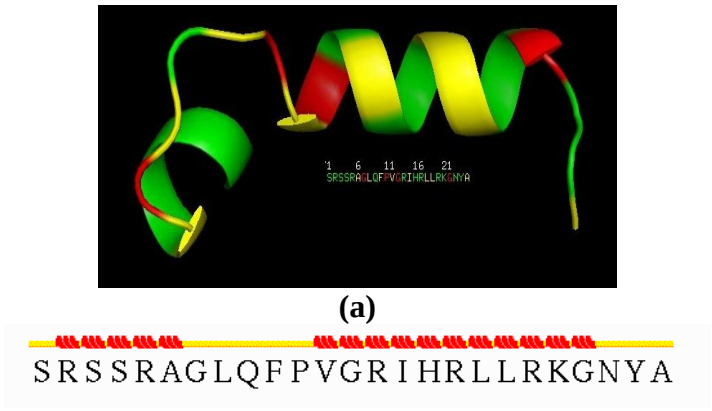


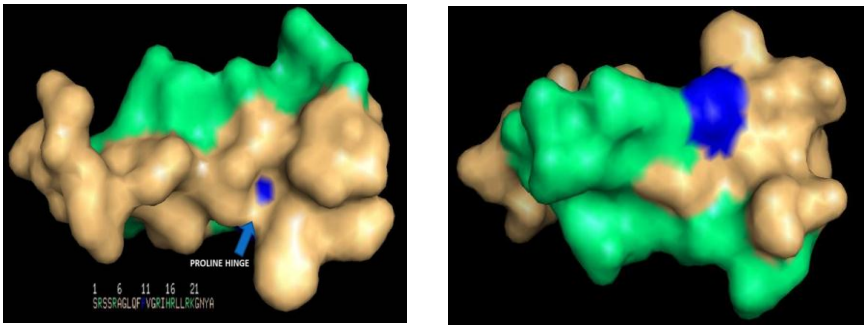
Fig 2.13. Schiffer-Edmundson Helical wheel representation of Molluskin generated using DNASTAR software. Hydrophobic and hydrophilic amino acids are seen occupying opposite positions.



(a)

(b)

Fig. 2.14. Secondary structure of Molluskin. **(a)** Secondary structure of Molluskin created in PyMol software. Green colour denotes hydrophilic region, yellow colour denotes hydrophobic regions. Glycine and Proline which cannot be assigned a proper status is denoted by red colour. **(b)** STRIDE analysis of secondary structure of Molluskin. The red regions represent α-helical arrangement while the yellow regions represent coils and turns.



View 1 **View 2**

Fig. 2.15. Tertiary Structure of Molluskin created in PyMol software. Green colour regions denote positively charged amino acids while brown coloured regions denote neutral ones. Proline hinge is labeled and proline is denoted in blue colour.

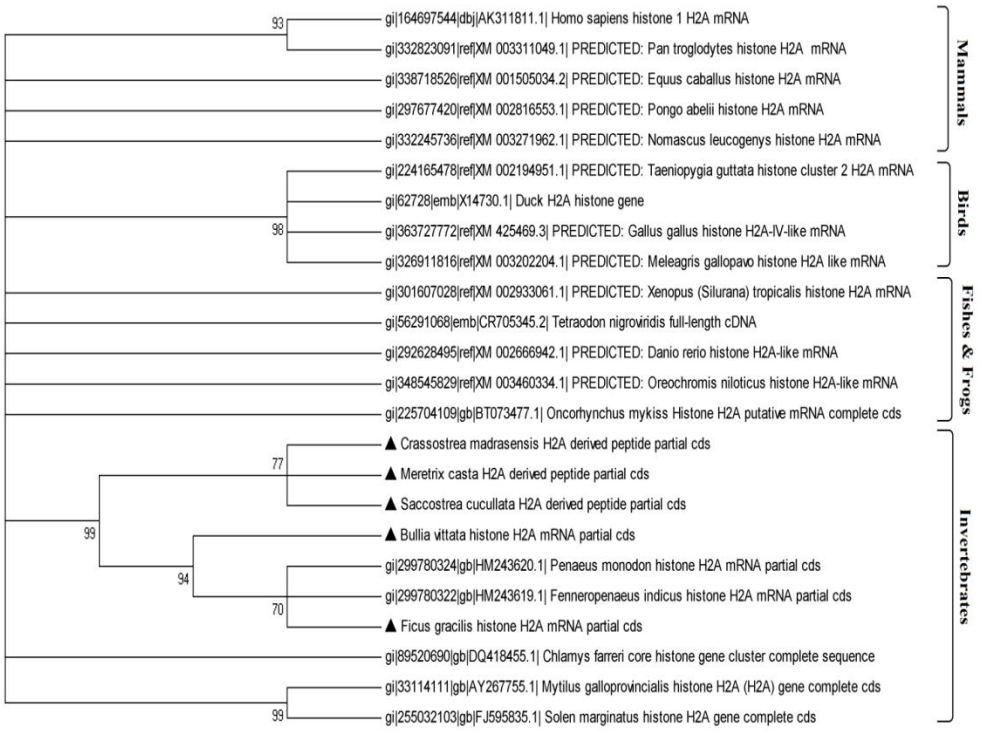


Fig 2.16. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the nucleotide sequence of histone H2A obtained from histone H2A amplified from *Crassostrea madrasensis*, *Saccostrea cucullata*, *Meretrix casta*, *Ficus gracilis* and *Bullia vittata* to the nucleotide sequences of previously reported histone H2A from various organisms downloaded from NCBI database.

3

Molecular Characterization and Phylogenetic Analysis of Histone Derived Antimicrobial Peptide Himanturin from Round Whip Ray, *Himantura pastinacoides*

3.1 Introduction

Elasmobranchs refer to a group of cartilaginous fishes which include sharks, skates and rays. Except for a few freshwater species, majority of Elasmobranchs are marine inhabitants. They exhibit rich diversity with regard to shape and size. Their size varies from the giant whale shark and the huge manta ray to tiny short nosed electric ray and dwarf lantern shark. Their shape varies from torpedo shaped sharks to flattened skates and rays and from weird looking hammer headed shark to inexplicable sawfish. In spite of these differences they share many common features like a skeleton made of cartilage, rows of replaceable teeth, 5-7 gill slits, skin made of small dermal denticles, rigid dorsal fin and an upper jaw not fused to the skull. Elasmobranchs are incredible hunters owing to their well-developed sense organs to detect smell and electric field. Our knowledge on the biology of Elasmobranch fishes is still very limited. Elasmobranchs are basal vertebrates with well-developed innate immune system. Their innate immunity plays a

key role in defending the organism against microbial pathogens and hence they are good model organisms to investigate novel antimicrobial peptides.

Antimicrobial peptides show wide divergence in their amino acid composition, size and conformational structures but exhibit striking similarity in their mode of action (Marshall and Arenas, 2003; Brogden, 2005; Pazgier et al., 2006; Labadie et al., 2007; Stromstedt et al., 2010; Lazaridis and Mihajlovic, 2010). They have retained their antimicrobial activity against a broad spectrum of pathogenic organisms, despite of their ancient and wide spread presence in nature (Zasloff, 2002; Marshall and Arenas, 2003). Remarkable specificity to prokaryotes with low toxicity to eukaryotic cells has favored their investigation and exploitation as new antibiotics (Zasloff, 1992). Fishes rely mainly on their innate immune defenses for protection against pathogenic organisms and hence should be considered as potential sources of antimicrobial peptides.

Fish possess a strong innate immune system which acts as the first line of defense against a broad spectrum of pathogens. HPLC based purification techniques have been widely used for identification of AMPs from fishes. One of the earliest works include isolation of three antimicrobial peptides of molecular weight 3.5 kDa, 4.5 kDa and 4.6 kDa from Hagfish intestine by Shinner et al. (1996). Pardaxin, a 33-amino-acid pore-forming polypeptide toxin isolated from the Red sea moses sole, *Purdachirus marmorutus*, was found to exhibit antibacterial activity against Gram positive bacteria (Oren and Shai, 1996). Cole et al. (1997) purified a novel 25-residue linear antimicrobial peptide, pleurocidin from the skin mucous secretions of the Winter flounder (*Pleuronectes americanus*). A novel 21-amino-acid antimicrobial peptide having a molecular mass of 2.5 kDa named misgurin, was isolated and characterized from the Loach, *Misgurnus anguillicaudatus* (Park et al., 1997).

Patrzykat et al. (2001) have reported a 26 amino acid N-terminal segment of Coho Salmon (*Oncorhynchus kisutch*) histone H1, termed histone derived fragment-1(HSDF-1), isolated from mucus and blood with antimicrobial activity. Another histone H1 derivative with antibacterial activity, termed as oncorhyncin-2 has been isolated from Rainbow Trout, *Oncorhynchus mykiss* skin secretions (Fernandez et al., 2004). In response to epidermal injury, *Parasilurus asotus,* a catfish, secreted a strong antimicrobial peptide into the epithelial mucosal layer. The 2 kDa AMP was found to be a derivative of histone H2A and was termed as parasin I (Park et al., 1998). Hipposin, one of the most potent histone H2A derived AMP was isolated from the skin mucus of Atlantic Halibut, *Hippoglossus hippoglossus* (Birkemo et al., 2003).

Almost all families of AMPs reported from fishes have been cloned and studied using gene based approach. These include pleurocidins from *Pseudopleuronectes americanus, Hippoglossoides platessoides, Hippoglossus hippoglossus* and *Limanda ferruginea*, hepcidin from *Morone chrysops, Pleuronectes americanus, Salmo salar, Chlorophthalmus bicornis*, *Zanclus cornutus* and *Oncorhynchus mykiss*, pardaxin from *Pardachirus marmoratus* and *Pardachirus pavoninus,* cathelicidin from *Salvelinus alpines, Gadus morhua, Salmo truttafario* and *Thymallus thymallus,* chrysophsin-1, 2 and 3 from *Chrysophrys major*, epinecidin from *Epinephelus coioides,* hipposin from *Hippoglossus hippoglossus,* NK-lysin from *Ictalurus punctatus* and onchorhyncin from *Oncorhynchus mykiss* (Reviewed by Patrzykat and Douglas, 2003; Yount et al., 2006; Smith et al., 2010; Rajanbabu and Chen, 2011).

Traditionally, histones are known as major components of the nucleosome structures in eukaryotic cells. Histone proteins play a key role in the innate immune defense of organisms by forming AMPs (Kawasaki and

Iwamuro, 2008). Proteolytic enzymes play a key role in the formation of histone derived AMPs. Histone derived AMPs with potent activity has been isolated and reported from various organisms (Park et al.,1996; Park et al.,1998; Richards et al., 2001; Birkemo et al., 2003; Fernandes et al., 2004; Patat et al., 2004; Li et al., 2007; Sook et al., 2008; De Zoysa et al., 2009). Buforin I isolated from Asian Toad *Bufo bufo* was the first report of a histone H2A derived AMP (Park et al., 1996; De Zoysa et al., 2009). In the case of marine invertebrates, histone derived AMPs have been reported from Pacific White Shrimp (Patat et al., 2004), Scallop (Li et al., 2007), Disk Abalone (De Zoysa et al., 2009) etc.

From fishes histone derived antimicrobial peptides have been reported from Catfish *Parasilurus asotus* (Park et al., 1998), Atlantic salmon *Salmon salar* (Richards et al., 2001), Rainbow Trout *Oncorhynchus mykiss* (Fernandes et al., 2004), and Atlantic Halibut *Hippoglossus hippoglossus* (Birkemo et al., 2003). Present study was carried out to identify novel antimicrobial peptides from Rays.

3.2 Materials and Methods

3.2.1 Sample Collection

Live Round Whip Ray, *Himantura pastinacoides* was obtained from local fishermen who caught the fish off Vypeen, Kochi, Kerala (Fig. 3.1). Samples were transported to laboratory in live condition. *Himantura pastinacoides* belongs to Super Class: Pisces; Class: Chondrichthyes; Sub-class: Elasmobranchii; Order: Rajiformes and Family: Dasyatidae.

3.2.2 RNA Extraction

Blood was collected from the lamellar artery near gill region of Round whip ray using specially designed capillary tubes (RNase free) rinsed in precooled anticoagulant solution (RNase free 10 % sodium citrate, pH 7). Total RNA was extracted from the blood usin TRI® Reagent. For details on RNA extraction procedure refer section 2.2.2 of Chapter 2.

3.2.3 cDNA Synthesis

Single stranded cDNA was synthesized from 5 µg of good quality RNA using reverse transcription. For details on cDNA synthesis refer section 2.2.3 of Chapter 2.

3.2.4 PCR Amplification

Amplification of a Hipposin- like antimicrobial peptide from cDNA of *Himantura pastinacoides* was done using sense primer (5′-ATGTCCGGRMG MGGSAARAC-3′) and antisense primer (5′-GGGATGATGCGMGTCTTCTT GTT-3′) (Birkemo et al., 2003). PCR amplification of 1 µl of cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (New England Biolabs, USA). The thermal profile used was an initial denaturation at 94° C for 2 minutes followed by 35 cycles of 94° C for 15 seconds, 60° C for 30 seconds and 68° C for 30 seconds and a final extension at 68° C for 10 minutes.

3.2.5 Agarose Gel Electrophoresis

The PCR product (10 µl) was analyzed on 1.5 % agarose gel. See section 2.2.5 of Chapter 2 for details on agarose gel electrophoresis.

3.2.6 Sequencing

Sequencing of purified PCR product was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, Kakkanadu, Kochi, India.

3.2.7 Sequence Analysis and Molecular Characterization

The nucleotide sequence was analyzed and assembled using GeneTool software. Translation of the nucleotide sequence was carried out followed by BLAST analysis of both nucleotide and deduced amino acid sequences. Further analysis of the amino acid sequence was performed using various computer based programs as explained in section 2.2.7 of Chapter 2.

3.2.8 Phylogenetic Analysis

Nucleic acid sequences of histone H2A genes reported from various organisms were retrieved from GenBank database and a NJ phylogenetic tree was constructed. For details refer section 2.2.8 of Chapter 2.

3.2.9 Taxonomic Identification Using Molecular Tools

3.2.9.1 DNA Isolation

For taxonomic identification of the species, DNA was isolated from the gills of *H. pastinacoides* using TRI® Reagent (Sigma), following the manufacturer's instructions. Briefly, 10 gm of tissue was homogenized with TRI Reagent (1 ml) in a tissue homogenizer. The homogenate was transferred to 1.5 ml micro centrifuge tubes (MCTs) and allowed to stand for 5 mins at room temperature. To the homogenate was added 0.2 ml chloroform, shaken

vigorously for 15 seconds and allowed to stand at room temperature for 15 mins. It was then centrifuged at 12,000 x g for 15 mins at 4^{o} C. Centrifugation separated the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase overlaying the interphase was carefully removed and DNA was precipitated from the interphase and organic phase by adding 0.3 ml of 100 % ethanol per 1 ml of TRI reagent used in sample preparation. It was then mixed by inversion and allowed to stand for 2–3 minutes at room temperature. This was followed by centrifugation at 2,000 × g for 5 minutes at 4° C. The supernatant was discarded and the DNA pellet was washed twice in 1 ml of 0.1 M trisodium citrate, 10 % ethanol solution. DNA pellet was allowed to stand (with occasional mixing) for at least 30 minutes during each washing step. Afterwards it was centrifuged at 2,000 × g for 5 minutes at 4° C. DNA pellet was resuspended in 75 % ethanol (1 ml) and allowed to stand for 10–20 minutes at room temperature followed by centrifugation at 2,000 × g for 5 minutes at 4° C. The DNA pellet was dried for about 30 at room temperature and dissolved in 8 mM NaOH (mild alkaline solution assures complete dissolution of the DNA pellet) with repeated slow pipetting with a micropipette. Centrifuged at 12,000 × g for 10 minutes to remove any insoluble material and transferred the supernatant to a new tube.

3.2.9.2 Determining the Quality and Quantity of DNA

The purity and quality of the DNA was visualized using electrophoresis on 0.8 % agarose gel. The concentration of isolated DNA was estimated using a UV-Vis Spectrophotometer (Hitachi U-2900). DNA was quantified and qualified by measuring optical density (O.D) at 260 and 280 nm in a UV spectrophotometer (U-2900, Hitachi). The ratio of absorbance at 260 nm and 280 nm is an indication of DNA quality. Good quality DNAs with absorbance

ratio (A260: A280) ≥1.8 were used for amplification. Optical density when measured at 260 nm, 1 O.D of DNA = 50 µg/ml, and hence, DNA concentration was calculated as:

DNA concentration (µg/ml) = O.D at 260 nm x Dilution factor x 50

3.2.9.3 PCR Amplification of DNA (CO1 gene)

The DNA was diluted to a final concentration of 100 ng/µl. The Cytochrome Oxidase-I (CO1) gene was amplified in a 25 µl reaction volume containing the above said PCR reagents (Section 3.2.4) in same concentration. One µl of genomic DNA was used as template. The primers used for the amplification of COI gene were LCO (5′- TCGACTAATCATAAAGATATG GGCCAC -3′) and HCO (5′-ACTTCAGGGTGACCGAAGAATCAGAA -3′**)** (Miller et al., 1998). The thermal regime consisted of an initial denaturation at 95^{o} C for 5 minutes followed by 35 cycles of 95^{o} C for 45 seconds, 50^{o} C for 30 seconds and 72^{o} C for 45 seconds and a final extension at 72^{o} C for 10 minutes.

3.2.9.4 Agarose Gel Electrophoresis

Agarose Gel Electrophoresis of PCR product was carried out in the same way as explained in section 2.2.5 of Chapter 2.

3.2.9.5 Sequencing

Amplicons obtained were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, India.

3.2.9.6 Sequence Analysis

The nucleotide sequences of CO1 gene was analyzed, and assembled using GeneTool software. Homology search of the nucleotide sequence was performed using BLASTn suite of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

3.3 Results

3.3.1 Taxonomic Identification

The LCO and HCO primers amplified a 710 bp region of the mitochondrial cytochrome oxidase subunit I gene (Fig. 3.2). The nucleotide sequence obtained from the amplicon is shown in Fig. 3.3. BLAST analysis of nucleotide sequence obtained from amplicon using BLASTn suite of NCBI (http://www.ncbi.nlm.nih.gov/blast) confirmed the identity of the Ray as *Himantura pastinacoides* showing 97% similarity to GenBank ID: EU398852.1 *Himantura pastinacoides*. The nucleotide sequence was submitted to GenBank Database (GenBank ID: **JN98236**). The genus *Himantura* belongs to Family Dasyatidae, Order Rajiformes, Sub-Class Elasmobranchii and Class Chondrichthyes.

3.3.2 Identification and Molecular Characterization of Histone H2A Sequence

3.3.2.1 Sequence Analysis

A 250 bp amplicon was obtained from PCR amplification of the cDNA using histone H2A specific primer (Fig. 3.4). A 204 bp fragment cDNA encoding 68 amino acids from the mRNA of blood cells of *H. pastinacoides*

was obtained by RT-PCR (Fig.3.5). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptide belonged to histone H2A family. The nucleotide and deduced amino acid sequences were deposited in GenBank database (GenBank ID: **HQ720150**).

Multiple-sequence alignment of the amino acid sequence of the peptide with previously reported histone H2A derived AMPs revealed that the first 39 amino acid sequence from N-terminal of the deduced peptide showed similarity to histone H2A derived AMPs i.e., Hipposin, Buforin I, Buforin II, Abhisin and those reported from *Oncorhynchus mykiss, Litopenaeus vannamei* and *Chlamys farreri* (Fig. 3.6). This 39 amino acid sequence from *H. pastinacoides* was termed as 'Himanturin' and here onwards will be denoted by the term.

The 39 amino acid Himanturin was found to be rich in arginine (15.4 %), glycine (12.8 %), alanine (12.8 %), leucine (10.3 %), valine (10.3 %) and lysine (7.7 %) as reported in all other histone H2A derived AMPs. Himanturin was found to have a predicted molecular weight of 4.27 kDa and a theoretical isoelectric point (p*I*) of 11.73 as predicted by PROTPARAM software. Himanturin was found to have one negative residue (Glu) as against nine positive residues (Arg+Lys). The peptide was predicted to have a net charge of +9.8, +8.1 and +4.5 at pH 4, 7 and 10 respectively by Protein Calculator v3.3. Hydrophobicity of the peptide was found to be +34.68 Kcal/mol (35 %) as predicted by PepDraw.

Analysis of Himanturin using Protean module of the DNASTAR Lasergene sequence analysis software suite revealed that the peptide will have a concentration of 1.43 mg/ml for an absorbance of 1 OD measured at 280 nm and 1 µg of the peptide would contain 234.36 pMoles. Hydrophobic amino

acids constituted 31.68 % while polar amino acids represented 19.45 % of the total weight of Himanturin

Schiffer-Edmundson helical wheel analysis of Himanturin clearly showed a perfect amphipathic nature (Fig. 3.7). Hydrophilic residues such as serine (S), lysine (K), histidine (H), arginine (R), asparagine (N), glutamine (Q) and hydrophobic residues such as valine (V), alanine (A), tyrosine (Y), leucine (L), isoleucine (I), methionine (M) were found to occupy positions opposite to each other in the helical wheel. This type of arrangement for hydrophobic and hydrophilic amino acid is also a confirmation of the helical structure of peptide.

The secondary structural analysis of Himanturin was carried out using secondary structure assignment program, STRIDE (http://webclu.bio.wzw.tum.de/cgi-bin/stride/stridecgi.py). The analysis showed that the peptide, Himanturin contains 56.41 % α-helical region (22 amino acid residues), 15.38 % turns (6 amino acid residues) and 28.21 % coils (11 amino acid residues). No β-sheet region has been noticed in the secondary structure of Himanturin (Fig. 3.8). The α-helical arrangement is present in Himanturin from Arg 5 to Ala 9, Val 15 to Gly 25 and Ala 33 to Tyr 38. Himanturin exhibits turns from Asn 26 to Val 31. Coils are present from Lys 1 to Ser 4, Gly 10 to Pro 14 and Gly 32 and Leu 39. Three dimensional arrangement of the peptide generated in PyMOL using the data in pdb format obtained from SWISS-MODEL is presented in Fig. 3.9.

Analysis of Himanturin for antimicrobial activity was carried out with Antimicrobial Peptide Database which predicts Himanturin to be an antimicrobial peptide with a protein binding potential of 2.24 kcal/mol.

3.3.2.2 Phylogenetic Analysis

Bootstrap (1000 repetition) distance tree calculated by NJ method confirmed the similarity of the obtained nucleotide sequence to the histone H2A nucleotide sequences previously reported from various organisms (Fig.3.10). The phylogenetic tree branched into three clusters/clades, one of higher vertebrates (mammals and birds), one comprising of lower vertebrates (fishes and frogs) and the last one comprising of vertebrates. The nucleotide sequence obtained from *H. pastinacoides* clustered along with the clade formed by fishes and frogs.

3.4 Discussion

Fragments of histone H2A protein with antimicrobial properties have been reported from a number of animals including fishes. These Histone H2A derived antimicrobial peptides are reported to have activity against both Gram-positive and Gram-negative bacteria and fungi (Park et al., 1996; Park et al., 1998; Richards et al., 2001; Birkemo et al., 2003; Patat et al., 2004; Li et al., 2007; Kawasaki and Iwamuro, 2008; De Zoysa et al., 2009). Studies revealed that in Asian Toad, *Bufo bufo gargarizans* and in Catfish, *Parasilurus asotus*the histone derived antimicrobial peptides are formed by the action of proteolytic enzymes on intact histone proteins. To study the activity of proteolytic enzymes on 68 amino acid histone H2A sequence obtained from Round Whip Ray, the sequence was analyzed using PeptideCutter tool (http://web.expasy.org/peptide_cutter/). The PeptideCutter tool predicts enzymes Pepsin, Proteinase K, Thermolysin, and Chymotrypsin to have a potential cleavage site at amino acid position 39 from the N-terminus and their activity at position 39 would result in the formation of a 39 amino acid

Himanturin. These results indicate that the N-terminus of histone H2A of *Himantura pastinacoides* could act as an active antimicrobial peptide participating in the innate immunity of the fish when required.

In Drosophila secretion of antimicrobial peptides is mediated by two distinct pathways, the Toll pathway and Immune Deficiency (IMD) pathway. The Toll pathway is activated primarily in response to fungal and Gram positive bacterial infections, whereas the IMD pathway is activated predominantly in response to Gram negative and other Gram positive bacterial infections (Lemaitre et al., 1997; Hoffmann and Reichhart, 2002). Toll activates expression of antifungal peptide genes, Dorsomycin and Metchnikowin, whereas, IMD induces transcription of genes, which encode the antibacterial peptides i.e., Diptericins, Cecropins, Drosocins and Attacins. A pathway similar to that of Drosophila IMD, termed as LvIMD was reported from *Litopenaeus vannamei*. Expression of LvIMD mRNA is influenced by LPS and Gram negative *Vibrio alginolyticus* and expression of LvIMD could induce a 3 fold increase in the expression of PEN 4 (Wang et al., 2009c). Presence of Toll-like receptor in *Litopenaeus vannamei* (Lv Toll1) was first reported by Yang et al. (2007). Two more Toll-like receptors, Lv Toll2 and Lv Toll3 were reported by Wang et al. (2012). In comparison to Lv Toll1 and Lv Toll3, Lv Toll2 was found to be more significant in the activation of AMP promoters in *Litopenaeus vannamei* (Wang et al., 2012). Mechanisms similar to these might be involved in the cleavage of precursor derived antimicrobial peptides. Mechanism by which histone proteins are expressed and enzymatically cleaved into fragments with antimicrobial activity are not been studied and are yet to be clarified.

In case of 51-mer Hipposin, fragment containing 1 to 19 amino acid residues from the N terminal did not exhibit marked antimicrobial activity,

whereas fragment consisting of 16-39 amino acid (similar to buforin II) had such activity indicating that this part of Hipposin possesses antimicrobial sequence motif and the activity was found to be enhanced by the presence of the fragment having 40-51 amino acid residues (Birkemo et al., 2004). Fragment consisting of 4-39 amino acid residues from N-terminal of Himanturin exhibit striking similarity to 16-39 amino acid fraction of Hipposin which has been shown to contain the antimicrobial sequence motif. Almost all previously reported histone H2A derived AMPs have fragments similar to this fraction of Hipposin and it could be deduced that their activity is mainly due to this region. Fraction 4-39 of Himanturin is similar to 16 to 51 amino acid fraction of hipposin except for Thr at position 16 of Hipposin which has been replaced by Ser (position 4) in Himanturin and His at position 41 of Hipposin being replaced by Glu (position 29) in Himanturin. Presence of Ser instead of Thr at position 16 of Hipposin can also be seen in Abhisin and histone H2A derived AMPs reported from *Litopenaeus vannamei* and *Chlamys farreri*.

Broad spectrum antimicrobial activity has been reported for previously identified histone H2A derived antimicrobial peptides. Hipposin and Parasin I are the most studied Histone H2A derived antimicrobial peptides of marine origin. Hipposin exhibit strong antibacterial activity which could be detected down to concentrations of 1.6 µg/ml (Birkemo et al., 2003). Potent antibacterial and antifungal activity have been reported for Parasin I also. Abhisin reported from Disk abalone, *Haliotis discus* was found to be active against bacteria, yeast and THP-1 leukemia cancer cells. Abhisin was non-toxic to normal cells (De Zoysa et al., 2009). Histone H2A derived AMPs exhibit strong activity against aquatic and human pathogens. Himanturin exhibit strong structural and sequence similarity to these highly potent

antimicrobial peptides and hence Himanturin is expected to have a similar activity and mode of action.

The antimicrobial property of Himanturin was confirmed by the helical structure analysis and the total hydrophobic surface. The secondary structure analysis showed that Himanturin contains many coils and α-helices. Peptides with α-helix regions do have a significant possibility of exhibiting antimicrobial property. Furthermore, Himanturin was analyzed using Schieffer-Edmundson helical wheel program which clearly indicated the peptide to be a α-helical molecule, having hydrophobic and hydrophilic residues on opposing sides. Moreover, Himanturin was found to be rich in cationic amino acids and hence contain a net positive charge, which is important for an AMP. The α-helical structure, hydrophobic surfaces and net positive charge clearly indicate that Himanturin has the potential for interacting with microbial membranes and thereby acting as antimicrobial peptides. To confirm its antimicrobial property, Himanturin was analyzed using predictor tool of Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). The database predicts Himanturin to be an Antimicrobial peptide since it form alpha helices and has at least 7 residues on the same hydrophobic surface which allows the peptide to interact with membranes.

The phylogenetic relationship of *Himantura pastinacoides* to other organisms is shown in Fig. 3.10. The molecular phylogenetic tree based on nucleotide sequences of previously reported histone H2A sequences demonstrate that the members of the family are derived from a common ancestor by a series of evolutionary changes. The boot strap distance tree calculated reveals that *Himantura pastinacoides* cluster under the clade formed by Fishes and Amphibians. Histone genes evolve very slowly and therefore, evolutionary analyses of histones should be informative with regard

to the phylogenetic relationships of distantly related organisms (Thatcher and Gorovsky, 1994). At the nucleotide level, the variability in histone genes appears to be the result of a larger amount of non-synonymous variation, which affects to a lesser extent, the structural domain of the protein comprising the histone fold (Lopez et al., 2008). Because the topology of major histone H2A phylogeny is similar to the eukaryotic phylogeny, histone H2A can be used as a molecular marker for classification. More data on Ray histone H2A sequences will decipher the relationship of Ray H2A to other vertebrate and invertebrate H2A.

Conclusion

A peptide containing antimicrobial sequence motif from the histone H2A of Round Whip Ray, *Himantura pastinacoides* was identifiedand named as Himanturin. The 39 amino acid Himanturin exhibit high similarity to other histone H2A derived AMPs with proven antimicrobial activity with regard to sequence and structure. The physicochemical properties of Himanturin are in agreement with those of traditional antimicrobial peptides in terms of small size, cationic charge and hydrophobicity. These striking similarities to other AMPs strongly endorse Himanturin to be an antimicrobial peptide. Himanturin has the potential to be developed into an effective antimicrobial agent with broad application potential. The experimental and computational results discussed in the chapter provide first insight into the role of histones in the innate immune defenses of Elasmobranch fishes. These fishes remain an under explored group with regard to antimicrobial peptide research.

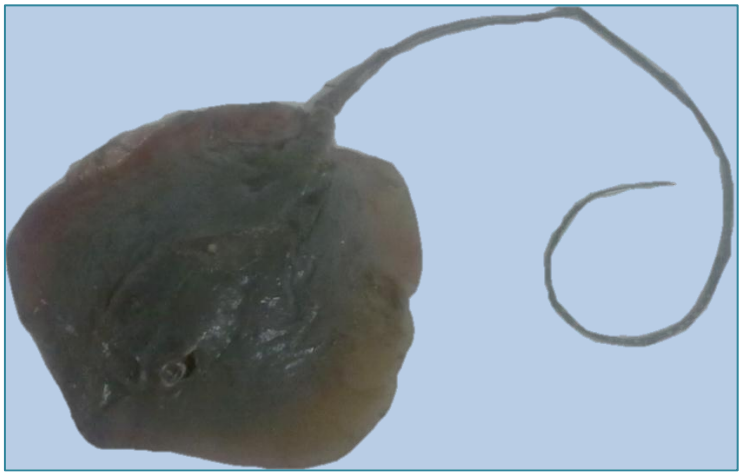


Fig. 3.1. Round Whip Ray, *Himantura pastinacoides* caught from Vypeen, Cochin.

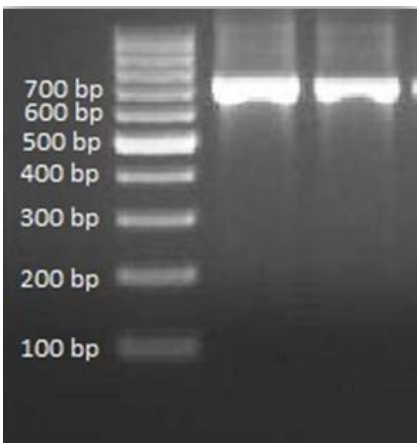


Fig. 3.2. Amplicons of CO1 gene 710 bp obtained from DNA isolated from *H. pastinacoides*

AGTTAAGCCAACCAGGCGCACTACTCGGTGATGATCAGATCTACAATGTGAT
TGTTACCGCCCATGCCTTCGTGATAATCTTCTTTATAGTAATACCTATCATAA
TTGGGGGTTTTGGTAACTGGCTCGTTCCCCTAATAATCGGCGCCCCGGATATG
GCCTTTCCCCGAATAAACAACATAAGTTTCTGGCTTCTGCCGCCCTCCTTTCT
GCTACTTTTAGCCTCTGCTGGGGTTGAAGCCGGGGCTGGGACGGGTTGGACT
GTTTATCCCCCACTAGCTGGCAACCTAGCACATGCTGGAGCTTCAGTAGACCT
AGCAATCTTCTCATTACACCTGGCTGGTGCTTCTTCTATCCTAGCCTCCATTA
ACTTTATTACTACAATTATCAACATAAAACCACCAGCAATCTCACAATATCA
GACACCCCTCTTTGTCT

Fig. 3.3. Nucleotide sequence of CO1 gene fragment amplified from *H. pastinacoides*

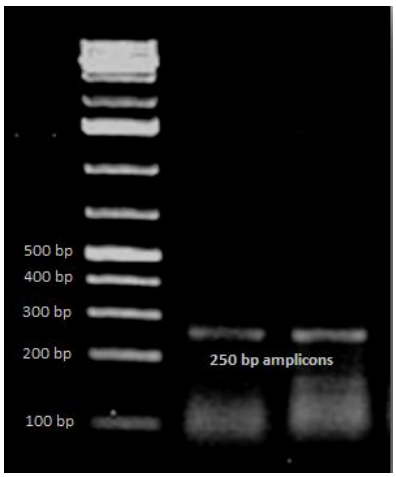


Fig. 3.4. Amplicons of size 250 bp (H2A) obtained from cDNA of *H. pastinacoides.*

```
aaggcaaagagccgttcttcccgggccgggctgcagttcccggtgggccgcgtccacagg
K  A  K  S  R  S  S  R  A  G  L  Q  F  P  V  G  R  V  H  R
ctgctgaggaagggcaactacgccgagcgggtgggcgccggggccccggtctacctggcc
L  L  R  K  G  N  Y  A  E  R  V  G  A  G  A  P  V  Y  L  A
gccgtgctcgagtacctgacagccgagatcctggagctggccggcaacgcggcccgggac
A  V  L  E  Y  L  T  A  E  I  L  E  L  A  G  N  A  A  R  D
aacaagaagacgcgcatcatcccc
N  K  K  T  R  I  I  P
```

Fig. 3.5. Nucleotide and deduced amino acid sequence of histone H2A region amplified from *H. pastinacoides*. Highlighted region is the sequence of putative AMP, Himanturin.

```
                    10        20        30        40        50
BuforinI    : -AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY---------- : 39
BuforinII   : ----------------TRSSRAGLQFPVGRVHRLLRK---------- : 21
Litopenaeu  : -AGRGK-GGKVKGKSKSRSSRAGLQFPVGRIHRLLRKGNY---------- : 38
Abhisin     : MSGRGK-GGKTKAKAKSRSSRAGLQFPVGRIHRLLRKGNYA---------- : 40
Sacallop_A  : MSGRGK-GGKVKGKAKSRSSRAGLQFPVGRIHRLLRKGNYA---------- : 40
Hipposin    : -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYL : 51
Rainbow_Tr  : -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL : 51
Himanturin  : ----------KAKSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL : 39
```

Fig. 3.6. Clustal W Multiple Sequence Alignment of Himanturin with previousily reported histone H2A derived AMPs from Asian Toad (Buforin 1 and Buforin 2), Pacific White Shrimp (*L. vannamei*), Disk Abalone (Abhisin) Scallop, Atlantic Halibut (Hipposin) and Rainbow Trout.

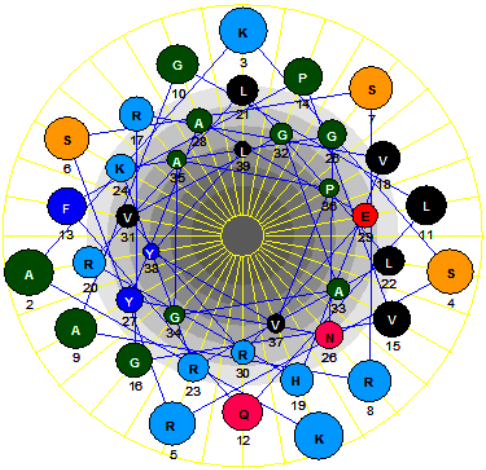
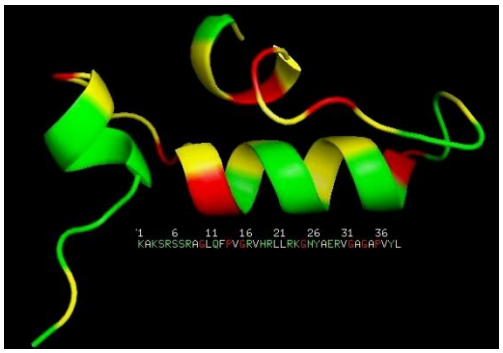


Fig. 3.7. Schiffer-Edmundson helical wheel analysis of Himanturin showing hydrophilic and hydrophobic amino acids occupying opposite position.

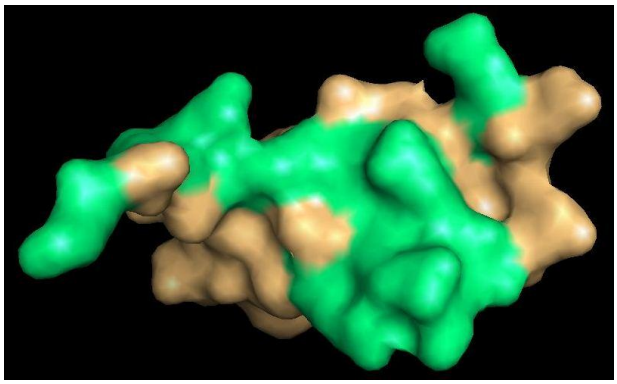


(a)

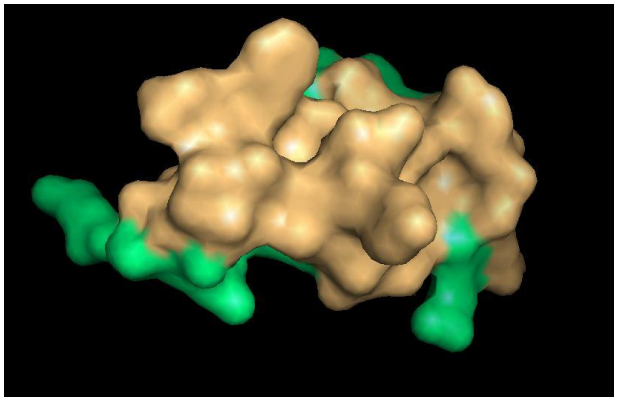
(b)

Fig. 3.8. Secondary structure of Himanturin. **(a)** Secondary structure of Himanturin created in PyMol software. Green colour denotes hydrophilic region, yellow colour denotes hydrophobic regions. Glycine and Proline which

cannot be assigned a proper status is denoted by red colour. **(b)** STRIDE analysis of secondary structure of Himanturin. The red regions represent α-helical arrangement while the yellow regions represent coils and turns.



