Mangrove plant *Ceriops tagal* as a potential source of anti White Spot Syndrome Virus preparations for *Penaeus monodon*

Thesis submitted to the

Cochin University of Science and Technology

In partial fulfilment of the requirements for the award of the degree of

Doctor of Philosophy

in

Marine Biotechnology

Under the Faculty of Marine Sciences

Department of Marine Biology Microbiology & Biochemistry

Cochin University of Science and Technology

By

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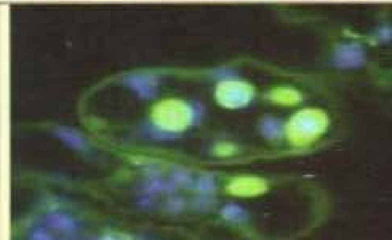
December 2009



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December-2009

"Recite: In the Name of thy Lord who created,
created man of a blood-clot.
Recite: And thy Lord is the Most Generous,
who taught by the Pen,
taught man that he knew not. ,,

-Holly Quran, Surah 96, verses 1-2 -

Certificate

This is to certify that the research work presented in this thesis entitled **"Mangrove plant *Ceriops tagal* as a potential source of anti White Spot Syndrome Virus preparations for *Penaeus monodon*"** is based on the original work done by Mr. Sudheer N.S under our guidance, at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682016, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in this thesis entitled **"Mangrove plant *Ceriops tagal* as a potential source of anti White Spot Syndrome Virus preparations for *Penaeus monodon*"** is based on the original work done by me under the joint guidance of Dr. Rosamma Philip, Senior Lecturer, Department of Marine Biology, Microbiology and Biochemistry and Co-Guidance of Prof. I.S. Bright Singh Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin 682016
December 2009

Sudheer N.S

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syndrome virus molecules from selected species of Marine Biota and development of an anti WSSV preparation"

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Sudheer N.S

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Chapter 1

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1.1 Introduction

Aquaculture was in practice since 2000 BC in the ancient Chinese and Roman Empire (Balon, 1995; Dunham et al., 2001) and grew to global dimensions in recant decades. The term aquaculture is defined as a “form of agriculture that involves the propagation, cultivation, and marketing of aquatic animals and plants in a controlled environment” (Swann, 1992). Aquaculture production has enlarged dramatically since the early 1980s and is becoming increasingly important as the demand for fish and fishery products have been increasing world wide as the returns from capture fishery have reached a plateau or showed declining trend. Aquaculture not only provides a sustainable source of protein but also provides meaningful livelihood to poor as it is practiced in the peri- urban or rural or remote areas (FAO 2006). From a production of less than 1 million tons in the early 1950s, it rose to 51.7 million tons, with a value of US $78.8 billions during 2006. Precisely aquaculture has been registering continued and rapid growth than any other food production sectors (FAO 2009).

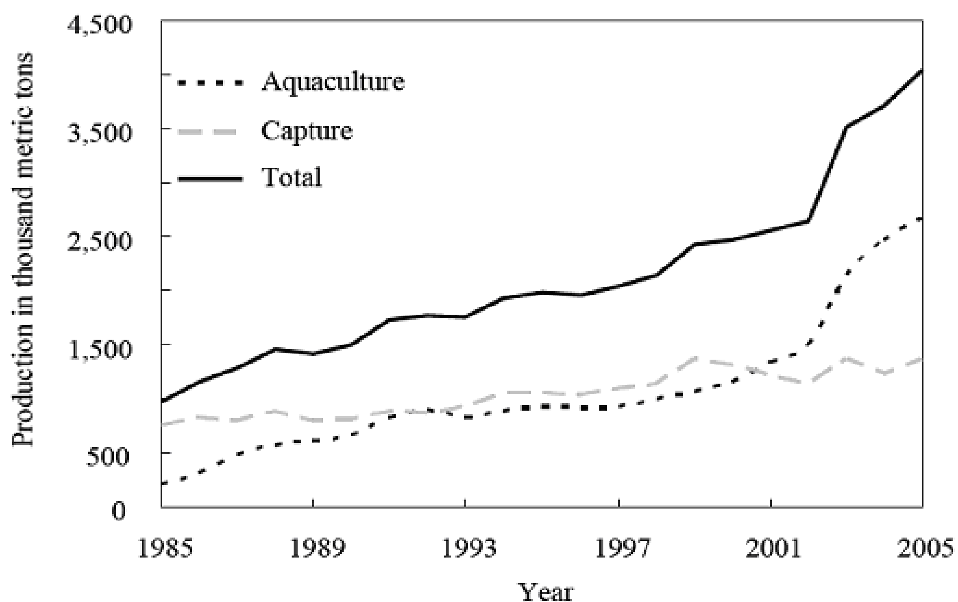

Fig. 1 Trends in Penaeid shrimp aquaculture production of the world compared to capture

India is the second largest producer of aquaculture products with an input of 27, 94, 636 tons with an annual average growth rate of 5.71% (FAO 2009). However, supply is still in short of demand and by 2025, it is aimed to attain 350% increase in production to meet the impending shortage (Hardy, 1999).

Globally, Penaeid shrimp culture ranks 6th in terms of quantity and second in terms of value amongst all taxonomic groups of aquatic animals cultivated (Fig.1) .Over the past three decades, shrimp farming in Asia has been expanding rapidly from of traditional, low-density polyculture to a vibrant export industry currently valued to more than US $ 8 billion (FAO 2006; 2005) (Fig.1) when compared to the cultured species in warm water countries, black tiger shrimp *Penaeus monodon* is the highly preferred species for cultivation, owing to its fast growth, easy availability of seed and above all the high market price it fetches (Pechmanee, 1997). India is in the 5th position in terms of aquaculture production of Penaeid shrimps with an annual production of 1, 43,170 metric ton as reported in 2005 (FAO 2005) (Table 1).

Table 1 Major Penaeid shrimp producing countries in Asia and American continents

Asia	Production in metric tons (MT)	Americas	Production in MT
China	1024049	Mexico	72279
Thailand	375320	Brazil	63134
Vietnam	327200	Ecuador	56300
Indonesia	279375	Colombia	18040
India	143170	Venezuela	16500
Bangladesh	63052	Belize	10433
Myanmar	48640	Peru	9809
Philippines	39909	Nicaragua	9633
Malaysia	33364	Panama	7098
Taiwan	14760	Costarica	5714

Source: FAO (2005)

Indian shrimp culture is dominated by *P. monodon* with the west coast accounting for 70% of the production. Majority of the culture systems in India are traditional in nature, however semi-intensive systems are also in operation in several States (Hein, 2002).

During the past three decades shrimp culture has been emerging as one of the major industries in tropical and subtropical areas of the world and as the major source of earning for poor coastal areas (Adger 1998). However, serious setbacks were witnessed in the industry during this period primarily due to the emergence of diseases (Rosenberry et al, 2002). They included viral, bacterial, ricketsial, fungal, protistan, and metazoan in etiology (Lightner, 1988, 1996; Brock and Lightner, 1990; Brock and LeaMaster, 1992; Flegel et al., 1992; Johnson, 1995). Among these etiological agents viral diseases were found most devastating in a global perspective. So far 20 shrimp viruses have been reported (Tan and Shi, 2008; Walker and Mohan, 2009) (Table 2). Major viral pathogens of shrimps include White Spot Syndrome Virus (WSSV), Monodon Baculo Virus (MBV), Yellow Head Virus (YHV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Hepatopancreatic Parvo Virus (HPV), Taura Syndrome Virus (TSV), Baculovirus Penaei (BP) and Baculovirus Midgut gland Necrosis Virus (BMNV) (Flegel, 2006). Among them WSSV is the most virulent pathogen ever reported which can cause total mortality within 7-10 days of its onset in shrimp culture systems (Lightner, 1996).