View 1



View 2

Fig. 3.9. Tertiary Structure of Himanturin created in PyMol software. Green colour regions denote positively charged amino acids while brown coloured regions denote neutral ones.

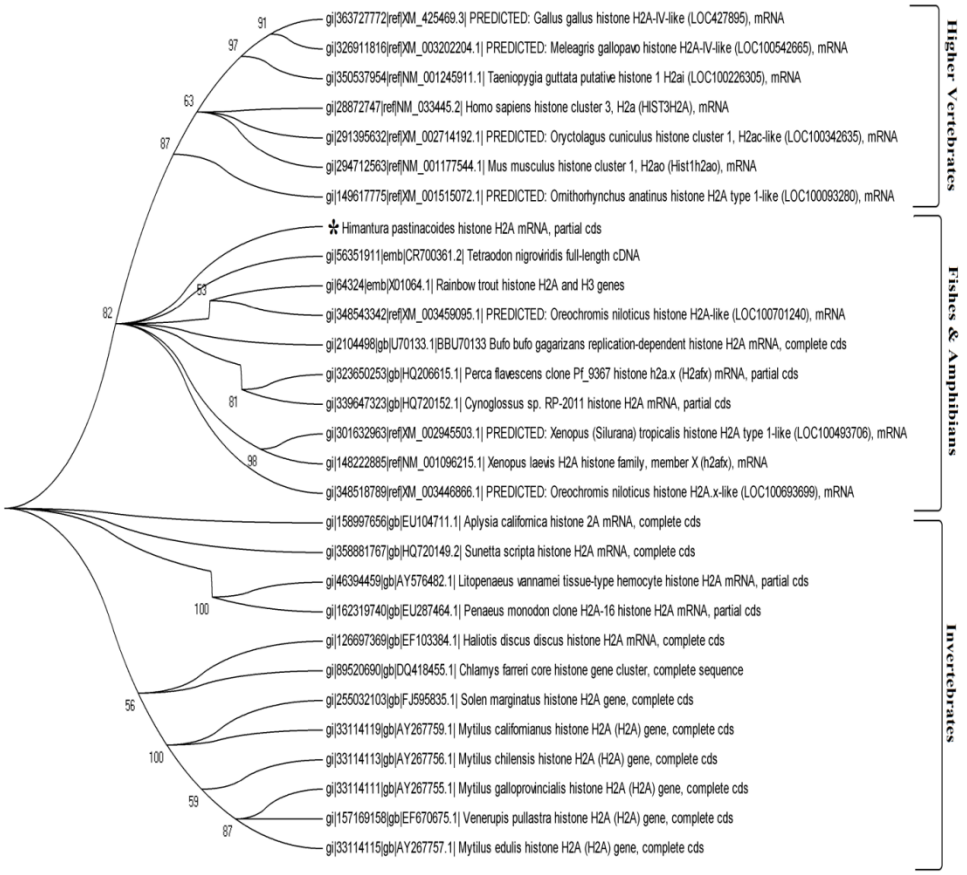


Fig. 3.10. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the nucleotide sequence of *H. pastinacoides* to the neucleotide sequences of previously reported histone H2A from different organisms. The nucleotide sequences of previously reported histone H2A were downloaded from NCBI site.

4

Molecular characterization and phylogenetic analysis of Histone derived Antimicrobial peptides (Harriottins) from Sicklefin Chimaera *Neoharriotta pinnata*

4.1 Introduction

The Sub-class Holocephali includes a group of cartilaginous fishes commonly called Chimaeras inhabiting deep seas from 200 m to more than 2000 m. Though cartilaginous fishes, they differ from Elasmobranchs in having smooth skin devoid of dermal denticles, a single gill opening (a gill cover is present over four gills), grinding tooth plates instead of teeth rows and an upper jaw fused to cranium. A true stomach and ribs are absent in these fishes. Chimeras have a very peculiar look characterized by disproportionately huge head, large eyes, a rabbit-like mouth, plate-like grinding teeth and a long and thin tail. Their movement is propelled by the sweeping action of large pectoral fins. Very little is known about the biology of Holocephalan fishes. These mysterious fishes have never been previously investigated with regard to antimicrobial peptides.

In the last few decades considerable number of gene coded AMPs, either inducible or constitutive, with broad spectrum activity against different types of pathogens, have been reported from wide range of organisms and their significance in innate immunity is becoming more and more appreciated. The specific immune mechanisms in the primeval vertebrates such as fish are less developed than those of higher vertebrates (Ellis, 1974; Manning, 1998) and are limited by temperature restraints on their metabolism (Bly & Clem, 1991). Therefore, fish rely highly on their innate immune mechanisms for protection against invading pathogens and this makes them a potential candidate for antimicrobial peptide research. N-terminus of histone H2A is rich in basic amino acids, a characteristic which allows histone H2A to act as a precursor for antimicrobial peptides (Li et al., 2007). As discussed in chapter 2 and 3, AMPs derived from the N-terminus domain of histone H2A have been reported from a number of marine fishes including catfish (Park et al., 1998), Atlantic Salmon (Richards et al. 2001), Atlantic Halibut (Birkemo et al., 2003) Rainbow Trout (Fernandes et al., 2002) and from teleost fishes *Tachysurus jella* and *Cynoglossus semifasciatus* (Chaithanya et al., 2013). Histone derived AMPs have also been reported from marine invertebrates including Pacific White Shrimp (Patat et al, 2004), Scallop (Li et al., 2007) and Abalone (De Zoysa et al., 2009).

Historically Holocephalan fishes have been poorly investigated scientifically and not much is known about their immune defenses. The role of antimicrobial peptides in the innate immune response of fishes belonging to the family Holocephali has never been studied in detail. The present study was carried out to get a deeper insight into the role of histone H2A derived AMPs in the immune response of Sicklefin Chimaera *Neoharriotta pinnata*. The evolutionary relationship of Holocephalan fishes to other organisms based on

the nucleotide sequence of cytochrome oxidase subunit 1and the divergence in molecular evolution of histone H2A in *N. pinnata* and related fishes have also been analysed.

4.2 Materials and Methods

4.2.1 Sample Collection

Live *N. pinnata* shown in Fig. 4.1 was caught from a depth of 500 m off Karaikkal Coast, Tamil Nadu (India) during Cruise No. 291 of Fisheries and Oceanography Research Vessel *Sagar Sampada* (Ministry of Earth Sciences, Govt. of India). High Speed Demersal Trawl (HSDT) net operated on-board was employed for capturing the species.

Neoharriotta pinnata belongs to Super Class: Pisces; Class: Chondrichthyes; Sub-class: Holocephali; Order: Chimaeriformes and Family: Rhinochimaeridae.

4.2.2 RNA Extraction

Blood was collected from the lamellar artery near gill region of the fish using specially designed capillary tubes (RNase free) rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7). Blood was homogenized in TRI® reagent (Sigma) and stored at -20^{o} C on-board in the Biological Laboratory facility of the research vessel. RNA was isolated from the blood preserved in TRI® reagent at the Molecular Biology Laboratory of Dept. of Marine Biology, Microbiology and Biochemistry. Homogenized blood of *N. pinnata* preserved in TRI® reagent was thawed at room temperature and total RNA was extracted and the quality and quantity was

determined. For detailed protocol of RNA extraction refer section 2.2.2 of Chapter 2.

4.2.3 cDNA Synthesis

Single stranded cDNA was reverse transcribed from good quality RNA isolated from the blood of Sicklefin Chimaera. See section 2.2.3 of Chapter 2 for details on cDNA synthesis.

4.2.4 PCR Amplification

Amplification of histone H2A sequence from cDNA of *N. pinnata* was done using Forward primer (5′-ATGTCCGGRMGMGGSAARAC-3′) and Reverse primer (5′-GGGATGATGCGMGTCTTCTTGTT-3′) (Birkemo et al., 2003). PCR amplification of 1 µl of cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (New England Biolabs). The thermal profile used was an initial denaturation at 94^o C for 2 minutes followed by 35 cycles of 94^o C for 15 seconds, 60^o C for 30 seconds and 68^o C for 30 seconds and a final extension at 68^o C for 10 minutes. PCR products were analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with SYBR® Safe and visualized under UV light.

4.2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was crried out using 10 µl of the PCR product. The gel was analyzed in a UV transilluminator. For details refer section 2.2.5 of Chapter 2.

4.2.6 Sequencing

Sequencing of purified PCR product was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, Kakkanadu, Kochi, India.

4.2.7 Sequence Analysis and Molecular Characterization

The nucleotide sequence was analyzed and assembled using GeneTool software. Deduced amino acid sequence was obtained from nucleotide sequence and then BLAST analysis of both nucleotide and deduced amino acid sequences were carried out. Further analysis of the amino acid sequence was performed using various computer based programs as explained in section 2.2.7 of Chapter 2.

4.2.8 Taxonomic Identification Using Molecular Tools

DNA was isolated from 10 gm of the gill tissue of *N. pinnata* using TRI® reagent. The cytochrome oxidase subunit I (CO1) gene was amplified using LCO and HCO primers (Miller et al., 1998). The PCR products were analyzed on 1.5 % agarose gel. Amplicons obtained were sequenced at SciGenom Sequencing Facility, India. Homology search of the nucleotide sequence was performed using BLASTn suite of National Center for Biotechnology Information. For details on taxonomic identification using molecular tool refer section 3.2.9 of Chapter 3.

4.2.9 Phylogenetic Analysis

Histone H2A amino acid sequences previously reported from various organisms were retrieved from NCBI database and multi aligned using

ClustalW and GeneDoc computer programmes. Similarly nucleotide sequences of CO1 gene of organisms representing various classes were downloaded from NCBI database and multi aligned using ClustalW and GeneDoc computer programmes. Phylogenetic tree was constructed by the Neighbour-Joining (NJ) method based on amino acid sequence of histone H2A and nucleotide sequence of cytochrome oxidase subunit I, using MEGA version 5.05. Confidence in estimated relationships of NJ tree topologies of both Histone H2A and CO1 genes were evaluated by a bootstrap analysis with 100 and 1,000 replicates with MEGA version 5.05. Kimura 2 parameter (K2P) model was used to construct NJ and ML tree for CO1 genes.

4.3 Results

4.3.1 Taxonomic Identification Based on CO1 gene

The LCO and HCO primers amplified a 710 bp region of the mitochondrial cytochrome oxidase subunit I gene (Fig. 4.2). The nucleotide sequence of the amplified region of CO1 gene is shown in Fig. 4.3. BLAST analysis of nucleotide sequences confirmed the identity of the organism as *N. pinnata* showing 99 % similarity to GenBank ID: HM239670.1 *Neoharriotta pinnata*. The nucleotide sequence was submitted to GenBank Database (GenBank ID: **JX297203**). The genus *Neoharriotta* belongs to Class Chondrichthyes, Sub-Class: Holocephali, Order: Chimaeriformes and Family: Rhinochimaeridae.

4.3.2 Identification and Molecular Characterization of Histone H2A Sequence

Reverse Transcription-PCR amplification of the mRNA from Sicklefin Chimaera yielded a 243 bp fragment cDNA encoding 81 amino acid residues (Fig. 4.4). The nucleotide sequence and the deduced amino acid sequence of histone H2A amplified from Sicklefin Chimaera are presented in Fig. 4.5. The nucleotide and deduced amino acid sequences were deposited in GenBank database (GenBank ID: **JX297204**). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptides belonged to histone H2A family.

Analysis of functional aspects and chemical properties of the histone H2A protein were carried out using reliable computer based programs. The PeptideCutter tool predicts proteolytic enzymes, chymotrypsin and pepsin to have a potential cleavage site at position 52 and 40 from N-terminus of histone H2A of Sicklefin Chimaera. Cleaving the protein at position 52 would release Harriottin-1, a peptide sharing similarity with Hipposin. Proteolytic activity of these enzymes at position 40 would result in the release of a peptide termed as Harriottin-2 which is similar to Buforin I reported from toad. Enzyme trypsin was found to have potential cleavage sites at position 16 and 37 from the N-terminus. Trypsin mediated processing of *N. pinnata* histone H2A would result in the formation of a 21 mer peptide, Harriottin-3 having a sequence resembling Buforin II. Diagrammatic representation of the cleavage site of enzymes and release of the three Harriottins is presented in Fig 4.6.

Sequence analysis of the peptides was carried out using ProtParam software which predicted Harriottin-1, 2 and 3 to have molecular weights of 5.56 kDa, 4.39 kDa and 2.44 kDa respectively and a theoretical isoelectric

point (p*I*) of 12.01, 12.41 and 12.60 respectively. All three Harriottins were found to have an overall net positive charge. At pH 4, 7 and 10, Harriottin-1 was found to have a charge of 14.8, 12.4 and 7.4; Harriottin-2 had a net positive charge of 14.1, 12.4 and 7.9 while Harriottin-3 displayed a net positive charge of 7.1, 5.4 and 4. Hydrophobicity of Harriottin-1, 2 and 3 were found to be +52.17 kcal/mol (30 %), +45.66 kcal/mol (25 %) and +24.37 kcal/mol (28 %) as predicted by PepDraw.

Multiple-sequence alignment of the amino acid sequences of Harriottin-1 with previously reported histone H2A derived AMPs revealed that the peptide showed similarity to previously reported histone H2A derived AMPs like Buforin I, Buforin II, Hipposin, Teleostin, Abhisin, and histone H2A derived AMPs reported from *Oncorhynchus mykiss*, *Litopenaeus vannamei* and *Chlamys farreri.* Sunettin, Molluskin and Himanturin reported in this study (Chapters 2 and 3) are also included (Fig. 4.7). Only Harriottin-1 was considered for multiple-sequence alignment as it covers sequences of both Harriottin-2 and Harriottin-3.

Analysis of Harriottins using Protean module of the DNASTAR Lasergene sequence analysis software suite revealed that Harriottin-1 and 2 will have a concentration of 1.87 mg/ml and 2.95 mg/ml for an absorbance of 1 OD measured at 280 nm, whereas, Harriottin-3 will not give any reading at 280 nm wavelength as it lacks Thr, Cys and Trp. The module further predicts that 1 µg of the Harriottin-1, 2 and 3 would contain 179.75 pmoles, 227.63 pmoles and 409.03 pmoles respectively.

Schiffer-Edmundson helical wheel modeling of the peptides using Protean module revealed a perfect amphipathic nature for Hariottin-1 to 3 (Fig. 4.8). Hydrophilic residues such as serine (S), lysine (K), histidine (H),

arginine (R), asparagine (N), glutamine (Q) and hydrophobic residues such as valine (V), tyrosine (Y), leucine (L), isoleucine (I), methionine (M) were found to occupy positions opposite to each other in the helical wheel. Such type of arrangement for hydrophobic and hydrophilic amino acid also confirms the helical nature of the peptides.

The secondary structure analysis of Harriottin-1 to 3 was carried out using secondary structure assignment program, STRIDE. Harriottin-1 was found to contain 43.14 % α-helical region (22 amino acid residues), 15.68 % turns (8 amino acid residues) and 41.18 % coils (21 amino acid residues). The STRIDE programme had excluded the initial amino acid Met and hence Met was not taken into consideration for calculating the percentage of structural characteristics of the peptide. The α-helical arrangement is present in Harriottin-1 from Arg 18 to Ala 22, Val 28 to Gly 38 and Ala 46 to Tyr 51. Harriottin-1 exhibits turns from Ser 2 to Gly 5 and Ala 41 to Val 44. Coils are present from Lys 6 to Ser 17 and from Gly 23 to Pro 27. Asn 39, Tyr 40, Gly 45 and Leu 52 are arranged as coils (Fig. 4.9). In case of Harriottin-2, the structural arrangement was found to contain 41.03 % α-helical region (16 amino acid residues), 10.25 % turns (4 amino acid residues) and 48.72 % coils (19 amino acid residues). Here also initial amino acid Met was not taken into consideration for calculating the percentage of structural regions as STRIDE programme had excluded it. The α-helical arrangement is present in Harriottin-1 from Arg 18 to Ala 22 and from Val 28 to Gly 38. Harriottin-2 exhibits turns from Ser 2 to Gly 5. Coils are present from Lys 6 to Ser 17 and from Gly 23 to Pro 27. Asn 39 and Tyr 40 are also coils (Fig. 4.10). The smallest of the three peptides, Harriottin-3 has 66.67 % α-helical region (14 amino acid residues) and 33.33 % coils (7 amino acid residues). However, turns are absent in Harriottin-3 (Fig. 4.11). No β-sheet region has been noticed in the secondary

structure of any of the three Harriottins (Fig. 4.9 to 4.11). The predicted tertiary structure of Hariottin-1 (Fig. 4.12), Harriottin-2 (Fig. 4.13) and Harriottin-3 (Fig. 4.14) were created in PyMol software.

Analysis of Harriottins for their antimicrobial activity was carried out with Antimicrobial Peptide Predictor Program (http://aps.unmc.edu/AP /main.php) which predicts them to be Antimicrobial peptides with a protein binding potential of 2.58 kcal/mol, 3.26 kcal/mol and 3.83 kcal/mol for Harriottin-1, 2 and 3 respectively.

4.3.3 Phylogenetic Analysis

4.3.3.1 Based on CO1 Gene

Phylogenetic relationship of *N. pinnata* to other organisms was established based on the nucleotide sequence comparisons of CO1. NJ tree represented in Fig 4.15 gets broadly divided into six clusters. Cluster one include mammals, cluster two includes three sub-clusters representing birds, teleost fishes and frogs, cluster three includes two sub-clusters of cartilaginous fishes, one representing sharks and the other representing skates and rays, cluster four include fishes belonging to Holocephali group, cluster five represent crustaceans and cluster six include molluscs. *N. pinnata* though closely related to Holocephalan fishes occupy a position in between the vertebrate and invertebrate groups.

4.3.3.2 Based on Histone H2A Gene

Bootstrap distance tree calculated using deduced amino acid sequence confirmed its similarity with previously reported histone H2A sequences deposited in Genbank database. Bootstrap distance tree was calculated using

NJ method. Phylogenetic relationship of histone H2A of *N. pinnata* to histone H2A of other organisms was found to be virtually identical in both NJ tree and ML tree. The phylogenetic distance tree based on amino acid sequence of histone H2A is represented in Fig 4.16. The phylogenetic tree gets divided into two main clusters. Cluster one denotes histone H2A sequences of vertebrates and cluster two represent that of invertebrates. The vertebrate group could be classified into three sub-clusters representing mammals, amphibians and fishes while invertebrate group could be classified in mollusc and crustacean sub-clusters. Birds, when included for construction of phylogenetic tree, grouped with both mammals and fishes (Fig. 4.17). In case of histone H2A also, *N. pinnata* was found to occupy a position in between the vertebrate and invertebrate clusters, though more closely related to vertebrates than invertebrates.

ClustalW Multiple alignment of Histone H2A protein of *N. pinnatta* with Histone H2A protein of other organisms (downloaded from NCBI database) was performed (Fig. 4.18). A few peculiarities were noted in Histone H2A protein of *N. pinnatta.* Histone H2A protein of *N. pinnatta* differs from other reported histone H2A proteins in having amino acid His at position 34 and Asp at position 42 from the N-terminus. In all other previously reported sequence of histone H2A, Lys and Glu is present at the corresponding position of His and Asp. Histone H2A protein of *N. pinnatta* has Val at position 31 and Thr at position 60 from the N terminus. Histone H2A reported in vertebrates are similar to *N. pinnatta* in having Val and Thr at corresponding positions, but histone H2A reported from invertebrates have Ile and Ala at corresponding. *N. pinnatta* has Val at position 63, a feature found in invertebrates as all histone H2A reported from them have Val at corresponding position. The scenario is different in case of all other vertebrates as they have

Ile at corresponding position. Histone H2A of *N. pinnatta* display similarities and dissimilarities with both vertebrates and invertebrates and thereby occupies a position in between the two.

4.4 Discussion

The present study describes characterization of Harriottin-1, 2 and 3, with 52, 40 and 21 amino acid peptides identified from histone H2A of *N. pinnata* and phylogenetic analysis of the organism based on CO1 and histone H2A. Replication-independent histone H2A having an mRNA with a poly (A) tail was amplified for the study using oligo (dT) Primers.

4.4.1 Analysis of Harriottins

All histone H2A derived AMPs reported to date from various sources are derived from N-terminal region of Histone H2A, thereby suggesting its importance in innate immune response of an organism. Histone H2A fragments with antimicrobial activity reported from vertebrates and invertebrates clearly indicate the role of histone H2A as a potential precursor for highly potent antimicrobial peptides. In Asian Toad *Bufo bufo gargarizans*, the intact histone H2A protein is secreted into the stomach and Buforin I is produced by the action of pepsin isozymes cleaving the Try 39 – Ala 40 bond of intact protein (Kim et al., 2000). Similarly in Cat Fish (*Parasilurus asotus*), parasin I is produced by cleavage of Ser 19-Arg 20 bond of histone H2A by cathepsin D found in skin mucus of the fish (Cho et al., 2002). PeptideCutter tool predicts proteolytic enzymes, trypsin, chymotrypsin and pepsin to have potential cleavage sites in the histone H2A of Sicklefin Chimaera which presented the possibility of formation of three fragments similar to previously

reported histone H2A derived AMPs. A 52 mer fraction similar to hipposin was termed as 'Harriottin-1'; a 40 mer fraction resembling Buforin I was termed as 'Harriottin-2' and a third 21 mer fractions comparable to Buforin II was given the name 'Harriottin-3'.

Harriottins exhibited high sequence similarity with previously reported histone H2A derived AMPs. Harriottins are highly cationic peptides with amphipathic nature and α-helical structure, characteristic to all histone H2A derived AMPs. Harriottin-1, 2 and 3 were found to be rich in arginine (15, 18 and 23 %), glycine (17, 18 and 10 %), serine (8, 10 and 14 %), valine (10, 8, and 10 %) alanine (11, 8 and 5 %) as reported in all other histone H2A derived AMPs.

Histone H2A derived antimicrobial peptides are known to exhibit broad spectrum activity against bacteria and fungi. Hipposin and Buforins are the most studied histone H2A derived antimicrobial peptides. Hipposin exhibited strong antibacterial activity against several Gram positive and Gram negative bacteria and activity could be detected down to a concentration of 1.6 µg/ml (Birkemo et al., 2003). Harriottin-1 has a sequence and structure similar to Hipposin and therefore would have a similar activity. Buforins are among one of the most potent antimicrobial peptides. In addition to their broad spectrum activity against bacteria and fungi (Park et al., 1996), they also possess anti-endotoxic and anticancer activities (Cho et al., 2009). Harriottin-2 and 3 would be expected to match the activity of Buforin I and II respectively, by virtue of their sequence and structure. Buforin II does not cause significant membrane permeabilization (Kobayashi et al., 2000) but brings about the lysis of bacterial cells by readily entering the cells in vivo and interacting with intracellular nucleic acids (Park et al., 2000; Uyterhoeven et al., 2008). NMR structural studies showed that proline at position 11 serves as a hinge between

a C-terminus helix and an N-terminal region with an extended helical structure (Yi et al., 1996).

This sole proline residue (Pro_{11}) of Buforin II is necessary for effective translocation across cell membrane (Kobayashi et al., 2000; Park et al., 2000). Presence of proline at position 11 and the resulting proline hinge detected as in Buforin II was found to be characteristic feature of Harriottin-3. Presence of proline hinge seems to indicate that the antimicrobial activity of Harriottin-3 lies in its ability to interact with nucleic acid rather than membrane permeabilization.

Antimicrobial peptides are also viewed as agents with therapeutic potential against cancer cells (Hoskin & Ramamoorthy, 2008). Buforin II exhibit selective cytotoxicity against cancer cells through interaction with cell surface gangliosides and once inside the cell they induce mitochondria-dependent apoptosis (Lee et al., 2008). Buforin II does not exhibit cytotoxic activity of any kind against normal mammalian cells (Takeshima et al., 2003). Having a structure similar to Buforin II makes Harriottin-3 potential candidates for anticancer research. Antimicrobial Peptide Predictor Program (http://aps.unmc.edu/AP/main.php) predicted Harriottins to be AMPs since Harriottin-1, 2 and 3 form alpha helices and possess 6, 4 and 4 residues respectively on the same hydrophobic surface which assist them to interact with membranes. Harriottin 1, 2 and 3 illustrate all the characteristic features of AMPs including high cationicity, higher hydrophobic residue and elevated protein binding potential, i.e., 2.58, 3.26 and 3.83 kcal/mol respectively.

4.4.2 Phylogenetic analysis based on CO1 gene

One of the commonly used molecular markers for taxonomic identification of a species is the CO1 gene, comparison of which provides a

reliable determination of the phylogenetic relationship of a species indicating its position in the evolutionary tree. The phylogenetic relationship of *N. pinnatta* based on nucleotide sequence of CO1 gene was analyzed by NJ method and ML method. As expected *N. pinnatta* was found to be closely related to Holocephalan fishes but was found to occupy a position between vertebrates and invertebrates. Order Chimaeriformes to which chimaeras belong include three families; Chimaeridae, Callorhinchidae and Rhinochimaeridae. Results of the phylogenetic analysis based on CO1 gene indicate that Rhinochimaeridae represented by *N. pinnatta* appears to be more primitive of the three.

4.4.3 Phylogenetic Analysis Based on Histone H2A

The phylogenetic relationship of histone H2A amino acid sequence of *N. pinnatta* to the amino acid sequence of previously reported histone H2A proteins from various organisms was carried out using NJ method and ML method. The molecular phylogenetic tree based on amino acid sequences of previously reported histone H2A derived AMPs demonstrates that the members of the family are derived from a common ancestor by a series of evolutionary changes. Selected H2A derived antimicrobial peptide sequences got divided into two major groups, i.e., vertebrates and invertebrates. The boot strap distance tree calculated reveals that histone H2A protein of *N. pinnatta* can align with the vertebrate group but the lineage is distant enough to conclude that it occupies a position between vertebrate and invertebrate groups. Birds when included in the phylogenetic tree did not form a group of their own and was found to align with fishes and mammals. Evolution of histone H2A is not clearly demarcated in birds and this indicates that the histone H2A has a highly conserved sequence. Even though the rate of evolution is slow in histone H2A, well-marked differences can be observed in

case of *N. pinnata*. Histone H2A protein of *N. pinnatta* diverges from other reported histone H2A proteins at amino acid position 34 and 42 from the N-terminus where it has His and Asp respectively. Lys and Glu is present at the corresponding position of His and Asp in all other reported sequences of histone H2A. Since Lys and His as well as Asp and Glu belong to the same characteristic group in amino acid classification, their interchange would not have much effect on the property of the protein. The region of histone H2A corresponding to amino acid position 16 to 51 in histone H2A of *N. pinnatta* is a highly conserved region in animal kingdom. *N. pinnatta* differ from histone H2A of other organisms in this region at two positions (position 34 and 42) which clearly indicates that Sicklefin Chimaera has followed a different path of evolution. *N. pinnatta* represent Holocephalan fishes which are believed to be branched off from their sister group of sharks and rays and have remained isolated ever since. This is quite evident from the results of phylogenetic analysis based on histone H2A amino acid sequence of *N. pinnatta* which show similarity to both vertebrates and invertebrates and at the same time differ from both of them. Since histone H2A sequences of cartilaginous fishes are scarce in GenBank database a detailed investigation was not possible. Histone genes represent much conserved regions and therefore evolutionary analyses of histones should provide important information with regard to the phylogenetic relationships of distant/ closely related organisms.

Conclusion

Three peptides containing antimicrobial sequence motif from the histone H2A of *N. pinnatta* were identified and named as Harriottin-1, 2 and 3. High similarity of Harriottins to potent histone H2A derived AMPs with proven antimicrobial activity, their physicochemical properties and molecular

structure which are in agreement with those of traditional antimicrobial peptides strongly endorse them to be antimicrobial peptides. The study was taken up as an initiator to investigate the role of histone derived AMPs in Holocephalan fishes and more research in this area would reveal new facets of innate immunity in this less understood group of fishes. The study gives a comparative account of CO1 and H2A nucleotide sequences in the molecular taxonomic identification of members of the animal kingdom. Birds get grouped both with fishes and mammals; but not with amphibians, which is really intriguing. The study also offers an insight into the evolutionary divergence of *N. pinnatta* with respect to CO1 gene andhistone H2A occupying an intermediate position with respect to invertebrates and invertebrates.

Fig. 4.1. *N. pinnatta* caught during Cruise No. 291 of FORV *Sagar Sampada*.

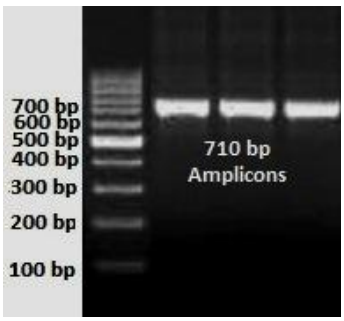


Fig. 4.2. Amplicons of size 710 bp obtained by PCR amplification of CO1 gene of *N. pinnatta.*

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GATGATCAAATTTATAATGTTATCGTGACTGCACATGCTTTCGTAATAATTTTCTTT
ATAGTAATACCAATTATAATCGGCGGATTTGGAAATTGACTTGTTCCCTTAATAATC
GGTGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTCTGACTACTTCCT
CCTTCTTTTCTTCTTCTCCTAGCTTCAGCAGGAGTAGAAGCAGGAGCAGGAACCGGC
TGAACTGTTTACCCCCCTTTAGCAAGTAATTTAGCCCACGCTGGAGCATCTGTTGAT
TTAGCTATTTTTTCTCTTCATTTAGCAGGTGCTTCTTCAATTCTAGCTTCTATTAAT
TTTATTACTACAATTATTAATATAAAACCACCATCAATCACCCAATATCAGACTCCT
TTATTTGTCTGATCTATTTTAATTACTACAGTCTTACTTCTCCTATCTTTACCTGTC
TTAGCTGCTGGTATCACGATACTACTCACAGACCGTAATCTTAATACAACTTTTTTT
GACCCCGCAGGAGGAGGAGATCCTATTTTATATCAACATTTATTTTGATTTTTT

Fig. 4.3. Nucleotide sequence of CO1 gene amplified from *N. pinnatta*

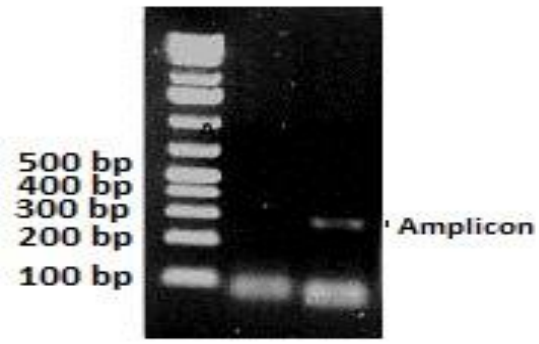


Fig. 4.4. Amplicon of size 243 bp obtained from cDNA of *N. pinnatta* using histone H2A specific primer.

```
atgtccggacgagggaagactggcggtaaagtccgagccaaggccaagtcccgctcctcc
 M  S  G  R  G  K  T  G  G  K  V  R  A  K  A  K  S  R  S  S
cgggccgggctgcagttccccgtgggccgcgtccacaggcatctccggaaaggcaactat
 R  A  G  L  Q  F  P  V  G  R  V  H  R  H  L  R  K  G  N  Y
gctgatcgcgtgggtgccggagccccggtctatctggccgccgtgctcgagtatctgacg
 A  D  R  V  G  A  G  A  P  V  Y  L  A  A  V  L  E  Y  L  T
gctgaggtgctggagctggcggggaacgccgcccgcgacaacaagaagacgcgcatcatc
 A  E  V  L  E  L  A  G  N  A  A  R  D  N  K  K  T  R  I  I
ccc
 P
```

Fig. 4.5. Nucleotide and deduced amino acid sequences of histone H2A amplified from *N. pinnatta.*

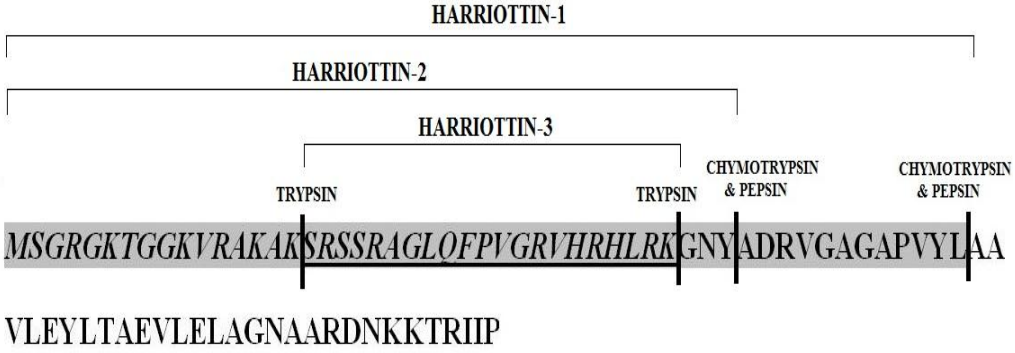


Fig. 4.6. Diagrammatic representation of potential cleavage site of proteolytic enzymes resulting in formation of Harriottin 1, 2 and 3. The first 52 amino acid fragment from N-terminus (from Met 1 to Leu 52) form Harriottin-1; 40 amino acid fragment from Met 1 to Tyr 40 form Harriottin-2 and 21 amino acid fragment from Ser 17 to Lys 37

Fig. 4.7. Clustal W Multiple-sequence alignment of the amino acid sequences of Harriottin-1 with previously reported histone H2A derived AMPs like Buforin I, Buforin II, Hipposin, Himanturin, Teleostin, Abhisin, Sunettin and histone H2A derived AMPs reported from *Oncorhynchus mykiss*, *Litopenaeus vannamei* and *Chlamys farreri*.

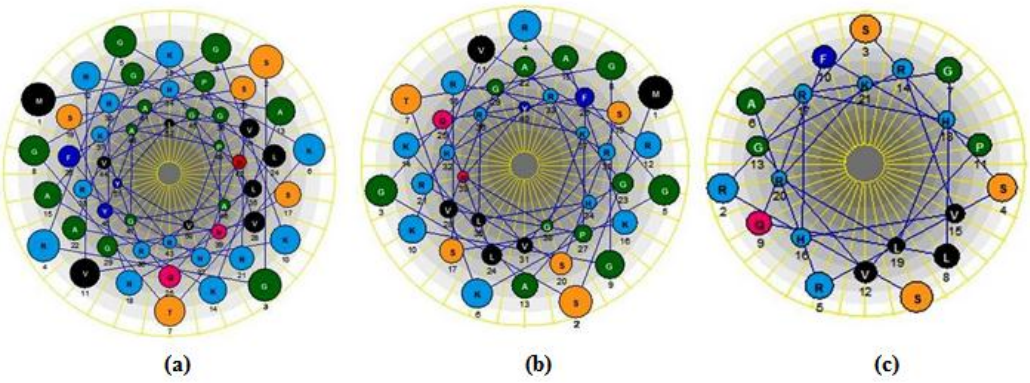
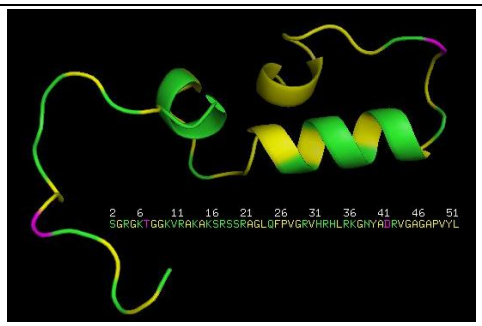


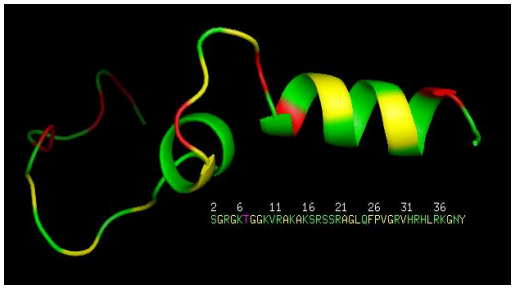
Fig. 4.8. Schiffer-Edmundson helical wheel diagram confirming the amphipathic and α-helical nature of (a) Harriottin-1, (b) Harriottin-2 and (c) Harriottin-3. Hydrophilic and hydrophobic amino acids are seen occupying opposite positions.



(a)

(b)

Fig. 4.9. Secondary structure of Harriottin-1. **(a)** Secondary structure of Harriottin-1 created in PyMol software. Green colour denotes hydrophilic region, yellow colour denotes hydrophobic regions. Glycine and Proline which cannot be assigned a proper status is denoted by red colour. **(b)** STRIDE analysis of secondary structure of Harriottin-1. The red regions represent α-helical arrangement while the yellow regions represent coils and turns.



(a)

(b)

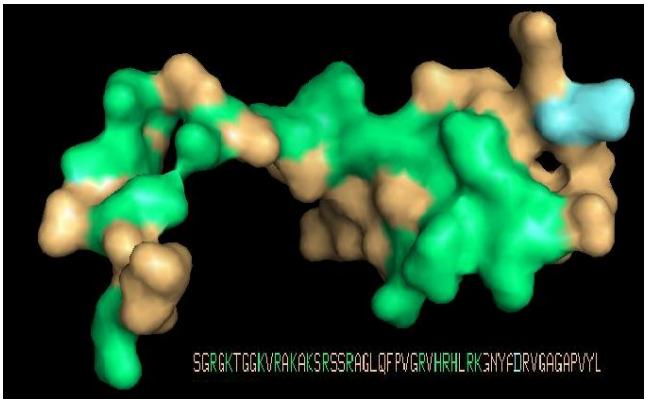
Fig. 4.10. Secondary structure of Harriottin-2. **(a)** Secondary structure of Harriottin-2 created in PyMol software. Green colour denotes hydrophilic

region, yellow colour denotes hydrophobic regions. Glycine and Proline which cannot be assigned a proper status is denoted by red colour. **(b)** STRIDE analysis of secondary structure of Harriottin-2. The red regions represent α-helical arrangement while the yellow regions represent coils and turns.