Table 2 Major Viral pathogens of penaeid shrimp (Rahman, M.M, 2007)

Family	Virus
DNA virus	
Parvoviridae	Infectious hypodermal and hematopoeitic necrosis virus (IHHNV) Hepatopancreatic parvovirus (HPV) Spawner-isolated mortality virus (SMV) Lymphoidal parvo-like virus (LPV)
Baculoviridae	Baculovirus penaei (BP) Monodon baculovirus (MBV) Baculovirus midgut gland necrosis virus (BMNV) Type C baculovirus of *Penaeus monodon* Hemocyte infecting non-occluded baculo-like virus
Iridovidae	Shrimp iridovirus (IRIDO)
Nimaviridae	White spot syndrome virus (WSSV)
RNA Virus	
Picornaviridae	Taura syndrome virus (TSV)
Roniviridae	Yellow head virus (YHV) Gill associated virus (GAV) Lymphoid organ virus (LOV)
Reoviridae	Reo-like virus (REO) type II and IV
Rhabdoviridae	Rhabdovirus of penaeid shrimp (RPS)
Togaviridae	Lymphoid organ vacuolization virus (LOVV)
Totiviridae	Infectious myonecrosis virus (IMNV)
Bunyaviridae	Mourilyan virus (MOV)
unclassified	Monodon slow growth syndrome (MSGS)

1.2 Geographical distribution of the WSSV

First appearance of WSSV was in northern Thailand during 1992, which caused massive mortality of shrimps (Chou et al., 1995). During 1993 the causative agent was isolated from a disease out break in Japan in *Penaeus japonicus.* By this time it had spread to all shrimp growing nations and got recognized as the most serious pathogen of crustaceans (Escobedo-Bonilla et al., 2008; Claydon et al., 2004; Seiffert et al., 2006; Chang et al., 1999; Flegel and Fegan, 2002; Durand et al., 2000; Walker and Mohan, 2009). Its presence in the wild population of shrimp, crabs, crayfish transformed the culture operations most risky and, the rate of success of any system turns out to be beyond prediction.

1.3 WSSV hosts and carriers

White spot syndrome virus has a broad host range within decapod crustaceans having almost all cultured species of crustaceans being susceptible to the virus which includes at least 18 cultured and/or wild penaeid shrimps (Wongteerasupaya et al., 1996, Durand et al., 1997; Lu et al., 1997; Chou et al., 1998; Lightner et al., 1998; Park et al., 1998). The major species of Penaeid shrimps naturally infected by the virus are *P. monodon, P. Chinensis, P. penicillates, P. indicus, P. japonicus* (Inouye et al., 1994; Takahashi et al., 1996), *P. setiferus* (Lightner et al., 1997) and *Metapenaeus dobsoni, Parapenaeopsis stylifera, Solenocera indica* (Hossain et al., 2001).WSSV has been reported from non penaeids also. Crabs are one of the largest groups of Decapod which are being infected by the virus; 38 crab species have been reported to be infected by WSSV (Lo et al., 1996b; Kanchanaphum et al., 1998; Kou et al., 1998; Sahul-Hameed et al., 2001, 2003). Crabs like *Charybdis annulata, C. cruciata, Macropthalmus sulcatus, Gelasimus marionis nitidus, Metopograpsus messor* have been described as carriers of WSSV (Hossain et al., 2001). Eight caridean species (Sahul-Hameed, et al., 2000; Shi, et al., 2000; Pramod-Kiran et al., 2002), seven species of lobsters (Chang et al., 1998; Rajendran et al., 1999),

seven species of crayfishes (Wang et al., 1998a; Corbel et al., 2001; Jiravanichpaisal et al., 2001; Edgerton 2004; Jiravanichpaisal et al., 2004), six non-decapod crustacean species (Supamattaya et al., 1998; Otta et al., 1999; Hossain, et al., 2001) were also reported to be WSSV positive. Other than arthropods, phyla Chaetognata, Rotifera (Yan et al., 2004; Ramı´rez-Douriet, et al., 2005; Yan et al., 2007) polychaete worms (Supak et al., 2005; Vijayan et al., 2005) also were found to be positive to WSSV on diagnostic PCR. However, this does not mean that they were susceptible to WSSV infection. Some aquatic insect larvae have been found to be WSSV-positive by PCR (Lo et al., 1996b; Flegel, 1997; Ramı´rez-Douriet et al., 2005) Rotifers, polychaetes, non decapodal crustacean including *Artemia salina,* non-crustacean aquatic arthropods such as sea slaters (*Isopoda*) and *Euphydradae* insect larvae, as well as micro algae can act as vectors of WSSV. All these species can accumulate high concentrations of viable WSSV, although there were no evidences of virus replication in them (Liu et al., 2007; Yan et al., 2004; Vijayan et al., 2005; Chang et al., 2002; Li et al., 2003; Lo et al., 1996b). All these findings point to the severity of the situation warranting development of very effective anti WSSV principles to protect shrimp culture from the virus.

1.4 Mode of transmission

There are two modes of transmission of the virus such as horizontal and vertical. Horizontal transmission includes the one through water, vectors and carriers, contaminated live feed organisms, and anthropogenic activities. Transmission through water is prevalent as the virus can sustain in sea water for 3-4 days without host (OIE, 2006). Rapid transmission of WSSV in culture systems do occur from infected shrimp through cannibalism of weak moribund animals (Chang et al., 1996, Sanchez-Martinez et al., 2007). The possibility of viral transmission through contaminated soil cannot be ruled out since a recent study could demonstrate (Natividad et al., 2008) through nested PCR amplification of WSSV DNA in pond soil even after 5 days at 70 °C. Birds (Vanpatten et al., 2004),

different arthropods including insects (Lo et al., 1996b), and other aquatic organisms (Kanchanaphum et al., 1998; Supamataya et al., 1998) can act as vectors of WSSV. There are reports of live feed organisms like *Artemia* (Li et al., 2003), rotifers (Yan et al., 2007) lobsters, crabs (Rajendran et al., 1999), polychaet worms (Vijayan et al., 2005) etc. acting as source of WSSV infection. Among the anthropogenic sources the untreated infected shrimp by- products from processing plants, untreated aquaculture tools etc. may play greater role in transmitting the disease (Sanchez-Martinez et al., 2007). Vertical transmission from parents to offspring is well established in WSSV. There are reports about transmission of WSSV by infected gonads, oogonia and follicle cells in *P.momodon* ovarian tissues (Kou et al., 1997; Mohan et al., 1997). Lo et al., 1997 reported absence of WSSV in mature eggs suggesting that infected ones get killed by the virus even before maturation.

1.5 Taxonomy

Initially the virus was reported as a member of the genus non- occluded baculovirus, subfamily Nudibaculovirinae and family Baculoviridae, based on morphology, size, and site of assembly and nucleic acid. Later, The International Committee on Taxonomy of Viruses (ICTV) assigned this as an unassigned invertebrate virus status (Murphy et al., 1995). Earlier it was believed that the virus was a member of baculovirus, because of its resemblance in morphology and pathology and similarity of a 12 kbp fragment of the 200kbp genome to the baculovirus. Eight open reading frames were apparent including genes for the large and small subunits of ribonucleotide reductase. Phylogenetic analysis showed that these genes did not share an immediate common ancestor with a member of a novel genus in the family baculoviridae, or a possible representative of the family. In 1999 Van Hulten, suggested the name Whispovirus (as a singulum for White spot). Based on sequence analysis of several enzyme genes including the ribonucleotide reductase large and small subunits, the chimeric thymidine-thymidylate kinase and the DNA polymerase demonstrated that the WSSV was distantly related to the other known viral families (Chen et al., 2002;

Liu et al., 2001; Tsai et al., 2000; Van Hulten et al., 2000; Van Hulten et al., 2001; Vlak et al., 2005; Witteveldt et al., 2001). Based on these studies, the ICTV on its 8th report assigned WSSV in a new virus family Nimaviridae. This family consists of a single genus (Whispovirus) and WSSV is the only species in it (Mayo 2002; Vlak et al., 2005).

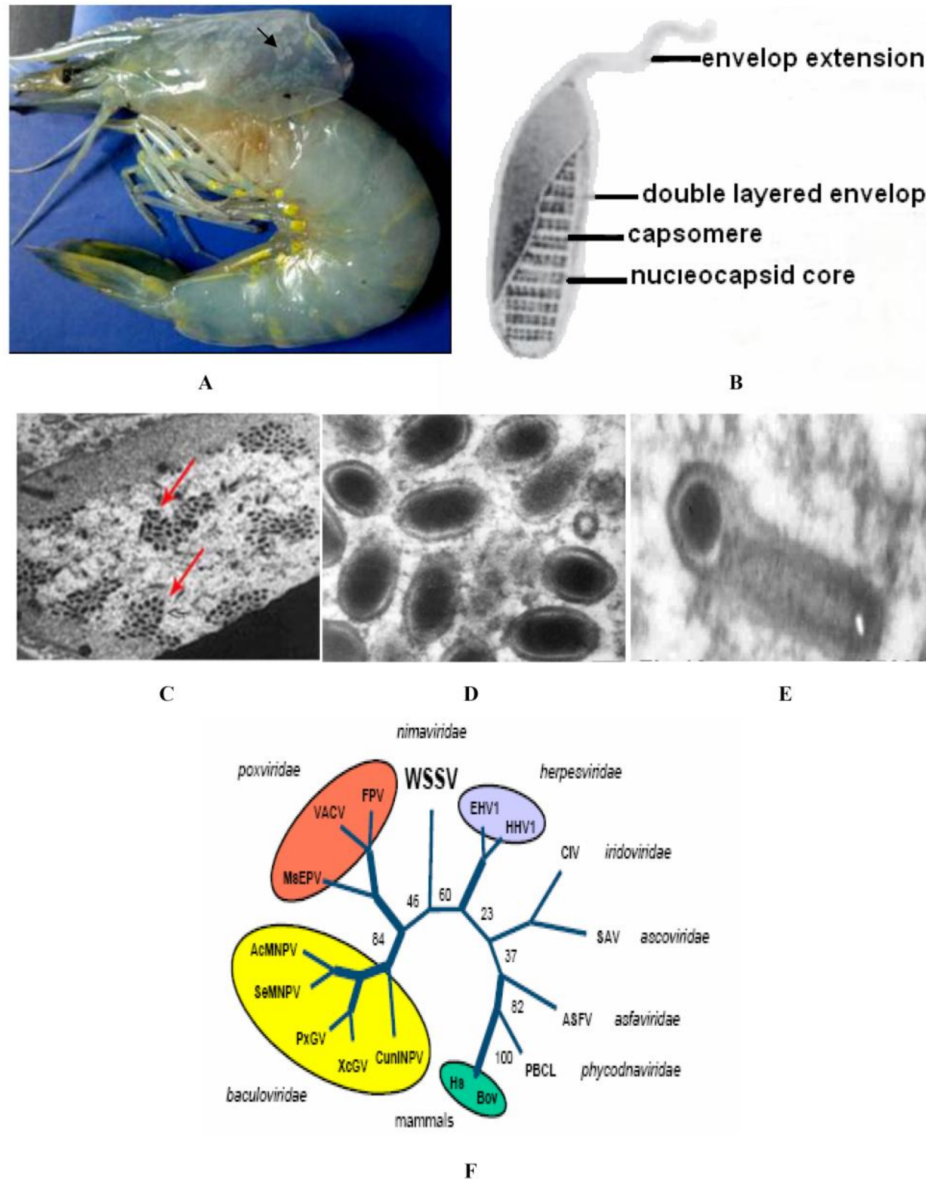

Fig. 2 **A)** WSSV infected *P.monodon* **B)**Schematic diagram of WSSV virion **C)** WSSV virions in nucleus(Manjusha et al., 2009), **D&E)** WSSV different stages of infection cycle (Manjusha et al., 2009) **F)** Phylogenetic tree of WSSV (Vlak et al.,2002)

1.6 Morphology and Ultra structure

WSSV is a Bacilliform no-occluded enveloped virus (Chen, 1995; Wang et al 1995; Wongteersupaya et al, 1995). The virion of WSSV is a large, ovoid particle. Electron microscopic studies revealed that the virions range between 210 and 380 mm in length and 167 - 170nm in width (Park et al., 1998; Rajendran et al., 1999; Tan and Shi, 2008) with a tail like appendage at the end (Wongteersupaya et al, 1995; Durand et al., 1997; Tan and Shi 2008). So far, neither the function nor the composition of this appendage is known. The virus contains one nucleo capsid with a typical striated appearance and composed of 5 major and 39 minor structural proteins (Tsai et al., 2004). However, a recent study conducted based on shot gun identification and i TRAQ differentiation technique it has been found that WSSV is with total of 45 structural proteins (Li et al., 2007). According to recent studies it is estimated that WSSV is assembled by at least 59 structural proteins, among them 35 are envelop protein and 9 nucleocapsid (Tan and Shi. 2008). The virion consists of a rod-shaped nucleocapsid layer surrounded by a loose fitting trilaminar envelope, which consists mainly of the WSSV encoded proteins VP28 and VP19 (Durand et al., 1997; Nadala et al, 1998; van Hulten et al., 2000a; van Hulten et al, 2000b). VP28 is most likely located on the surface of the virus particle and plays key role in WSSV infection (van Hulten et al., 2001b). The nucleocapsid is superficially segmented in appearance. It is formed by stocks of rings which are formed by regular spaced globular subunits of about 8nm in diameter, arranged in two parallel rows (Durand et al., 1997; Nadala et al., 1998). The nucleocapsid contains the viral genome and consists mainly of the WSSV encoded proteins such as VP664,VP51C,VP60B and VP15 (Durand et al., 1997., van Hulten et al., 2002; Witteveldt et al., 2001; Zhang et al., 2002). The VP664 is large viral structural protein responsible for striated appearance of the nucleocapsid (Leu et al., 2005). The nucleocapsid cylinder is closed at one extremity by a smaller segment that forms a slightly rounded end and the opposite end is squared in shape

(Durand et al., 1997,). The area between the nucleocapsid and the envelope varies from about 2 to 7.5 nm. The external wall of the nucleocapsid is 6 nm thick and core of the nucleocapsid is a highly electron dense area (Durand et al., 1997).

1.7 Stability of the virus

WSSV can be inactivated in 120 minutes at 50°C and less than 1 minute at 60 °C (Nakano et al., 1998). The virus is viable for at least 30 days at 30 °C in sea water under laboratory conditions (Momoyama, 1998) and is viable in ponds for at least 3-4 days

1.8 Genome

WSSV has a circular, super coiled, and double stranded (ds) DNA, of about 300 kbp. It is one of the largest sequenced animal viral genomes available (van Hulten et al., 2001a; Escobedo-Bomilla et al., 2008). The DNA molecule is with an AT content of 59% which is homogenously distributed (Tan and Shi., 2008). The genomes of three WSSV geographical isolates have been sequenced completely. This include, a) 292.9 kb isolated from Thailand (WSSV-TH) (AF440570), b) 307.2 kb isolated from Taiwan (WSSV-TW) and c) 305.1 kb isolate from China AF332093 (WSSV-CN) (van Hulten et al., 2001a;Yang et al., 2001;Chen et al., 2002a). Between the isolates of WSSV few RFLPs were reported indicating some genetic variation. Recent study on 81 Indian WSSV isolates by Pradeeep et al., (2008) found that the Indian isolate of WSSV carries a 10,970bp deletion in the ORF 23/24 region relative to WSSV-TH and WSSV TH-96-II. On analysis of the ORF 14/15 they could find two novel strains of WSSV with unique sequences which could have evolved by recombination. None of the isolates had a transposase sequence or VP35 gene as reported for Taiwan isolates. They suggested that the Indian strains are in close relation with Thailand strains. This is suggestive of the movement of putative ancestor from Thailand to other parts of the world including India. The WSSV- TH genome shows 184 ORF, coding between 50 or more proteins (Lo et al., 1999; Yang et al., 2001)

where as WSSV CN has 181 ORF. The major differences among the three genomes of WSSV are two polymorphic regions of about 14 kbp (Sanchez-Martinez et al., 2007). Sequence analysis shows that the WSSV genome contains between 531 and 684 ORF with an ATG initiation codon on which 181-184 ORF are likely to encode functional proteins with size between 51 and 6077 amino acids, which represents 925 of the genetic information contained in the genome (van Hulten et al., 2001a; Yang et al., 2001). However, only 21-29% of such ORFs have been shown to encode WSSV protein or share identity with other known proteins (Escobedo Bonilla et al., 2008). Only 12 of the 184 ORFs (6%) could be assigned a putative function involved in DNA replication, nucleotide metabolism and protein modification (van Hulten et al., 2001a). The WSSV genome is further characterized by the presence of 9 direct repeat regions with different sizes designated as homologous regions (h*rs* 1-9) (Tan and Shi, 2008). These *hr*s (Homologous regions) are dispersed throughout the WSSV genome and consist of three to eight identical repeat units of 250bp or parts there of. The *hr*s are largely located in intergenic regions, although several short ORFs are annotated within the WSSV *hrs* (van Hulten et al., 2001a). An internal ribosome entry site (IRES) element is also reported in WSSV genome which has efficiently co expressed a glutathione S- transferase and a GFP protein arranged in a dicistronic mRNA *in vitro* (Han and Zhang, 2006).

The WSSV genome can be divided into a) structural genes which encode for envelop and nucleocapsid or integument, b) functional genes involved in the virus proliferation and life cycle function, c) the latency related genes whose expression can be detected even though the structural genes might not be active, d) temporal regulatory genes which participate at specific times during infection (Sanchez-Martinez et al., 2007).