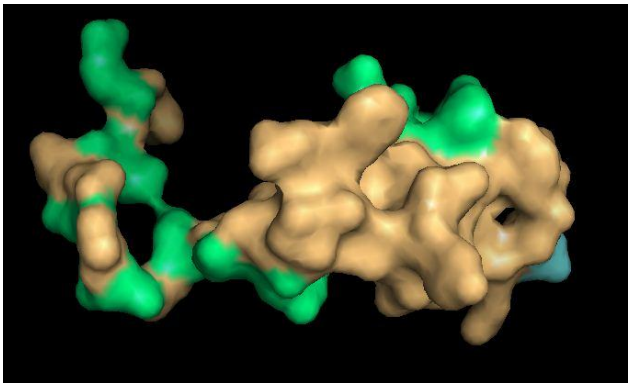
(a)

(b)

Fig. 4.11. Secondary structure of Harriottin-3. **(a)** Secondary structure of Harriottin-3 created in PyMol software. Green colour denotes hydrophilic region, yellow colour denotes hydrophobic regions. Glycine and Proline which cannot be assigned a proper status is denoted by red colour. **(b)** STRIDE analysis of secondary structure of Harriottin-3. The red regions represent α-helical arrangement while the yellow regions represent coils and turns.

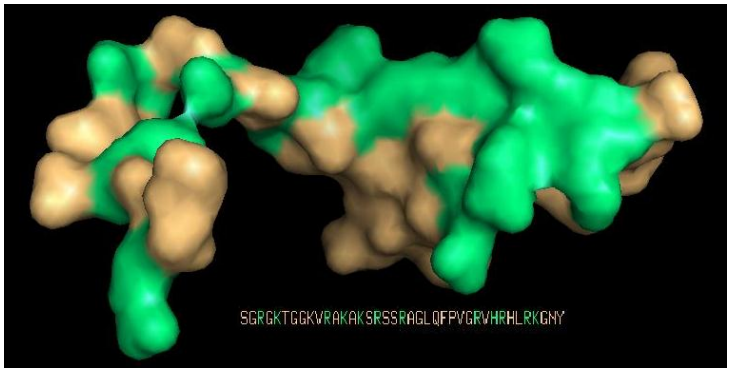


View 1

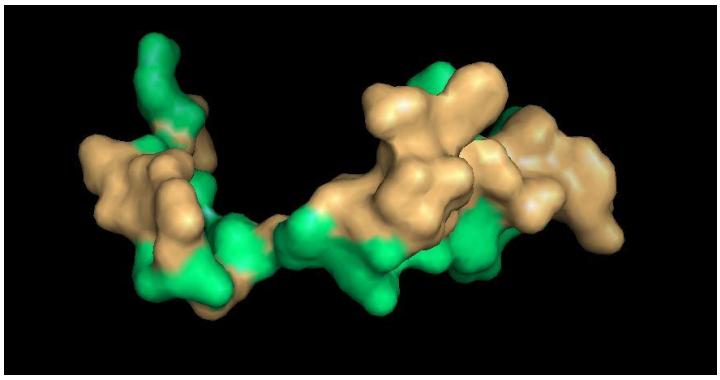


View 2

Fig. 4.12. Tertiary Structure of Harriottin-1 created in PyMol software. Green colour regions denote positively charged amino acids; blue coloured region denotes negatively charged amino acids while brown coloured regions denote neutral ones.

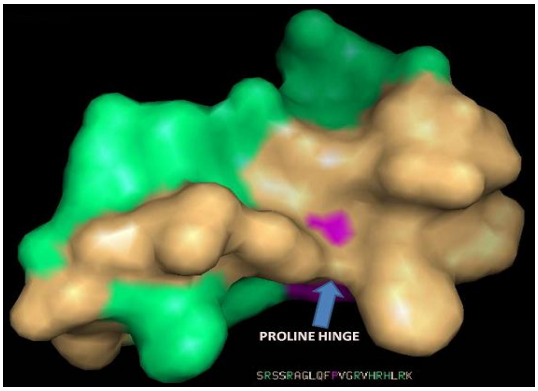


View 1

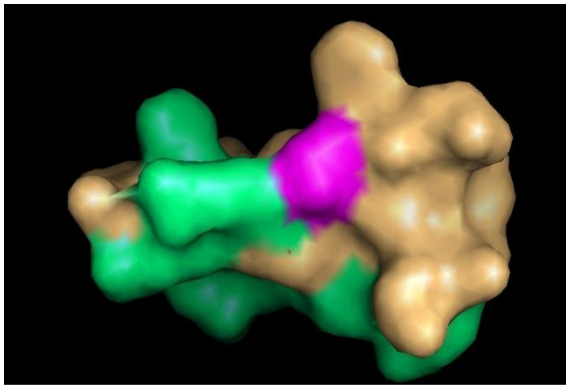


View 2

Fig. 4.13. Tertiary Structure of Harriottin-2 created in PyMol software. Green colour regions denote positively charged amino acids while brown coloured regions denote neutral ones.

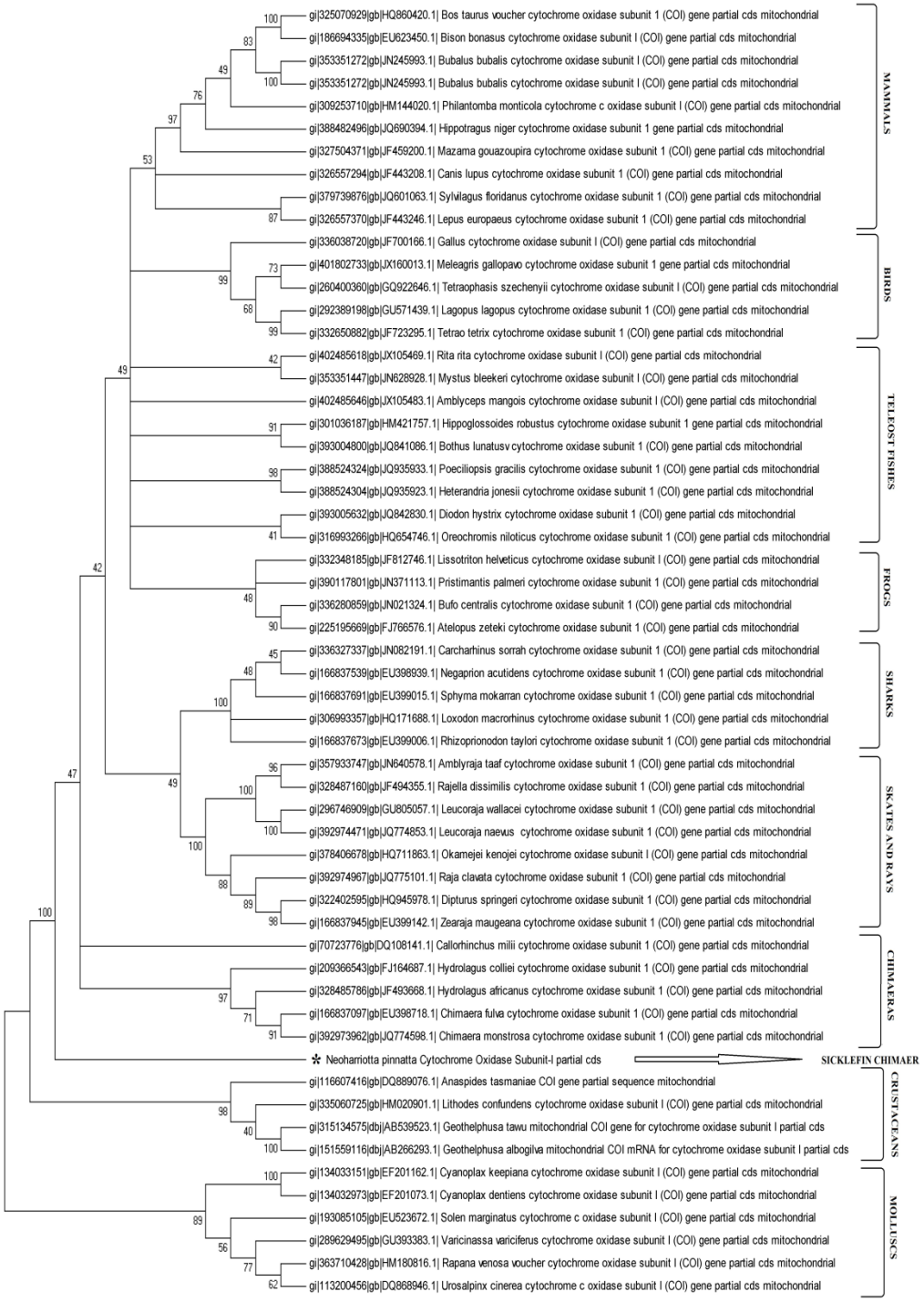


View 1



View 2

Fig. 4.14. Tertiary Structure of Harriottin-3 created in PyMol software. Green colour regions denote positively charged amino acids while brown coloured regions denote neutral ones. Proline is show in red colour and the hinge formed by the presence of proline is labeled.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gi|353351272|gb|JN245993.1| Bubalus bubalis cytochrome oxidase subunit I (COI) gene partial cds mitochondrial
gi|309253710|gb|HM144020.1| Philantomba monticola cytochrome c oxidase subunit I (COI) gene partial cds mitochondrial
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gi|327504371|gb|JF459200.1| Mazama gouazoupira cytochrome oxidase subunit 1 (COI) gene partial cds mitochondrial
gi|326557294|gb|JF443208.1| Canis lupus cytochrome oxidase subunit 1 (COI) gene partial cds mitochondrial
gi|379739876|gb|JQ601063.1| Sylvilagus floridanus cytochrome oxidase subunit 1 (COI) gene partial cds mitochondrial
gi|326557370|gb|JF443246.1| Lepus europaeus cytochrome oxidase subunit 1 (COI) gene partial cds mitochondrial
MAMMALS
gi|336038720|gb|JF700166.1| Gallus cytochrome oxidase subunit I (COI) gene partial cds mitochondrial
gi|401802733|gb|JX160013.1| Meleagris gallopavo cytochrome oxidase subunit 1 gene partial cds mitochondrial
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gi|332650882|gb|JF723295.1| Tetrao tetrix cytochrome oxidase subunit 1 (COI) gene partial cds mitochondrial
BIRDS
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gi|353351447|gb|JN628928.1| Mystus bleekeri cytochrome oxidase subunit I (COI) gene partial cds mitochondrial
gi|402485646|gb|JX105483.1| Amblyceps mangois cytochrome oxidase subunit I (COI) gene partial cds mitochondrial
gi|301036187|gb|HM421757.1| Hippoglossoides robustus cytochrome oxidase subunit 1 gene partial cds mitochondrial
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TELEOST FISHES
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FROGS
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SHARKS
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CHIMAERAS
Neoharriotta pinnatta Cytochrome Oxidase Subunit-I partial cds
SICKLEFIN CHIMAER
gi|116607416|gb|DQ889076.1| Anaspides tasmaniae COI gene partial sequence mitochondrial
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CRUSTACEANS
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gi|113200456|gb|DQ868946.1| Urosalpinx cinerea cytochrome c oxidase subunit I (COI) gene partial cds mitochondrial
MOLLUSCS

Fig. 4.15. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating the phylogenetic relationship of N. *pinnatta* based on the nucleotide sequence of cytochrome oxidase subunit-1 gene to CO1 gene sequences of other organisms downloaded from NCBI site.

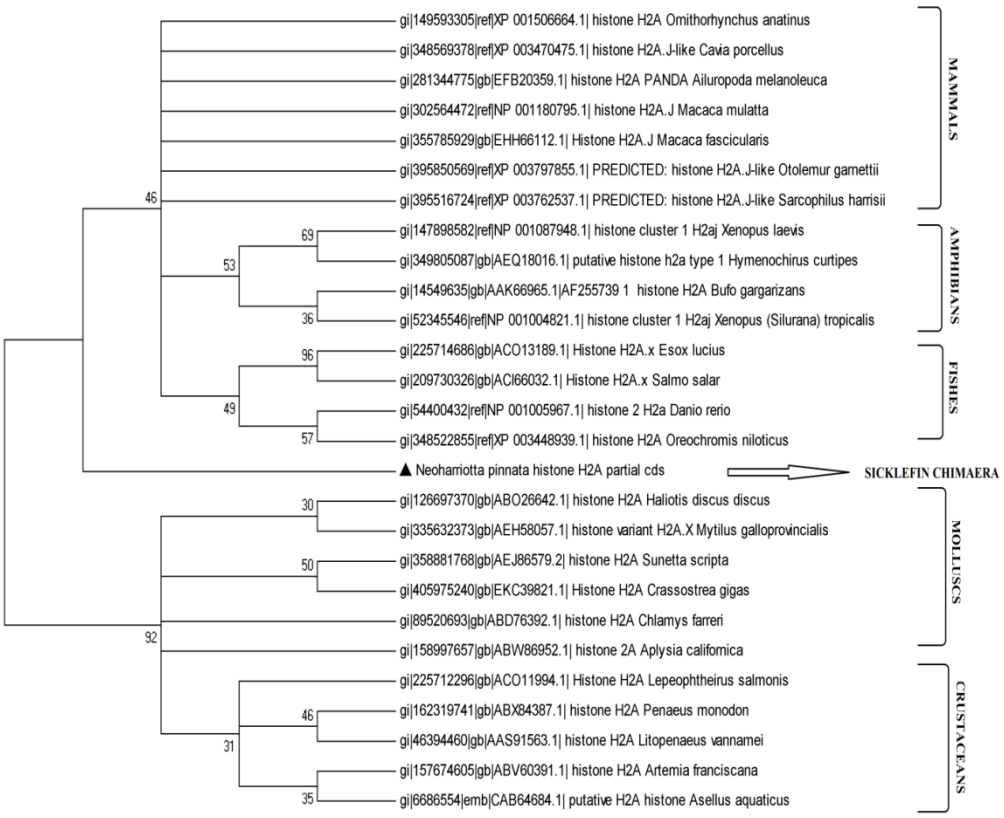


Fig. 4.16. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the amino acid sequence of histone H2A of *N. pinnatta* to the amino acid sequences of previously reported histone H2A from different organisms. Previous records of histone H2A sequences were downloaded from NCBI database.

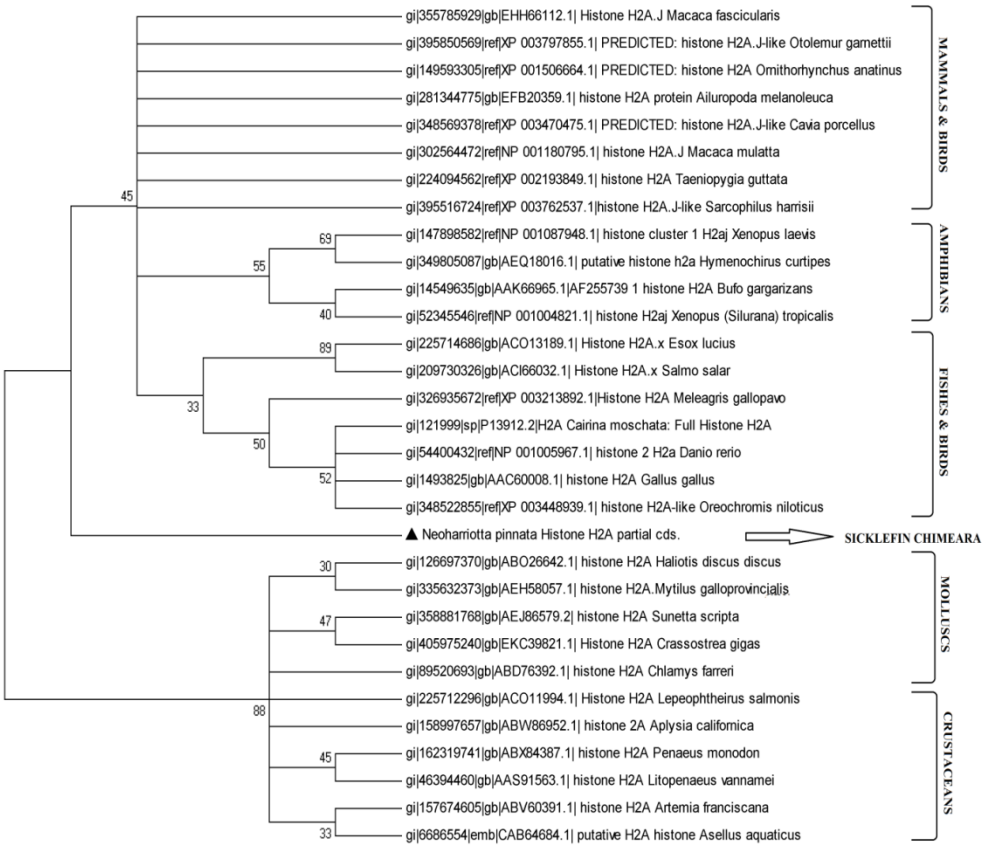


Fig. 4.17. A bootstrapped neighbor-joining tree obtained (including histone H2A sequences of birds) using MEGA version 5.05 illustrating relationships between the amino acid sequence of histone H2A of *N. pinnatta* to the amino acid sequences of previously reported histone H2A from invertebrtaes, fishes, amphibians, birds and mammals downloaded from NCBI database.

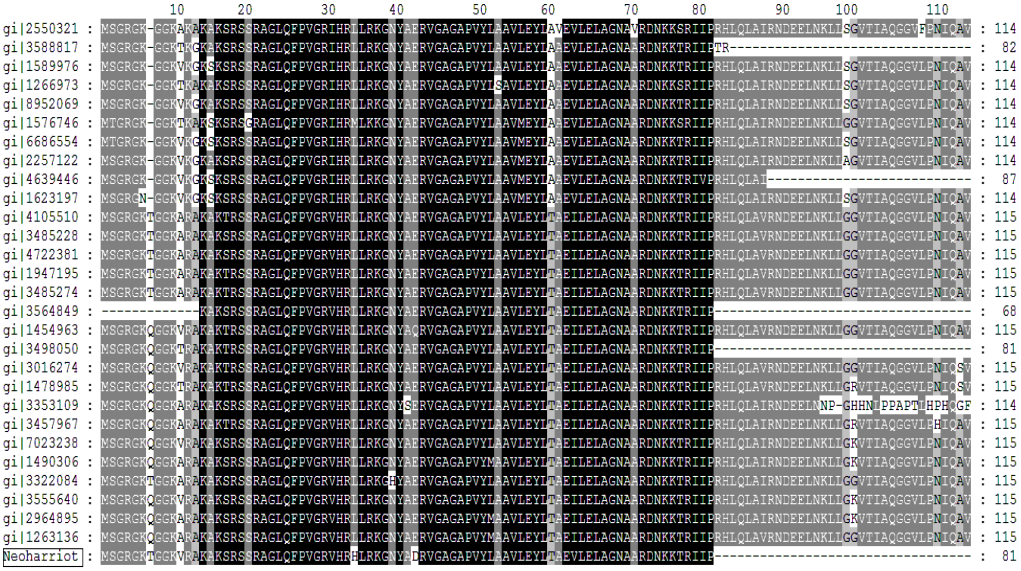


Fig. 4.18. ClustalW multiple alignment of amino acid sequence of histone H2A of *N. pinnatta* to the amino acid sequence of histone H2A previously reported from various organisms.

5

Recombinant Expression of Harriottin-1 and 2 and Testing its Antibacterial Activity.

5.1 Introduction

Antimicrobial peptides are fast emerging as one of the most attractive new tools to tackle the alarming increase in incidence of multiple drug resistant pathogens. It is an exceedingly difficult task for pathogens to develop resistance against AMPs due to their peculiar mode of action which involves binding and disrupting cell membranes. It is less likely that genetic mutations leading to alteration in overall structural make-up of bacterial plasma membrane would occur to counteract AMP action (Lee et al., 2002). Therefore it can be safely said that AMPs will remain resistant to bacterial adaptability for a very long time. Several hundreds of AMPs have been discovered so far and various biopharmaceutical companies are in the process of developing AMPs as potential novel therapeutic agents (Zhang and Falla, 2010).

During the initial stages of AMP research, AMPs were obtained either through purification from their original hosts or through chemical synthesis. Isolation of AMPs from host tissue requires several fractionation and purification steps. Also, large quantity of host tissue is needed to obtain

sufficient quantity of AMP required to carry out microbiological assays. Using purification techniques over 700 AMPs have been reported from various organisms (Mookherjee and Hancock, 2007). Requirement of large quantities of source material, low yield and tedious nature of purification techniques render this method of AMP isolation unfavourable. On the other hand chemical synthesis of peptides using the established solid-phase techniques is faster but expensive. It also has limitations with respect to complex posttranslational modifications.

In recent years, recombinant DNA technologies have been successfully employed to produce many AMPs in heterologous hosts (Parachin et al., 2012). This method allows purification of larger quantities of recombinant AMPs. Employing genetically modified microorganisms to produce recombinant proteins/peptides is cost effective and facilitates rapid growth on inexpensive substrates and controllable laboratory conditions (Terpe, 2006; Li et al., 2010c). Another advantage of the recombinant DNA technology is the availability of a wide range of vectors and host strains.Recombinant production of AMPs is becoming increasingly popular with the realization that using recombinant techniques will accelerate research and thereby their potential clinical applications (Xu et al., 2006).

Expression of AMPs have been carried out using a range of host cells but *Escherichia coli* has been established as one of the most popular recombinant host due to its rapid growth rate andwell-established expression systems (Xu et al., 2006; Ingham and Moore, 2007). Large availability of commercial expression vectors, well-established DNA manipulation protocols, and extensive knowledge on genetics, biochemistry and physiology are other advantages which favour *E. coli* as the most preferred expression host for AMPs (Sorensen and Mortensen, 2005). However, effective expression of

AMPs in a bacterial host requires overcoming certain challenges which include AMP activity on the host cell itself, susceptibility of AMP to host proteases and instability of AMP owing to its size and chemical properties (Li, 2011). These obstacles are often overpowered by the use of fusion proteins (Piers et al., 1993; Li et al., 2010c; Francis et al., 2010). Commonly used fusion tags are hexa histidine, thioredoxin, maltose-binding protein, and glutathione-S transferase. These fusion partners also facilitate downstream purification of recombinant AMPs.

The use of bacterial hosts in the recombinant expression of AMPs is not only limited to linear AMPs but also to AMPs with complex structure including those possessing di-sulphide bridges. A few examples of heterologously expressed functionally active linear AMPs include moricin (Hara and Yamakawa, 1996), histatin-5 (Tsai et al., 1996), buforin-II (Lee et al., 1998), sarcotoxin IA (Skosyrev et al., 2003), cathelicidin (Moon et al., 2006; Li et al., 2006; Krahulec et al., 2010) and examples of AMPs with more complex structural requirements include lactoferricin (Kim et al., 2006), cecropin (Xu et al., 2007), Crustin (Supungul et al., 2008; Amparyup et al; 2008) and β-defensins (Huang et al., 2008; Huang et al., 2009). From these examples it is evident that bacterial hosts have been successfully employed to produce and analyze AMPs belonging to diverse structural classes.

Recombinant expression of Histone derived AMPs has been carried out in case of buforin-II and histone H2A derived AMP reported from Scallop, *Chlamys farreri*. Buforin II is a potent 21 amino acid antimicrobial peptide with strong antibacterial activity discovered in the stomach tissue of the Asian toad *Bufo bufo gargarizans* (Park et al., 1996). Mass production of recombinant buforin-II was carried out as tandem repeats in *E. coli* and the multimers were enzyme cleaved to obtain buforin-II (Lee et al., 1998).

Recombinant buforin-II thus obtained had an antimicrobial activity identical to that of natural buforin-II. Expression and purification of recombinant buforin-IIb, a buforin-II derivative, was carried out in an *Escherichia coli* expression system (Pyo et al., 2004; Wang et al., 2011). The antimicrobial activity of recombinant buforin-IIb was similar to that of chemically synthesized one (Pyo et al., 2004). A 39 amino acid AMP derived from the N–terminus region of Scallop histone H2A was cloned and expressed in *Pichia pastoris* GS115, a yeast expression system (Li et al., 2007). The recombinant histone derived AMP from Scallop exhibited activity against both Gram positive and Gram negative bacteria.

In the present study, recombinant expression of two histone H2A derived antimicrobial peptides, Harriottin-1 and Harriottin-2 identified from Sicklefin Chimaera *Neoharriotta pinnatta* (refer chapter 4) was carried out in an *Escherichia coli* expression system. The recombinant peptides were purified, quantified and tested for antibacterial activity. This is the first report of recombinant expression of antimicrobial peptides from cartilaginous fishes.

5.2 Materials and Methods

5.2.1 Target AMPs

The antimicrobial peptides, Harriottin-1 and Harriottin-2 identified from the histone H2A of Sicklefin Chimaera, *Neoharriotta pinnata* was considered for recombinant expression. Harriottin-1 is a 5.56 kDa peptide having 52 amino acid residues and Harriottin-2 is a 4.39 kDa peptide having 40 amino acid residues. Both Harriottin-1 and 2 were found to have an α-helical structure, a net positive charge and an amphipathic nature. Details of Harriottin-1 and 2 is given in Chapter 4.

5.2.2 Designing Primers with Restriction Sites

Restriction primers were designed specifically to obtain the active peptide region of Harriottin-1 and Harriottin-2. *Nco*1 having a restriction site sequence CCATGG was added to the 5' end and *Hind*III (AAGCTT) to the 3'end of the peptides. Histidine tag (a stretch of 6 His amino acids) was also added to 5' end. The designed primers have the sequence as given in Table 1.

Table. 1. Forward and reverse restriction primer sequences designed to specifically amplify Harriottin-1 and Harriottin-2.

Sl.No.	AMP	Primer Sequence
1	Harriottin-1	Forward:5'-CCATGGGCCATCATCATCATCATTCCGGACGAGGGAAGA-3' Reverse:5'-GAAGCTTTCACAGATAGACCGGGGCT-3'
2	Harriottin-2	Forward:5'-CCATGGGCCATCATCATCATCATTCCGGACGAGGGAAGA-3' Reverse:5'-CGAAGCTTTTAATAGTTGCCTTTCCGGA-3'

5.2.3 PCR amplification of active peptide region

Total RNA isolated from the blood of *N. pinnata* as per section 4.2.2 of Chapter 4 was used for the study. The cDNA synthesis from the total RNA was carried out (see section 4.2.3 of Chapter 4). The active peptide regions corresponding to Harriottin-1 and 2 were amplified from the cDNA using the primers with restriction sites as specified in Table 1. Briefly, PCR was conducted in a 25 µl reaction volume containing 2.5 µl of 10x standard Buffer with 2.5 mM dNTP (2.5 µl), 1 µl (10 pmol) of forward and reverse primer and

1 µl (0.5U) of DNA Taq polymerase and 1 µl cDNA template. The PCR amplification programme consisted of heating the mixture to 95° C for 5 min followed by 35 cycles of denaturation at 94° C for 15 sec, annealing at 60°C for 30 sec and extension at 68° C for 30 sec with a final extension at 68° C for 10 min. Aliquots (10 µl) of PCR products were analyzed by 1.2 % agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light. The gel was documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

5.2.4 Cloning into pGEM®-T easy vectors

The pGEM®-T Easy cloning vectors are linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing re-circularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (eg. Taq DNA polymerase). These vectors are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates. The A tailed PCR products were ligated with pGEM-T easy vector (Promega, USA) by following the manufacturer's instructions. Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T vector (50 ng μl^{-1}), 3.5 µl PCR product, 1 µl ligation buffer (10X), 1 µl ligase (3 Weiss units μl^{-1}) and 4 µl MilliQ were incubated at 22° C for two hours. This allowed the ligation of PCR products with pGEM-T easy vector.

5.2.5 Transformation into *E. coli* DH5α

The frozen (-80^{o} C) *E. coli* DH5α competent cells were thawed in ice for 5-10 min. A sterile 1.5 ml vial was immersed in ice and added10 µl of ligated product followed by 100 µl of competent cells. The vial was gently flicked to uniformly mix the contents and again placed on ice for 20 min. The cells were then given heat shock for 45 sec by placing the vial in a water bath maintained exactly at 42° C. Immediately after the heat shock, vial was immersed in ice for 2 min and added 600 µl of super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone ; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose). The vial containing cells transformed with ligation product was incubated for 1 to 1.5 h at 37° C with shaking at 250 rpm. Plated 100 µl of transformed culture onto duplicate Luria-Bertani (LB) plates containing ampicillin (100 µg ml^{-1}), X-gal (80 µg ml^{-1}) and IPTG (100 mM). The plates were incubated overnight (12-16 h) at 37° C. Positive colonies were identified using blue white screening.

5.2.6 PCR confirmation of gene insert in the selected clones

The white coloured positive clones were selected and patched on to LB plates containing ampicillin (100 µg ml^{-1}), X-gal (80 µg ml^{-1}) and IPTG (100 mM) to reconfirm the transformation. All individually streaked colonies were subjected to colony PCR using vector primers (T7 forward and SP6 reverse) designed from either side of the multiple cloning site of the vector so that,the primer could amplify the insert from either side. The 25 µl reaction PCR reaction mixture contained 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1µl *Taq* polymerase (0.5 U µl^{-1}), pinch of colony, 1 µl of T7 forward and SP6 reverse primers each (10 pmol µl^{-1}) and the mixture was made up to 25 µl with MilliQ. PCR programme used for the amplification was 95° C for 5 min

followed by 35 cycles of denaturation at 94° C for 15 sec, annealing at 57° C for 45 sec, extension at 72° C for 1 min, followed by final extension at 72° C for 10 min. Ten µl of PCR product was analyzed on 1.2 % agarose gel, stained with ethidium bromide, visualized and documented using Gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA). The clones with the required size inserts were segregated for further use.

5.2.7 Propagation of *E. coli* containing the plasmid vectors

E. coli DH5α cells with pGEM-T- Harriottin-1 and pGEM-T- Harriottin-2 plasmids were propagated in LB broth media supplemented ampicillin (100 µg μl^{-1}) and incubated at 37° C with shaking at 250 rpm (12hrs).

5.2.8 Plasmid extraction

Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) by following manufacturer's instructions. Briefly, an aliquot of 10 ml culture after overnight incubation was pelletized at 12,000 x g for 1 min. The pellet was resuspended in 200 µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and centrifuged at 12,000 x g for 10 min to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centifuged at 12,000 x g for 1 min. Plasmid DNA bound to the column was washed twice with wash solution to remove any unbound materials. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100 µl 10 mM Tris-Cl and centrifuged at 12,000 x g for 1 min and stored at -20º C. Plasmid DNA obtained was analysed by 1.2 % agarose gel electrophoresis to detect the presence of the target inserts (Harriottin 2 and Harriottin 3).

5.2.9 Plasmid Sequencing

Plasmids were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit using T7 and SP6 primers on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, Kakkanad (Cochin, India).

Sequences were analyzed in GeneTool software for the presence of His tag, restriction site sequence and for correct frame of the nucleotides.

5.2.10 Restriction digestion of cloned plasmid with *Nco*1 and *Hind*III

The purified plasmid vectors were restriction digested with *Nco*1 and *Hind*III enzymes to release His labeled active peptide region gene. For restriction digestion 100 µl of purified plasmid was incubated with 10 µl of reaction buffer and 0.5 units each of *Nco*1 and *Hind*III for 1 hour at 37° C and then the enzymes were inactivated by elevating the temperature to 65° C for 20 min. The restriction digestion was confirmed by 1.2 % agarose gel electrophoresis.

5.2.11 Purification of released active peptide gene sequence

The restriction digested plasmids were loaded onto 1.2 % agarose gel (25 µl per well) and the released peptide gene sequences were gel purified using GenElute™ Gel extraction kit (Sigma, USA) following manufacturer's instructions. Briefly, the agarose gel that contained DNA fragment of appropriate size was excised and transferred to 2.0 ml pre-weighed vials. Weight of the excised gel was taken and 3 gel volumes of gel solubilization solution was added. It was incubated at 60º C for 10 min with repeated vortexing. After incubation, 1 gel volume of 100 % isopropanol was added and mixed gently until it became homogenous. This solubilized gel solution

was loaded into the binding column that was pre-treated with column preparation solution and centrifuged at 12,000 x g for 1 min. An aliquot of 700 µl wash solution was added and centrifuged for 1 min at 12,000 x g, repeated the centrifugation and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2 ml vial) and added 50 µl of elution buffer (10 mM Tris-HCl, pH 9.0) centrifuged at 12,000 x g for 1 min and stored at -20º C. The concentration of DNA was measured spectrophotometrically at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan).

5.2.12 Cloning into pET32a+ translation vector and transformation into *E. coli* DH5α

The pET32a+ translation vector was restriction digested with *Nco*1 and *Hind*III enzymes. The protocol followed was same as described above. The restriction digested vector was gel purified using GenElute™ Gel Extraction kit (Sigma, USA). Restricted and purified active peptide genes were ligated to pET32a+ translation vector (Novagen, UK) by following the manufacturer's instructions. Briefly, 10 µl ligation mixture containing 1.0 µl pET32a+ vector (50 ng μl^{-1}), 4.0 µl PCR product, 1 µl ligation buffer (10x), 1 µl T4 DNA ligase (1U μl^{-1}) and 3.0 µl MilliQ were incubated at 22° C for two hours. This allowed for the ligation of PCR products with pET32a+ translation vector. The transformation into *E. coli* DH5α was carried out as discussed earlier (section 5.2.5).

5.2.13 PCR confirmation of gene insert in the selected clones

The clones were selected and patched on LB/ampicillin (100 µg ul^{-1}) plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers. The PCR reaction mixture

containing 2.5 µl 10x buffer, 2.5 µl dNTPs (2.5 mM), 1µl *Taq* polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of T7-Forward (5'-TAATACGACTCACTATAGGG-3') and T7-Reverse (5'-CTAGTTATTGCTCAGCGGTG-3') primers each, was made up to 25 µl with MilliQ. The PCR programme used for the amplification was 95° C for 5 min followed 35 cycles of denaturation at 94°C for 15 sec, annealing at 52° C for 20 sec, extension at 72° C for 1 min, followed by final extension at 72° C for 10 min. Ten (10) µl of PCR products was analyzed by 1.2 % agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

5.2.14 Propagation of confirmed clone and plasmid extraction

After confirmation, the transformed *E. coli* DH5α containing recombinant vector constructs (with Harriottin-1 and Harriottin-2 insert) was propagated in 10 ml LB ampicillin (100 µg μl^{-1}) medium at 37° C, with shaking at 250 rpm. Plasmid extraction was carried out as detailed above (section 5.2.8).

5.2.15 Plasmid sequencing

The cloned plasmids were sequenced at SciGenom Sequencing Facility, Kakkanad (Kochi, India) and the sequences were analyzed in GeneTool for the in-frame confirmation of the genes with the ATG of the pET32a+ system.

5.2.16 Rosetta-gami™ B (DE3) pLysS competent cell preparation

Rosetta-gami™ B (DE3) pLysS belongs to Rosetta-gami B host strains. Rosetta-gami B combines the key features of BL21, Origami, and Rosetta to

enhance both the expression of eukaryotic proteins and the formation of target protein disulfide bonds in the bacterial cytoplasm. Rosetta-gami B strains are derived from Origami B cells, a kanamycin-sensitive K-12 strain carrying the *trxB* and *gor* mutations for disulfide bonds formation in the cytoplasm. The Rosetta strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and GGA on a compatible chloramphenicol-resistant plasmid, pRARE. The codons are under the control of their native promoter. By supplying these rare codons, the Rosetta strains provide for "universal" translation, where translation would otherwise be limited by the codon usage of *E. coli*. BL21 *lacZY* deletion mutant allows precise control with IPTG.

For preparing competent cells, Rosetta-gami^{TM} B (DE3) pLysS (Novagen) cells were streaked on LB agar plate for obtaining single colonies. A single colony was inoculated in 10 ml LB media and grown overnight at 37° C with shaking at 150 rpm. An aliquot of 5 ml of overnight culture was inoculated into 50 ml LB and incubated at 37° C for 2 hrs at 250 rpm. The re-inoculation helps to get *E.coli* cells in their log phase. The cells (50 ml) were centrifuged at 6000 rpm for 20 min at 4° C. The supernatant decanted and cells were resuspended by gentle vortexing with 0.1 M $CaCl_2$ (1/4th original culture volume). The resuspended cells were placed in ice for 45 min with intermittent swirling and mixing. The cells were centrifuged at 6000 rpm for 20 min at 4° C. The supernatant was decanted and cell pellet was resuspended in 1 ml of 0.1M $CaCl_2$. The competent cells formed are stored in -80° C with addition of 10-15 % glycerol.

5.2.17 Transformation into *E. coli* Rosetta-gami™ B (DE3) pLysS competent cells

The competent cells [Rosetta-gami™ B (DE3) pLysS] were thawed by placing on ice for 5-10 min. Added 5 µl of positive plasmid constructs to a sterile 1.5 ml vial already on ice. Transferred 50-100 µl of competent cells into the 1.5 ml vials (containing +ve plasmid construct) on ice. Gently flicked the tubes to mix the contents uniformly and placed them on ice for 20 min. Heat shocked the cells for 90 seconds in a water bath maintained exactly at 42° C. Immediately, returned the tubes to ice for 2 min. An aliquot of 600 µl super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose) was added to the tubes containing cells transformed with ligation mixture. Incubated for 1.5 hrs at 37° C with shaking at 250 rpm. Plated 100 µl of each transformation culture onto duplicate LB plates supplemented with ampicillin. Plates were then incubated overnight (12-16 h) at 37° C. Colonies obtained were patched onto LB plates supplemented with ampicillin.