The structural genes of WSSV include the genes encoding structural proteins VP28 and VP 19 (van Hulten et al., 2001a; Yang et al., 2001; Shekar and Ravichandran 2007). VP28 is the major envelope protein which has

important role in infection. The presence of multiple glycosylation sites of VP 28 are surmised to contribute in the recognition of receptors from shrimp cell surface (Yi et al., 2004). However, this has not yet been proved. The amino acid sequence of another structural protein which matched with the ORF 1050 (AF411634), VP 281 which encodes 31.5 kDa protein, homologous to VP 292 from the same genome also have been reported (Huang et al., 2002a, b). Liang et al. (2005) reported VP 281 as the viral attachment protein. VP76 is a recently characterized 76 kDa envelop protein encoded by ORF 220 from WSSV – CN and ORF 112 from WSSV-TH, which have a conserved domain of a Class 1 cytokine receptor (Huang et al., 2005). ORF 340 encodes another structural protein VP 31 (Li et al., 2005b). Li et al. (2006a) reported another structural protein VP110 encoded by WSSV genome. The genes encoding Nucleocapsid proteins like VP26 (Xie et al., 2005), VP 15 (Witaveldt et al., 2005), VP 664 (Leu et al., 2005) have been located in WSV Genome. The structural proteins are synthesized later during the infection and generally have a degenerate TIS motif (A/TNAC/G) located 25 nucleotides down stream of an A/T rich region which is similar to TIS motifs in arthropods (Tsai et al., 2004; Marks, 2005).

Many of the functional genes of the WSSV have been studied for their role in viral multiplication cycle. These genes coding for proteins includes enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase (Chen et al., 2002a), a nonspecific nuclease (Witteveldt et al., 2001; Li et al., 2005 a), a small and large subunit of ribonucleotide reductase (van Hulten et al., 2000a; Tsai et al., 2000b) thymidine kinase, thymidylate kinase, a chimeric thymidine – thymidylate kinase (Tsai, et al., 2000a), a thymidylate synthase (Li et al. 2004b), a dUTPase (Liu & Yang 2005) and two Protein kinases PK (van Hulten and Vlak, 2001; van Hulten et al., 2001a; Yang et al., 2001). Wang et al., 2004 identified an ORF 390 as a novel apoptotic gene. This ORF displays two capsase cleavage sites. He suggested that the ORF 390 in WSSV function as an apoptotic suppresser. Transcriptional analysis of genes coding for proteins required

in DNA replication and nucleotide metabolism are synthesized early during virus replication. Early transcribed WSSV genes in general have a TATA box 20–30 nucleotides upstream of the transcription initiation site (TIS) (A/C) TCANT (Chen et al., 2002a; Liu et al., 2005; Marks 2005).

Three early latency related genes were found to be involved in WSSV latency. These three genes were identified using microarray technique in SPF Shrimp. The ORFs corresponding 151,366 and 427 code for these latency related genes (Khadijah et al., 2003).

The immediate early genes are considered as temporal regulatory genes. These genes do not require viral protein to be transcribed and are expressed using the host molecular machinery in the first hours of infection. These genes include ORF 126, ORF 242and 418 named as *ie*1, *ie2, ie3* (Liu et al., 2005). Recently a viral gene WSSV 447 has also found as an early gene, which plays a key role in DNA replication and virus proliferation. Transcription experiments carried out at 4 hour post infection effectively suggest that it is an early gene coding for GTP binding activity protein (Han et al., 2007).

1.9 Proteomics of WSSV

With the completion of the WSSV genomic sequencing, attention has been focused on the functional analysis of the encoded proteins. A better understanding of WSSV structural proteins and the localization in the virion will shed more light on virus assembly, its infection pathway and the discovery of antiviral drugs. The large size and the complexity of the genome indicate that there are many other proteins which have not yet been identified by conventional methods. This is evident in SDS-PAGE analysis of WSSV which exhibited large number of protein bands (Tsai et al., 2004; Tan and Shi, 2008). Even though several structural proteins like VP 35, VP 28, VP 26, VP24, VP19, and VP15 were successfully studied using methods like SDS-PAGE and Western blotting and / protein- n terminal sequencing (Chen et al., 2002b; Hameed et al., 1998; Van

Hulten et al., 2000b) methods such as immunogold electron microscopy (IEM) was also used to locate 14 proteins including VP28, VP26, VP31, VP51C, VP36B, VP68, VP41A, VP12B, VP180, VPI24, VP39, VP110, and VP24 as envelop proteins and VP 466 as nucleocapsid protein. (Huang et al., 2002a; Huang et al., 2002b; Li et al., 2004b, 2005b, 2006a ; 2006b; Xie et al., 2006; Zhang et al., 2004; Zhang et al., 2002a; Zhang et al., 2002 b; Zhu et al., 2005; Zhu et al., 2006). In 2007 Li et al., (2007) published their proteomic study of WSSV structural proteins by using i TRAQ technology. According to them the WSSV is assembled by at least 59 structural proteins, which included 35 envelop proteins (including integument protein) and 9 nucleocapsid proteins. During the same year a new envelop protein WSV 010 was identified by Shotgun proteomic approach using offline coupling of LC system with MALDI-TOF/TOF MS/MS (Chen et al., 2007).

Many attempts have been made on the functional aspects of major WSSV viral proteins. The WSSV nucleocapsid contains mainly the WSSV encoded proteins VP664 and VP15. The VP 664 is the large viral structural protein found, responsible for striated appearance of the nucleocapsid (Leu et al., 2005). The VP 664 gene is a late transcribed gene, suggesting that its protein contribute to the assembly and morphogenesis of the virion. VP 15 is a basic protein with no hydrophobic regions, is a histon like double stranded DNA binding protein that tends to binds with DNA suggesting that VP15 is involved in packing the viral genome within the nucleocapsid (Witteveldt et al., 2005).

Envelop proteins play vital role in infection, including binding to receptors or penetrate into host cells by membrane fusion. VP28 is the most abundant envelop protein located on the surface of the virus particle, supposed to play a key role in WSSV binding to shrimp cells as an attachment protein facilitating virus entry into cytoplasm (van Hulten et al., 2000b; Yi et al., 2004). It has been reported that VP28 can bind to shrimp cells in low pH environment and interact with host cells through PmRab7, a membrane protein from shrimp haemocyte

cycle which have high homology to the small GTP binding protein Rab7 (Sritunyalucksana et al., 2006b). Another envelope protein VP 110 was identified by IEM and Western blot, which interacts with host cell suggesting that an RTG motif of VP 110 could play a role in WSSV infection (Li et al., 2006a).VP 36A is also considered as a tegument protein (Tsai et al., 2004). A collagen-like protein is also located in WSSV envelop (Li et al., 2004a).VP 26 has been identified as a tegument protein which is supposed to be associated with viral penetration due to its actin binding motif that facilitate the attachment of the virus to the shrimp cell membrane (Xi and Yang 2005; Tsai et al., 2006). Xi and Yang (2006) reported that VP28 interact with VP 24 and VP 26 by forming a complex. Chen et al. (2007) reported that WSSV 010 also interact with VP 24 which indicated that VP 24 might act as linker for VP28, VP26 and VP 110 to form a complex which plays an important role in viral morphogenesis and infection. A study by Zhou et al, 2009 found that four major WSSV envelop proteins, VP28, VP26, VP24, and VP19 can bind to form a multiprotein complex. The major envelop proteins identified involved in infection are VP28, VP31, VP66A, and VP36B (VP281), VP466, VP68, and VP76 (Huang et al., 2002a; 2002b;2002c; Li et al., 2005b, 2006b; van Hulten et al., 2001b; Wu et al., 2005). From the above studies it could be concluded that there were multiple proteins involved in WSSV infection rather than one alone.

The non structural proteins include mainly the enzymes involved in the nucleotide metabolism like DNA polymerase, Riobonucleotide reductase subunits, thymidine-thymidylate kinase, thymidylate synthase and dUTPase (van Hulten et al., 2001a; Chen et al., 2002a; Tsai et al., 2000a, 2000b; Yang et al., 2001) and Protein kinase (van Hulten and Vlak, 2001). The WSSV DNA polymerase gene defines a polypeptide of 2351 amino acids residues which contains DNA polymerase and exonuclease domains. The WSSV DNA *pol* is much larger than other known viral DNA *pol*s. Ribonucleotide reductase and Thymidine kinase are important for the synthesis of the DNA precursors: RR for *de novo* pathway and

TK for the salvage pathway. The presence of Ribonucleotide reductase suggests that WSSV uses its own enzyme to reduce all 4 ribonucleoside diphosphates in the *de novo* bio synthesis pathway of the deoxy ribonucleotides (Tsai et al., 2000b; van Hulten et al., 2000a).The WSSV *tk-tmk* is a unique unusual gene, which codes a chimeric protein of 388 aminoacid residues with homology to two proteins thymidine kinase (TK) and thymidylate kinase (TMK). WSSV TK is found to be similar to eukaryotic cystolic TKs in function which makes it difficult to develop a drug targeting against WSSV TK (Tzeng et al., 2002). Another enzyme essential for WSSV replication is dUTP pyrophosphatase which maintains the level of dUTP : dTTP ratio as reported from WSSV-CN (Liu and Yang, 2005).