5.2.18 Recombinant protein expression of Hariottin-1 and Harriottin-2

Single colonies of recombinant pET32a+ Hariottin-1 and Harriottin-2 constructs in Rosetta-gami™ B (DE3) pLysS were inoculated into 5 ml LB both media supplemented with ampicillin (100 µg $µl^{-1}$) and kanamycin (50 µg $µl^{-1}$). It was incubated at 37° C at 250 rpm overnight. One ml of the overnight culture was added to 100 ml LB both media supplemented with ampicillin (100 µg $µl^{-1}$) and kanamycin (50 µg $µl^{-1}$) and further incubated at 37° C for 3-4 hrs until an OD_{600} of 0.6 (Optimum OD for protein expression) was obtained. The OD_{600} was monitored frequently during the growth phase of the

culture by removing aliquots aseptically. Just prior to induction 5 ml culture was aliquoted, that served as un-induced control. IPTG was added to a concentration of 100 mM to the induced culture. The cultures (induced and uninduced) were incubated further for 3 hrs at 37° C with shaking at 250 rpm. From third hour onwards 2 ml aliquot of the culture (induced) was removed, every hour for seven hours to check for the time period when maximum expression is obtained. The cells were pelletized at 12000 x g for 2-5 min at room temperature. The pellets were stored at -20° C until further processing.

5.2.19 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of cell pellets

The cell pellets were analyzed for recombinant protein expression on 16% SDS- PAGE. A pinch of pellet from both induced and un-induced samples were boiled in 10 µl sample buffer (150 mM Tris-Cl, pH 7, 12 % SDS, 30 % glycerol, 6 % mercaptoethanol, 5 % Coomassie brilliant blue R-250) for 15 min. The samples were given a short spin and the supernatant was subjected to 16 % SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The protein was separated and analyzed using 4 % stacking gel and 16 % resolving (running) gel. Electrophoresis was performed in 1x Tris (pH 8.9) as anode buffer and 1x Tris-tricine (pH 8.3) as cathode buffer at a voltage of 50 V in stacking gel and 120 V in resolving gel (4-gel Mini-PROTEAN® Tetra cell protein electrophoresis unit, BioRad, USA). After electrophoretic separation, gel was stained in coomasie brilliant blue stain R-250 (0.5 % coomasie brilliant blue R-250, 40 % methanol and 10 % acetic acid in distilled water) and de-stained in de- staining solution 1(10 % methanol and 10 % acetic acid in distilled water) and photographed using Gel-DOC™ XR+ imaging system (BioRad, USA). Recombinant protein expression was determined by comparing the presence and absence of protein profile with that

of un-induced cells. Molecular weight of protein was determined by comparing with standards (BioLit low range protein marker 2.5/3 – 40 kDa).

5.2.20 Ni-NTA column purification of 6x His-tagged recombinant peptides under denaturing conditions

Qiagen Ni-NTA superflow cartridges pre-filled with 1 ml Ni-NTA superflow and ready to use for purification of 6x His-tagged proteins was used for the purification of recombinant Harriottins. The peptides were purified under denaturing condition. Cell lysate was prepared by adding 700 µl of buffer B (7 M Urea, 100 mM NaH_2PO_4, pH 8.0) to cell pellet collected from 5 ml culture. Prior to this the pellet was thawed at RT for 15 min. Cell pellet in buffer B was vortexed continuously for 15 min. It was then centrifuged at 6000 x g for 30 min. The supernatant was formed by the cell lysate which was stored at -20^o C. A 10 ml syringe was filled with buffer B. The syringe was attached to the cartridge inlet. The cartridge was equilibrated with 10 ml column volumes of buffer B. The flow rate was maintained at 1 ml min^{-1}. The syringe was removed and filled with cell lysate. The cell lysate was applied to the cartridge at a flow rate of 1 ml min^{-1}. The cartridge was washed with 10 column volumes of buffer C (wash buffer-8 M Urea, 100 mM NaH_2PO_4, pH 6.3) at the same flow rate as earlier. The recombinant protein was eluted with 5-6 column volumes of buffer E (elution buffer- 8 M urea, 100 mM NaH_2PO_4, pH 4.5). The elute was stored at -20^o C for further use.

5.2.21 SDS PAGE analysis to check purity

The lysate, flow through, wash elute and final elutes were analyzed on 16 % SDS-PAGE to confirm the complete purification steps.

5.2.22 Concentrating and refolding of the gel extracted elutes.

The eluted samples were concentrated to one tenth of original volume using Millipore's Amicon Ultra-Centrifugal 3 kDa cut-off membrane by centrifuging at 5000 x g for 30 mins. The concentrated samples were reconstituted to the original volume using the refolding buffer (Tris-Cl pH 8-50 mM, EDTA- 0.1 mM and NaCl- 0.15M) and centrifuged at 5000 x g for 30 mins. The concentrated sample was given 10-12 washes with the refolding buffer to remove the denaturing salts and thus refold the protein to its native form.

5.2.23 Enterokinase digestion of refolded Proteins

Enterokinase was used to cleave the fusion proteins and release recombinant AMP sequence. Enterokinase from porcine intestine (E0885-40UN) supplied by Sigma was used to release the recombinant AMP sequence from fusion proteins. To 1 ml of refolded proteins, 2 units of enterokinase was added and incubated at 37^{o} C for 14-16 hours.

5.2.24 SDS PAGE analysis

Ten microliter of enterokinase treated sample was analyzed on SDS-PAGE to check for enterokinase activity. Refolded protein without enterokinase treatment was loaded as reference to the enerokinase treated sample.

5.2.25 Purification of recombinant AMPs

The enterokinase treated sample was centrifuged in Millipore's Amicon Ultra-Centrifugal 3 kDa cut-off membrane at 5000 x g for 30 min. The recombinant AMP sequence having lower molecular weight will pass through

the filter while high molecular weight fusion proteins will be retained by the filter. The filtrates containing recombinant Harriottin-1 and 2 were collected separately and sored at -20^{o} C for further analysis. The EDTA in refolding buffer often interfere with the antibacterial assay and hence the refolding buffer (containing dissolved Harriottin-1 and 2) was replaced with 50 mM Tris-HCl using the procedure explained in secion 5.2.22.

5.2.26 Qubit analysis of recombinant Harriottins

The recombinant Hariottin-1 and 2 were quantified with Quant-iT^{TM} protein assay kit using Qubit fluorometer (Invitrogen, UK). The assay is accurate for initial sample concentrations from 12.5 μg ml^{-1} to 5 mg ml^{-1} and exhibits low protein-to-protein variation. The assays were performed at room temperature, and the signal was stable for 3 hrs.

The Quant-iT working solution was made by diluting the Quant-iT protein reagent 1: 200 in Quant-iT protein buffer. The Quant-iT working solution was mixed well without any air bubbles and 190 μl was aliquoted to 0.5 ml tubes. An aliquot of 10 μl each of the recombinant peptides was added to the respective tubes and mixed by mild vortexing with care not to generate air bubbles (air bubbles cause error in the readings). The tubes were incubated for 15 min at room temperature. The samples were measured using the Quant-iT protein programme. The readings were recorded. The Qubit fluorometer gave values for Quant-iT protein assay kit in μg ml^{-1}. This value corresponded to the concentration after the sample was diluted into the assay tube.

The sample concentration was calculated using the following equation:

Concentration of sample = QF value x (200/X)

Where, QF value = the value given by the Qubit Fluorometer,

X = the number of microliters of sample added to the assay tube.

5.2.27 Antibacterial Assay

5.2.27.1 Bacterial strains used for assay

Antibacterial activity of the recombinant Hariottins was tested against two strains of Gram positive bacteria viz., *Bacillus cereus* (MCCB 101) and *Staphylococcus aureus* (MTCC 3061) and five stains of Gram negativebacteria viz., *Edwardsiella tarda* (MTCC 2400), *Pseudomonas aeruginosa* (MCCB 119), *Aeromonas hydrophila* (MCCB 113), *Vibrio cholera* (MCCB 129) and *Vibrio parahaemolyticus* (MCCB 133).

5.2.27.2 Preparation of bacterial suspension

The bacterial strains were tested for purity by repeated streaking on Nutrient agar plates. The isolated colonies obtained were picked and streaked onto sterile nutrient agar slants. The slants were incubated at 37^{o} C for 24 hours. Succeeding incubation, sufficient quantity of 50 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) was added to the slants. The bacterial cells were scraped off using sterile inoculation loop and mixed well for uniformity. The Optical Density (OD) was measured at 600 nm and the OD was so adjusted that every 10 µl of bacterial suspension contained approximately 10^3 colony forming units (cfu).

5.2.27.3 Liquid growth inhibition assay

Antibacterial assay of recombinant Hariottin-1 and 2 was carried out in 96 well, microwell plates using Liquid Growth Inhibition assay as described by Huang et al. (2006) with modifications. Briefly, 10 µl of recombinant peptides was mixed with 10 µl of the bacterial suspension (10^3 cells ml^{-1}) in

microtiter wells. Blank constituted 10 µl of the HEPES buffer and 10 µl of 50 mM Tris-HCL solution. The control constituted 10 µl of 50 mM Tris-HCl mixed with 10 µl of the bacterial suspension. Ten (10 µl) of the bacterial suspension mixed with 10 µl of ampicillin solution (1 mg/ml) served as positive control. After an incubation of 2 hours at room temperature, 80 µl of the nutrient broth was added to each well, followed by incubation of approximately 5 to 6 hours at 37^{o} C. The incubation was succeeded by the addition of 25 µl of 5 µg ml^{-1} solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). It was further incubated on a shaker at room temperature for 30 minutes during which the live cells will reduce MTT to formazan. The reaction was stopped by adding 125 µl of acidified isopropanol (0.1 N HCl in isopropanol) and was incubated on a shaker at room temperature for 30 minutes. Absorbance was measured at a wavelength of 570 nm and reference wavelength of 630 nm using a Microplate reader (Tecan, USA). Inhibition % was calculated as:

Inhibition % = 100 – Growth %.

where, Growth % = (OD of wells with bacteria + peptides/OD of wells with bacteria only) x 100.

The inhibition of growth in wells containing the AMP is determined by comparing it with the bacterial growth in control wells. The growth of bacteria in wells containing AMPs divided by bacterial growth in control wells will give a value which is multiplied by 100 to obtain growth percentage of bacteria in wells containing AMPs. Hundred minus the growth percentage of bacteria in wells containing AMPs will give the inhibition %.

Five concentrations (prepared by 2 fold dilutions of peptides) of both Harriottin-1 and Harriottin-2 were tested against seven bacterial strains for

testing the Minimum Inhibition Concentration (MIC) of the peptides. MIC was estimated for Harriottin-1 and Harriottin-2 by Probit analysis using PriProbit computer based program.

5.3 Results

5.3.1 PCR amplification and cloning of active peptide region

Restriction primers with restriction sites (for *Nco*1 and *Hind*III) and His tag were designed to amplify the corresponding active peptide region (gene) of Harriottin-1 and 2 (Table 1). PCR amplification of cDNA of *Neoharriotta pinnata* with restriction primers specific to Hariottin-1 and Harriottin-2 yielded amplicons of approximate size 180 bp and 150 bp respectively (Fig. 5.1). The PCR products were cloned into pGEM®-T Easy cloning vectors and transformed into *E. coli* DH5α competent cells. Transformed cells were plated on to LB plates supplemented with antibiotics and positive colonies were identified based on blue white screening. The white coloured colonies obtained were patched onto LB plates and the presence of plasmid with insert was confirmed by colony PCR using T7 forward and SP6 reverse primers. PCR amplified amplicons of size 282 bp (156 + 126 = 282 bp) and 246 bp (120 + 126 = 246 bp) for Harriottin-1 and 2 respectively (Fig. 5.2).

5.3.2 Isolation of cloned plasmid and its restriction digestion with *Nco*1 and *Hind*III

The ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer for the extracted plasmids carrying Harriottin-1 and Hariottin-2 inserts were close to 1.8 (1.73 and 1.67 respectively) and the purity was further confirmed by agarose gel electrophoresis (Fig. 5.3). The cloned

plasmid was sequenced to check for frame shift if any. Sequencing of the cloned plasmids revealed in-frame sequence of Harriottin-1 and Harriottin-2 and thereby confirming the insert and frame of inserts (Fig. 5.4 a-b). Presence of His tag and restriction site sequence in the insert was also confirmed. After the confirmation of the insert to be in-frame in the cloned plasmid, the plasmid was digested with *Nco*1 and *Hind*III restriction enzymes. Agarose gel analysis of the restriction digested products confirmed the release of inserts, 180 bp Harriottin-1 and 150 bp Harriottin-2 from the plasmids carrying Harriottin-1and Harriottin-2 respectively (Fig. 5.5 a-c).

5.3.3 Cloning into pET32a+ translation vector and transformation into *E. coli* DH5α

Amplicons of size 906 bp (156 + 750 = 906 bp) and 870 (120 + 750 = 870 bp) were obtained from colony PCR of transformed cells carrying recombinant vectors pertaining to Harriottin-1 and 2 respectively (Fig. 5.6). Plasmid was extracted from positive colonies and send for sequencing.

5.3.4 Transformation into Rosetta-gami™ B (DE3) pLysS competent cell

Transformed Rosetta-gami™ B (DE3) pLysS clones (carrying Harriottin-1 and Harriottin-2 genes) could be obtained on LB agar plates with Ampicillin.

5.3.5 Recombinant expression of Harriottin-1 and Harriottin-2

Maximum expression was obtained from 4th hour to 6th hour for both Hariottin-1 and Harriottin-2 (Fig. 5.7 a-c). Bulk production (1000 ml culture) of recombinant Harriottin-1 and 2 was carried out and cells were harvested

after 5 hours of IPTG induction. The recombinant expression was confirmed using SDS-PAGE.

5.3.6 Purification and quantification of recombinant peptides

Proteins of size 23.56 kDa (5.56 kDa of Harriottin-1 + 18 kDa of Fusion proteins) and 22.39 kDa (4.39 kDa of Harriottin-2 + 18 kDa of Fusion proteins) were obtained for Harriottin-1 and Harriottin-2 (Fig. 5.8; a-b). The released recombinant peptides were observed on SDS-PAGE (Fig 5.9). Concentration of Harriottin-1 and 2 was found to be 25 µg/ml (1.89 µM) and 26 µg/ml (2.51 µM) respectively.

5.3.7 Antimicrobial assay

Liquid Growth Inhibition assay was used to determine the antibacterial potency of recombinant Harriottin-1 and Harriottin-2 (Fig. 5.10). Harriottin-1 exhibited antibacterial activity against *Vibrio cholera* and *Vibrio parahaemolyticus* with estimated MICs of 104.8 µM and 32.11 µM respectively. Harriottin-1 was ineffective against *Bacillus cereus, Staphylococcus aureus, Edwardsiella tarda, Pseudomonas aeruginosa* and *Aeromonas hydrophila* at all tested concentrations. The antibacterial activity of Harriottin-1 against *Vibrio cholera* and *Vibrio parahaemolyticus* is shown in Fig. 5.11 a-b.

In case of Harriottin-2 also, antibacterial activity was observed against *Vibrio cholera* and *Vibrio parahaemolyticus* with estimated MICs of 12.09 µM and 12.93 µM respectively. The antibacterial activity of Harriottin-2 against *Vibrio cholera* and *Vibrio parahaemolyticus* at all tested concentrations is shown in Fig. 5.12 a-b. Harriottin-2 inhibited the growth of *P. aeruginosa* by 25.23 % at a concentration of 2.51 µM and by 18.42 % at

1.25 µM concentration. The peptides also inhibited the growth of *E. tarda* by 15.31 % and *A. hydrophila* by 12.56 % at maximum tested concentration (2.51 µM). However, no antibacterial activity was observed (at any of the concentrations) against the tested Gram positive bacteria.

5.4 Discussion

The role of histones as antimicrobial peptides in the innate immune responses of both invertebrates and vertebrates is being increasingly recognized. The AMPs produced from Histone H2A has not yet been reported in Chimaera fishes and Harriottins happens to be the first such report. In this study, recombinant production of Harriottin-1 and 2 in Rosetta-gami™ B (DE3) pLysS *E. coli* expression system was carried out to investigate their antibacterial activity. Though *E. coli* is a good expression host, there is a possibility of recombinant AMPs exhibiting activity against the host. To avoid toxicity of recombinant Harriottins on *E. coli* host and to get sufficient quantity of recombinant peptides, pET32a+ expression vector which has His, Trx and S fusion tags was used. The 6x His tag plays an important role in purification of the recombinant peptide. During purification using Ni-NTA column, the peptides carrying 6x His tag (recombinant peptides) will bind to the matrix of Ni-NTA column which can be latter eluted using appropriate eluent. As expected, the resulting recombinant Harriottins were highly expressed in Rosetta-gami™ B (DE3) pLysS.

Most antimicrobial peptides share many common physicochemical properties. The cationic charge, which is the most common feature of AMPs, allows them to bind to negatively charged bacterial membrane. Both the Harriottins are rich in basic amino acids, which give a net positive charge to

Harriottin-1 and 2. The amphipathic nature of the peptide is also considered to be a crucial factor in determining its antimicrobial character (Shai, 2002). Amphipathic peptides cause membrane permeation after binding to the outer membrane of bacterial cell. The histone derived AMPs, Harriottin-1 and Harriottin-2 were predicted to have an amphipathic nature and α-helical structure by Schiffer–Edmundson wheel analysis.

The genes coding for Harriottin-1 and 2 were labelled with His tag to facilitate purification of the recombinant peptides. The recombinant peptides were expressed at 37^{o} C. IPTG induction was carried out at OD_{600} = 0.6 as it is optimum for protein expression. Also, when protein production is induced at an earlier stage of growth, lysis of bacterial cells would be easier (Moon et al., 2007). The cell pellets were lysed under denaturing condition and the recombinant Harriottins were purified on Ni-NTA column, to which proteins with His tag alone would bind. The purity was found to be satisfactory which suggests that the wash buffer (buffer C - 8 M Urea, 100 mM NaH_2PO_4, pH 6.3) used was efficient enough to wash away most of the impurities bound to Ni-NTA column. The stepwise reduction of urea with refolding buffer provided and extensive washing as well as the refolding of the recombinant Harriottins. The refolding buffer was subsequently replaced with 50 mM Tris-Cl solution so as to remove EDTA which would otherwise hinder with the antimicrobial assay results. The denatured Harriottins were successfully refolded as judged by the clearness of the solution (misfolded proteins would precipitate in short period of time) and cleavage by enterokinase enzyme.

An attempt was made in this study to understand the antibacterial activity of purified recombinant Harriottin-1 and Harriottin-2 by employing liquid growth inhibition assay. Both Harriottin-1 and 2 exhibited strong antibacterial activity against two (*V. cholerae* and *V. parahaemolyticus*) of the

four tested Gram negative bacteria, whereas, no activity was observed against the tested Gram positive bacteria. However, Harriottin-2 was found to be better than Harriottin-1 in terms of antibacterial activity. The antibacterial activity was high at higher concentration and was found to reduce gradually with reduction in concentration. The MIC of Harriottin-2 in case of *V. cholerae* was almost nine times less than Harriottin-1 and in case of *V. parahemolyticus* it was three fold lesser than Harriottin-1. The recombinant Harriottin-2 was also found to reduce the growth of *P. aeruginosa*, *E. tarda* and *A. hydrophila*. No such activity was observed for Harriottin-1 against these bacteria. These results clearly indicate that Harriottin-2 is a better antimicrobial peptide than Harriottin-1 in terms of antibacterial activity. Harriottin-1 is lengthier than Harriottin-2 at the C-terminus region by 12 amino acids, that is from Ala-41 to Lys-52. The difference in antibacterial activity of Harriottin-1 and 2 indicates that the removal of 12 amino acids from the C-terminus of Harriottin-1 would result in an enhancement of antibacterial activity.

The antimicrobial activity of histone H2A derived antimicrobial peptides have been studied by many authors (Kawasaki and Iwamuro, 2008). The results obtained for antibacterial assay, in the present study is different from most of the previous reports. Hipposin, which is similar to Harriottin-1 in terms of amino acid sequence length, exhibited antimicrobial activity against all of the tested Gram positive and Gram negative bacteria (Birkemo et al., 2003). Antibacterial activity against the tested Gram positive and Gram negative bacteria was also exhibited by Scallop histone H2A derived AMP (Li et al., 2007), Buforin I (Cho et al., 2009) and Abhisin (Zoysa et al., 2009). These three peptides are similar in amino acid sequence length to Harriottin-2. However, it needs to be taken into consideration that the Gram positive and

Gram Negative bacterial strains used were different in each of the above mentioned studies. Harriottin-1 which demonstrated antibacterial activity against *V. cholerae* and *V. parahaemolyticus* was found to have absolutely no effect on the growth of *P. aeruginosa* and *E. tarda*. Hence, the possibility of Harriottins exhibiting antibacterial activity against Gram positive bacterial strains other than *B. cereus* and *S. aureus* cannot be overruled. Moreover, naturally isolated or chemically synthesized peptides were used for antimicrobial assay in case of Buforin I, Abhisin and Hipposin. In case of Scallop histone H2A derived AMP, recombinant peptide expressed in *Pichia pastoris* GS115 was tested for antibacterial activity. Method of production of AMP for testing its antibacterial activity might play a significant influence on its antimicrobial potency. Birkemo et al. (2003) has reported that type of antimicrobial assay used has an influence on the potency of AMPs to some of the tested organisms. Their findings clearly indicate that the results obtained with different assay procedures need not be entirely consistent. Minor differences in amino acid sequence of the peptides may also influence its antimicrobial potency. Harriottin-2, Buforin I and Abhisin have same length but exhibit minor differences in their amino acid sequence (refer section 4.4 of chapter 4). Antibacterial activity against the tested *Vibrio* strains was exhibited by both Harriottiin-2 and Buforin I (Cho et al., 2009), whereas, no activity against the tested *Vibrio* strains was observed in case of Abhisin even at a concentration of 250 µg/ml (Zoysa et al., 2009). Therefore, it can be assumed that the difference in amino acid sequence of Harriottins to other reported histone H2A derived AMPs might have rendered Harriottins to be more specific towards *Vibrio* strains. Harriottin-1 and 2 exhibited all common features of peptides with antimicrobial activity and the recombinant Harriottins displayed antibacterial activity against the tested *Vibrio* strains,

suggesting the possible role of N-terminus region of Histone H2A in the innate immune responses of *Neoharriotta pinnata*.

Conclusion

In the present study, two histone H2A derived antimicrobial peptide sequences named Harriottin-1 and Harriottin-2, identified from Sicklefin Chimaera, *Neoharriotta pinnata* was cloned and expressed in Rosetta-gami™ B (DE3) pLysS *E. coli* expression system. The recombinant Harriottins were purified and assayed for their antibacterial property. Both Harriottin-1 and Harriottin-2 were found to exhibit antibacterial activity against *V. cholerae* and *V. parahemolyticus*. Results of the study indicate the possible role of histone H2A in the immune responses of Holocephali fishes. Harriottin-1 and Harriottin-2 have the potential to be developed as antibacterial agents against *Vibrio* strains pathogenic to humans and fishes. The use of recombinant DNA technology is a promising method to produce bulk quantity for AMPs for clinical research. Recent advances in technology have led to the development of new cloning methods for overexpression and purification of recombinant AMPs. Further improvement in these technologies is expected as clinical interest shifts from antibiotics to antimicrobial peptides.

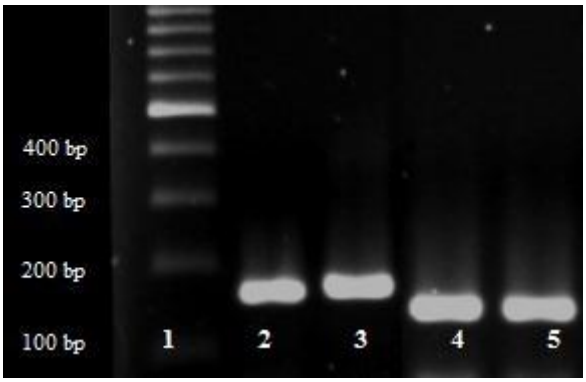


Fig. 5.1

Fig. 5.1. PCR amplification of Harriottin-1 and Harriottin-2 using specific primers. Lane 1 denotes 100 bp marker, Lane 2 and 3 show 150 bp amplicons of Harriottin-1 and Lane 4 and 5 denotes 120 bp amplicons of Harriottin-2.

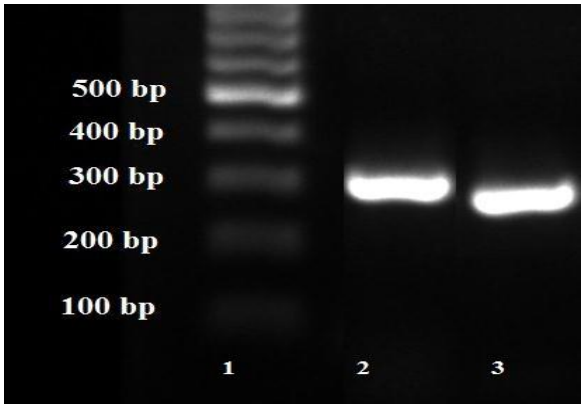


Fig. 5.2

Fig. 5.2. PCR amplification (colony PCR) of Harriottin-1 and Harriottin-2 insert in pGEMT vector construct using T7 forward and SP6 reverse primers. Lane 1 denotes 100 bp marker, Lane 2 show 282 bp (156 + 126 = 282 bp) amplicon of Harriottin-1 and Lane 3 denotes 246 bp (120 +126 =246 bp) amplicon of Harriottin-2.

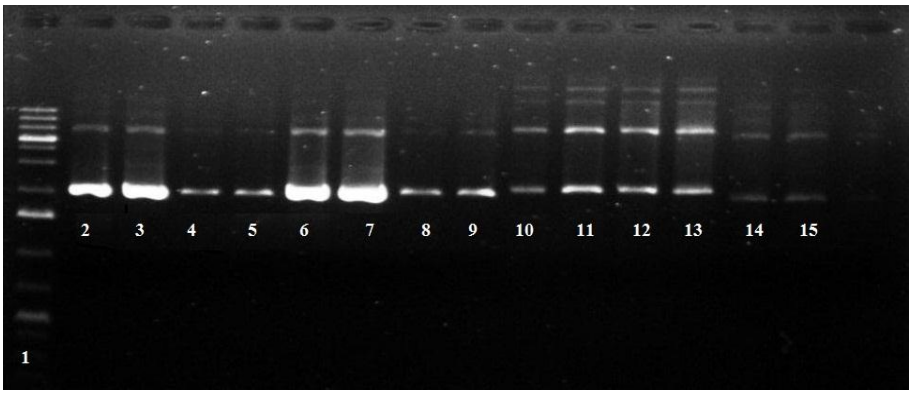


Fig. 5.3. Plasmid extracted from positive clones of pGEMT vector constructs. Lane 1 shows 100 bp marker, Lane 2 to 7 plamids with Hariottin-1 insert and Lane 8 to 15 denotes plasmid with Harriottin-2 insert.

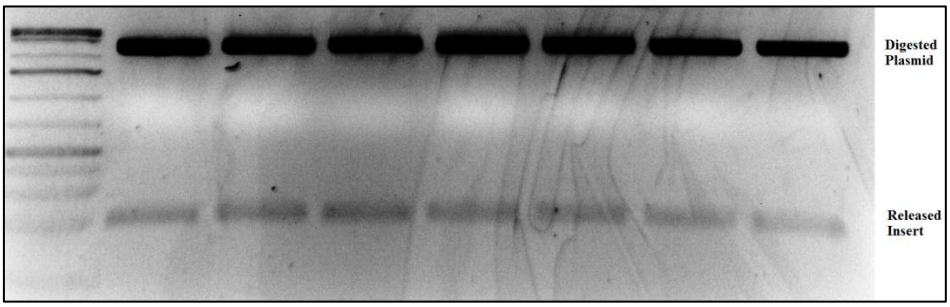
```
gccatgggccatcatcatcatcatcattccggacgagggaagactggcggtaaagtccga
 A  M  G  H  H  H  H  H  H  S  G  R  G  K  T  G  G  K  V  R
gccaaggccaagtcccgctcctcccgggccgggctgcagttccccgtgggccgcgtccac
 A  K  A  K  S  R  S  S  R  A  G  L  Q  F  P  V  G  R  V  H
aggcatctccggaaaggcaactatgctgatcgcgtgggtgccggagccccggtctatctg
 R  H  L  R  K  G  N  Y  A  D  R  V  G  A  G  A  P  V  Y  L
taaaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaac
 -  K  L  A  A  A  L  E  H  H  H  H  H  H  -  D  P  A  A  N
```

(a)

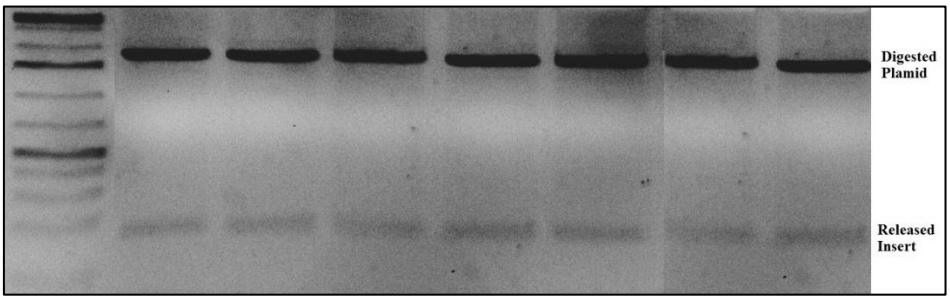
```
gccatgggccatcatcatcatcatcattccggacgagggaagactggcggtaaagtccga
 A  M  G  H  H  H  H  H  H  S  G  R  G  K  T  G  G  K  V  R
gccaaggccaagtcccgctcctcccgggccgggctgcagttccccgtgggccgcgtccac
 A  K  A  K  S  R  S  S  R  A  G  L  Q  F  P  V  G  R  V  H
aggcatctccggaaaggcaactattaaaagcttgcggccgcactcgagcaccaccaccac
 R  H  L  R  K  G  N  Y  -  K  L  A  A  A  L  E  H  H  H  H
```

(b)

Fig. 5.4. The in-frame sequence of (a) Harriottin-1 and (b) Harriottin-2 in pGEMT vector constructs. The regions highlighted in blue is the in-frame amino acid sequence and the inframe amino acid region is given in red. The hexa His label is highlighted in yellow.



(a)



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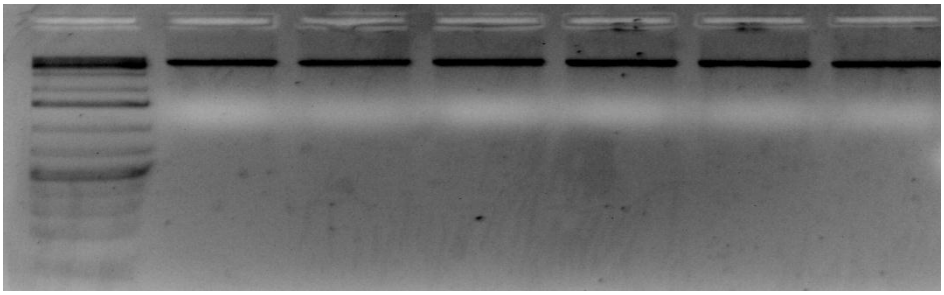


(c)

Fig. 5.5. Agarose gel image of plasmid digested with *Nco*1 and *Hind*III restriction enzymes. (a) Released Harriottin-1 insert; (b) Released Harriottin-2 insert; (c) pET vector restricted digested with *Nco*1 and *Hind* III enzymes.

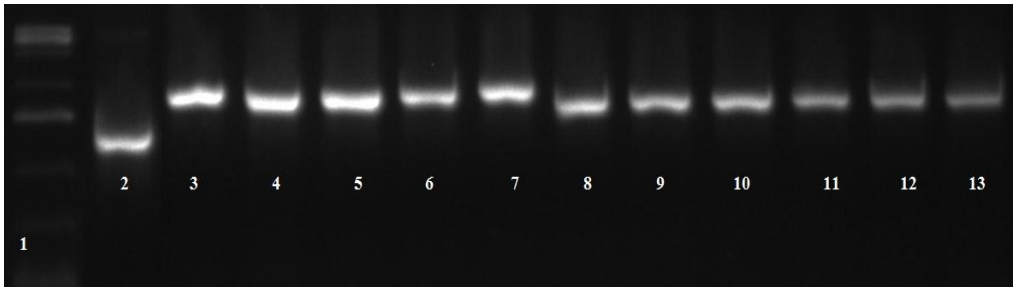
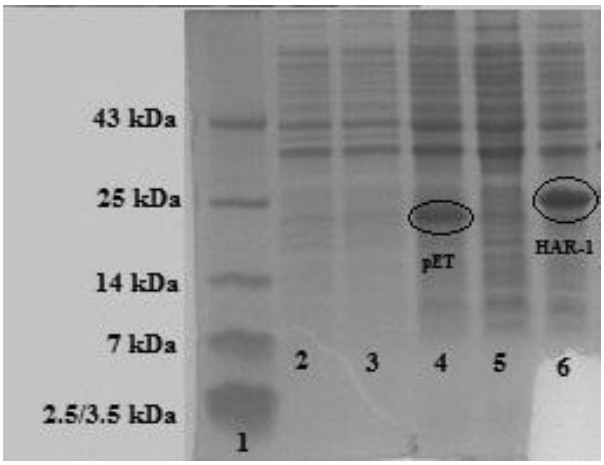
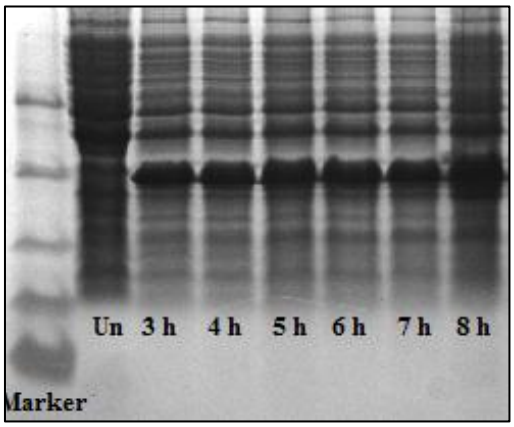


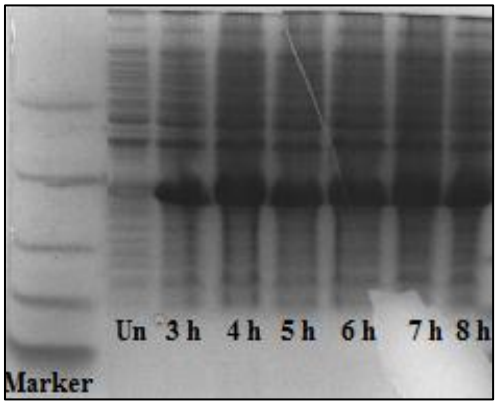
Fig. 5.6. PCR amplification (colony PCR) of pET32a+ vector construct with Harriottin-1 and Harriottin-2 insert using T7 forward and T7 reverse primers. Lane 1 denotes 100 bp marker, Lane 2 show pET32a+ vector without insert, Lane 3 to 7 show amplified pET32a+ vector with Harriottin-1 insert (156 + 750 = 906 bp) amplicon of Harriottin-1 and Lane 8 to 13 denotes 870 bp (120 + 750 = 870 bp) amplicon of pET32a+ with Harriottin-2 insert.



(a)



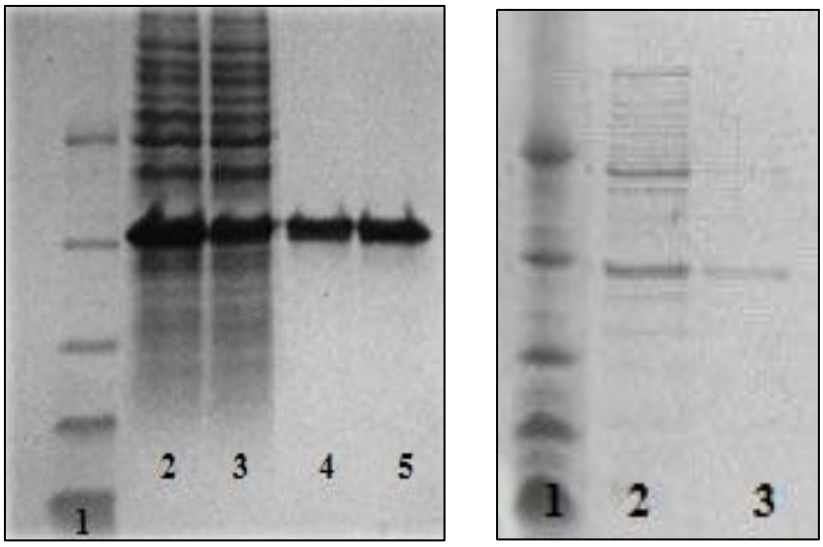
(b)



(c)

Fig. 5.7. SDS-PAGE gel image of recombinant expression of Harriottin-1 and 2. (a) shows difference in protein profile of Rosetta gami cells producing recombinant proteins and cells not producing recombinant proteins. Lane 1: 100 bp marker, Lane 2 Rosetta gami cells without any vector, Lane 3: Uninduced cells with pET vector, Lane: 4 IPTG induced cells producing recombinant vector protein (marked by a circle), Lane 5: uninduced cells having pET vector with Harriottin-1 insert, Lane 6: IPTG induced cells producing recombinant Harriottin-1 fused with vector protein (marked by a

circle). (b) and (c) show recombinant expression of Harriottin-1 and Harriottin-2 respectively at each hour from third hour to eight hour after IPTG induction. The thick bands of size 23.56 kDa in (b) represent Harriottin-1 and thick bands at 22.39 kDa in (c) represent Harriottin-2.



(a) **(b)**

Fig. 5.8. SDS-PAGE gel showing Ni-NTA purified recombinant proteins. (a) shows purified Harriottin-1. Lane 1: 100 bp marker, Lane 2 and 3: Cell lysate with recombinant Harriottin-1, Lane 4 and 5: purified recombinant Harriottin-1. (b) shows purified Harriottin-2. Lane 1: 100 bp marker, Lane 2: Cell lysate with recombinant Harriottin-2, Lane 3: Ni-NTA purified recombinant Harriottin-2.