1.10 Virulence and Pathogenesis of WSSV

WSSV can cause total mortality of a culture stock within 3-7 days of the onset of disease. Differences have been observed in virulence between isolates of WSSV from different regions and between host species. Lan et al. (2002) found that in cray fish *P. clarkii* WSSV containing a 305 kb genome gave total mortality of the stock earlier to WSSV with a 4.8 kb deletion. However, Marks (2005) came out with a different opinion that differences in virulence and competitive fitness are dependent on the genome size. In his study a putative ancestral WSSV isolate (WSSV-TH-96-II) with the largest genome size recorded (312 kbp) showed a lower virulence [median lethal time (LT50) = 14 days] and competitive fitness compared with another WSSV isolate (WSSV-TH) with a smaller genome size (292 kbp) (LT50 = 3.5 days). He could conclude that WSSV isolates with a smaller genome size may represent an advantage for virus replication. Wang et al., 1999b carried out challenge studies with six isolates of WSSV in post-larvae of *L. vannamei* and juveniles of *F. duorarum* inoculated *per os*. According to them the Texas isolate was the most virulent, while the Washington isolate (from crayfish) was the least virulent. In *F. duorarum,* the cumulative mortality was 60% with the Texas isolate and 35% with the WSSV isolate from crayfish.

Virulence can vary according to the life stage of the host also (Momoyama et al., 1999; Wang et al., 1999a). WSSV infection could not be induced in the early larval stages of *P. monodon* (nauplii, zoea, mysis) by immersion and oral challenge (Yoganandhan et al., 2003b). Apparently, shrimp becomes susceptible to infection from PL 6 (Venegas et al., 1999), PL 10 (Flegel 2007) or PL 30 onwards (Pérez et al., 2005).The mode of challenge also gives different results. Differential host passaging also alters pathogenicity and induces genomic variation in white spot syndrome virus (Waikhom et al., 2006). Three white spot syndrome virus (WSSV) isolates from Thailand and Vietnam (WSSV Thai-1, WSSV Thai-2, and WSSV Viet) were compared for Mortality patterns and quantified the WSSV positive cells in tissue after injection in *Penaeus vannamei* juveniles. There were significant correlations between virulence and number of infected cells in gill tissue (Rahman et al., 2008).

Sudha et al. (1998) categorized natural outbreaks of WSSV into per acute, acute to sub acute and chronic forms, where mortality occurred within 2-3 days, 7-10 days and 15-28 days, respectively. The portals of entry of virus have not yet been clearly defined (Escobedo-Bonilla et al., 2008). Li et al. (2007) described the cell surface molecule integrin as a cellular receptor for WSSV entry. In feeding challenge the primary sites of viral replication in *P.monodon* were found to be epithelial cells of the stomach and the cells in the gills, in the integument and in connective tissue of hepatopancreas (Chang et al., 1996). On *M. japonicus* it was epithelial cells of midgut trunk which allowed the virus to cross underlying basal lamina (Di Leonardo et al., 2005). Immersion challenge in *P.monodon* showed many positive cells in gill and few in mid gut epithelium. On Electron microscopy epithelial cells in the mid gut were VP28 positive in supranuclear vacuoles early during infection (8 hpi), suggesting lysis of WSSV particles.VP28-positive nuclei were never seen in the epithelial cells of the mid gut (Arts et al., 2007). Infectivity titer and oral inoculation procedures were standardized (Escobedo-Bonilla

et al., 2005; 2006) and primary sites of WSSV replication was found to be epithelial cells in the fore gut, cells in the gills, and also cells in the antennal gland (Escobedo-Bonilla et al., 2007).

There were different opinions about the spread of the virus from primary replication site to the other target organs. One opinion is that the virus circulates through haemolymph to target organs (Wang et al., 2002, Di Leonardo et al., 2005). In other studies circulating haemolymph was found to be refractory to WSSV (van De Braak et al., 2002; Shi et al., 2005; Escobedo-Bonilla et al., 2007). These results suggest that the WSSV circulates through haemolymph in cell free form (Escobedo-Bonilla et al., 2007; 2008).

Organs of ectodermal and mesodermal origin are the main target of WSSV. This includes epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eye- stalk, compound eye, heart, gonads, body cuticular epithelium, hematopoietic cells, and cells associated with nervous system, pleopods, pereiopods, testes and ovaries (Durand et al.,1996; Chang et al., 1998; Kou et al., 1998; Lo et al., 1997; Rajendran et al., 1999; Wang et al., 1999a; Wongteerasupaya et al., 1995; Chang et al., 1996; Sahul Hameed et al., 1998; Rajan et al., 2000; Yoganandhan et al., 2003a; Escobedo Bonilla et al., 2007). Quantitative pathogenic analyses suggest that the major target tissues for replication are gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ and antennal glands (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007). Epithelial cells of organs of ectodermal origin such as the hepatopancreas anterior and posterior mid gut ceaca and midgut trunk are refractory to WSSV (Sahul Hameed et al., 1998). Hepatopancreas and heart are infected only in the connective tissues (Chang et al., 1996; Lo et al., 1997). During late stage of infection the epithelia of stomach, gills, and integument become damaged and lead to multiple organ dysfunction leading to death (Chang et al., 1996; Wang et al., 1999a; Escobedo-Bonilla et al., 2008).

1.11 Molecular basis of virus host interaction

Until recent years there was less information available on the molecular basis of viral cycle during WSSV infection. However, recent developments in bioinformatics and molecular biology could lead to the discovery of several genes and proteins involved in the infection of WSSV. Infection of the virus starts with the attachment of the virions on to the cell surface receptors. In the case of WSSV, recently there were more attempts to study protein - protein interaction during WSSV infection (Sritunyalucksana, et al., 2006b; Li et al., 2006b; Wang et al., 2008). Earlier works have shown that WSSV envelope protein VP28, is involved in systemic infection of WSSV (Van Hulten,et al., 2001b) and is able to bind to the surface of shrimp cells (Yi et al., 2004; Witteveldt et al., 2004a). Sritunyalucksana, et al., (2006) reported the interaction of VP28 with a specific shrimp protein, a 25-kDa protein with high homology to the small GTP-binding protein Rab7, and named it as P. *monodon* Rab7 (*Pm*Rab7). Silencing of *Pm*Rab7 inhibited WSSV- VP28 RNA and protein expression and also inhibited replication of YHV in the YHV infected shrimp suggesting that *Pm*Rab7 is a common cellular factor required for WSSV or YHV replication in shrimp (Ongvarrasopone et al., 2008). Li et al. (2006) proposed that more than one protein was involved in the interaction with the host. The WSSV proteins VP 36A, VP36B and VP 31 having RGD motif have been shown to have a role in viral interaction with the host. Presence of RGD motifs are important because these peptides mediate cell recognition and infectivity of a variety of viral pathogens like adenovirus 2, coxsackievirus A9, human herpesvirus 8, hepatitis A virus and foot-and-mouth disease virus etc (Roivainen et al 1996; Chavez et al., 2001; Hippenmeyer et al., 2002; Jackson et al., 2002; Wang et al., 2003; Williams et al., 2004; Shayakhmetov et al., 2005). In WSSV, there are 6 proteins with RGD motif (Yang et al., 2001). However, whether the RGD motifs in these proteins play a key role needs to be elucidated through further research.

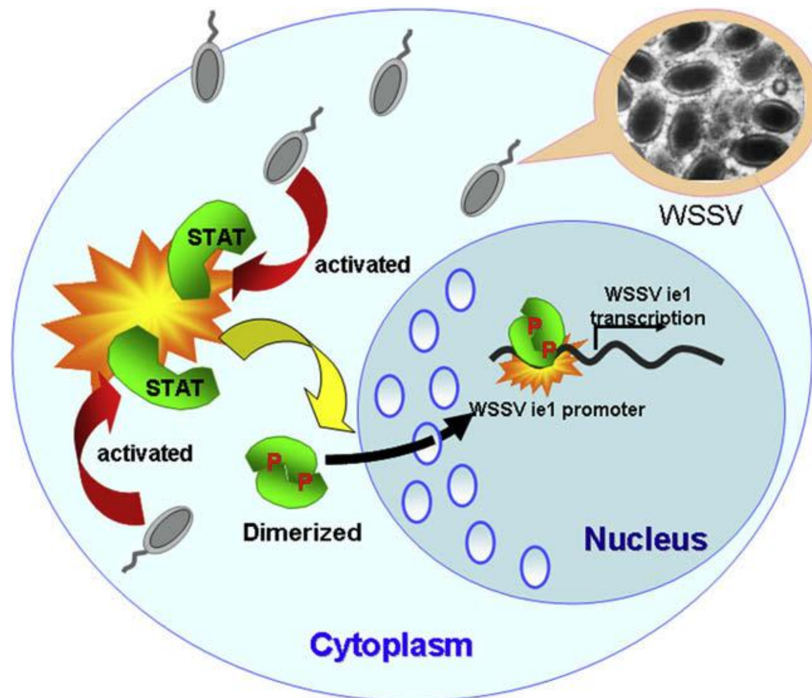

Fig.3 Diagrammatic representations of STAT activation in Shrimp according to Liu, et al., 2009

Recently, information about the immediate early genes which are active from the onset of WSSV infection has been elucidated. Three immediate early genes *ie1*, *ie2* and *ie3* have been identified by micro array and reveres transcriptase PCR analysis (Liu et al., 2005) and it was found that these genes had very high promotor activity (He et al. 2008). Recent studies could reveal that WSSV used a shrimp STAT as a transcription factor to enhance viral gene expression in host cells (Liu et al., 2007). WSSV *ie1* immediate early gene promoter is found to be active in shrimp; it was found highly expressed through out the infection cycle. WSSV *ie1* contains a STAT binding motif which is important for *ie1* promoter activity. It was found that levels of activated *Pm* STAT were higher in WSSV infected animals than WSSV free animals. Chen et al., 2008 have cloned and cDNA produced and the homology with other STATS were studied and found that it belonged to insect STAT family. The presence of functional domain suggested that shrimp STAT had similar function

and regulatory mechanisms with the well known STAT isolated from other model organisms. The study also found that WSSV did not direct JAK/STAT pathway, but it was benefited from STAT activation in shrimp host. Liu et al. (2009) suggested that stressors activate shrimp STAT (signal transducer and activator of transcription), which is then annexed by the virus and used to activate the promoter of the immediate early gene WSSV *ie1*. WSSV *ie1* protein also exhibits transactivation, dimerization and DNA biding activity (Liu et al., 2008; 2009) (Fig.3). Like most of the insect baculovirus, WSSV *ie1* transcription is mediated by host RNA polymerase II. This was proved when cycloheximide, protein synthesis inhibitor treated shrimps did not prevent expression of *ie1* genes when infected with virus. Where as known *ie* genes like dnapol, rr*1*, pk, tk - tmk, and endonu were getting inhibited when shrimps were treated with 250 mg/kg CHX (Liu et al., 2005). During infection by the large DNA viruses, such as baculoviruses and herpesviruses, gene expression is regulated such that the immediate-early (ie or a) genes are transcribed first, followed by the expression of the early (e or h) and late (l or g) genes, respectively (Blissard, 1996; Blissard and Rohrmann, 1990; Friesen and Miller 1986; Honess and Roizman 1974). Genes which are involved in WSSV infection cycle are genes coding for proteins including enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase (Chen et al., 2002a), a nonspecific nuclease (Witteveldt et al., 2001; Li et al., 2005 a), a small and large subunit of ribonucleotide reductase (van Hulten et al., 2000a; Tsai et al., 200b) thymidine kinase, thymidylate kinase, a chimeric thymidine–thymidylate kinase (Tsai, et al., 2000a), a thymidylate synthase (Li et al., 2004b) a dUTPase (Liu & Yang 2005) and two Protein kinases PK (van Hulten & Vlak 2001; Yang et al., 2001). Transcriptional analysis revealed that these genes coding for proteins required in DNA replication and nucleotide metabolism are synthesized early during virus replication (Chen et al., 2002a; Liu et al., 2005; Marks 2005). A recent study proposed that a viral ubiquitin ligase WSSV222 is required for efficient white spot syndrome virus replication in shrimp (He et al., 2009). The 3' RACE studies on poly adenylation

sites of WSSV suggested the possibility of WSSV using the cellular enzymes for polyadneylation of its mRNA for gene expression. The same study also found that internal ribosome entry site (IRES) elements might be used to translate the proteins of WSSV (Kang et al., 2009; Leu et al., 2009a,b). The possibility of WSSV using IRES to regulate translation was first proposed by Han and Zhang (2006). This kind of gene regulation mechanism is analogues to the operon in prokaryotes (Leu et al., 2009 a,b). Mechanism involved in the viral latency has also been studied. Lu et al. (2005) suggested that the latency related *orf* 427 is transcribed in the very late phase during viral lytic infection since the promoter of *orf* 427 could not drive the expression of an *egfp* reporter gene independently. And, they suggested that *orf* 427 functions in maintaining WSSV in the latent phase, but not required for virus re activation.

Many approaches have been made to study the change in host gene expression after infection. It was found that there were up-regulation or down regulation of immune related genes including pattern recognition protein, antimicrobial peptides, genes involved in PO system, and proteinase inhibitor (Destoumieux et al., 2000; Jarasrassamee et al., 2005; Liu et al., 2005; Cheng et al., 2005; Liu et al., 2006; Arts et al., 2006; Wongpanya et al, 2007; Liu et al., 2007) Wang et al. (2008) indicated the role of these genes in antiviral immune response in shrimps against WSSV. Recently it was found that STAT path way is involved in shrimp immune response to WSSV. It is an important finding because, in lower animals like insects the innate immune responses are largely orchestrated by 3 signaling pathways Toll, Imd and JAK-STAT (Souza-Neto et al., 2009). JAK-STAT pathway is one among the immune gene activating signaling pathway in lower animals like insects. (Souza-Neto et al., 2009). The STAT Pathway was found to be mediating Late-Phase Immunity against Plasmodium in the Mosquito *Anopheles gambiae* (Gupta et al., 2009).

Rojtinnakron et al. (2002) showed that WSSV infection stimulate defense related genes in shrimps. In a study using substractive hybridization it was found

that the genes b-1-3-D-Glucan binding protein, hemocyanin, lectin, ferritin, oxygenase and chitinase were found to be abundant in WSSV infected shrimps (Pan et al., 2005). Liu et al. (2006) has carried out an expression study in Cray fish during WSSV infection. They could find that an antilipopolysaccharide factor which interfered with WSSV replication *in vitro* and *in vivo*. In this study knock down of ALF bt RNAi resulted in higher rate of viral replication suggesting that ALF could not protect Cray fish from WSSV infection. A WSSV related Centaurin-α1 homologue named *MjCent* was found to be upregulated in WSSV infected and down regulated in WSSV resistant *Marsupeneaus japonicus* suggesting close relationship between *MjCent* and WSSV invasion and host defense of the shrimp (Wang et al., 2009). A proteomic study of shrimp after WSSV infection (Wang et al., 2007) showed increased level of key glycolytic enzymes, in the electron transport chain and kinases involved in the nucleic acid synthesis suggesting that WSSV up regulate the synthesis of ATP and nucleic acids to promote its rapid multiplication. WSSV infection also appears to up regulate a protein involved in calcium homeostasis (sarco/ER-type calcium pump-ER Ca 2^+ - ATPase) a cellular signaling protein and a voltage dependent anion channel (VDAC). Proteins with decreased level included several digestive enzymes, two calcium binding proteins (SCO and Calponin) and small ubiquitin-like modifier.

Induction of apoptosis in WSSV infected shrimps is well documented. It was found that non infected cells undergo apoptosis while infected cells become unapoptotic during WSSV infection to protect against further infection (Wongprasert et al., 2003). An apotoptic protein (ORF390: WVS 390) is found to function as apotoptic suppressor (Wang et al., 2004). Leu et al. (2009a,b) suggested that the ORF 390 is a direct *Penaeus monodon* capsase inhibitor which cleaves capsase 3 cleavage site of ORF 390 and form a complex and the activity gets blocked. Recently (Wang et al., 2008) a DNA mimic histone binding protein ICP11 has been found to be involved in the WSSV infection which deprives the

cell nucleus of histone proteins, and makes the host cell DNA vulnerable to damage and leading to disruption of genetic machinery of the nucleus.

1.12 Diagnosis

For better treatment of any disease effective, accurate and rapid diagnostic techniques are necessary. This is true with WSSV also. As a supportive profession to penaeid culture shrimp pathology originated 30 years ago (Lightner and Redman, 1998). Today this has become a major section in aquaculture industry, which employs recent advancements in molecular biology and immuonology. The diagnosis of WSSV includes classical methods like gross observation, histology, electron microscopy, and most advanced techniques in molecular biology and immunology as well.

1.12.1 Classical methods

Classical method of diagnosis include gross and clinical signs, with the most commonly used laboratory tests like direct observation, microscopic observation, isolation of etiological agents, histological techniques (Bell and Lightner, 1988; Lightner, 1996; Lightner and Redman, 1998).