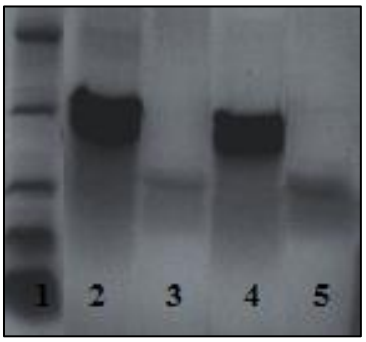


Fig. 5.9. SDS-PAGE gel image showing the release of recombinant Harriottins from vector fusion proteins. Lane 1: 100 bp marker, Lane 2: untreated recombinant protein includes fusion protein and Harriottin-1, Lane 3 enterokinase treated recombinant protein, Harriottin-1 is released and hence the fusion protein band indicates lower molecular weight, Lane 4: untreated recombinant protein includes fusion protein and Harriottin-2, Lane 5: enterokinase treated recombinant protein, Harriottin-2 is released and hence the fusion protein band indicates lower molecular weight.

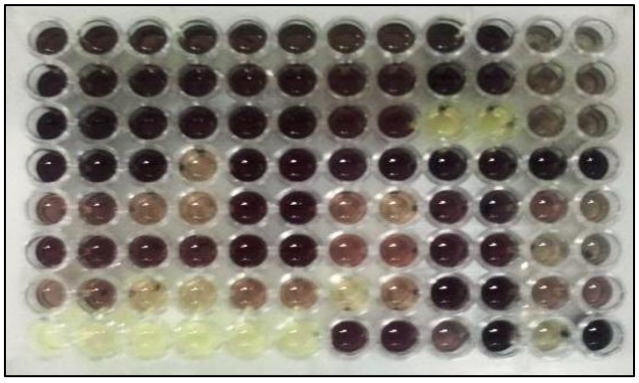
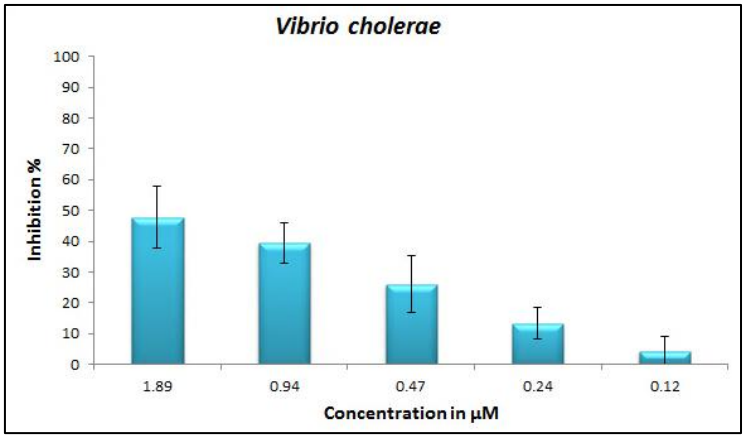
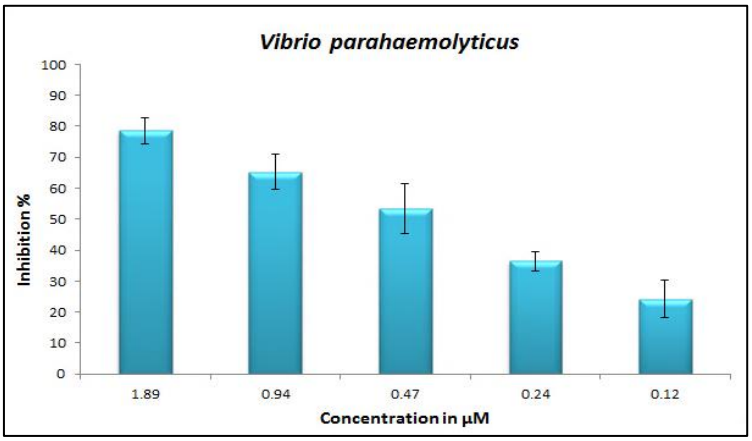


Fig. 5.10. Liquid growth inhibition assay carried out in 96 well microtitre plates. Live bacterial cells reduce MTT to purple coloured formazan. The intensity of purple colour is propotinal to bacterial density. Yellow coloured wells represent no or negligiable bacterial growth.

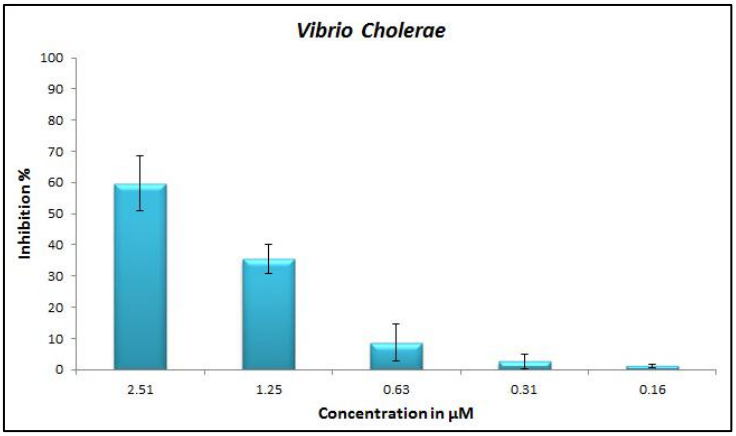


(a)

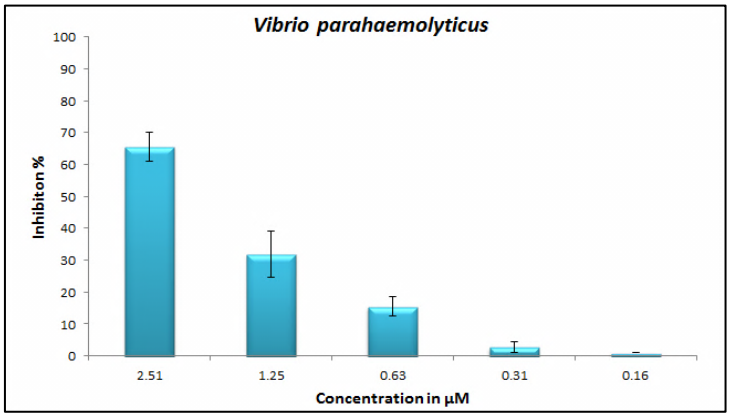


(b)

Fig. 5.11. Antibacterial activity of Harriottin-1 at various tested concentrations against (a) *V. cholera* and (b) *V. parahaemolyticus.*



(a)



(b)

Fig. 5.12. Antibacterial activity of Harriottin-1 at various tested concentrations against (a) *V. cholera* and (b) *V. parahaemolyticus.*

6

Antibacterial and Anticancer Activity of Synthetic Harriottin-3

6.1 Introduction

Bacterial resistance to antibiotics has become a global public health issue with life threatening implications. The ongoing emergence of bacterial resistance to antibiotics continues to spur worldwide research to discover novel anti-infective as alternatives for traditional antibiotics (Gordon et al., 2005). The time required by bacterial strains to develop complete resistance against antibiotics may be much shorter than we expect. Hence, the time is right to develop novel approaches to overcome antibiotic resistance. Currently, antimicrobial peptides enjoy an increasing attention as future antibiotic alternative. They are critical defense molecules that can render protection against pathogenic invasions. Even though, the most studied targets of AMPs are Gram positive and Gram negative bacteria, other targets like fungi, protozoa and enveloped virus have also been described (Hancock and Diamond, 2000; Giuliani et al., 2007). In spite of their immense potential as future magic drugs, AMPs has not yet entered into drug markets mainly because of their toxicity, susceptibility to proteolysis and manufacturing cost.

Nevertheless, antimicrobial peptides are the most promising prospect to be developed as new generation therapeutics.

The potential of antimicrobial peptides as anticancer agents is also being explored (Gasper et al., 2013). Cancer is caused by the growth and spreading of abnormal cells in an uncontrolled manner. Worldwide, millions of people are affected by cancer which remains a major cause of death. In last few decades many efforts have been devoted in developing effective therapy to treat cancer. However, the available chemotherapy methods have a relatively low success rate and also present a risk of reoccurrence (Harris et al., 2011). Numerous chemotherapeutic drugs like DNA-alkylating agents, antimetabolites, and hormone antagonists have been developed to treat cancers. These anticancer agents exhibit insufficient selectivity and subsequently an unspecific targeting of normal mammalian cells with several harmful side effects (Kalyanaraman et al., 2002; Al-Benna et al., 2011). This is mainly because of the inability of current drugs to differentiate between cancer cells and proliferating normal cells, thereby killing both (Gaspar et al., 2013). New anticancer agents need to be identified to overcome the limitations of currently available anticancer drugs and therapies. Antimicrobial peptides have also received attention as potential anticancer agents. Antimicrobial peptides exhibiting activity against cancer cells are often referred to as anticancer peptides (ACPs). Cancer cells have an altered cell membrane composition characterized by presence of anionic molecules such as the phospholipid phosphatidylserine (PS), O-glycosylated mucins, sialylated gangliosides and heparin sulfate, conferring them a net negative charge which is in contrast to zwitterionic nature of normal cells (Hoskin and Ramamoorthy, 2008; Schweizer, 2009). These negatively charged cell membranes of cancerous cell are ideal targets for ACPs (Huang et al., 2014; Oelkrug et al.,

2015). Thus antimicrobial peptides have the potential to be developed into both antimicrobial and anticancer agents.

Our knowledge on AMPs is limited to the small number of antimicrobial peptides which has been found and studied. It is assumed that there are numerous AMPs, still to be discovered from diverse group of organisms. Isolating AMPs sufficient enough to carry out an in-depth analysis from its natural source is not very feasible. Hence, alternative methods of producing AMPs like recombinant expression and/or chemical synthesis are preferred for such studies. Chemical synthesis of peptides is performed by two main techniques, *viz*., Solution phase synthesis (SPS) and Solid phase peptide synthesis (SPPS). In case of SPS, short fragments of required peptide are synthesized first and then they are coupled together to form a long peptide. One of the advantages of SPS is that the intermediate products can be purified and because of his the final desired peptide formed is highly pure (Vigneaud et al., 1953). SPS is easy and inexpensive but the long reaction time required is certainly a disadvantage (Chandrudu et al., 2013).

In SPPS method a resin is used as a support to which growing peptide is attached. The first amino acid (the C-terminus amino acid of the desired peptide) carrying a temporary protecting group on the reactive side chain (amino group) is attached covalently to an insoluble resin. In the next step the protecting group is removed (deprotection) and the resin is washed. Then the next amino acid (with protected amino group) is linked to the deprotected amino acid attached to the resin to form a protected dipeptide. The dipeptide is deprotected and subsequent steps are repeated until the desire peptide sequence is completed (Merrifield, 1963). Boc (t-Butoxycarbonyl) and Fmoc (9-fluorenylmethyloxycarbonyl) protecting groups have often been used for side chain protection (Anderson and McGregor, 1957; Carpino and Han, 1970)

and are removed by trifluoroacetic acid or 20 % piperidine in dimethylformamide, respectively. A limitation of this technique is the cost of synthesis.

As antimicrobial peptides are small sized peptides, SPS is generally employed for accomplishing their chemical synthesis. Several AMPs like Cecropin (Andreu et al., 1983), Mussel defensins (Mitta et al., 1999), Temporin (Yang et al., 2004), Cathelicidin (Chang et al., 2005), Maximin (Wang et al., 2005), Epinecidin (Yin et al., 2006), Arenicin (Ovchinnikova et al., 2007), Arasin 1 (Stensvag et al., 2008), Hepcidin (Pan et al., 2010), Anti-lipopolysaccharide factor (Pan et al., 2010) and Brevinin (Yaghoubi, 2013) have been synthesized using SPS for assessing their antimicrobial potency. Solid phase synthesis of histone derived antimicrobial peptides for antimicrobial analysis was carried out in case of Parasin I identified from *Parasilurus asotus* (Park et al., 1998), Hipposin identified from *Hippoglossus hippoglossus* (Birkemo et al., 2003), Oncorhyncin II identified from *Oncorhynchus mykiss* (Fernandes et al., 2004) and Abhisin identified from *Haliotis discus* (Zoysa et al., 2009).

Three histone H2A derived antimicrobial peptide sequences named Harriottin-1 (52 amino acids), Harriottin-2 (40 amino acids) and Harriottin-3 (21 amino acid) was identified from Sicklefin Chimaera, *Neoharriotta pinnata* (refer chapter 4). In the present study, the smallest of the three peptides, Harriottin-3, was chemically synthesized using SPS. Antibacterial assay of the synthesized Harriottin-3 was carried out against Gram positive and Gram negative bacteria. Anticancer assay against 60 human cancer cell lines and cytotoxicity assay against Human epithelial type 2 (HEp2) cells was also carried out for synthetic Harriottin-3.

6.2 Materials and Methods

6.2.1 Target Peptide

Harriottin-3, an antimicrobial peptide identified from the histone H2A sequence of *Neoharriotta pinnata* was chemically synthesized to test its antibacterial and anticancer activity. The 21 amino acid Hariottin-3 has a molecular weight of 2.44 kDa. The amphipathic nature coupled with net positive charge and α-helical structure gives a strong indication of antimicrobial nature of Harriottin-3.

6.2.2 Chemical Synthesis of Harriottin-3

Chemical synthesis of Harriottin-3 was carried out at VCPBIO Limited, Shenzhen, China by Solid phase procedure of Fmoc chemistry. The peptides were synthesized from C-terminus to N-terminus of the peptide sequence. Crude peptides were purified by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). The molecular masses and purities of the purified peptides were verified by mass spectrum (MS) analysis and HPLC, respectively. The synthetic peptide was supplied in lyophilized powdered form.

6.2.3 Mass Spectrometry Analysis

Mass spectrometry was performed at VCPBIO Limited, China. The analysis was performed using a Thermo Finnigan LCQ Duo mass spectrometer with an electrospray source and Xcaliber software. Sample was dissolved in 50 % (v/v) acetonitrile and 50 % of 0.1 % trifluoracetic acid (v/v) and analyzed by Electrospray Ionization (ESI). The spectra were obtained in

the continuous acquisition mode, scanning from m/z 400 to 2000 at a scan time of 5 s.

6.2.4 Purity determination using HPLC

The purity of synthesized Hariottin-3 was determined using HPLC (at VCPBIO, China). For HPLC purification sample was dissolved in 50 µl of 0.1% trifluoracetic acid. A volume of 10 µl of the sample was injected to HPLC. Inertsil ODS-SP column of dimensions 4.6 mm x 250 mm was used for purification. Solvent system used comprised of Solvent A (0.1 % trifluoroacetic in 100 % acetonitrile) and Solvent B (0.1 % trifluoroacetic in 100 % H_2O). Step gradient system was used for purification. Initially a solvent gradient of 15 % Solvent A and 85 % solvent B was maintained for 0.01 min, followed by a gradient of 40 % solvent A and 60 % solvent B for 25 min. Finally a flow of 100 % solvent A was maintained for 25 min. A flow rate of 1 ml/min was maintained throughout the purification. The eluent was monitored at 220 nm wavelength.

6.2.5 Antibacterial assay

6.2.5.1 Bacterial strains used for assay

Anttibacterial activity of the synthetic Harriottin-3 was tested against two strains of Gram positive bacteria viz., *Bacillus cereus* (MCCB 101) and *Staphylococcus aureus* (MTCC 3061) and five stains of Gram negative bacteria viz., *Edwardsiella tarda* (MTCC 2400), *Pseudomonas aeruginosa* (MCCB 119), *Aeromonas hydrophila* (MCCB 113), *Vibrio cholera* (MCCB 129) and *Vibrio fluvialis* (MCCB 130).

6.2.5.2 Preparation of bacterial suspension

The bacterial suspension was prepared in HEPES buffer and the dilution was adjusted to get 10^3 cells per 10 µl of the suspension. See section 5.2.27.2 of chapter 5 for details.

6.2.5.3 Liquid growth inhibition assay

Protocol used for liquid growth inhibition assay is described in section 5.2.27.3 of chapter 5.

For determining the MIC, initially six concentrations (152.56, 76.28, 38.14, 19.07, 9.54 and 4.77 µM concentrations) of synthetic Harriottin-3 were tested for antibacterial activity against seven bacterial strains. In case of bacterial strains, where more than 50% growth was not observed at the minimum tested concentration (4.77 µM), antibacterial assays were repeated with lower concentrations of the synthetic peptide (2.38, 1.19, 0.6, 0.3, and 0.15 µM concentrations). MIC was estimated by Probit analysis using PriProbit computer based program.

6.2.6 Anticancer Assay

Anticancer assay of synthetic Harriottin-3 against human cell lines was carried out at National Cancer Institute (NCI), Marryland, US. The National Cancer Institute (NCI) 60 human tumour cell line anticancer drug screen (NCI60) developed under Developmental Therapeutics Program (DTP) in the late 1980s functions as an in vitro drug-discovery tool providing service screen with an intention of supporting the cancer research community. The NCI60 screening operation utilizes 60 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney. Before the actual screening assay, the compound is

subjected to virtual screening using computer based virtual screening softwares. The structure of the compound in 'Mol' format is used for virtual screening. The compound will undergo actual screening process only if satisfactory results are obtained in virtual screening stage. The actual screening is a two-stage process, first the activity of 10 µM (single dose) of sample is evaluated against the 60 cell lines. The samples which exhibit significant growth inhibition are further evaluated at five concentration levels against the 60 cell lines.

The structure of Haariottin-3 in Mol format was send to NCI for virtual screening (Fig. 6.1). Afterwards obtaining satisfactory results for virtual screening (results not provided by NCI), NCI requested for 10-12 mg of Harriottin-3 for conducting single dose assay. The single dose (10 µM) of synthetic Harriottin-3 was assayed for its anticancer activity against 60 human cancer cell lines under NCI60 programme. The NCI60 protocol for anticancer screening was employed (at NCI) to test the anticancer activity of Harriottin-3. Briefly, the cell lines were grown in RPMI 1640 medium containing 5 % fetal bovine serum and 2 mM L-glutamine. The cells were then inoculated into 96 well microtiter plates in 100 µL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. After inoculation, the microtiter plates were incubated at 37° C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 hours.

After 24 hours incubation, two plates of each cell line were fixed *in situ* with TCA (Trichloro acetic acid) so as to keep a record of the cell population for each cell line at the time of adding the sample (Tz). Sample (Harriottin-3) was solubilized in dimethyl sulfoxide and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to a concentration of 10 µM with complete medium containing 50 µg/ml

gentamicin. Following addition of sample, the plates are incubated for an additional 48 hours at 37° C, 5 % CO_2, 95 % air, and 100 % relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were then fixed *in situ* by the gentle addition of 50 µl of cold 50 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4° C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each well, and plates were incubated for 10 minutes at room temperature. After staining, unbound dye was removed by washing five times with 1 % acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wave length of 515 nm. The methodology followed for suspension cells was almost same, except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µl of 80 % TCA (final concentration, 16 % TCA). Using the absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of sample (Ti)], the percentage growth was calculated as:

$$\text{Growth \%} = [(Ti-Tz)/(C-Tz)] \times 100$$

6.2.7 Cytotoxicity assay

Cytotoxicity assay of synthetic Harriottin-3 was carried out against Human epithelial type 2 (HEp2) cells. For conducting the assay, approximately 1×10^6 HEp2 cells were inoculated into wells of a 96 well tissue culture plate containing minimal essential medium (MEM). The tissue culture plate was then incubated for 12 hours at 37° C. Following incubation, the wells were washed with phosphate buffered saline and the medium was

exchanged with MEM containing a series of two fold dilutions of Hariottin-3 (102.4, 51.2, 25.6, 12.8, 6.4, 3.2, 1.6 and 0.8 µM concentrations). In control MEM without Harriottin-3 was added. It was then incubated for 24 hours at 37° C. Cells were observed for growth under inverted phase contrast microscope (Leica, Germany). Sulphorhodamine B (SRB) assay for Cytotoxicity was performed using Cytotox-PAN I, Xenometrix, Germany. Absorbance was read at 540 nm in microplate reader (Tecan, USA). The inhibition percentage was calculated as:

Inhibition % = 100 – [(OD of wells with peptide /OD of control) x 100]

The IC_{50} of Harriottin-3 against HEp2 cells was estimated using PriProbit computed based programme.

6.3 Results

6.3.1 Determination of mass and purity

Synthetic Harriottin-3 (12.8 mg) was supplied by VCPBIO Ltd, China in lyophilized powdered form. The Molecular weight of Harriottin-3 (2444.86 Da) was verified by ESI mass spectroscopy. The ESI mass spectrum is shown in Fig. 6.2. The mass spectrum shows the mass to charge ratio (m/z) from 400 to 2000 of all the ionized molecules present in the sample. The most abundant ion in the spectrum is seen at a mass to charge ratio of 612.25. This corresponds to Harriottin-3 ionized to +4 (rounded off MW = 2445 + 4H+ = 2449). The mass to charge ratio is thus 2449/4 = 612.25. Two other relatively abundant ions attributed to Harriottin-3 were m/z of 490 ionized to +5 (MW = 2445 Da + 5H+ = 2450; 2450/5 = 490) and 816 (MW = 2245 + 3 = 2248; 2248/3 = 816). After confirming that the peptide had correct molecular weight,

the purity of the peptide was determined by HPLC analysis. The HPLC chromatogram of synthetic Harriottin-3 is shown in Fig. 6.3. The peptide was found to be 74.63 % pure by the percent area of the main peak at retention time 8.298 minutes as seen in Fig. 6.3.

6.3.2 Antibacterial assay

Antibacterial assay of Harriottin-3 was carried out against two Gram positive and five Gram negative bacteria using liquid growth inhibition assay. Antibacterial activity was observed against all tested bacteria. In general, Gram positive bacteria were found to be more resistant to Harriottin-3 than Gram negative bacteria. Harriottin-3 exhibited an estimated MIC of 348.98 µM against *Bacillus cereus* and 231.7 µM against *Staphylococcus aureus*. In case of tested Gram negative bacteria, *Edwardsiella tarda* was the only bacteria to exhibit resistance against the peptide with an inhibition % of only 31.6 at the highest tested concentration. Since an inhibition of less than 50 % was obtained at the highest tested concentration, MIC was not estimated for *E. tarda*. Harriottin-3 exhibited an MIC of 38.14 µM, 76.28 µM and 9.54 µM against *Aeromonas hydrophila*, *Vibrio fluvialis* and *Vibrio cholera* respectively. *Pseudomonas aeruginosa* was found to be the most sensitive bacteria to Harriottin-3 (MIC of 2.38) among all tested bacteria. The antibacterial activity of Harriottin-3 against the tested bacteria is shown in Fig. 6.4 (a-g). Harriottin-3 demonstrated potent antibacterial activity against the tested bacteria and activity could be detected down to a concentration of 0.15 µM in case of *Vibrio cholera* and *Pseudomonas aeruginosa*.

6.3.3 Anticancer assay

Anticancer activity of Harriottin-3 against 60 human cancer cell lines was tested at NCI, US. The growth % of cancer cell lines in media containing

10 µM of Harriottin-3 was determined. The peptide exhibited selective anticancer activity against K-562 cell line (human erythroleukemic cell line). The growth of K-562 cells was reduced to 53.27 % in presence of 10 µM Harriottin-3. Minor reduction in growth was also observed in case of HCC-2998 (colon cancer) and BT-549 (Breast cancer) cell lines where growth was observed to be 90.62 % and 92.62 % respectively. The results of NCI60 screening of Harriottin-3 against 60 human cancer cell lines is represented in table. 6.1 and Fig. 6.5.

Table. 6.1. Growth % of tested human cancer cell lines (NCI60) against 10 µM of Harriottin-3.

Cell Lines		**Growth %**
Leukemia		
1)	CCRF-CEM	98.85
2)	HL-60(TB)	100.94
3)	K-562	53.27
4)	MOLT-4	98.41
5)	RPMI-8226	102.18
6)	SR	115.43
Non-Small Cell Lung Cancer		
1)	A549/ATCC	98.98
2)	HOP-62	100.71
3)	HOP-92	116.53
4)	NCI-H226	100.02
5)	NCI-H23	100.94
6)	NCI-H322M	104.98
7)	NCI-H460	102.84
8)	NCI-H522	100.50
Colon Cancer		
1)	COLO 205	106.24
2)	HCC-2998	90.62
3)	HCT-116	99.85

4)	HCT-15	102.58
5)	HT29	101.26
6)	KM12	107.84
7)	SW-620	107.97
CNS Cancer		
1)	SF-268	113.33
2)	SF-295	106.57
3)	SF-539	94.69
4)	SNB-19	110.86
5)	SNB-75	104.82
6)	U251	95.38
Melanoma		
1)	LOX IMVI	104.08
2)	MALME-3M	125.22
3)	M14	105.58
4)	MDA-MB-435	104.04
5)	SK-MEL-2	106.30
6)	SK-MEL-28	108.76
7)	SK-MEL-5	101.49
8)	UACC-257	100.42
9)	UACC-62	99.59
Ovarian Cancer		
1)	IGROV1	105.21
2)	OVCAR-3	105.81
3)	OVCAR-4	103.46
4)	OVCAR-5	103.18
5)	OVCAR-8	102.11
6)	NCI/ADR-RES	103.77
7)	SK-OV-3	106.88
Renal Cancer		
1)	786-0	98.54
2)	ACHN	101.05
3)	CAKI-1	100.50
4)	RXF 393	102.16
5)	SN12C	107.05
6)	TK-10	106.30

7)	UO-31	105.49
Prostate Cancer		
1)	PC-3	102.33
2)	DU-145	107.91
Breast Cancer		
1)	MCF7	104.71
2)	MDA-MB-231/ATCC	112.40
3)	HS 578T	112.24
4)	BT-549	92.62
5)	T-47D	103.33
6)	MDA-MB-468	114.36

6.3.4 Cytotoxicity assay

Cytotoxicity assay of Harriottin-3 was carried out against Human epithelial type 2 (HEp-2) Cells using Sulphorhodamine B (SRB) assay. The peptide was found to be cytotoxic to HEp2 cells at higher concentration. A reduction of 28.26 % was observed for HEp2 cells at the highest tested concentration of Harriottin-3 (102.4 µM). The IC_{50} of Harriottin-3 against HEp2 cells was estimated to be 239.2 µM. The cytotoxic activity exhibited by Harriottin-3 against HEp2 cells is represented in Fig. 6.6 (a-b).

6.4 Discussion

In the present study, antibacterial, anticancer and cytotoxicity assay of a 21 amino acid histone H2A derived AMP, designated as Harriottin-3, identified from Sicklefin Chimaera is described. The antimicrobial and anticancer activity of histone derived AMPs have been reported from various invertebrate and vertebrate species (Kawasaki and Iwamuro, 2008). However, there are no previous reports of an antimicrobial peptide derived from

Sicklefin Chimaera histone H2A. Harriottin-3 is a 21 amino acid α-helical peptide having a molecular weight of 2.44 kDa, a net positive charge (+ 7.1, + 5.4 and + 4.0 at pH 4, 7 and 10 respectively), p*I* of 12.6 and hydrophobicity of 28%. Also, the characteristic similarities of Hariottin-3 to other histone H2A derived AMPs suggest that histone H2A derived AMPs from both vertebrates and invertebrates have a common ancestral gene (discussed in detail in chapter 4).

Harriottin-3 is similar in amino acid length, sequence and structure to 21 amino acid histone H2A derived AMP, Buforin-II. Only a difference of two amino acids at position 1 (Ser in Harriottin-3 and Thr I Buforin-II) and 18 (His in Harrittin-3 and Lys in Buforin-II) was noted between Harriottin-3 and Buforin-II. It has been reported that Buforin-II kills bacterial cells and cancer cells by readily entering the cells in vivo and by interacting with intracellular nucleic acids (Park et al. 2000; Uyterhoeven et al. 2008; Lee et al., 2008). Structural studies have indicated that Pro_{11} serves as a hinge between a C-terminus helix and an N-terminal extended helical region (Yi et al. 1996). This Pro_{11} is required for cell penetration activity of Buforin-II (Park et al., 1998). Harriottin-3 also has a Pro at position 11 and share a structural similarity to Buforin-II. Hence it can be assumed that the antibacterial and anticancer activity of Harriottin-3 is due to its cell penetrating ability.

Peptides derived from N-terminus of histone H2A was reported to exhibit broad spectrum antibacterial activity against both Gram positive and Gram negative bacteria (Park et al., 1996; Park et al., 1998; Richards et al., 2001; Birkemo et al., 2003; Fernandes et al., 2004; Patat et al., 2004; Li et al., 2007; Sook et al., 2008; De Zoysa et al., 2009). The results obtained for Harriottin-3 was in agreement with the previous reports as it exhibited antibacterial activity against the tested Gram positive and Gram negative

bacteria. The antibacterial activity of Harriottin-3 was found to be concentration dependent as it increased with increase in peptide concentration. Its antibacterial activity was detected down to a concentration of 0.15 μM at which it inhibited the growth of *V. cholera* by 48.44 % and *P. aeruginosa* by 24.14 %. *E. tarda* was the most resistant bacteria against Hariottin-3. It exhibited a growth percentage of 68.4 even at the highest tested concentration (152.56 μM) of Harriottin-3. Histone H2A derived peptides reported from various organisms demonstrate potent antibacterial activity against Gram positive and Gram negative bacteria but its activity appears to be variable. Many previously reported histone H2A derived AMPs, like those from Trout (Fernandes et al., 2002), Pacific white shrimp (Patat et al., 2004), Scallop (Li et al., 2007) and Disk abalone (Zoysa et al., 2009) have shown to exhibit stronger antibacterial activity against Gram positive bacteria than Gram negative bacteria. No marked difference in the antibacterial activity against Gram positive bacteria and Gram negative bacteria was observed for Parasin I (Park et al, 1998) and Hipposin (Birkemo et al., 2003). Harriottin-3 was found to be more active against Gram negative bacteria than Gram positive bacteria. The reason for variability in antibacterial activity of N-terminus region of histone H2A reported from various species needs to be investigated for fully understanding the role of histone H2A in innate immunity.

In the study, the anticancer activity of synthetic Harriottin-3 against 60 human cancer cell lines was also determined. Harriottin-3 was found to be highly specific to K-562 cell line categorized under Leukemia cell lines. Harriottin-3 inhibited the growth of K-562 cells by 46.73 % at a concentration of 10 μM. At the same concentration, the peptide also reduced the growth of HCC-2998 (colon cancer cell line) by 9.38 % and BT-549 (breast cancer cell line) by 7.38 % which suggest that at higher concentration Harriottin-3 can

exhibit significant activity against HCC-2998 and BT-549 cells. Lee et al. (2008) studied the anticancer activity of buforin IIb, a synthetic derivative of buforin II against the same 60 human cancer cell lines. Buforin IIb was found to be active against all of the tested cancer cell lines with an IC_{50} ranging from 7.2 to 17.6 µg/ml. The negatively charged gangliosides present abundantly in the plasma membrane of cancerous cells were found to be the main target of buforin-IIb. Hence it can be said that gangliosides allow buforin IIb to distinguish cancer cells from normal cells. After interacting with gangliosides buforin IIb penetrate the cell membrane and bring about apoptosis by disrupting the mitochondrial membrane. However, the precise mechanism of buforin IIb-mediated apoptosis is not understood. Harriottin-3 shows a striking similarity to buforin IIb and hence is expected to identify and kill cancer cells by a mechanism similar to that of buforin IIb. It is not necessary that gangliosides alone be the target of Harriottin-3, but negative charged phosphatidylserine (PS) and/or heparan sulfate (HS) found on the cell membrane of cancer cells can also be its possible targets. Unlike buforin IIb, Harriottin-3 is highly specific to K-562 cells and hence has the potential to be developed into a drug aimed specifically at treating patients suffering from erythroleukemia.

One of the most desired properties of AMPs is its low or no toxicity to eukaryotic cells. The cytotoxicity of Harriottin-3 was determined against HEp2 cells using Sulphorhodamine B (SRB) assay. The IC_{50} of Harriottin-3 against HEp2 cells was estimated to be 239.2 µM which is almost 24 folds the concentration required to reduce the growth of K-562 cells (a human erythroleukemic cell line) by 46.73 %. The estimated IC_{50} against HEp2 cells was found to be much more than the MIC of Harriottin-3 against the tested Gram negative bacteria. The MIC of Harriottin-3 against the Gram positive

bacteria was found to be almost equal to or more than the IC_{50} against HEp2 cells. The antibacterial activity of Harriottin-3 against more Gram positive bacteria needs to be carried out before affirming its ability to restrain the growth of these bacterial strains without being toxic to normal cells. The results from the present study indicate that Harriottin-3 can only be a supplement to other drugs used for controlling the growth of Gram positive bacteria. However, the much lower MIC obtained for Gram negative bacteria suggest that Harriottin-3 have the potential to be developed into a therapeutic agent to specifically restrict the growth of Gram negative bacteria at safe levels of administration. The high specificity of Harriottin-3 to K-562 cells indicates the high potential of Harriottin-3 to be developed into an anticancer agent against erythroleukemia.

Conclusion

In the present study, the antibacterial and anticancer activity of a 21 amino acid AMP, named as Harriottin-3, identified from the histone H2A of Sicklefin Chimaera, *Neoharriotta pinnata* has been reported. Harriottin-3 demonstrated antibacterial activity against the tested Gram positive and Gram negative bacteria. Harriottin-3 was found to be much more active against Gram negative bacteria (MIC ranged from 2.38 to 76.28 µM) than Gram positive bacteria (MIC ranged from 231.7 to 348.98 µM). Harriottin-3 displays strong selective anticancer activity against K-562 human cancer cell line, reducing the growth of cancer cells by 46.73 % at a concentration of 10 µM. Cytotoxic assay of Harriottin-3 was conducted against HEp2 cells and the IC_{50} was estimated to be 239.2 which is much higher than the concentration required to reduce the growth of K-562 cells and the MICs obtained for Gram negative bacteria. These results suggest Harriottin-3 to be a potential anticancer and antibacterial agent and its precursor histone H2A might be

involved in the innate immune defenses of Sicklefin Chimaera. The 21 amino acid histone H2A derived AMP, Harriottin-3 has all the qualities required to be developed into an ideal therapeutic agent against erythroleukemia and infections caused by Gram negative bacteria.

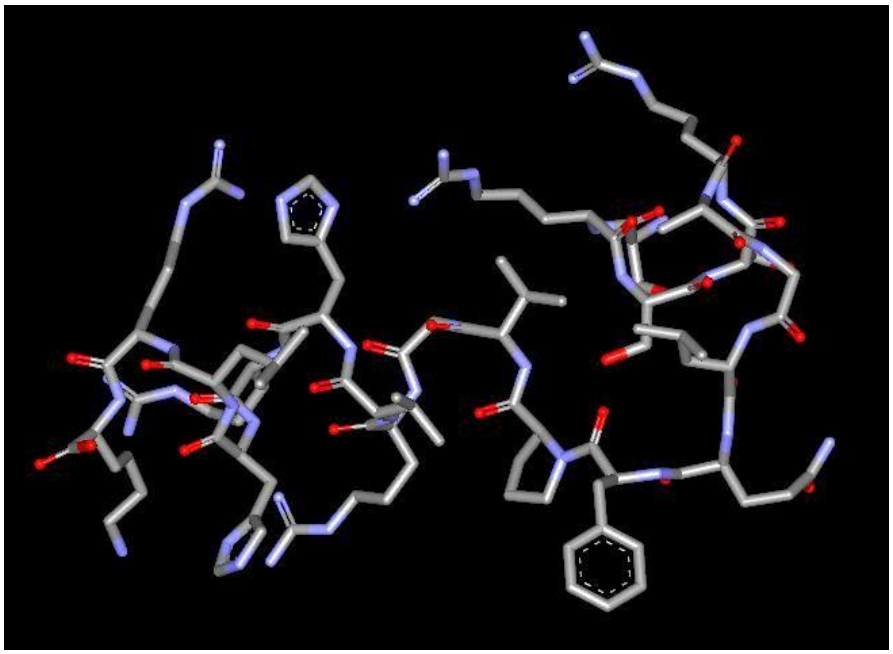


Fig. 6.1. The 2-D image of structure of Harriottin-3 (‘Mol’ format) send to NCI for virtual screening against cancer cells.

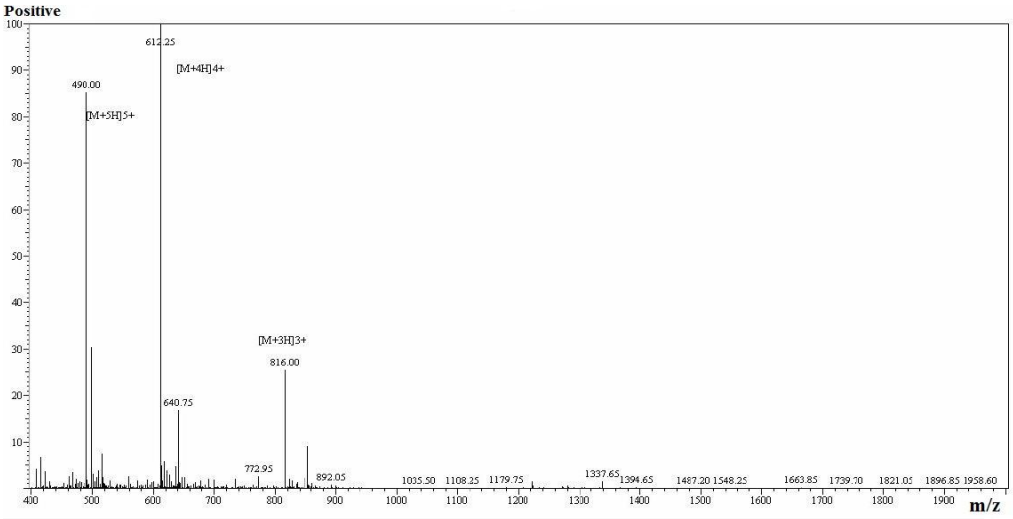


Fig. 6.2. ESI Mass Spectrum of Synthetic Harriottin-3. Most abundant ion in spectrum is seen at m/z of 612.25 [M+4H]4+ followed by 490.00 [M+5H]5+ and 816.00 [M+3H]3+.

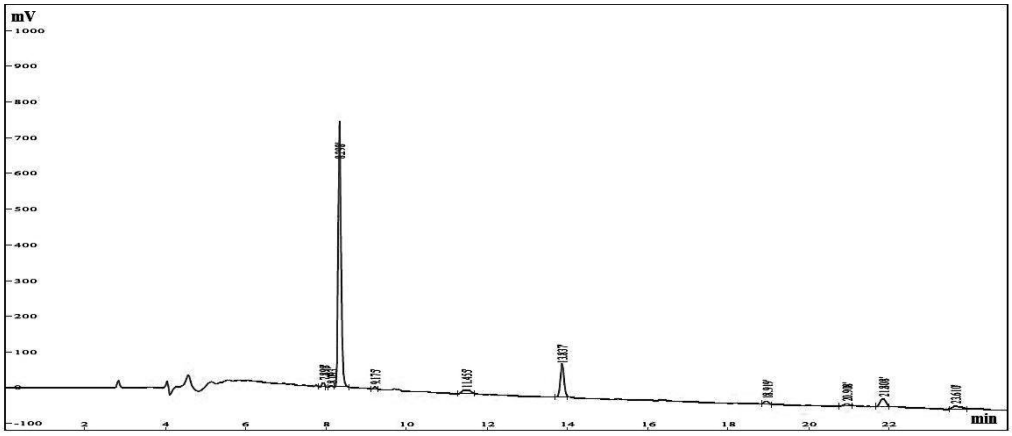
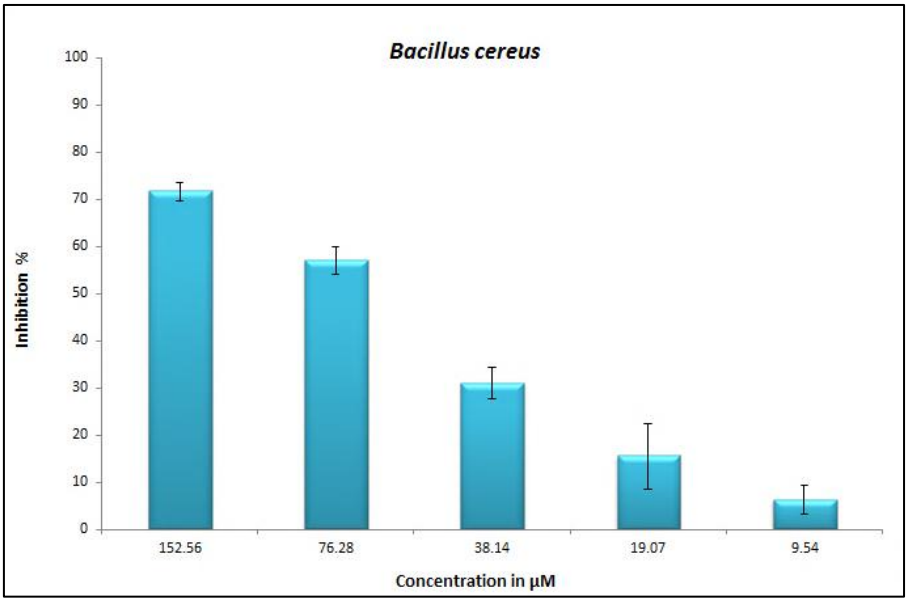
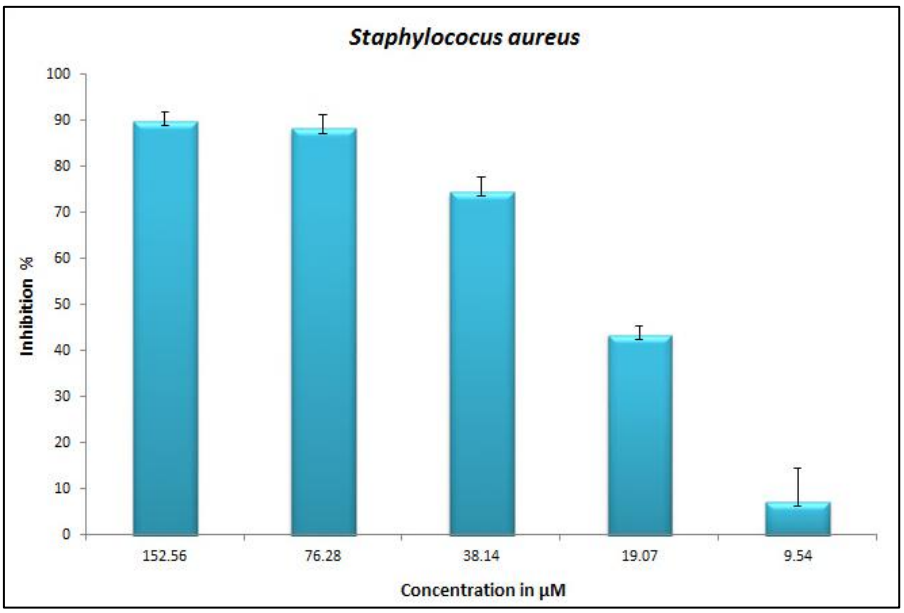


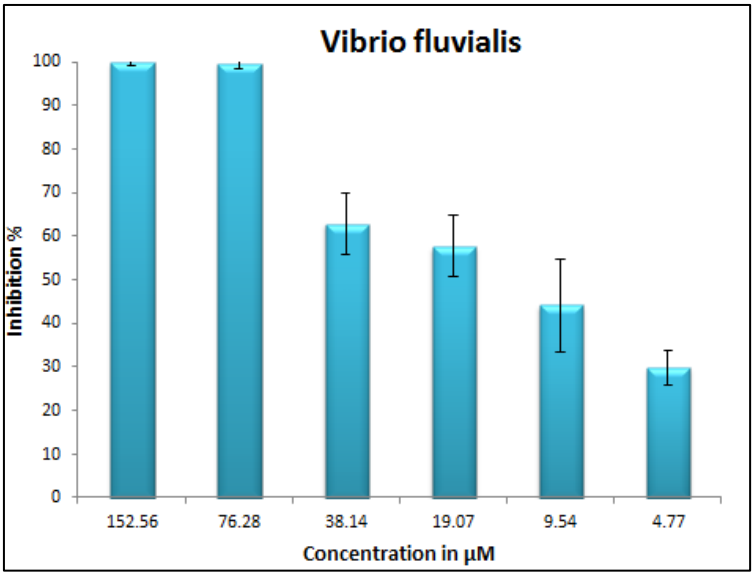
Fig. 6.3. HPLC chromatogram of synthetic Harriottin-3. The major peak is seen at retention time of 8.298 min. The peak has an area of 3706678 out of total area of 4966953 (by all peaks) which corresponds to a purity of 74.63 %.


Bacillus cereus
Inhibition %
100
90
80
70
60
50
40
30
20
10
0
152.56
76.28
38.14
19.07
9.54
Concentration in µM

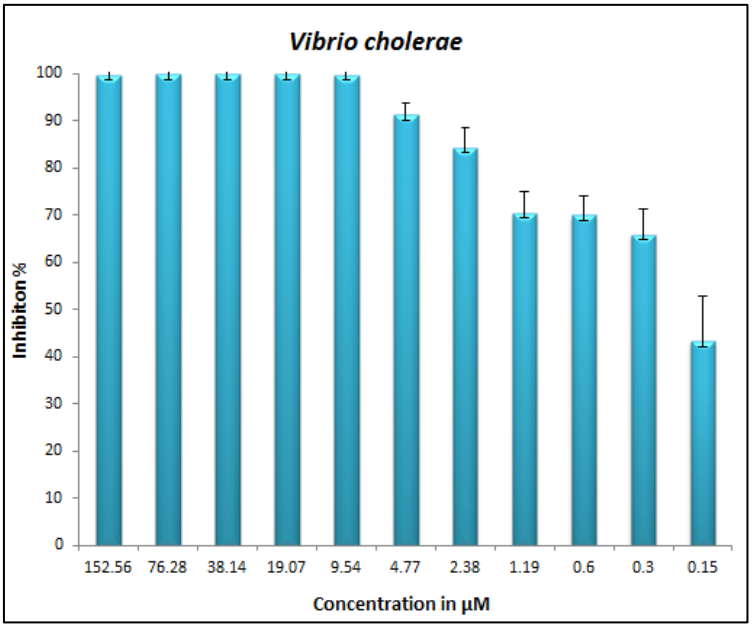
(a)


Staphylococus aureus
Inhibition %
100
90
80
70
60
50
40
30
20
10
0
152.56
76.28
38.14
19.07
9.54
Concentration in µM

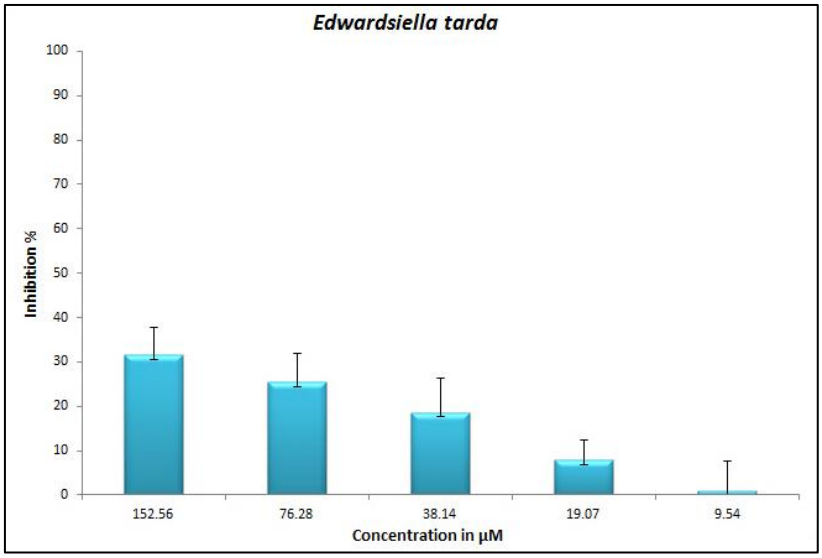
(b)


Vibrio fluvialis
Inhibition %
100
90
80
70
60
50
40
30
20
10
0
152.56
76.28
38.14
19.07
9.54
4.77
Concentration in µM

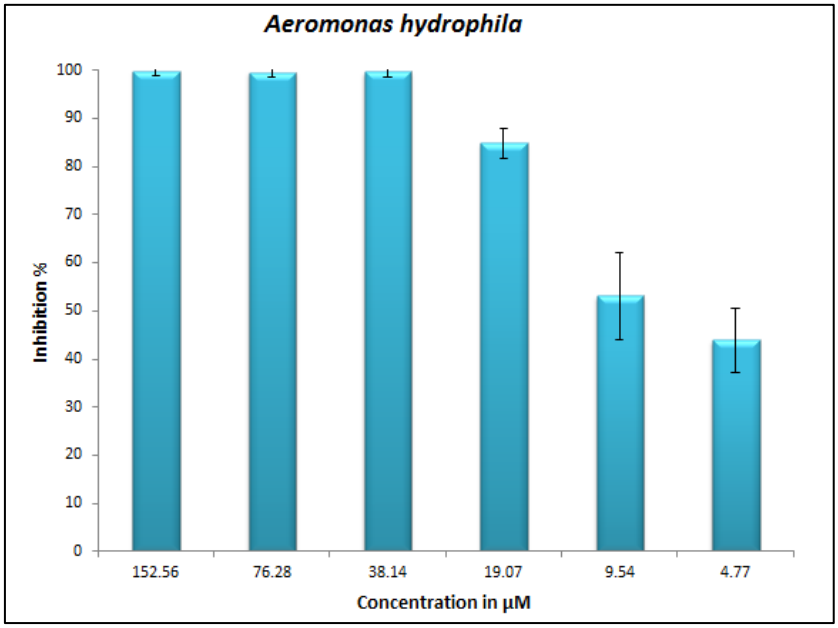
(c)


Vibrio cholerae
Inhibiton %
100
90
80
70
60
50
40
30
20
10
0
152.56
76.28
38.14
19.07
9.54
4.77
2.38
1.19
0.6
0.3
0.15
Concentration in µM

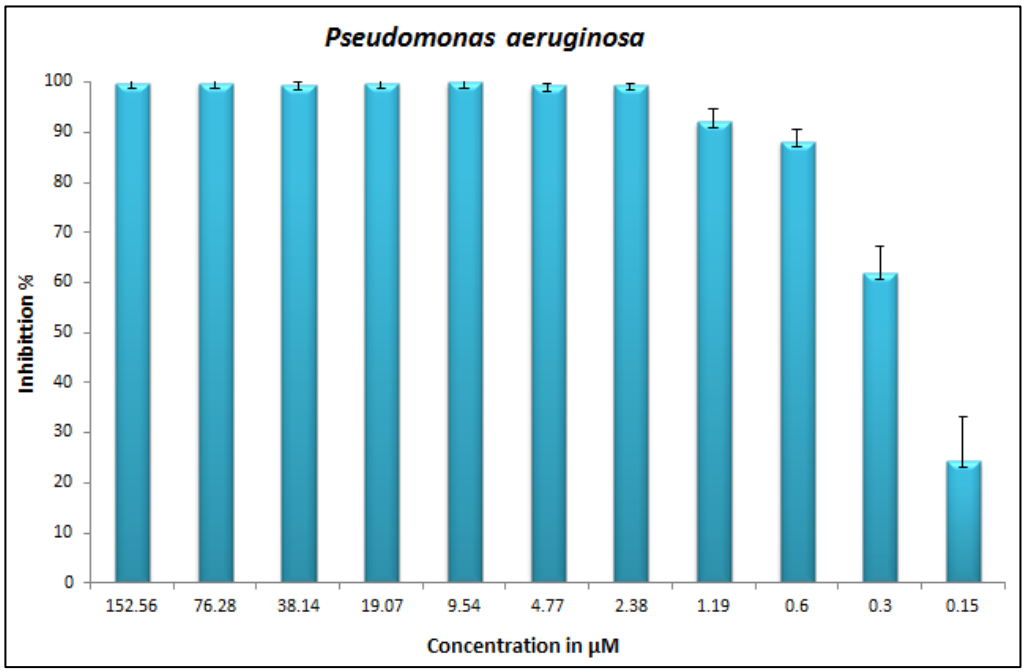
(d)


Edwardsiella tarda
Inhibition %
100
90
80
70
60
50
40
30
20
10
0
152.56
76.28
38.14
19.07
9.54
Concentration in µM

(e)


Aeromonas hydrophila
Inhibition %
100
90
80
70
60
50
40
30
20
10
0
152.56
76.28
38.14
19.07
9.54
4.77
Concentration in µM

(f)



(g)

Fig. 6.4. (a-g). Antibacterial activity of Harriottin-3 against the tested bacteria at various tested concentration is shown.

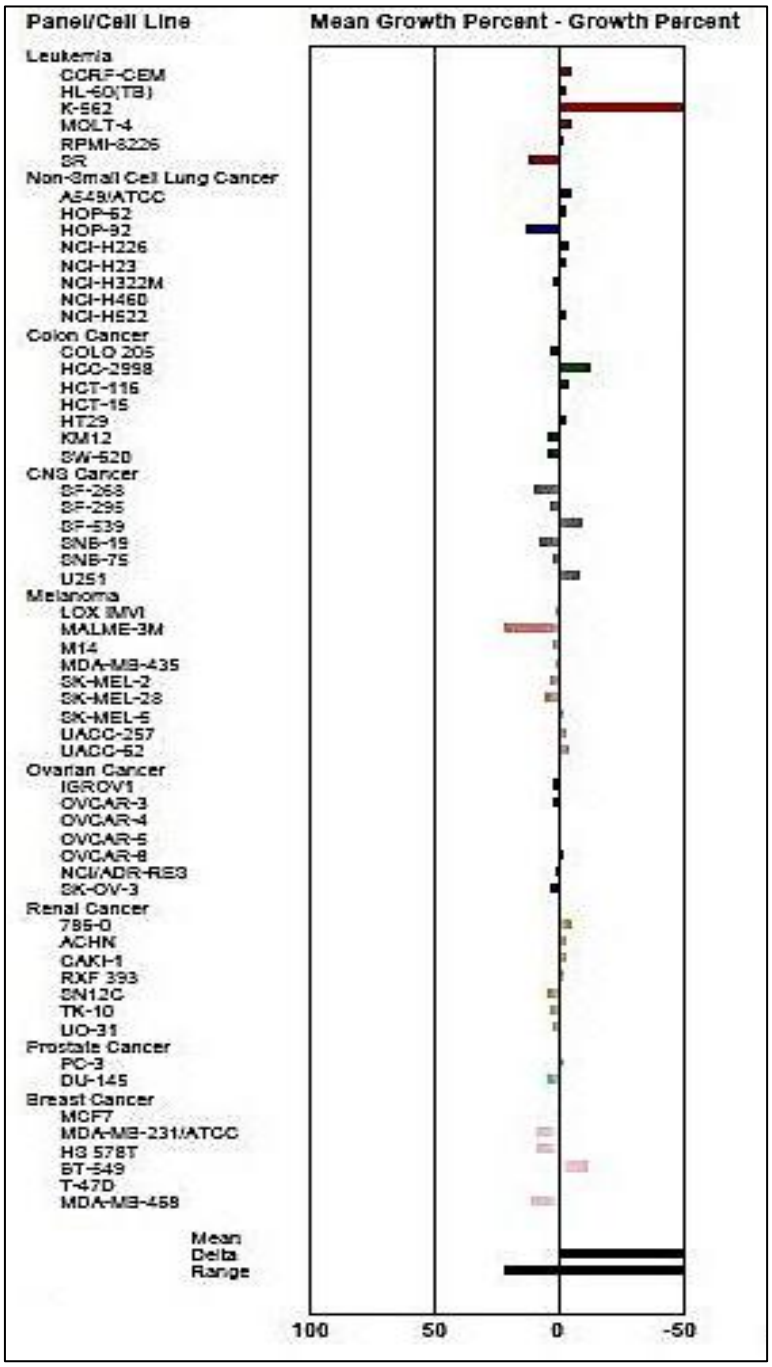
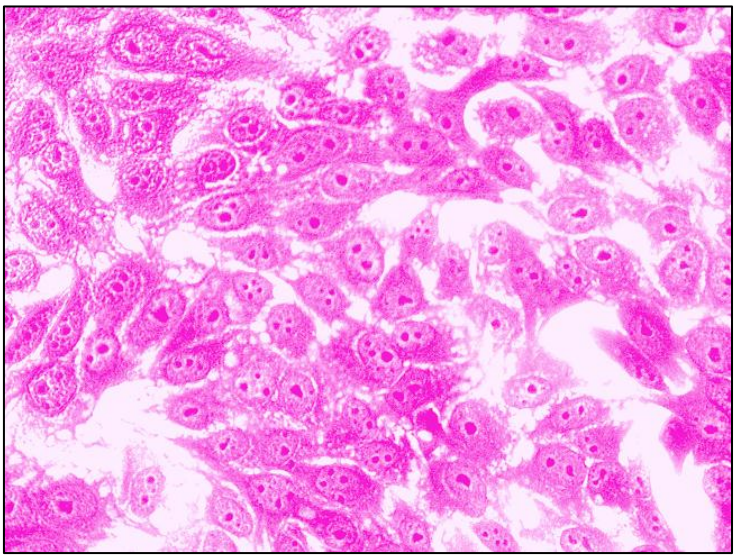
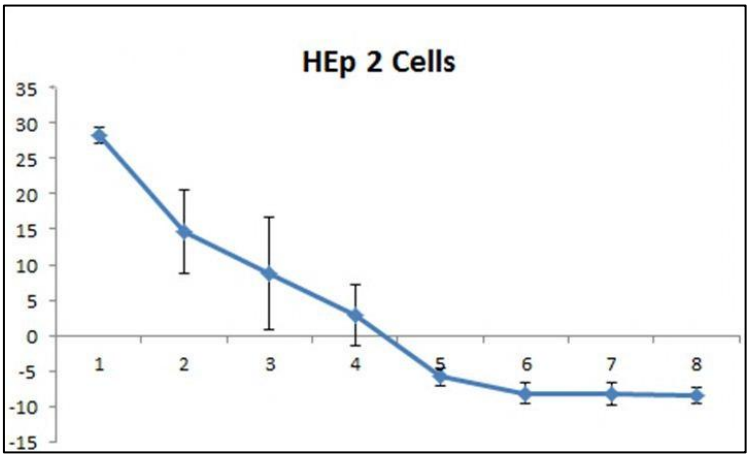


Fig. 6.5. Results of anticancer assay performed against 60 human cancer cell lines as provided by NCI, USA.



(a)



(b)

Fig. 6.6. Cytotoxicity assay of Harriottin-3 against HEp2 cells. (a) Sulforhodamine B bound with cellular proteins of HEp2 cells; (b) Activity of Harriottin-3 against HEp2 cells at various tested concentrations is shown

7

Summary and Conclusion

Antimicrobial peptides (AMPs) are receiving great attention as potential alternatives to overcome bacterial resistance to conventional antibiotics. The last two decades witnessed the discovery of hundreds of AMPs from plants, animals and microorganisms. These peptides are components of innate defense mechanisms exhibited by these organisms. Marine organisms ranging from lower invertebrates to teleost fishes are continuously exposed to high microbial density. These organisms produce a range of AMPs varying greatly in their size, structure and other physicochemical properties. Investigating novel antimicrobial peptides from marine organisms can provide new insight into the immune response of these organisms and a possibility of discovering new and effective drugs in medicine/aquaculture. In the present study, gene based approach was employed for identifying a number of histone H2A derived AMPs with novel sequences from molluscs and cartilaginous fishes thriving in marine environment.

The objectives of the study were:

- Detection and molecular characterization of histone derived Antimicrobial Peptides from marine organisms.

- Recombinant Expression of histone based AMP Genes and testing its bioactivity.
- Characterization and testing the bioactivity of histone derived synthetic antimicrobial peptides.

Salient Findings

- ✓ A 5.42 kDa, 51 amino acid AMP was identified from the histone H2A sequence obtained from Marine Clam *Sunetta scripta* and named as Sunettin (GenBank ID**: HQ720149**).
- ✓ Sunettin was found to have an α-helical structure, a net positive charge of +13.8, +12.1 and +6.9 at pH of 4, 7 and 10 respectively, hydrophobicity of 29 % and an isoelectric point of (pI) of 11.9.
- ✓ Phylogenetic analysis revealed that histones are derived from a common ancestor. Histone H2A sequence of *Sunetta scripta* grouped along with the cluster formed by invertebrate histones.
- ✓ A 2.84 kDa, 25 amino acid histone H2A derived AMP, Molluskin was identified from marine molluscs *viz.*, *B. vittata* (GenBank ID: **HQ720143**), *C. madrasensis* (GenBank ID: **HQ720145**), *F. gracilis* (GenBank ID: **HQ720146**), *S. cucullata* (GenBank ID: **HQ720147**) and *M. casta* (GenBank ID: **HQ720148**)
- ✓ Molluskin, which exhibited similarity to previously reported histone H2A derived AMPs, has an α-helical structure and a net positive charge of +7.1, +6.1 and +4.0 at pH 4, 7 and 10 respectively. A hydrophobicity of 32 % and p*I* of 12.18 was estimated for Molluskin.

- ✓ In phylogenetic tree constructed using histone H2A sequences, the sequences obtained from *C. madrasensis, S. cucullata, M. casta, F. gracilis* and *B. vittata* was found to align along with invertebrate histone sequences.

- ✓ A 4.87 kDa, 39 amino acid, histone H2A derived AMP named as Himanturin was identified from the histone H2A sequence of Round whip ray, *Himantura pastinacoides* (GenBank ID: **HQ720150)**.

- ✓ Physicochemical properties estimated for Himanturin revealed an α-helical structure with a hydrophobicity of 32 %, p*I* of 11.83 and a net positive charge of +9.8, +8.1 and +4.5 at pH 4, 7 and 10 respectively.

- ✓ In phylogenetic tree constructed using histone H2A sequences of the Round whip ray grouped along with the cluster formed by histone sequences of fishes and amphibians.

- ✓ The cytochrome Oxidase-I (CO1) gene sequence was amplified and sequenced to confirm the taxonomic identification of Round whip ray. The CO1 gene sequence was deposited to NCBI database (GenBank ID: **JN982361)**.

- ✓ Three AMP sequences named Harriottin -1 (5.56 kDa, 52 amino acids), Harriottin-2 (4.39 kDa, 39 amino acids) and Harriottin-3 (2.44 kDa, 21 amino acids) having sequence similarity with previously reported histone H2A derived AMPs were identified from histone H2A sequence of Sicklefin Chimaera, *Neoharriotta pinnata*.

- ✓ The histone H2A nucleotide sequence and deduced amino acid sequence obtained from Sicklefin Chimaera was deposited to NCBI database (GenBank ID**: JX297204)**.

- ✓ Harriottin-1, 2 and 3 were found to have an α-helical structure with a hydrophobicity of 30 %, 25 % and 28 % respectively and a p*I* of 12.01, 12.41 and 12.60 respectively. At pH 4, 7 and 10, Harriottin-1 was found to have a charge of +14.8, +12.4 and +7.4; Harriottin-2, a net positive charge of +14.1, +12.4 and +7.9 while Harriottin-3 displayed a net positive charge of +7.1, +5.4 and +4.

- ✓ The histone H2A nucleotide based phylogenetic analysis revealed Sicklefin Chimaera to occupy a position in between the invertebrate and vertebrate groups.

- ✓ The cytochrome Oxidase-I (CO1) gene sequencing (GenBank ID: **JX297203)** confirmed the taxonomic identification of the species to be *Neoharriotta pinnata*. The CO1 gene based phylogenetic analysis also showed *Neoharriotta pinnata* to occupy a position in between the groups formed by vertebrates and invertebrates.

- ✓ Recombinant expression of Harriottin-1 (5.56 kDa) and Harriottin-2 (4.39 kDa) was successfully carried out using pET32a+ expression vector system and Rosetta-gami^{TM} B (DE3) pLysS expression host system.

- ✓ Recombinant AMP, Harriottin-1 exhibited antibacterial activity against *V. cholerae* (MIC 104.8 μM) and *V. parahaemolyticus* (MIC 32.11 μM).

- ✓ Recombinant AMP, Harriottin-2 also exhibited antibacterial activity against *V. cholerae* (MIC 12.08 μM) and *V. parahaemolyticus* (MIC 12.93 μM).

- ✓ Synthetic AMP, Harriottin-3 (synthesized at VCPBIO Pvt Ltd, China) exhibited activity against the tested Gram positive [*B. cereus* (MIC 348.98 µM) and *S. aureus* (MIC 231.7 µM)] and Gram negative bacteria [*V. choleare* (MIC 9.54 µM), *V. fluvialis* (MIC 76.28 µM), *P. aeruginosa* (MIC 2.38 µM) and *A. hydrophila* (MIC 38.14 µM)].
- ✓ Synthetic Harriottin-3 exhibited anticancer activity in terms of inhibition of the growth of K562 human cancer cells by 46.7% at a concentration of 10 µM.
- ✓ Cytotoxicity assay against HEp2 cells revealed synthetic Harriottin-3 less cytotoxic and the IC_{50} was found to be 239.2 µM.

The marine environment, with its enormous biodiversity remains a largely untapped reservoir of bioactive peptides. The present study was carried out to identify histone derived novel AMP sequences from marine organisms. As an outcome of the study novel histone H2A derived sequences i.e., Sunettin and Molluskin were reported from marine Molluscs, Himanturin from Round Whip Ray and Harriottins (1, 2 and 3) from Sicklefin Chimaera. Recombinant AMPs, Harriottin-1 and Harriottin-2 exhibited significant antibacterial activity against the tested *Vibrio* spp. Synthetic AMP, Harriottin-3 demonstrated broad spectrum activity against the tested Gram positive and Gram negative bacteria and also exhibited anticancer activity against K562 human cancer cell line. These histone H2A derived peptides identified from marine organisms have the potential to be developed into antibacterial and anticancer agents. From among the histone derived AMPs identified in the present study, Harriottin-3 appears to be most promising candidate with potential for application in therapeutics and aquaculture. It is quite evident from available literature that histones do play a role in innate immune defenses of an organism. However, to

better understand the role of histone proteins in immunity, the research should be focused on deciphering the mechanism by which histone proteins of antimicrobial nature are specifically regulated at transcriptional/translational /protein cleavage level. Histone derived peptides are increasingly being regarded as lead molecules for therapeutic use against pathogens and cancer.

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1) *Sunetta scripta* histone H2A mRNA, complete cds
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   GenBank ID: **HQ720150.2**
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   GenBank ID: **HQ720148.1**
4) *Ficus gracilis* histone H2A mRNA, partial cds
   GenBank ID: **HQ720146.1**
5) *Saccostrea cucullata* histone H2A mRNA, partial cds
   GenBank ID: **HQ720147.1**
6) *Crassostrea madrasensis* histone H2A mRNA, partial cds
   GenBank ID: **HQ720145.1**
7) *Bullia vittata* histone H2A mRNA, partial cds
   GenBank ID: **HQ720143.1**
8) *Neoharriotta pinnata* histone H2A mRNA, partial cds
   GenBank ID: **JX297204.1**
9) *Himantura pastinacoides* cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
   GenBank ID: **JN982361.1**
10) *Neoharriotta pinnata* voucher RP2012-CR291 cytochrome oxidase subunit I gene, partial cds; mitochondrial
   GenBank ID: **JX297203.1**

Publications

List of Publications

1) Sathyan, N., Philip, R., Chaithanya, E. R., Kumar, P. R. A. & Antony, S. P. (2012). Identification of a histone derived, putative antimicrobial peptide Himanturin from round whip ray *Himantura pastinacoides* and its phylogenetic significance. *Results in Immunology* 2: 120-124.

2) Sathyan, N., Philip, R., Chaithanya, E. R. & Kumar, P. R. A., (2012). Identification and molecular characterization of Molluskin, a histone H2A derived antimicrobial peptide from representatives of phylum Mollusca. *ISRN Molecular Biology* 2012, pp 6; Article ID 219656.

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Short Communication

Identification of a histone derived, putative antimicrobial peptide Himanturin from round whip ray *Himantura pastinacoides* and its phylogenetic significance

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ABSTRACT

Histone H2A participates in host defense responses by producing antimicrobial peptides (AMPs). The present study deals with identification of a putative antimicrobial sequence, Himanturin from the histone H2A of Round Whip Ray, *Himantura pastinacoides*. A 204 bp fragment encoding 68 amino acid residues was amplified from cDNA of Round Whip Ray, *H. pastinacoides*. Himanturin exhibited high similarity to previously reported histone H2A derived AMPs indicating the presence of an antimicrobial sequence motif. Physicochemical properties of Himanturin suggest it to be a potential antimicrobial candidate.

1. Introduction

Antimicrobial peptides (AMPs) are an important component in the innate immune system of almost all multicellular organisms [11,4,28,35,13,18,9]. They are generally defined as low molecular weight, amphipathic peptides which are mostly cationic. AMPs show wide divergence in their amino acid composition, size and conformational structures but exhibit striking similarity in their mode of action [22,5,29,17,33,23]. They have retained their antimicrobial activity against a broad spectrum of pathogenic organisms, despite of their ancient and wide spread presence in nature [41,22]. Remarkable specificity to prokaryotes with low toxicity to eukaryotic cells has favored their investigation and exploitation as new antibiotics [40]. Aquatic organisms rely mainly on their innate immune defenses for protection against pathogenic organisms and hence should be considered as potential sources of antimicrobial peptides.

AMPs derived from histone proteins form an important category of peptide antibiotics [14]. Traditionally, histones are known as major components of the nucleosome structures in eukaryotic cells. Histone proteins play a key role in the innate immune defense of organisms by forming AMPs. These AMPs are derived from their histone precursors by the action of proteolytic enzymes. Histone derived AMPs with potent activity has been isolated and reported from various organisms [25,26,30,2,8,27,20,32,7]. Buforin I isolated from Asian toad *Bufo bufo* was the first report of a histone H2A derived AMP [25,7]. In the case of marine invertebrates, histone derived AMPs have been reported from Pacific White Shrimp *Litopenaeus vannamei* [27], Scallop *Chlamys farreri* [20] and Disk Abalone *Haliotis discus discus* [7]. From fishes histone derived antimicrobial peptides have been reported from Catfish *Parasilurus asotus* [26], Atlantic salmon *Salmon salar* [30], Rainbow Trout *Oncorhynchus mykiss* [8], and Atlantic Halibut *Hippoglossus hippoglossus* [2]. Present study was carried out to identify novel antimicrobial peptides from Rays as part of their innate immunity. Here we report the identification of an antimicrobial peptide sequence from the histone H2A of Round Whip Ray, *Himantura pastinacoides*. This is the first report of a histone H2A derived AMP from Elasmobranchs.

2. Materials and methods

2.1. Sample collection

Live Round Whip Ray, *H. pastinacoides* was caught off Vypeen, Kochi, Kerala, India. Samples were transported to laboratory in live condition. Blood was collected from the lamellar artery near gill region using specially designed capillary tubes (RNase free) rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7).

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2.2. cDNA synthesis

Total RNA was isolated from blood cells using TRI® reagent and following manufacturer's instructions. Purity and quality of RNA was checked on 0.8% agarose gel. RNA was quantified by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratios (A260:A280) equal to or greater than 1.8 were used for the present work. First strand cDNA was generated in a 20 μl reaction volume containing 5 μg total RNA, 1 × RT buffer, 2 mM dNTP, 2 mM oligo $(dT)_{20}$ 20 U of RNase inhibitor and 100 U of MMLV Reverse transcriptase (New England Biolabs, USA). The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min.

2.3. PCR amplification

Amplification of a Hipposin- like antimicrobial peptide from cDNA of *H. pastinacoides* was done using sense primer (5′-ATGTCCGGRMGMGGSAARAC-3′) and antisense primer (5′-GGGATGATGCGMGTCTTCTTGTT-3′) [2]. PCR amplification of 1 μl of cDNA was performed in a 25 μl reaction volume containing 1 × standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (New England Biolabs, USA). The thermal profile used was an initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. PCR product was analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with SYBR® safe and visualized under UV light. Sequencing of purified PCR product was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, India.

2.4. Taxonomic identification

For taxonomic identification of the species, genomic DNA was isolated from gills using 'salting out' technique as described by Miller et al. [24]. The concentration of isolated DNA was estimated using a UV–vis Spectrophotometer (Hitachi U-2900). The DNA was diluted to a final concentration of 100 ng/μl. The Cytochrome Oxidase-I (COI) gene was amplified in a 25 μl reaction volume containing the above said PCR reagents in same concentration. 1 μl of genomic DNA was used as template. The primers used for the amplification of COI gene were Forward (5′-TCGACTAATCATAAAGATATGGGCCAC-3′) and Reverse (5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) [38]. The thermal regime consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 45 s, 50 °C for 30 s and 72 °C for 45 s and a final extension at 72 °C for 10 min. Amplicons obtained were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, India.

The COI primers amplified a readable 600 bp region of the gene mitochondrial cytochrome oxidase subunit I (GenBank ID JN982361). BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) of nucleotide sequences confirmed the identity of the Ray as *H. pastinacoides* showing 97% similarity to GenBank ID: EU398852.1 *H. pastinacoides*.

2.5. Analysis of peptide properties

The nucleotide sequence and deduced amino acid sequence of peptide was subjected to BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Translation of the cDNA was performed using the Expert Protein Analysis System (http://au.expasy.org/). Physicochemical parameter of the deduced peptide was calculated by the ProtParam tool (http://cn.expasy.org/tools/protparam.html). Multiple sequence alignment of the peptide with previously reported histone derived AMPs from other animals, was performed with ClustalW. Phylogenetic tree was constructed by the Neighbor-joining (NJ) and Maximum Likelihood (ML) method based on nucleic acid sequences. Phylogenetic tree was drawn using MEGA version 5.05. Homology searches were performed using BLASTn and BLASTp at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Three dimensional structure of the peptide was predicted based on template PDB No. 1TzyA using SWISS-MODEL [1,10,31].

3. Results

A 204 bp fragment cDNA encoding 68 amino acids from the mRNA of blood cells of *H. pastinacoides* was obtained by RT-PCR (Fig. 1). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptide belonged to histone H2A family. The obtained nucleotide and deduced amino acid sequences were deposited in GenBank database (GenBank ID: HQ720150). Multiple-sequence alignment of the amino acid sequence of the peptide with previously reported histone H2A derived AMPs revealed that the first 39 amino acid sequence from N-terminal of the deduced peptide showed similarity to histone H2A derived AMPs i.e. Hipposin, Buforin I, Buforin II, Parasin I, Abhisin and those reported from *Oncorhynchus mykiss*, *L. vannamei* and *Chlamys farreri* (Fig. 2). This H2A derived peptide sequence from *H. pastinacoides* was termed as 'Himanturin' and here onwards will be denoted by the term. The 39 amino acid Himanturin was found to have a predicted molecular weight of 4.27 kDa and a theoretical isoelectric point (pI) of 11.73 as predicted by PROTPARAM software. Himanturin was found to be rich in arginine (15.4%), glycine (12.8%), alanine (12.8%), leucine (10.3%), valine (10.3%) and lysine (7.7%) as reported in all other histone H2A derived AMPs. Himanturin was found to have one negative residue (Glu) as against nine positive residues (Arg+Lys), thereby having a net charge of +8. Hydrophobicity of peptide was found to be +34.68 Kcal/mol (35%) as predicted by PepDraw. The hydrophilic index plot of Himanturin was analyzed using Kyte and Doolittle method [16]. The result showed the presence of both hydrophilic and hydrophobic domains in Himanturin, indicating the amphipathic nature of the peptide. N-terminus was found to be hydrophilic and C-terminus hydrophobic. Bootstrap distance tree calculated confirmed the similarity of the obtained nucleotide sequence to the

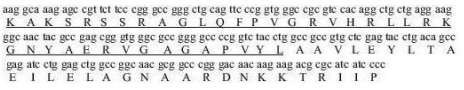

Fig. 1. Nucleotide and amino acid sequences of histone H2A. The underlined amino acid sequences indicate Himanturin from Round Whip Ray. *H. pastinacoides*.

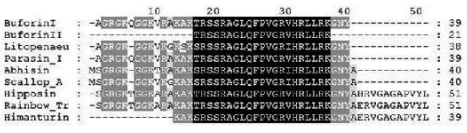

Fig. 2. ClustalW multiple alignment of Himanturin (*H. pastinacoides*) with hipposin (*Hippoglossus hippoglossus*), Buforin I and II (*Bufo bufo gargarizans*), Parasin I (*Parasilurus asotus*), Rainbow Trout H2A (*Oncorhynchus mykiss*), Litopenaeus AMP (*Litopenaeus vannamei*), Scallop AMP (*Chlamys farreri*) and abhisin (*Haliotis discus*).