1.12.2 Gross observation

Gross observation of the animal can give an idea about the infection. The animals become lethargic, stop feeding and preening activity. The body becomes reddish- pink in color. Characteristic white spot appear on carapace and if the infection is acute it spreads to the other parts of body (OIE 2008). All theses signs appear at later stage of disease. The presence of white spot in carapace alone does not conclude that the shrimps are infected with WSSV; since bacterial infection can also lead to the formation of white spot in the carapace (Wang et al., 1999a; Cyrille et al., 2000; Sahoo et al., 2005; Walker and Mohan, 2009). It is difficult to diagnose the disease in its early stage only by gross observation.

1.12.3 Wet mount demonstration

Wet mount demonstration of hypertrophied nuclei in squash preparations of the gills and/or cuticular epithelium, which may be stained or unstained, unstained wet-mount preparations of haemolymph by dark field microscopy is another way of diagnosing the disease. This is the simplest of the microscopic techniques and is recommended for people with limited expertise in WSSV (OIE 2008). Recently a rapid gill staining technique for early diagnosis of WSSV was developed (Rao et al., 2003) which is useful for checking of cultured shrimps at regular intervals for the possible occurrence of white spot disease.

1.12.4 Histopathology

During earlier days, histopathology was very widely used for diagnosing wssv by which tissue tropism, specifically to tissues and organs of mesodermal and ectodermal origin could be demonstrated (Wongteerasupaya et al., 1995; 1996; Flegel. 1996a; Rajendran et al., 1999; Yoganandhan et al., 2003a; Vijayan et al. 2003). WSSV can be demonstrated histlogically by observing hematoxylin eosin stained tissue sections. This include demonstration of nuclear hypertrophy, cellular, degeneration, multifocal necrosis and hemolytic encapsulation in the infected tissues (Wongteerasupaya et al., 1995; 1996; Chang et al., 1996; Rajendran et al., 1999; Lo et al., 1997; Karunasagar et al., 1997; Flegel 1996 b; Mohan et al., 1998; 1997; Lo et al., 1996b; Sudha et al., 1998; Wang et al., 1997; Wang et al., 2002.). Tissues of ectodermal origin such as subcuticular epithelium, connective tissue, hematopoietic tissue, antennal gland and nervous tissue are severely infected by the virus. These tissues show intracellular hypertrophy of nuclei in the cells of necrotic tissues which demonstrate different stages of viral infection. Eosinophilic intranuclear inclusions surrounded by marginated basophilic chromatin will appear in early stage of the infection followed by enlargement of the eosinophilic intracellular inclusions and finally the swollen nuclei filled with a prominent pale basophilic inclusion, which occupy most of the cytoplasm of the infected cell (Kasornchandra et al., 1998; Takahshi et al., 2000). Karyorrhexis and cellular disintegration may occur which

lead to the formation of necrotic areas characterized by vacuolization (Karunasagar et al., 1997; Kasornchandra et al., 1998; Wang et al, 1999 a) Rajendran et al. (2005) developed a non lethal rapid histology protocol for detecting WSSV in frozen as well as paraffin fixed tissues. According to them the infection could be diagnosed 18 h post infection. They suggested that this technique could be adopted for screening brood stock for generation of SPF and SPR populations Since pleopod and eye stock are used, the process is considered non destructive. Frequent histologic observation of the shrimps in culture ponds can asses the development of the disease in the ponds.

1.12.5 Molecular Diagnostics

Prior to advent of molecular methods, diagnosis of disease relied largely on culture of causative organisms in media or cells, analysis of phenotypic or serological properties of the pathogen or histological examination of the effects on host tissue. Nucleic acid technology presents the opportunity to detect the pathogen directly, targeting the genetic material replacing culture, serological or histological techniques (Wagener, 1997; McKeever and Rege, 1999). The need for rapid and sensitive diagnostic methods has led to the application of modern biotechnology to penaeid shrimp disease. DNA based detection methods for the most important viral pathogens have been reported in literature and a few DNA based diagnostic methods are commercially available (FAO 2005). Development and application of nucleic acid based diagnosis in the diagnosis of shrimp disease is very recent, and the first probe was developed by Lightner for detecting the shrimp virus IHHNV (Lightner et al., 1992; Mari et al., 1993). DNA based probes for white spot disease virus have been developed by several laboratories (Chang et al., 1996; Durand et al., 1996; Zhan et al., 2000).

In acute phase of WSSV infection clinical signs and histological changes could be observed. But during chronic phase clinical signs and histopathological signs are not seen and in this case WSSV could be detectable only by sensitive methods like PCR. (Lo et al., 1996; 1997; Hossain et al., 2001). At present PCR

based diagnostic techniques are being widely used because of its high sensitivity. Many primers have been developed so far (Lo et al., 1996A; Takashashi et al., 1996; Nunan and Lightner, 1997; Kasornchandra et al., 1998; Ota et al., 1999). This has been used for detection of WSSV in brood stock and post larvae, for investigation of WSSV transmission route among different hosts and for epidemiological studies (Lo et al., 1996 a, b; 1997; Peng et al., 1998; Chou et al., 1998., Hameed et al., 2002, Hoa et al., 2005). In India two commercial kits have been developed and are being marketed by Mangalore Biotech (P) Ltd., Mangalore and Bangalore Genei (P) Ltd., Bangalore respectively. Internationally several such kits have been made available for the detection of white spot syndrome virus through PCR (Kim et al., 1998; Nunan et al., 1998; Peng et al., 1998; Hsu et al., 1999; Lo et al., 1996a; Kaitpathomchai et al., 2001; Tang and Lightner, 2000; Tan et al., 2001; Islam et al., 2007).

Different variations of PCR methods like, Single step, Nested, Real time, Quantitative, Semi quantitative, Multiplex PCRs for different pathogens along with WSSV and for different isolates are available now. This has improved sensitivity, versatility and flexibility of the PCR diagnostic techniques for detection of WSSV. Tapay et al., 1999 developed primers for PCR based on the sequence of a cloned fragment of the white spot disease virus genome and used the primers to detect white spot disease virus from both experimentally and naturally infected shrimp. They developed one step and two step PCR protocol as a very sensitive and specific alternative protocol to western blot assay for the detection of white spot virus. Kaitpathomchai et al. (2001) developed a non stop single tube, semi nested PCR technique for grading the sensitivity. Another method to quantify WSSV DNA through competitive PCR could be accomplished by Tang and Lightner, (2000). A two step nested PCR has been developed by Lo et al. (1996a) and Kimura et al. (1996). Islam et al. (2007) developed a nested PCR protocol for WSSV screening for major crustaceans inhabiting shrimp farms in Bangladesh. Nested PCR has been reported to

increase sensitivity of detection by 10^3-10^4 times and are able to detect 10-50 DNA copies (Lo et al, 1996a). In a recent study (Natividad et al., 2008) a nested PCR could amplify WSSV DNA in pond soil even after 5 days at 70 °C. It was found that the sensitivity increased with the amplicon size (Hossain et al., 2004). It is common to observe WSSV by nested PCR in apparently healthy *P.monodon* that go through normal crop. The sensitivity of the PCR method is such that it could detect as little as 5 fg of WSSV DNA (20 viral particle) in crude extracts of PL, Pleopods, Haemolymph from larger shrimps (Kaiatpathomachie et al., 2001). Tsai et al. (2002) developed multiplex RT-PCR method for detection of WSSV and TSV. A real time PCR detection technique was developed for WSV and IHHNV using SYBR Green chemistry by Dhar et al. (2001). Recently a multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed for simultaneously detecting six major shrimp viruses including yellow-head virus (YHV), white spot syndrome virus (WSSV), Taura syndrome virus (TSV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV) (Khawsak et al., 2008). It was found that various PCR methodologies gave different results depending up on whether first step, or two step method was used, whether internal or external control were kept and whether appropriate host tissue could be used (Walker and Cowley, 2000). Even farmers usually complain that identical samples analyzed in 2 different laboratories have given confusing results. To examine this situation Sritunyalucksana et al. (2006a) carried out a comparison of PCR methods for white spot syndrome virus and could establish that Real time PCR could detect approximately 5 copies per reaction where as 1000 copies were needed for common one step PCR and 50 for a common single tube nested PCR. Park et al. (1998) could amplify PCR products from WSSV infected shrimp using primers developed for RV-PJ but not with the primers developed by Lo et al. (1996a). They also found that Korean virus looked similar to WSSV found in Thailand and not RV-PJ of Japan. The sequences of the PCR products were identical to sequence of RV-PJ fragment. These point towards the

possibility of mutations in WSSV or presence of less virulent or virulent strains (FAO 2005).