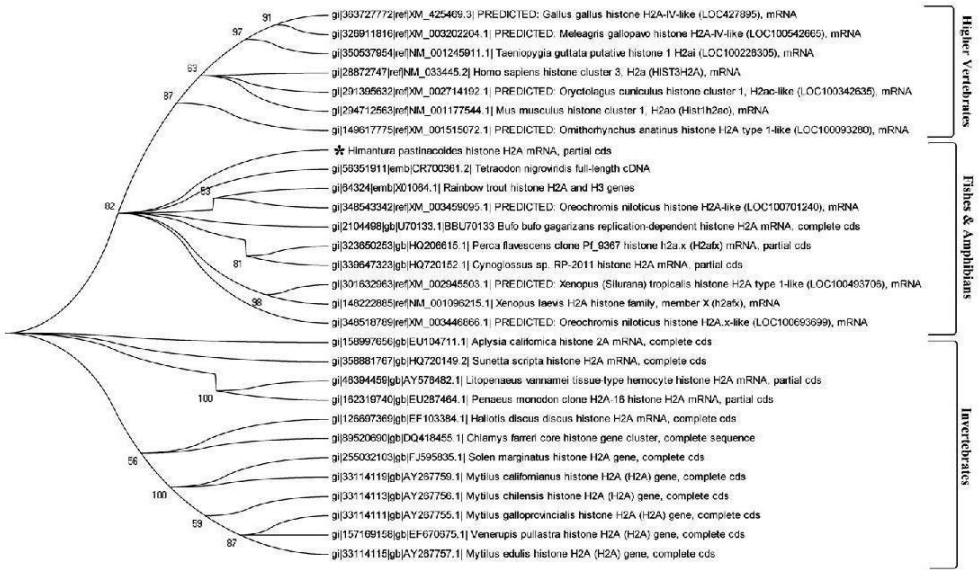

Fig. 3. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the nucleotide sequence of *H. pastinacoides* to the neucleotide sequences of previously reported histone H2A from different organisms: *Gallus gallus*, *Meleagris gallopavo*, *Taeniopygia guttata*, *Homo sapiens*, *Oryctolagus cuniculus*, *Mus musculus*, *Ornithorhychus anatinus*, *Tetradon nigroviridis*, *Rainbow trout*, *Oreochromus niloticus*, *Cynaglossus* sp., *Perca flavescens*, *Gasterosteus aculeatus*, *Oreochromis niloticus*, *Bufo bufo gagarizans*, *Xenopus tropicalis*, *Xenopus laevis*, *Aplysia californica*, *Sunetta scripta*, *Litopenaeus vannamei*, *Penaeus monodon*, *Lepeophtheirus salmonis*, *Haliotis discus*, *Chlamys farreri*, *Solen marginatus*, *Mytilus californianus*, *Mytilus chilensis*, *Mytilus galloprovincialis*, *Mytilus edulis* and *Venerupis pullastra*.

Fig. 4. Predicted 3-dimentional structure of Himanturin from Round Whip Ray *H. pastinacoides*.

previously reported histone H2A nucleotide sequences (Fig.3). SWISS-MODEL predicted an alpha helical structure for Himanturin (Fig.4). Analysis of Himanturin for its antimicrobial activity was carried out with Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php).

4. Discussion

Histone H2A derived antimicrobial peptides reported from various animals have activity against both Gram-positive and Gram-negative bacteria and fungi [25,26,30,2,27,20,14,7]. In Asian Toad, *Bufo bufo gargarizans*, the intact histone H2A protein is secreted into the stomach and Buforin I is produced by the action of pepsin isozymes cleaving the Try 39 – Ala 40 bond of intact protein [15]. Similarly in Catfish (*Parasilurus asotus*), Parasin I is produced by cleavage of Ser19-Arg20 bond of histone H2A by Cathepsin D found in skin mucus of the fish [6]. Action of enzyme pepsin on 68 amino acid sequence obtained from Round Whip Ray was analyzed using PeptideCutter tool (http://web.expasy.org/peptide_cutter/), which predicts pepsin to have a potential cleavage site at amino acid position 39 thereby resulting in formation of Himanturin. These findings suggest the possibility that N-terminus of histone H2A of *H. pastinacoides* could be an active antimicrobial peptide assisting in innate immunity of the Ray. In *Drosophila* secretion of antimicrobial peptides is mediated by two distinct pathways, the Toll pathway and Immune Deficiency (IMD) pathway. The Toll pathway is activated primarily in response to fungal and Gram positive bacterial infections, whereas the IMD pathway is activated predominantly in response to Gram negative and other Gram positive bacterial infections [19,12]. Toll activates expression of antifungal peptide genes, Dorsomycin and Metchnikowin, whereas, IMD induces transcription of genes, which encode the antibacterial peptides i.e., Diptericins, Cecropins, Drosocins and Attacins. A pathway similar to that of Drosophila IMD, termed as LvIMD was reported from *L. vannamei*. Expression of LvIMD mRNA is influenced by LPS and Gram negative *Vibrio alginolyticus* and expression of LvIMD could induce a 3 fold increase in the expression of PEN 4 [36]. Presence of Toll-like receptor in *L. vannamei* (Lv Toll1) was first reported by Yang et al. [39]. Two more Toll-like receptors, Lv Toll2 and Lv Toll3 were reported by Wang et al. [37]. In comparison to Lv Toll1 and Lv Toll3, Lv Toll2 was found to be more significant in the activation of AMP promoters in *L. vannamei* [37]. Mechanisms

similar to these might be involved in the cleavage of precursor derived antimicrobial peptides.

In case of 51-mer Hipposin, fragment containing 1 to 19 amino acid residues from the N terminal did not exhibit marked antimicrobial activity, whereas fragment consisting of 16–39 amino acid (similar to buforin II) had such activity indicating that this part of Hipposin possesses antimicrobial sequence motif and the activity was found enhanced by the presence of the fragment having 40–51 amino acid residues [3]. Fragment consisting of 4–39 amino acid residues from N-terminal of Himanturin exhibit striking similarity to 16–39 amino acid fraction of Hipposin which has been shown to contain the antimicrobial sequence motif. Almost all previously reported histone H2A derived AMPs have fragments similar to this fraction of Hipposin and it could be deduced that their activity is mainly due to this portion. Fraction 4–39 of Himanturin is similar to 16 to 51 amino acid fraction of hipposin except for Thr at position 16 of Hipposin which has been replaced by Ser (position 4) in Himanturin and His at position 41 of Hipposin being replaced by Glu (position 29) in Himanturin. Presence of Ser instead of Thr at position 16 of Hipposin can also be seen in Abhisin and histone H2A derived AMPs reported from *L. vannamei* and *Chlamys farreri*. Histone H2A AMPs reported from marine organisms exhibit broad spectrum antimicrobial activity. Hipposin and Parasin I are the most studied Histone H2A derived antimicrobial peptides. Hipposin showed strong antibacterial activity against several Gram positive and Gram negative bacteria and the activity could be detected down to concentrations of 1.6 μg/ml [2]. Parasin I was found to exhibit a minimum inhibitory concentration of 1 to 4 μg/ml against an array of Gram positive and Gram negative bacteria without any haemolytic activity [26]. Parasin I was also found to exhibit antifungal activity against *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Candida albicans* with minimum inhibitory concentration of 2 μg/ml [26]. Abhisin, an antimicrobial peptide derived from histone H2A of Disk Abalone *Haliotis discus* was found to be active against Listeria *monocytogenes* (G+), *Vibrio ichthyoenteri* (G−), and yeast, *Pityrosporum ovale*. Abhisin treatment (50 μg/ml) decreased the viability of THP-1 leukemia cancer cells approximately by 25% without any effect on the normal Vero cells, suggesting that Abhisin has cytotoxicity against cancer cells but not normal cells [7]. Histone H2A derived AMPs exhibit strong activity against aquatic and human pathogens. Himanturin exhibit strong similarity to these highly potent antimicrobial peptides. Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) predicts Himanturin to be an Antimicrobial peptide since it form alpha helices and have at least 7 residues on the same hydrophobic surface which allows the peptide to interact with membranes.

The phylogenetic relationship of *H. pastinacoides* to other organisms is shown in Fig. 3. The molecular phylogenetic tree based on nucleotide sequences of previously reported histone H2A sequences demonstrate that the members of the family are derived from a common ancestor by a series of evolutionary changes. The boot strap distance tree calculated reveals that *H. pastinacoides* cluster under the group Fishes and Amphibians. Histone genes evolve very slowly and therefore, evolutionary analyses of histones should be informative with regard to the phylogenetic relationships of distantly related organisms [34]. At the nucleotide level, the variability in histone genes appears to be the result of a larger amount of non-synonymous variation, which affects to a lesser extent, the structural domain of the protein comprising the histone fold [21]. Because the topology of major histone H2A phylogeny is similar to the eukaryotic phylogeny, histone H2A can be used as a molecular marker for classification. More data on Ray histone H2A sequences will decipher the relationship of Ray H2A to other vertebrate and invertebrate H2A.

5. Conclusion

A peptide containing antimicrobial sequence motif from the histone H2A of Round Whip Ray, *H. pastinacoides was* identified and named as Himanturin. High similarity of Himanturin to other histone H2A derived AMPs with proven antimicrobial activity and its physicochemical properties in agreement with those of traditional antimicrobial peptides strongly endorse it to be an antimicrobial peptide. Since the peptide is reported from a "food grade" source, the Round Whip Ray, it has the potential to be developed into an effective antimicrobial agent with broad application potential.

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Research Article

Identification and Molecular Characterization of Molluskin, a Histone-H2A-Derived Antimicrobial Peptide from Molluscs

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Antimicrobial peptides are humoral innate immune components of molluscs that provide protection against pathogenic microorganisms. Among these, histone-H2A-derived antimicrobial peptides are known to actively participate in host defense responses of molluscs. Present study deals with identification of putative antimicrobial sequences from the histone-H2A of back-water oyster *Crassostrea madrasensis*, rock oyster *Saccostrea cucullata*, grey clam *Meretrix casta*, fig shell *Ficus gracilis*, and ribbon bullia *Bullia vittata*. A 75 bp fragment encoding 25 amino acid residues was amplified from cDNA of these five bivalves and was named "Molluskin." The 25 amino acid peptide exhibited high similarity to previously reported histone-H2A-derived AMPs from invertebrates indicating the presence of an antimicrobial sequence motif. Physicochemical properties of the peptides are in agreement with the characteristic features of antimicrobial peptides, indicating their potential role in innate immunity of molluscs.

1. Introduction

Invertebrates mainly rely on their innate immune defenses to battle a variety of invading microbial pathogens. Among these molecules the antimicrobial peptides (AMPs) play an important role in the humoral innate immune mechanism of invertebrates. AMPs have received increasing attention in recent years as their contribution to host defense mechanisms and their potential as new pharmaceutical substances is becoming increasingly appreciated. This is mainly because of the broad spectrum activity of AMPs and the rapid development of microbial resistance to conventional antibiotics [1]. AMPs derived from histone proteins form an important category of peptide antibiotics [2]. Histone-derived antimicrobial peptides with potent activity have been isolated and reported from various organisms [3–11]. In the case of marine invertebrates histone-H2A-derived AMPs have been reported from Pacific white shrimp *Litopenaeus vannamei* [8], scallop *Chlamys farreri* [9], and abalone *Haliotis discus discus* [11]. From fishes histone-derived antimicrobial peptides have been reported from catfish *Parasilurus asotus* [4], Atlantic salmon *Salmo salar* [5], Atlantic halibut *Hippoglossus hippoglossus* [6], rainbow trout *Oncorhynchus mykiss* [7], and round whip ray *Himantura pastinacoides* [12]. Present study was carried out to identify histone-derived antimicrobial peptides from molluscs, namely, back water oyster *Crassostrea madrasensis*, rock oyster *Saccostrea cucullata*, grey clam *Meretrix* casta, fig shell *Ficus gracilis*, and ribbon bullia *Bullia vittata*.

2. Materials and Methods

2.1. Haemolymph Collection. Live *C. madrasensis* and *M. casta* were collected from Vembanad estuary, Kochi (Kerala, India) and *S. cucullata*, *F. gracilis*, and *B. vittata* from the coastal waters of Kochi. Samples were transported to laboratory in live condition. Haemolymph was collected from byssus muscles of bivalves and foot region of gastropods using 1 mL syringe rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7).

2.2. Isolation of Total RNA and cDNA Synthesis. Total RNA was isolated from haemolymph using TRI reagent. Purity

and quality of RNA were checked on 0.8% agarose gel. RNA was quantified by spectrophotometry at 260 and 280 nm. Only RNA with absorbance ratio (A260 : A280) equal to or greater than 1.8 was used for the analysis. First strand cDNA was generated in a 20 μL reaction volume containing 5 μg total RNA, 1x RT buffer, 2 mM dNTP, 2 mM oligo d(T20), 20 U of RNase inhibitor, and 100 U of MMLV Reverse Transcriptase. The reaction was conducted at 42°C for 1 h followed by an inactivation step at 85°C for 15 min. Gene-specific primers forward (5′-atgtctggacgaggaaagggagga-3′) and reverse (5′-tacttggcaggtttctgggtct-3′) were used to amplify a product of 945 bp constitutively expressed gene, the beta-actin as an internal control.

2.3. PCR Amplification. Amplification of histone-H2A-derived antimicrobial peptide sequence from cDNA of molluscs was done using forward primer (5′-gaattcatgtct-ggacgaggaaaggg-3′) and reverse primer (5′-gcggccgcatag-tttcccttacggagcaga-3′). PCR amplification of 1 μL of cDNA was performed in a 25 μL reaction volume containing 1x standard taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1 U Taq DNA polymerase. The thermal profile used was an initial denaturation at 94°C for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 30 seconds and a final extension at 68°C for 10 minutes. PCR products were analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with SYBR safe and visualized under UV light. Amplicons obtained were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 377 DNA sequencer at SciGenom Sequencing Facility, India.

2.4. Sequence Analysis. The nucleotide sequence and deduced amino acid sequence were subjected to BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Translation of the cDNA was performed using the Expert Protein Analysis System (http://au.expasy.org/). Multiple sequence alignment of the peptide with previously reported histone-derived AMPs from other animals was performed with ClustalW. Homology searches were performed using BLASTn and BLASTp at National Center for Biotechnol-ogy Information (http://www.ncbi.nlm.nih.gov/). Physicochemical parameters of the deduced peptide were calculated by the ProtParam tool (http://cn.expasy.org/tools/protparam.html). The pdb data was generated by SWISS-MODEL [13–15] and three-dimensional arrangement of the peptide was created using PyMOL. Phylogenetic tree was constructed based on nucleic acid sequences by the neighbour-joining (NJ) method using MEGA version 5.05.

3. Results

A 75 bp fragment cDNA encoding 25 amino acids from the mRNA of the five molluscs was obtained by RT-PCR (Figure 1). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptides belonged to histone-H2A family. The H2A sequence obtained was similar for all the five molluscs (2 oysters, 1 clam, and 2 gastropods). The obtained nucleotide and deduced amino acid sequences were deposited in GenBank database: *B. vittata* (GenBank ID: HQ720143), *C. madrasensis* (GenBank ID: HQ720145), *F. gracilis* (GenBank ID: HQ720146), *S. cucullata* (GenBank ID: HQ720147), and *M. casta* (GenBank ID: HQ720148). Multiple sequence alignment of the amino acid sequence with previously reported histone-H2A-derived AMPs revealed that the 25 amino acid sequence of the deduced peptide showed similarity to previously reported histone-H2A-derived AMPs like Buforin I, Buforin II, Hipposin, Himanturin, Abhisin, Sunettin, and those reported from *Litopenaeus vannamei* and *Chlamys farreri* (Figure 2). This H2A-derived peptide sequence amplified from *C. madrasensis*, *M. casta*, *S. cucullata*, *F. gracilis*, and *B. vittata* was termed as "Molluskin." Sequence analysis of the peptide was carried out using ProtParam software which predicted Molluskin to have a molecular weight of 2.84 kDa and a theoretical isoelectric point (pI) of 12.18. ProtParam estimated the half life of peptide to be 1.9 hours in mammalian reticulocytes, more than 20 hours in yeast and more than 10 hours in *E. coli*. Molluskin was found to be rich in arginine (20%), leucine (12%), serine (12%), glycine (12%), and alanine (8%) as reported in all other histone-H2A-derived AMPs. The 25 amino acid peptide was found to have a net charge of +6. Hydrophobicity of Molluskin was found to be +21.92 kcal/mol (32%) as predicted by PepDraw. Analysis of Molluskin using Protean module of the DNASTAR Lasergene sequence analysis software suite revealed that the peptide will have a concentration of 1.91 mg/mL for an absorbance of 1 OD measured at 280 nm and 1 μg of the peptide would contain 352.08 pmoles. Hydrophobic amino acids constituted 29.61% while polar amino acids represented 23.47% of the total weight of Molluskin. Schiffer-Edmundson helical wheel modeling of the peptide using Protean module revealed clustering of hydrophobic and hydrophilic/basic residues on opposing sides of the helical wheel (Figure 3). This result suggests an amphipathic nature and an α-helical structure for the Molluskin. Three-dimensional arrangement of the peptide generated in PyMOL is presented in Figure 4. Analysis of Molluskin for antimicrobial activity was carried out with antimicrobial peptide database (http://aps.unmc.edu/AP/main.php) which predicts Molluskin to be an antimicrobial peptide with a protein-binding potential of 2.96 kcal/mol. Bootstrap distance tree calculated confirmed the similarity of nucleotide sequences to the previously reported histone-H2A nucleotide sequences (Figure 5).

4. Discussion

In invertebrates, humoral immunity mainly consists of antimicrobial agents present in the circulating body fluid [16]. Therefore identifying novel antimicrobial peptides provides information crucial for elucidating invertebrate innate immunity. Molluskin exhibited significant similarity with previously reported histone-H2A-derived AMPs as indicated in Figure 2. Molluskin has a Ser at N-terminus region. H2A-derived antimicrobial peptides reported from

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tcc cgc tct tct cgc gct gga ctt cag ttc ccc gtg ggt cgt atc cac cgt ctg ctc cgt
 S   R   S   S   R   A   G   L   Q   F   P   V   G   R   I   H   R   L   L   R
aag gga aac tat gcg
 K   G   N   Y   A
```

FIGURE 1: Nucleotide and amino acid sequences of histone-H2A-derived antimicrobial peptide, Molluskin from *C. madrasensis*, *S. cucullata*, *M. casta*, *F. gracilis*, and *B. vittata*.

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                    10        20        30        40        50
Molluskin:  ----------SRSSRAGLQFPVGRIHRLLRKGNYA----------
Buforin II: ----------TRSSRAGLQFPVGRVHRLLRK----------
Buforin I:  -AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY----------
Litopenaeus AMP: -AGRGK-GGKVKGKSKSRSSRAGLQFPVGRIHRLLRKGNY----------
Abhisin:    MSGRGK-GGKTKAKAKSRSSRAGLQFPVGRIHRLLRKGNYA----------
Scallop AMP: MSGRGK-GGKVKGKAKSRSSRAGLQFPVGRIHRLLRKGNYA----------
Himanturin: ----------KAKSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL--
Trout AMP:  -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL--
Hipposin:   -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL--
Sunettin:   MSGRGK-GGKTKGKAKSRSSRAGLQFPVGRIHRLLRKGNYAERVGAGAPVYL—
```

FIGURE 2: ClustalW multiple alignment of Molluskin (*C. madrasensis*, *S. cucullata*, *M. casta*, *F. gracilis*, and *B. vittata*) with Buforin I and II (*Bufo bufo gargarizans*), Hipposin (*Hippoglossus hippoglossus*), rainbow trout H2A (*Oncorhynchus mykiss*), Litopenaeus AMP (*Litopenaeus vannamei*), scallop AMP (*Chlamys farreri*), Abhisin (*Haliotis discus*), Sunettin (*Sunetta scripta*), and Himanturin (*Himantura pastinacoides*).

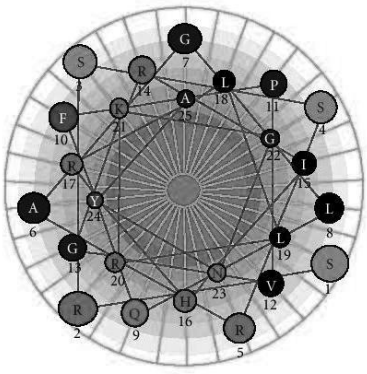

FIGURE 3: Schiffer-Edmundson helical wheel diagram demonstrating probable amphipathic α-helical conformation of Molluskin.

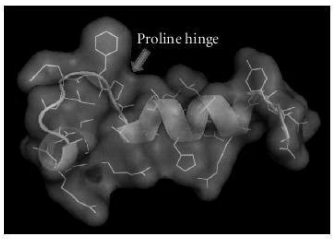

FIGURE 4: Predicted 3-dimentional structural arrangement of Molluskin using PyMol. Presence of proline hinge is highlighted.

other invertebrates and Himanturin reported from round whip ray also possess Ser at the corresponding position, but in case of all other vertebrates, Thr is present in position of Ser. Molluskin possesses Ile at position 15 from N-terminus. This is the same for all invertebrates as they possess Ile at the corresponding position, whereas in vertebrates Val occupies the position instead of Ile. Since Ser and Thr are hydrophilic and Ile and Val are hydrophobic and since they do not contribute to the charge of the peptide, their interchange will have no or very little effect on the activity of the peptides. All these antimicrobial peptides are derived from the N-terminal region of H2A histone suggesting its role in the innate immunity of an organism.

Histone-H2A-derived antimicrobial peptides are cleaved from their precursors mainly by the action of proteolytic enzymes. In Asian toad *Bufo bufo gargarizans*, the intact histone-H2A protein is secreted into the stomach and Buforin I is produced by the action of pepsin isozymes cleaving the Try 39-Ala 40 bond of intact protein [17]. Similarly in catfish (*Parasilurus asotus*), parasin I is produced by cleavage of Ser19-Arg20 bond of histone-H2A by cathepsin D found in skin mucus of the fish [18]. To understand the action of proteolytic enzymes on histone-H2A protein of molluscs, we considered a full length histone-H2A protein sequence of *Sunetta scripta* (GenBank ID: HQ720149) previously reported by us (in press: Sathyan et al. 2012; Identification of a histone-derived, putative antimicrobial peptide sunettin from marine clam *Sunetta scripta*. Blue Biotechnology Journal). The sequence was analyzed using PeptideCutter tool (http://web.expasy.org/peptide_cutter/). PeptideCutter tool predicts extracellular digestive enzyme, trypsin to have potential cleavage site at amino acid position 15 and enzymes proteinase K and Asp-N endopeptidase to have a potential cleavage site at amino position 40 from the N-terminus. Proteolytic activity of these enzymes will result in the formation of a peptide like the Molluskin. Enzyme

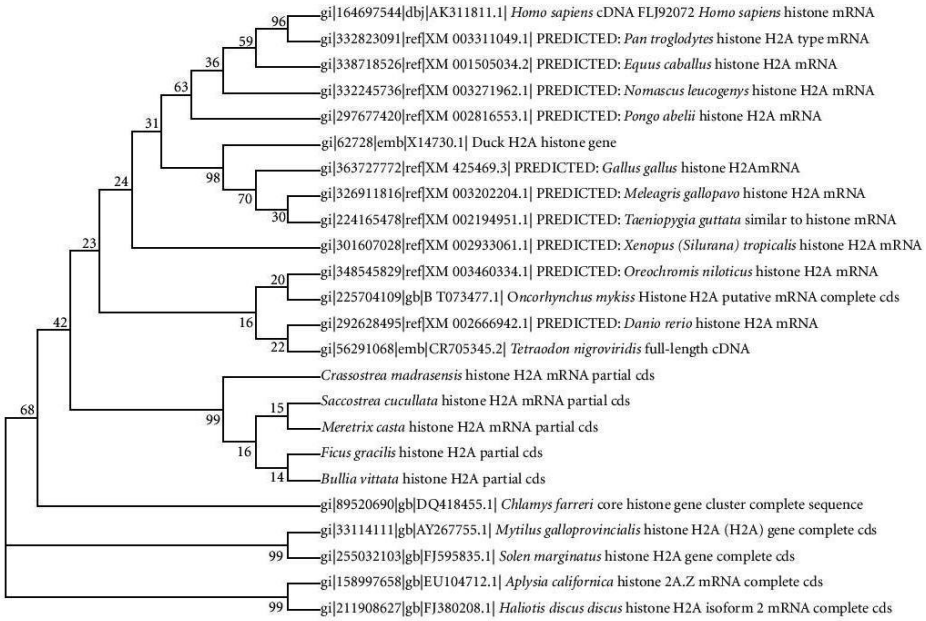

FIGURE 5: A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the nucleotide sequence of *Crassostrea madrasensis*, *Saccostrea cucullata*, *Meretrix casta*, *Ficus gracilis*, and *Bullia vittata* to the nucleotide sequences of previously reported histone-H2A from different organisms.

pepsin was found to have a cleavage site at amino acid position 39 from the N-terminus, which would release a peptide similar to Buforin 1, Abhisin, and also to the histone-H2A-derived AMPs from *Litopenaeus vannamei* and *Chlamys farreri*. These findings suggest that the proteolytic enzymes could transform the N-terminus of histone-H2A in molluscs into an active antimicrobial peptide assisting in the innate immunity of the organisms. AMPs derived from precursors are less understood in case of marine invertebrates and therefore the study would provide a better understanding of their innate immune responses. Two distinct pathways, the Toll pathway and Immune Deficiency (IMD) pathway, mediate the secretion of antimicrobial peptides in *Drosophila*. Infections mainly due to fungal and Gram-positive bacterial attack activate the Toll pathway, whereas the IMD pathway is activated, predominantly, in response to infections by Gram-negative and other Gram-positive bacteria [19, 20]. A pathway similar to that of *Drosophila* IMD, termed as LvIMD, was reported from a marine invertebrate *Litopenaeus vannamei* [21]. Existence of Toll receptors was also reported in *Litopenaeus vannamei* [22, 23]. Mechanisms similar to these might be involved in the cleavage of precursor-derived antimicrobial peptides and detailed studies in this area would open up new frontiers in AMP research.

Broad spectrum activity against bacteria and fungi was exhibited by histone-H2A-derived antimicrobial peptides reported from various sources. Antimicrobial peptides are also viewed as agents with therapeutic potential against cancer cells [24]. Hipposin and Buforins are the most studied Histone-H2A-derived antimicrobial peptides. Hipposin exhibited strong antibacterial activity against several Gram-positive and Gram-negative bacteria and activity could be detected down to a concentrations of 1.6 μg/mL [6]. Buforins are among one of the most potent antimicrobial peptides. In addition to their broad spectrum activity against bacteria and fungi [3], they also possess antiendotoxic and anticancer activities [25]. General mode of action of antimicrobial peptides is to kill cells through membrane disruption. Buforin II differs from this generalization as it does not cause significant membrane permeabilization [26]. Instead, Buforin II appears to readily enter bacterial cells *in vivo* [27] and lipid vesicles *in vitro* [26] showing that the peptide can traverse the cell membrane without any receptor. Once inside, it is believed to cause bacterial cell lysis by interacting with intracellular nucleic acids [28]. No cytotoxic activity against normal mammalian cells was observed for Buforin II [29]. NMR structural studies showed that proline at position 11 serves as a hinge between a C-terminal helix

and N-terminal-extended helical structure [30]. This sole proline residue (Pro_{11}) of Buforin II is necessary for effective translocation across cell membrane [26, 27]. Presence of a proline hinge as in Buforin II was also found to be a characteristic feature of Molluskin (Figure 4). We suppose that the presence of proline hinge clearly indicates that the antimicrobial activity of Molluskin lies in its ability to interact with nucleic acid rather than membrane permeabilization. Buforin II exhibits selective cytotoxicity against cancer cells through interaction with cell surface gangliosides and once inside the cell they induced mitochondria-dependent apoptosis [31]. Structural similarity of Molluskin to Buforin II may imply potential anticancer activity. Antimicrobial activity of Molluskin was further confirmed by antimicrobial peptide database (http://aps.unmc.edu/AP/main.php) which predicts it to be an AMP since it forms alpha helices and has at least 5 residues on the same hydrophobic surface which allows the peptide to interact with membranes. Molluskin shows the characteristic features of AMPs including high cationicity (+6), higher hydrophobic residue (32%), and 2.96 kcal/mol protein-binding potential.

The molecular phylogenetic tree based on nucleic acid sequences of previously reported histone-H2A-derived AMPs demonstrates that the members of the family are derived from a common ancestor by a series of evolutionary changes (Figure 5). The boot strap distance tree calculated reveals that nucleotide sequences of Molluskin from all five molluscs align with the molluscan group. Histone genes evolve very slowly and therefore, evolutionary analyses of histones should be informative with regard to the phylogenetic relationships of distantly related organisms [32].

5. Conclusion

Peptide having antimicrobial sequence motif was identified from the histone-H2A of *C. madrasensis*, *S. cucullata*, *M. casta*, *F. gracilis*, and *B. vittata* and was named as Molluskin. High similarity of Molluskin in terms of physicochemical properties and molecular structure to other histone-H2A-derived AMPs with proven antimicrobial activity strongly endorse it to be an antimicrobial peptide. This work was undertaken to study the presence of histone-derived AMPs in molluscs depicting its possible role in innate immunity. Synthesizing histone-derived AMPs for commercial applications would be a highly promising endeavor as an alternative to the conventional antibiotics which elicit drug resistance in microbes and impose tremendous ecological damage to the environment both terrestrial and aquatic. Since Molluskin is a very short peptide, it has the potential to be developed into an effective antimicrobial agent for use in aquaculture and medicine.

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IDENTIFICATION OF A PUTATIVE ANTIMICROBIAL PEPTIDE SEQUENCE, SUNETTIN FROM MARINE CLAM, SUNETTA SCRIPTA

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ABSTRACT

Histone H2A is reported to participate in host defense response by producing novel antimicrobial peptides (AMPs). The present study deals with identification of a putative antimicrobial sequence, Sunettin from the histone H2A of Marine clam, Sunetta scripta. A 242 bp fragment encoding 82 amino acid residues was amplified from cDNA of Sunetta scripta. Sunettin exhibited high similarity to previously reported histone H2A derived AMPs indicating the presence of an antimicrobial sequence motif. Physico chemical properties of Sunettin are in agreement with the characteristic features of antimicrobial peptides, indicating its potential role in innate immunity of marine clam.

Keywords: Antimicrobial peptide; Sunetta scripta; Innate immunity; Histone H2A; Sunettin; Clam

1. INTRODUCTION

Antimicrobial peptides (AMPs) play a significant role in the innate immune defenses of both vertebrates and invertebrates [1-4]. AMPs derived from histone proteins form an important category of peptide antibiotics [5]. Histone H2A derived antimicrobial peptides with potent activity has been isolated and reported from various organisms [6-14]. Buforin I

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isolated from Asian toad Bufo bufo was the first report of a histone H2A derived AMP [6, 14]. In case of marine invertebrates histone H2A derived AMPs have been reported from Pacific white shrimp Litopenaeus vannamei [11], scallop Chlamys farreri [12] and abalone Haliotis discus discus [14]. Present study was carried out to identify novel antimicrobial peptides from clams to understand their innate immunity. Here we report the identification of a antimicrobial peptide sequence from the histone H2A of marine clam, Sunetta scripta. This is the first report of an antimicrobial peptide from Sunetta scripta.

2. MATERIALS AND METHODS

2.1. Haemolymph Collection

Live Sunetta scripta was caught off Calicut, Kerala (India). Samples were transported to laboratory in live condition. Haemolymph was collected from byssus muscles using 1 ml syringe rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7).

2.2. cDNA Synthesis

Total RNA was isolated from haemolymph using TRI® reagent and following manufacturer's instructions. Purity and quality of RNA was checked on 0.8% agarose gel. RNA was quantified by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratios (A260:A280) equal to or greater than 1.8 were used for the present work. First strand cDNA was generated in a 20 µl reaction volume containing 5 µg total RNA, 1x RT buffer, 2 mM dNTP, 2 mM oligo d(T20), 20 U of RNase inhibitor and 100 U of MMLV Reverse transcriptase (New England Biolabs,USA). The reaction was conducted at 42° C for 1 h followed by an inactivation step at 85° C for 15 min.

2.3. PCR Amplification

Amplification of a Hipposin like antimicrobial peptide from the cDNA of clam was carried out using sense primer (5′-ATGTCCGGRMGMGGSAARAC-3′) and antisense primer (5′-GGGATGATGCGMGTCTTCTTGTT-3′) [9]. PCR amplification of 1 µl of cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (New England Biolabs).

The thermal profile used was an initial denaturation at 94°C for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 30 seconds and a final extension at 68°C for 10 minutes. PCR product was analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with SYBR® safe and visualized under UV light. Purified PCR products were sequenced with an ABI Prism Sequencing kit (BigDye Terminator Cycle) at SciGenom, India.

2.4. Sequence Analysis

The nucleotide sequence and deduced amino acid sequence of peptide was subjected to BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/blast).Translation of the cDNA was performed using the Expert Protein Analysis System (http://au.expasy.org/).

Physico chemical parameter of the deduced peptide was calculated by the ProtParam tool (http://cn.expasy.org/tools/protparam.html). Multiple sequence alignment of the peptide with previously reported histone derived AMPs from other animals was performed with ClustalW. Phylogenetic tree was constructed by the Neighbour-joining (NJ) method and the Maximum Likelihood (ML) method based on amino acid sequences. Phylogenetic tree was drawn using MEGA version 5.05.

Homology searches were performed using BLASTn and BLASTp at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Three dimensional structure of the peptide was predicted using SWISS-MODEL [15-17].

3. RESULTS

A 249 bp fragment cDNA encoding 82 amino acids from the mRNA of Sunetta scripta was obtained by RT-PCR (Figure 1). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptide belonged to histone H2A family. The obtained nucleotide and deduced amino acid sequences were deposited in GenBank database (GenBank ID: HQ720149).

Multiple-sequence alignment of the amino acid sequence of the peptide with previously reported histone H2A derived AMPs revealed that the first 51 amino acid sequence at the N-terminal of the deduced peptide showed similarity to histone H2A derived AMPs i.e. hipposin, buforin I, buforin II, abhisin and those reported from Oncorhynchus mykiss, Litopenaeus vannamei and Chlamys farreri (Figure 2).

This H2A derived peptide sequence from Sunetta scripta was termed as 'Sunettin'. Bootstrap distance tree was calculated and confirmed the similarity of Sunettin to the previously reported histone H2A derived AMPs (Figure 3).

The 51 amino acid Sunettin was found to have a predicted molecular weight of 5.43 kDa and a theoretical isoelectric point (pI) of 11.9 as predicted by PROTPARAM software. Sunettin was found to be rich in arginine (13.7%), glycine (19.6%), alanine (9.8%), leucine (11.3%), valine (10.3%) and lysine (11.8 %) as reported in all other histone H2A derived AMPs. Sunettin was found to have a net charge of +12. Hydrophobicity of peptide was found to be +50.02 kcal/mol (29%) as predicted by PepDraw. The hydrophilic index plot of Sunettin was analyzed using Kyte and Doolittle method [18].

The result showed that Sunettin contains both hydrophilic and hydrophobic domains, which indicated that the peptide may be an amphipathic molecule. N-terminus was found to be hydrophilic and C-terminus hydrophobic. SWISS-MODEL predicted an alpha helical structure for Sunettin (Figure 4).

Analysis of Sunettin for its antimicrobial activity was carried out with Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) which predicts Sunettin to be an Antimicrobial peptide with a protein binding potential of 2.3 kcal/mol.

```
atgtctggacgaggaaagggtggaaagacaaagggaaaggcaaagagccgctcttctcgc
M  S  G  R  G  K  G  G  K  T  K  G  K  A  K  S  R  S  S  R
gctggacttcagttccccgtgggtcgtatccaccgtctcctacgtaagggtaactacgcc
A  G  L  Q  F  P  V  G  R  I  H  R  L  L  R  K  G  N  Y  A
gagagagttggagctggtgcacctgtgtatctcgccgccgtgctcgagtacttggccgcc
E  R  V  G  A  G  A  P  V  Y  L  A  A  V  L  E  Y  L  A  A
gaagttttggaattggctgcaacgccgcacgagacaacaagaagacgcgcatcatcccc
E  V  L  E  L  A  G  N  A  A  R  D  N  K  K  T  R  I  I  P
actcgatga
T  R  *
```

Figure 1. Nucleotide and amino acid sequences of histone H2A. The underlined amino acid sequences indicate Sunettin from Marine clam, Sunetta scripta.

```
                    10        20        30        40        50
Buforin-I   : -AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY---------- : 39
Litopenaeu  : -AGRGK-GGKVKGKSKSRSSRAGLQFPVGRIHRLLRKGNY---------- : 38
Abhisin     : MSGRGK-GGKTKAKAKSRSSRAGLQFPVGRIHRLLRKGNYA--------- : 40
Scallop-AM  : MSGRGK-GGKVKGKAKSRSSRAGLQFPVGRIHRLLRKGNYA--------- : 40
Hipposin    : -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYL : 51
Trout-AMP   : -SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL : 51
Sunettin    : MSGRGK-GGKTKGKAKSRSSRAGLQFPVGRIHRLLRKGNYAERVGAGAPVYL : 51
```

Figure 2. ClustalW multiple alignment of Sunettin (S. scripta) with Hipposin (Hippoglossus hippoglossus), Buforin I (Bufo bufo gargarizans), Trout AMP (Oncorhynchus mykiss), Litopenaeus AMP (Litopenaeus vannamei), Scallop AMP (Chlamys farreri) and Abhisin (Haliotis discus).

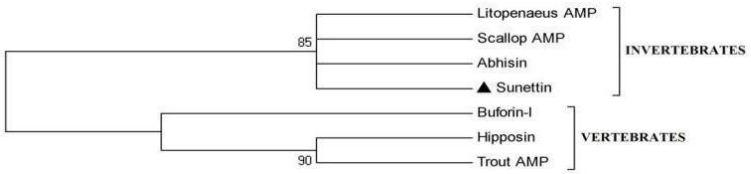

Figure 3. A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the deduced amino acid sequence of Sunettin (S. scripta) to the amino acid sequences of previously reported histone H2A derived: Hipposin (Hippoglossus hippoglossus), Buforin I (Bufo bufo gargarizans), Trout AMP (Oncorhynchus mykiss) Litopenaeus AMP (Litopenaeus vannamei) Scallop AMP (Chlamys farreri) and Abhisin (Haliotis discus).

Figure 4. Predicted 3-dimentional structure of Sunettin from Marine clam, S. scripta.

4. DISCUSSION

Sunettin showed significant similarity with previously reported histone H2A derived AMPs. All these peptides are derived from the N-terminal region of H2A histone suggesting its role in the innate immunity of an organism. Invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses to defend themselves against pathogens. In invertebrates, humoral immunity mainly consists of antimicrobial agents present in the circulating body fluid [2]. Therefore identifying novel antimicrobial peptides provide information crucial for elucidating invertebrate innate immunity. AMPs derived from precursors are less understood in case of marine invertebrates and therefore the study would provide a better understanding of innate immune responses in invertebrates.

Histone H2A derived antimicrobial peptides are cleaved from their precursors mainly by the action of proteolytic enzmes. In Asian toad, Bufo bufo gargarizans, the intact histone H2A protein is secreted into the stomach and buforin I is produced by the action of pepsin isozymes cleaving the Try 39 – Ala 40 bond of intact protein [19]. Similarly in cat fish (Parasilurus asotus), parasin I is produced by cleavage of Ser19-Arg20 bond of histone H2A by cathepsin D found in skin mucus of the fish [20]. An inducible trpsin-like serine proteinase, the tryptase which has a preferential cleavage site in histone H2A molecule, 20 residues downstream N-terminus was reported from mast cell granules [21]. These findings suggest that N-terminus of histone H2A of Sunetta scripta could be an active antimicrobial peptide assisting in innate immunity of the clam.

Histone H2A derived antimicrobial peptides reported from various organisms exhibit broad spectrum activity against both Gram-positive and Gram-negative bacteria and fungi. Hipposin, Parasin I and Buforin I are the most studied Histone H2A derived antimicrobial peptides. Hipposin showed strong antibacterial activity against several Gram positive and Gram negative bacteria and activity could be detected down to concentrations of 1.6 µg/ml [9]. Parasin I was found to exhibit a minimum inhibitory concentration of 1 to 4 µg/ml against an array of Gram positive and Gram negative bacteria without any haemolytic activity [7]. Minimum inhibitory concentration in case of Buforin I was found to be 4 to 8 µg/ml against both categories of bacteria [22]. Both Parasin I and Buforin I were found to exhibit antifungal activity against Cryptococcus neoformans, Saccharomyces cerevisiae and Candida albicans with minimum inhibitory concentration of 2 µg/ml and 4 µg/ml respectively [7, 22]. Abhisin, an antimicrobial peptide derived from histone H2A of disk abalone Haliotis discus was found to be active against Listeria monocytogenes (G+), Vibrio ichthyoenteri (G-) bacteria, and fungi (yeast) Pityrosporum ovale. Abhisin treatment (50 µg/ml) decreased the viability of THP-1 leukemia cancer cells approximately by 25% without any effect on the normal vero cells, suggesting that abhisin has cytotoxicity against cancer cells but not normal cells [14]. Histone H2A derived AMPs exhibit strong activity against aquatic and human pathogens. Sunettin exhibit strong similarity to these highly potent antimicrobial peptides. Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) predicts Sunettin to be an Antimicrobial peptide since it form alpha helices and can have at least 7 residues on the same hydrophobic surface which allows the peptide to interact with membranes. Sunettin shows the characteristic features of antimicrobial peptides including high cationicity (+12), higher hydrophobic residue and 2.3 kcal/mol protein binding potential.

The phylogenetic relationship of Sunettin to other H2A derived antimicrobial peptides is shown in Figure 3. The molecular phylogenetic tree based on amino acid sequences of previously reported histone H2A derived AMPs demonstrates that the members of the family are derived from a common ancestor by a series of evolutionary changes. H2A derived antimicrobial peptide sequences get divided into two major groups i.e. vertebrates and invertebrates. The boot strap distance tree calculated reveals that Sunettin aligns with the invertebrate group.

CONCLUSION

A peptide containing antimicrobial sequence motif from the histone H2A Marine clam, Sunetta scripta was identified and named as Sunettin. High similarity of Sunettin to other histone H2A derived AMPs with proven antimicrobial activity and its physico chemical properties in agreement with those of traditional antimicrobial peptides strongly endorse it to be an antimicrobial peptide. Since Sunettin come from a 'food grade' source, it has the potential to be developed into an effective antimicrobial agent for use in aquaculture and pharmacy.

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Research Article

Characterization of Histone H2A Derived Antimicrobial Peptides, Harriottins, from Sicklefin Chimaera *Neoharriotta pinnata* (Schnakenbeck, 1931) and Its Evolutionary Divergence with respect to CO1 and Histone H2A

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Antimicrobial peptides (AMPs) are humoral innate immune components of fishes that provide protection against pathogenic infections. Histone derived antimicrobial peptides are reported to actively participate in the immune defenses of fishes. Present study deals with identification of putative antimicrobial sequences from the histone H2A of sicklefin chimaera, *Neoharriotta pinnata*. A 52 amino acid residue termed Harriottin-1, a 40 amino acid Harriottin-2, and a 21 mer Harriottin-3 were identified to possess antimicrobial sequence motif. Physicochemical properties and molecular structure of Harriottins are in agreement with the characteristic features of antimicrobial peptides, indicating its potential role in innate immunity of sicklefin chimaera. The histone H2A sequence of sicklefin chimera was found to differ from previously reported histone H2A sequences. Phylogenetic analysis based on histone H2A and cytochrome oxidase subunit-1 (CO1) gene revealed *N. ptinnata* to occupy an intermediate position with respect to invertebrates and vertebrates.

1. Introduction

Antimicrobial peptides (AMPs) are ubiquitous and multipotent components of humoral innate immune response of most living organisms against invasion by pathogens [1]. The characteristics of naturally occurring AMPs, such as relatively small size (12–50 amino acids), cationicity, and amphipathicity allow them to interact with and penetrate into the membranes by the formation of transmembrane ion permeable pores or by a detergent-like manner, resulting in the leakage of the cytoplasmic components and cell death [2]. In the last two decades a considerable number of gene coded AMPs, either inducible or constitutive, with broad spectrum activity against different types of pathogens, have been reported from wide range of organisms, and their significance in innate immunity is becoming more and more appreciated. The specific immune mechanisms in the primeval vertebrates such as fish are less developed than those of higher vertebrates [3, 4] and are limited by temperature restraints on their metabolism [5]. Therefore, fish rely highly on their innate immune mechanisms for protection against invading pathogens and this makes them a potential candidate for antimicrobial peptide research.

Histone derived antimicrobial peptides form an important category of AMPs and is reported from a number of vertebrates and invertebrates [6]. N-terminus of histone H2A is rich in basic amino acids, a characteristic which allows histone H2A to act as a precursor for antimicrobial peptides [7]. In case of marine fishes AMPs derived from the N-terminus domain of histone H2A have been reported from

a number of species including catfish *Parasilurus asotus* [8], Atlantic salmon *Salmo salar* [9], Atlantic halibut *Hippoglossus hippoglossus* [10], rainbow trout *Oncorhynchus mykiss* [11], round whip ray *Himantura pastinacoides* [12], and recently from two marine teleost fishes, *Tachysurus jella* and *Cynoglossus semifasciatus* [13]. Histone derived AMPs have also been reported from marine invertebrates including Pacific white shrimp *Litopenaeus vannamei* [14], scallop *Chlamys farreri* [7], abalone *Haliotis discus*, Marine Clam *Sunetta scripta* [15], and from few other marine molluscs [16].

The role of antimicrobial peptides in the innate immune response of fishes belonging to the family Holocephali has not been yet studied in detail. The present study was carried out to get a deeper insight into the role of histone H2A derived AMPs in the immune response of sicklefin chimaera *Neoharriotta pinnata*. Here we report the identification and characterization of antimicrobial peptide sequences derived from histone H2A of *N. pinnata*. This is the first report of histone derived antimicrobial peptides from Holocephali group of fishes. The paper also describes in detail the divergence in molecular evolution of histone H2A in *N. pinnata* and related fishes. Also the evolutionary relationship of Holocephalan fishes to other organisms based on the nucleotide sequence of cytochrome oxidase subunit I have been discussed in detail.

2. Methodology

2.1. Sample Collection. Live *N. pinnata* was caught from a depth of 500 m off Karaikkal Coast, Tamil Nadu, India, during Cruise number 291 of Fisheries and Oceanography Research Vessel *Sagar Sampada* (Ministry of Earth Sciences, Government of India). High Speed Demersal Trawl (HSDT) net operated on-board was employed for capturing the species. Blood was collected from the lamellar artery near gill region using specially designed capillary tubes (RNase free) and rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7). Blood was homogenized in TRI reagent (Sigma) and stored at −20°C on-board in the Biological Laboratory facility of the research vessel.

2.2. RNA Isolation and cDNA Synthesis. Total RNA was isolated from blood cells using TRI reagent (Sigma) and following manufacturer's instructions. Purity and quality of RNA were checked on 0.8% agarose gel. First strand cDNA was generated in a 20 μL reaction volume containing 5 μg total RNA, 1x RT buffer, 2 mM dNTP, 2 mM oligo d(T20), 20 U of RNase inhibitor, and 100 U of MMLV reverse transcriptase (New England Biolabs, USA). The reaction was conducted at 42°C for 1h followed by an inactivation step at 85°C for 15 min. Gene-specific primers forward (5′-GATCATGTTCGAGACCTTCAACAC-3′) and reverse (5′-CGATGGTGATGACCTGTCCGTC-3′) were used to amplify a product of 389 bp constitutive expression gene, the beta-actin as an internal control to verify the RT-PCR reaction.

2.3. PCR Amplification. Amplification of histone H2A derived antimicrobial peptide sequence from cDNA of *N. pinnata* was done using forward primer (5′-ATGTCC GGRMGMGGSAARAC-3′) and reverse primer (5′-GGG ATGATGCGMGTCTTCTTGTT-3′) [10]. PCR amplification of 1 μL of cDNA was performed in a 25 μL reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer, and 1 U Taq DNA polymerase (New England Biolabs). The thermal profile used was an initial denaturation at 94°C for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 30 seconds and a final extension at 68°C for 10 minutes. PCR products were analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with SYBR Safe and visualized under UV light.

2.4. TA Cloning and Sequencing. The purified PCR products were ligated into the pTZ57R/T easy clone vector and transformed using competent *E. coli* cells, JM107 as per manufacturer's protocols (InsTAclone PCR Cloning Kit, Fermentas). Transformed bacteria were cultured in Luria Bertaini agar plates containing ampicillin, IPTG, and X-gal at 37°C for 24 h, and the recombinant clones with the inserts were selected by blue white screening. The white colonies were selected and streaked on to fresh ampicillin plates and screened using vector specific primers, M13 F (5′-GTAAAACGACGGCCAG-3′) and M13 R (5′-CAGGAAACAGCTAT GAC-3′) and histone H2A sequence specific primers. For M13 primers the thermal profile used was 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds and a final extension at 72°C for 10 minutes. Amplicons obtained were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, India.

2.5. Taxonomic Identification. For taxonomic identification of the species genomic DNA was isolated using TRI reagent (Sigma). The concentration of isolated DNA was estimated using a UV spectrophotometer (Hitachi U-2900). The DNA was diluted to a final concentration of 100 ng/μL. The cytochrome oxidase-I (COI) gene was amplified in a 25 μL reaction volume containing the above said PCR reagents in same concentration. 1 μL of genomic DNA was used as template. The primers used for the amplification of COI gene were LCO1490 (5′-GGTCAACAAATC ATAAAGATATTGG-3′) and HC02198 (5′-TAAACTTCA GGGTGACCAAAAAATCA-3′) [17]. The thermal regime consisted of an initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, 50°C for 30 seconds, and 72°C for 45 seconds and a final extension at 72°C for 10 minutes. Amplicons obtained were sequenced using ABI Prism Sequencing kit (BigDye Terminator Cycle) at SciGenom, India.

2.6. Data Analysis. The homologue searching of the nucleotide sequence was performed with the Basic Local Alignment Search Tool (BLAST) through NCBI server (http://www.ncbi.nlm.nih.gov/blast). The nucleotide sequence was translated into amino acid sequence by the

DNA-Protein translation tool provided by Expert Protein Analysis System, ExPASy (http://au.expasy.org/). Phylogenetic tree was constructed by the neighbour-joining (NJ) method and maximum likelihood (ML) method based on amino acid sequence of histone H2A and nucleotide sequence of cytochrome oxidase subunit I, using MEGA version 5.05. Confidence in estimated relationships of ML and NJ tree topologies was evaluated by a bootstrap analysis with 100 and 1,000 replicates with MEGA version 5.0. Kimura 2 parameter (K2P) model was used to construct NJ and ML tree for CO1 genes. The cleavage sites of proteolytic enzymes on the deduced amino acid sequence were predicted using PeptideCutter Tool (http://web.expasy.org/peptide_cutter/). Molecular weight, isoelectric point, and stability of each peptide sequence were calculated using ProtParam software (http://web.expasy.org/protparam/). The primary structure of deduced amino acid sequences was compared with previously reported histone H2A derived AMPs from other species by using the multiple sequence alignment program CLUSTALW. Charge over a range of pH and concentration of peptides was calculated using Protein Calculator v 3.3 (http://www.scripps.edu/~cdputnam/protcalc.html) and hydrophobicity using PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/index.html). Three-dimensional arrangement of peptide was created in PyMOL software using data generated by SWISS-MODEL [18–20].

3. Results

The CO1 primers amplified a 710 bp region of the gene mitochondrial cytochrome oxidase subunit I (GenBank ID JX297203). BLAST analysis of nucleotide sequences confirmed the identity of the organism as *N. pinnata* showing 99% similarity to GenBank ID: HM239670.1 *Neoharriotta pinnata*. Phylogenetic relationship of *N. pinnata* to other organisms was established based on the nucleotide sequence comparisons of CO1. Phylogenetic relationship of *N. pinnata* to other organisms was virtually identical in both NJ tree and ML tree. NJ tree represented in Figure 1 gets broadly divided into six clusters. Cluster one includes mammals, cluster two includes three subclusters representing birds, teleost fishes, and frogs, cluster three includes two subclusters of cartilaginous fishes, one representing sharks and the other representing skates and rays, cluster four includes fishes belonging to Holocephali group, cluster five represents crustaceans, and cluster six includes molluscs. *N. pinnata* though closely related to Holocephalan fishes occupies a position in-between the vertebrate and invertebrate groups.

RT-PCR amplification of the mRNA from Sicklefin Chimaera yielded a 243 bp fragment cDNA encoding 81 amino acid residues. The obtained nucleotide and deduced amino acid sequences were deposited in GenBank database (GenBank ID: JX297204). BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptides belonged to histone H2A family. Bootstrap distance tree calculated using deduced amino acid sequence confirmed its similarity with previously reported histone H2A sequences deposited in GenBank database. Bootstrap distance tree was calculated using NJ method and ML method. Phylogenetic relationship of histone H2A of *N. pinnata* to histone H2A of other organisms was found to be virtually identical in both NJ tree and ML tree. The phylogenetic distance tree based on amino acid sequence of histone H2A is represented in Figure 2. The phylogenetic tree gets divided into two main clusters. Cluster one denotes histone H2A sequences of vertebrates, and cluster two represents that of invertebrates. The vertebrate group could be classified into three subclusters representing mammals, amphibians, and fishes, while invertebrate group could be classified in mollusc and crustacean sub-clusters. Birds, when included for construction of phylogenetic tree, grouped with both mammals and fishes (Figure 3). In case of histone H2A also, *N. pinnata* was found to occupy a position in between the vertebrate and invertebrate clusters, though more closely related to vertebrates than invertebrates. Histone H2A protein of *N. pinnatta* differs from other reported histone H2A proteins in having amino acid His at position 34 and Asp at position 42 from the N-terminus. In all other previously reported sequence of histone H2A, Lys and Glu are present at the corresponding position of His and Asp. Histone H2A protein of *N. pinnatta* has Val at position 31 and Thr at position 60 from the N-terminus. Histone H2A reported in vertebrates is similar to *N. pinnatta* in having Val and Thr at corresponding positions, but histone H2A reported from invertebrates has Ile and Ala at corresponding positions. *N. pinnatta* has Val at position 63, a feature found in invertebrates, as all histone H2A reported from them have Val at corresponding position. The scenario is different in case of all other vertebrates as they have Ile at corresponding position. Histone H2A of *N. pinnatta* displays similarities and dissimilarities with both vertebrates and invertebrates and thereby occupies a position in-between the two (for details see supplementary Figure in Supplementary Material available online at http://dx.doi.org/10.1155/2013/930216).

The nucleotide sequence and the deduced amino acid sequence of histone H2A amplified from sicklefin chimaera are presented in Figure 4. Analysis of functional aspects and chemical properties of the histone H2A protein were carried out using reliable computer based programs. The PeptideCutter tool predicts proteolytic enzymes, chymotrypsin, and pepsin to have a potential cleavage site at position 52 and 40 from N-terminus of histone H2A of sicklefin chimaera. Cleaving the protein at position 52 would release Harriottin-1, a peptide sharing similarity with Hipposin. Proteolytic activity of these enzymes at position 40 would result in the release of a peptide termed as Harriottin-2 which is similar to Buforin I reported from toad. Enzyme trypsin was found to have potential cleavage sites at position 16 and 37 from the N-terminus. Trypsin mediated processing of *N. pinnata* histone H2A would result in the formation of a 21 mer peptide, Harriottin-3 having a sequence resembling Buforin II. Diagrammatic representation of the cleavage site of enzymes and release of the three Harriottins is presented in Figure 5. Sequence analysis of the peptides was carried out using ProtParam software which predicted Harriottin-1, -2, and -3 to have molecular weights of 5.56 kDa, 4.39 kDa, and 2.44 kDa, respectively, and a theoretical isoelectric point

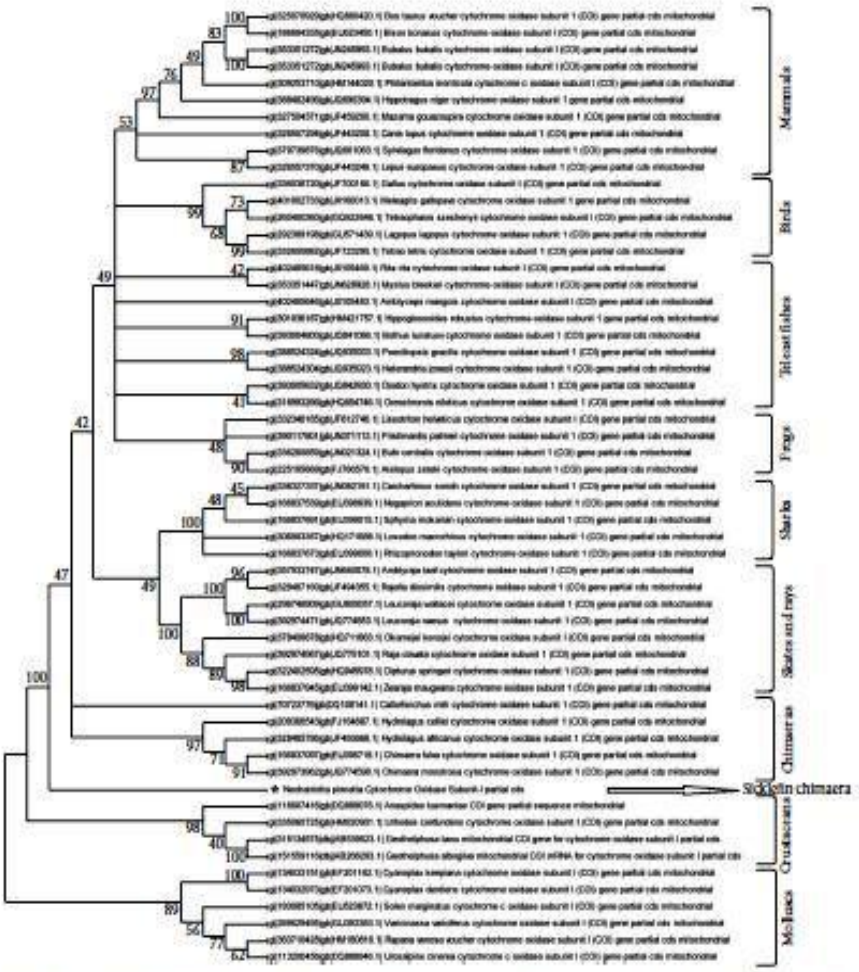


FIGURE 1: A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating the phylogenetic relationship of *N. pinnata* based on the nucleotide sequence of cytochrome oxidase subunit-1 gene.

(pI) of 12.01, 12.41, and 12.60, respectively. ProtParam software estimated the half-life of Harriottin-1, -2, and -3 to be more than 20 hour in yeast and more than 10 hours in *E. coli*. In case of mammalian reticulocytes Harriottin-1 and -2 were estimated to have a half-life of 30 hours, whereas Harriottin-3 was found to have a half-life of 1.9 hours. Multiple-sequence alignment of the amino acid sequences of Harriottin-1 with previously reported histone H2A derived AMPs revealed that the peptide showed similarity to previously reported histone H2A derived AMPs like Buforin I, Buforin II, Hipposin, Himanturin, Abhisin, Sunettin, and histone H2A derived AMPs reported from *Oncorhynchus mykiss*, *Litopenaeus vannamei*, and *Chlamys farreri* (Figure 6). Only Harriottin-1 was considered for multiple-sequence alignment as it covers sequences of both Harriottin-2 and Harriottin-3. All three Harriottins were found to have an overall net positive charge. At pH 4, 7, and 10, Harriottin-1 was found to have a charge of 14.8, 12.4, and 7.4; Harriottin-2 had a net positive charge of 14.1, 12.4, and 7.9, while Harriottin-3 displayed a net positive charge

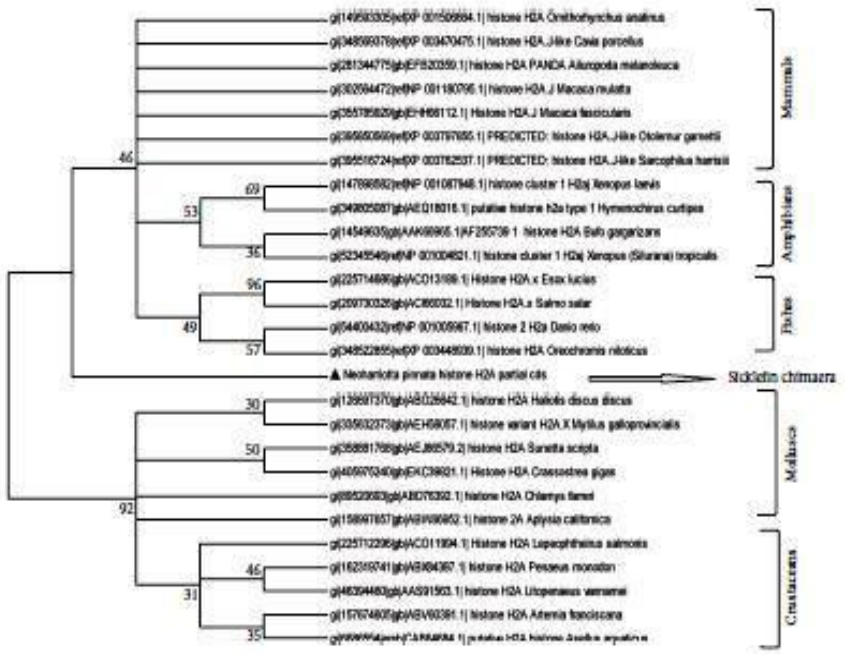


FIGURE 2: A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the amino acid sequence of histone H2A of *N. pinnatta* to the amino acid sequences of previously reported histone H2A from different organisms.

of 7.1, 5.4, and 4. Hydrophobicity of Harriottin-1, -2, and -3 were found to be +52.17 kcal/mol (30%), +45.66 kcal/mol (25%), and +24.37 kcal/mol (28%) as predicted by PepDraw. Analysis of Harriottins using Protean module of the DNASTAR Lasergene sequence analysis software suite revealed that Harriottin-1 and -2 will have a concentration of 1.87 mg/mL and 2.95 mg/mL for an absorbance of 1 OD measured at 280 nm, whereas Harriottin-3 will not give any reading at 280 nm wavelength, as it lacks Thr, Cys, and Trp. The module further predicts that 1 μg of the Harriottin-1, -2, and -3 would contain 179.75 pmoles, 227.63 pmoles, and 409.03 pmoles, respectively. Schiffer-Edmundson helical wheel modeling of the peptides using Protean module revealed clustering of hydrophobic and hydrophilic/basic residues on opposing sides of the helical wheel (Figure 7). This result suggests an amphipathic nature and an α-helical structure for the Harriottins. Three-dimensional arrangement of Harriottins is shown in Figure 8. Analysis of Harriottins for their antimicrobial activity was carried out with Antimicrobial Peptide Predictor Program (http://aps.unmc.edu/AP/main.php) which predicts them to be antimicrobial peptides with a protein binding potential of 2.58 kcal/mol, 3.26 kcal/mol, and 3.83 kcal/mol for Harriottin-1, -2, and -3, respectively.

4. Discussion

The present study describes characterization of Harriottin-1, -2, and -3, with 52, 40, and 21 amino acid peptides identified from histone H2A of *N. pinnata* and phylogenetic analysis of the organism based on CO1 and histone H2A. One of the commonly used molecular markers for taxonomic identification of a species is the CO1 gene, comparison of which provides a reliable determination of the phylogenetic relationship of a species indicating its position in the evolutionary tree. The phylogenetic relationship of *N. pinnatta* based on nucleotide sequence of CO1 gene was analyzed by NJ method and ML method. As expected *N. pinnatta* was found to be closely related to Holocephalan fishes but was found to occupy a position between vertebrates and invertebrates. Order Chimaeriformes to which chimaeras belong include three families: Chimaeridae, Callorhinchidae, and Rhinochimaeridae. Results of the phylogenetic analysis based on CO1 gene indicate that Rhinochimaeridae represented by *N. pinnatta* appears to be more primitive of the three.

The phylogenetic relationship of histone H2A amino acid sequence of *N. pinnatta* to the amino acid sequence of previously reported histone H2A proteins from various organisms

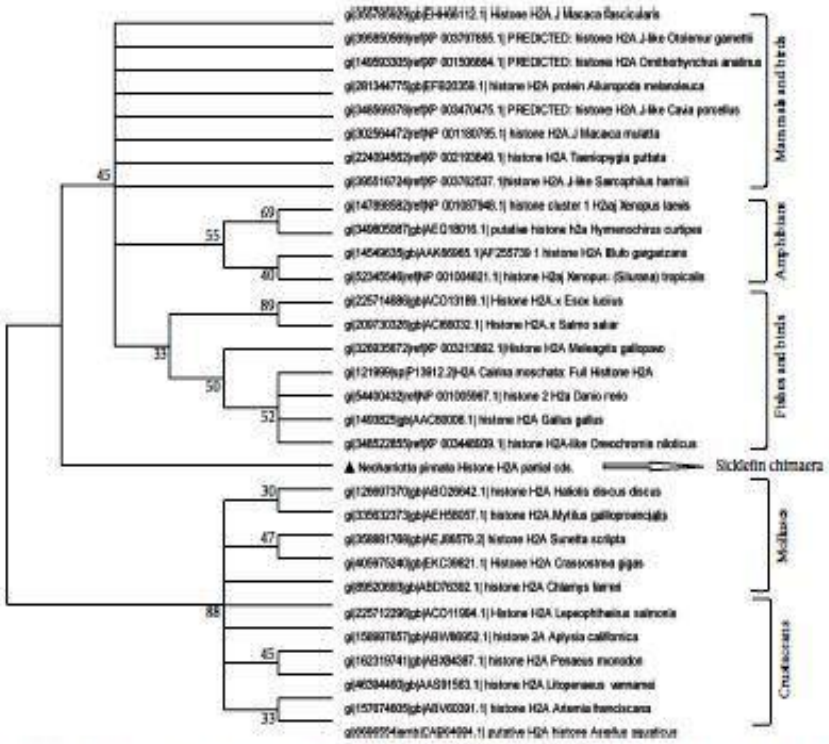


FIGURE 3: A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the amino acid sequence of histone H2A of *N. pinnatta* to the amino acid sequences of previously reported histone H2A from invertebrates, fishes, amphibians, birds, and mammals. Birds can be seen aligned with mammals and fishes.

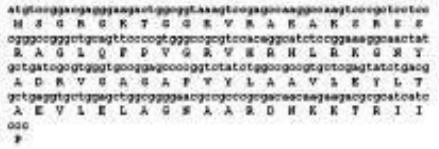


FIGURE 4: Nucleotide and amino acid sequence of histone H2A protein from *N. pinnatta*.

was carried out using NJ method and ML method. The molecular phylogenetic tree based on amino acid sequences of previously reported histone H2A derived AMPs demonstrates that the members of the family are derived from a common ancestor by a series of evolutionary changes. Selected histone H2A derived antimicrobial peptide sequences got divided into two major groups, that is, vertebrates and invertebrates. The boot strap distance tree calculated reveals that histone H2A protein of *N. pinnatta* can align with the vertebrate group, but the lineage is distant enough to conclude that it occupies a position between vertebrate and invertebrate groups. Birds when included in the phylogenetic tree did not form a group of their own and was found to align with fishes and mammals. Evolution of histone H2A is not clearly demarcated in birds and this indicates that the histone H2A has a highly conserved sequence. Even though the rate of evolution is slow in histone H2A, well-marked differences can be observed in case of *N. pinnata*. Histone H2A protein of *N. pinnatta* diverges from other reported histone H2A proteins at amino acid position 34 and 42 from the N-terminus where it has His and Asp, respectively. Lys and Glu is present at the corresponding position of His and Asp in all other reported sequences of histone H2A. Since Lys and His as well as Asp and Glu belong to same characteristic group in amino acid classification, their interchange would not have much effect on the property of the protein. The region of histone H2A corresponding to amino acid position 16 to 51 in histone H2A of *N. pinnatta* is a highly conserved region in animal kingdom. *N. pinnatta* differs from histone

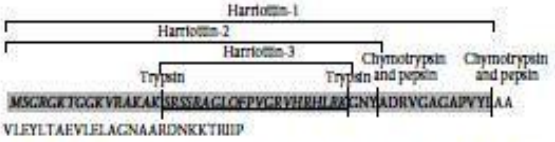


FIGURE 5: Potential cleavage site of proteolytic enzymes resulting in formation of Harriottin-1, -2, and -3 is demonstrated.

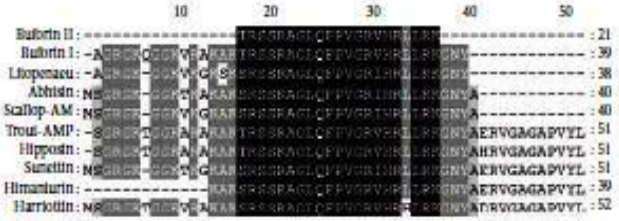
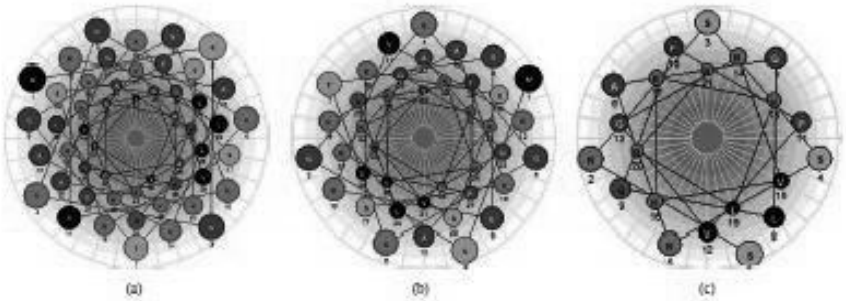


FIGURE 6: ClustalW multiple alignment of Harriottin-1 (*N. pinnatta*) with Buforin I and II (*Bufo bufo gargarizans*), Hipposin (*Hippoglossus hippoglossus*), Rainbow Trout H2A (*Oncorhynchus mykiss*), Litopenaeus AMP (*Litopenaeus vannamei*), Scallop AMP (*Chlamys farreri*), Abhisin (*Haliotis discus*), Sunettin (*Sunetta scripta*), and Himanturin (*Himantura pastinacoides*).

H2A of other organisms in this region at two positions (position 34 and 42) which clearly indicates that sicklefin chimaera has followed a different path of evolution. *N. pinnatta* represents Holocephalan fishes which are believed to be branched off from their sister group of sharks and rays and have remained isolated ever since. This is quite evident from the results of phylogenetic analysis based on histone H2A amino acid sequence of *N. pinnatta* which shows similarity to both vertebrates and invertebrates and at the same time differs from both of them. Since histone H2A sequences of cartilaginous fishes are scarce in GenBank database a detailed investigation was not possible. Histone genes represent much conserved regions, and therefore evolutionary analyses of histones should provide important information with regard to the phylogenetic relationships of distant/closely related organisms.

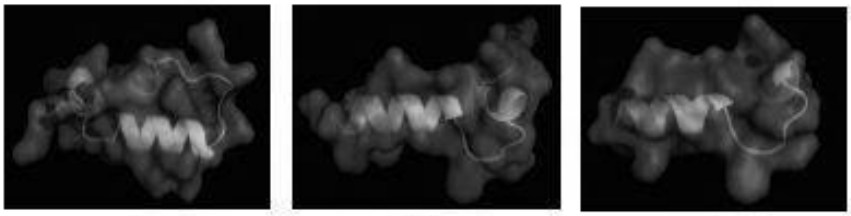
Harriottins exhibited high sequence similarity with previously reported histone H2A derived AMPs. Harriottins are highly cationic peptides with amphipathic nature and α-helical structure, characteristic to all histone H2A derived AMPs. Harriottin-1, -2, and -3 were found to be rich in arginine (15, 18, and 23%), glycine (17, 18, and 10%), serine (8, 10, and 14%), valine (10, 8, and 10%), and alanine (11, 8, and 5%) as reported in all other histone H2A derived AMPs. All histone H2A derived AMPs reported to date from various sources are derived from N-terminal region of histone H2A, thereby suggesting its importance in innate immune response of an organism. Histone H2A fragments with antimicrobial activity reported from vertebrates and invertebrates clearly indicate the role of histone H2A as a potential precursor for highly potent antimicrobial peptides. In Asian Toad *Bufo bufo gargarizans*, the intact histone H2A protein is secreted into the stomach, and Buforin I is produced by the action of pepsin isozymes cleaving the Try 39-Ala 40 bond of intact protein [21]. Similarly in Cat Fish (*Parasilurus asotus*), parasin I is produced by cleavage of Ser19-Arg20 bond of histone H2A by cathepsin D found in skin mucus of the fish [22]. PeptideCutter tool predicts proteolytic enzymes trypsin, chymotrypsin, and pepsin to have potential cleavage sites in the histone H2A of sicklefin chimaera which presented the possibility of formation of three fragments similar to previously reported histone H2A derived AMPs. A 52-mer fraction similar to hipposin was termed as Harriottin-1; a 40-mer fraction resembling Buforin I was termed as Harriottin-2, and a third 21-mer fraction comparable to Buforin II was given the name Harriottin-3.

Histone H2A derived antimicrobial peptides are known to exhibit broad spectrum activity against bacteria and fungi. Hipposin and Buforins are the most studied histone H2A derived antimicrobial peptides. Hipposin exhibited strong antibacterial activity against several Gram-positive and Gram-negative bacteria, and activity could be detected down to a concentration of 1.6 μg/mL [10]. Harriottin-1 has a sequence and structure similar to Hipposin and therefore would have a similar activity. Buforins are among one of the most potent antimicrobial peptides. In addition to their broad spectrum activity against bacteria and fungi [23], they also possess antiendotoxic and anticancer activities [24]. Harriottin-2 and -3 would be expected to match the activity of Buforin I and II, respectively, by virtue of their sequence and structure. Buforin II does not cause significant membrane permeabilization [25] but brings about the lysis of bacterial cells by readily entering the cells in vivo and by interacting with intracellular nucleic acids [26, 27]. NMR



(a) (b) (c)

FIGURE 7: Schiffer-Edmundson helical wheel diagram demonstrating probable amphipathic α-helical conformation of Harriottin-1 (a), Harriottin-2 (b), and Harriottin-3 (c).



(a) (b) (c)

FIGURE 8: Predicted 3-dimentional structural arrangements of Harriottin-1 (a), Harriottin-2 (b), and Harriottin-3 (c) generated using PyMol software.

structural studies showed that proline at position 11 serves as a hinge between a C-terminus helix and an N-terminal region with an extended helical structure [28]. This sole proline residue (Pro_{11}) of Buforin II is necessary for effective translocation across cell membrane [25, 26]. Presence of proline at position 11 and the resulting proline hinge as in Buforin II was also detected to be a characteristic feature of Harriottin-3. Presence of proline hinge seems to indicate that the antimicrobial activity of Harriottin-3 lies in its ability to interact with nucleic acid rather than membrane permeabilization. Antimicrobial peptides are also viewed as agents with therapeutic potential against cancer cells [29]. Buforin II exhibits selective cytotoxicity against cancer cells through interaction with cell surface gangliosides, and once inside the cell they induce mitochondria-dependent apoptosis [30]. Buforin II does not exhibit cytotoxic activity of any kind against normal mammalian cells [31]. Having a structure similar to Buforin II makes Harriottin-3 potential candidates for anticancer research. Antimicrobial Peptide Predictor Program (http://aps.unmc.edu/AP/main.php) predicted Harriottins to be AMPs, since Harriottin-1, -2, and -3 form alpha helices and possess 6, 4, and 4 residues, respectively, on the same hydrophobic surface which assist them to interact with membranes. Harriottin-1, -2, and -3 illustrate all the characteristic features of AMPs including high cationicity, higher hydrophobic residue, and elevated protein binding potential, that is, 2.58, 3.26, and 3.83 kcal/mol, respectively.

5. Conclusion

Three peptides containing antimicrobial sequence motif from the histone H2A of *N. pinnatta* were identified and named as Harriottin-1, -2, and -3. High sequence similarity of Harriottin-1, 2 and 3 to previously reported potent histone H2A derived AMPs and their similarity to traditional antimicrobial peptides in physicochemical properties strongly endorse Harriottins to be considered as peptides with antimicrobial activity. The study was taken up as an initiator to investigate the role of histone derived AMPs in Holocephalan fishes, and more research in this area would reveal new facets of innate immunity in this less understood group of fishes. The study gives a comparative account of CO1 and H2A nucleotide sequences in the molecular taxonomic identification of members of the animal kingdom. Birds get grouped both with fishes and mammals, but not with amphibians, which is really intriguing. The study also offers an insight into the evolutionary divergence of *N. pinnatta* with respect to CO1 gene and histone H2A occupying an intermediate position with respect to invertebrates and invertebrates.

Acknowledgment

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