Other diagnostic methods for WSSV detection include the miniarray method (Quere, et al., 2002), which allows one-step multiple detection of WSSV by hybridization of a PCR product onto a nylon membrane and the visualization of the hybrids by an antibody, increasing pathogen detection considerably. Another method is the loop-mediated isothermal amplification (LAMP), a novel, sensitive and rapid technique with a detection limit of up to 1fg, very sensitive when compared with the 10 fg detection threshold by nested PCR (Kono et al., 2004).

1.12.6 Immuno diagnostics

Apart from molecular diagnosis, immunological tests are widely used for the detection of WSSV in crustaceans. This includes ELISA, Dot Blot enzyme immuno assay and Western blot analysis. (Poulos et al., 2001; You et al., 2002; Anil et al., 2002; Chaivisuthangkura et al., 2006). A sensitive immuno dot assay for WSSV was developed using the specific rabbit polyclonal antiserum developed from truncated version of WSSV 27.5 KD an enveloped protein (You et al., 2002). A dot blot nitrocellulose enzyme immuno assay was developed against a WSSV by Nadala and Loh, (2000) and Western blot by Nadala et al. (1997) and Bruce et al. (1993). Monoclonal antibodies were developed against VP28 protein and used to develop immuno-blot assay using an immunocomb to detect WSSV from field samples (Zhan et al., 1999; Poulos et al., 2001; Shih et al., 2001; Anil et al., 2002; Makesh et al., 2006; Patil et al., 2008). The test developed had an analytical sensitivity of 625 pg and a diagnostic sensitivity of 100% compared to single step polymerase chain reaction (PCR). The Department of Aquaculture, College of Fisheries, Mangalore, India has developed a simple, rapid and sensitive monoclonal antibody (MAb)-based immunodot test kit 'RapiDot' for detection of WSSV and the same was compared with PCR method (Patil et al., 2008) and was found to be comparable with first step PCR. It was found that immunological dot blot assays are easy to perform and also useful for field level application than other

methods which require sophisticated equipments and well equipped laboratories. ELISA has been developed to detect WSSV (Hameed et al, 1998). Liu et al. (2002), developed monoclonal antibodies (MAbs) specific to WSSV envelope protein (28 kDa) and used for developing Ac-ELISA. According to them approximately 400 pg of purified WSSV sample and 20 pg of r-28 could be detected by Ac-ELISA, which is comparable in sensitivity to PCR assay but more sensitive than Western blot in the detection of purified virus. A one-step immunochromatographic assay for detecting WSSV in shrimp is also available (Ko, et al., 2003).

1.13 Management of WSV in Culture Systems

Till now there is no effective treatment or prophylactic measure available to control the outbreak of this virus in culture ponds. Like any viral diseases there are no adequate treatments available against WSSV (Witteveldt et al., 2004b). Once the virus is introduced into the system it spreads rapidly and uncontrollably (Yi et al., 2003). And, hence the better management of culture operation is the most suitable machinery to save the crop from the virus outbreak. The management measures recommended to prevent WSSV outbreak in culture systems include bio-secured culture system operation, vaccination, use of antiviral natural products and chemotherapeutics, immunostimulants, antimicrobial peptides, RNA interference (RNAi) Technology, controlling water temperature/salinity etc.

Biosecurity can be defined as the concept of protecting cultured shrimps from contamination by diseases and of preventing the spread of diseases (Menasveta, 2002; Lightner, 2005). It includes the development of (Specific Pathogen Free)SPF shrimp stock, exclusion of pathogens from brood stock in hatcheries and farms, 'zero' water exchange culture ,water treatment before filling in grow out, hygiene of workers and the use of quality feed (Lightner 2005). Success of effective biosecurity measures include effective treatment of water, successful pond eradication, precise screening of broodstock and post

larvae before stocking, better quarantine operation etc. Since the major routs of infection are the infected water and carrier shrimps (Flegel et al., 1995) the best approach is disinfection and elimination of potential carriers. Pratanpipat et al. (1996) suggested formalin as an effective disinfectant against WSSV. According to them 70 ppm formalin is enough to prevent transmission through water. Washing the naupli with or without disinfectant can reduce the input of WSSV and MBV before stocking them into larval rearing tank (Chen, 1992). Success in preventing the introduction of diseased shrimp in a farm does not depend only on the testing of PL before stocking. Sampling errors or misdiagnosis may occur (Fegan and Clifford 2001). In case diseased shrimp are introduced, quarantine might be useful to reduce the risk of transmission. Removal of dead shrimp from the farm, a quality diet and better feed management can also reduce the risk.

Earlier, it was believed that invertebrates like shrimps lack true adaptive immune response (Soderhall and Thornqvist, 1997). However, specific memory exists in innate immune systems and real mechanisms have not been evaluated (Kurtz and Franz, 2003 and Kurtz, 2005). According to Smith et al. (2003) the term vaccination may not be appropriate in shrimp, as they have only an innate immunity though the term has been used in different papers. 'Resistance' includes evidence of defense, clearance activities and ultimately elimination of the virus. 'Tolerance' implies a persistent infection, which is transmissible with or without detectable signs of viral pathology (Flegel, 2001). Wu and Muroga (2004), has shown that when Kuruma shrimp *P.japonicus* was exposed to WSSV it became resistant to subsequent challenges with the virus. This was thought to be because of humoral neutralizing factor in the so called 'immune shrimp'. There were several attempts to immunize different shrimp species with administration of viral proteins or recombinant viral proteins VP19, VP26, VP28, VP31, VP292 to provide protection against WSSV (Namikoshi et al., 2004; Witteveldt et al., 2004a, 2004b; Du et al., 2006a; Vaseeharan et al., 2006; Witteveldt et al., 2006; Jha et al., 2006; 2007; Rout et al., 2007). Intramuscular

immunization of the WSSV envelop protein VP 19 and VP 28 in *P.monodone* resulted in an increased survival ($P<0.05$) (Witteveldt et al., 2004a). How ever, mixed results were obtained with these vaccination studies. Rout et al., 2007, reported that there was no vaccination effect with nucleocapsid protein VP15, VP35. Recombinent rVP28 was found to be effective to reduce mortality in *P. monodon* and *Procambarus clarkii* (Namikoshi et al., 2004; Witteveldt et al., 2004b; Jha et al., 2007) but comparatively less effective in *P. vannamei* (Witteveldt et al., 2006). rVP28 expressed in BmN cells was more effective in protecting crayfish than expressed in *E. coli* (Du et al., 2006a). Monoclonal antibody (MAbs) against rVP28 (Musthaq et al., 2006; Natividad et al., 2007) or polyclonal antibodies (PAbs) against rVP (19+28) (Li, et al., 2005) were found to neutralize WSSV. Natividad et al. (2007) reported that neutralization efficacy of MAb against VP28 was found to be inoculation dose dependent and was less effective *in vitro.* Robalino et al. (2006) tested a series of monoclonal and polyclonal antibodies targeting vp28 for their ability to neutralize WSSV infectivity. They could observe strong inactivation of WSSV by rabbit sera in a manner independent of anti-VP28 antibodies possibly of some components other than antibodies in the rabbit antiserum to neutralize the WSSV proteins. Administration of formalin inactivated WSSV was also found to be protecting shrimps from subsequent challenge with WSSV in *P. japonicus*, *P. indicus* and *P. vannamei* (Namikoshi et al., 2004; Singh et al., 2005; Melena et al., 2006). However studies conducted by Namikoshi et al. (2004), with heat inactivated WSSV was found to be non effective. Recently a binary ethylenimine (BEI)-inactivated WSSV could protect *Procambarus clarkii* against white spot syndrome virus (Zhu et. al., 2009). They compared protecting ability of both heat inactivated and BEI inactivated WSSV and suggested that protective efficacy of BEI-inactivated WSSV lies on the integrity of envelop proteins of the inactivated WSSV. Till now there are no evidences about the practical use of these vaccines in field conditions. Lot more studies are required in this area.

Recently it has been hypothesized that during viral infections shrimps and other arthropods use endogenous RT (Reverse transcriptase) and recognize mRNA of both RNA /DNA viruses and use the integrase (IN) to randomly insert short cDNA sequence into their genomes and some of these sequence result in production of immuno specific RNA (imRNA) capable of stimulating RNAi that suppresses viral propagation, and individuals with protective inserts would pass these on to the next generation, together with similar protective inserts for other viruses that could be amalgamated rapidly in individual offspring by random assortment of chromosomes (Flegel 2009).

There are reports available on the use of natural products for controlling WSSV. Rao 1996 proposed use of antiviral herbal powder made from *Phyllanthus niruri* at a dosage of 1-2g/kg feed for three days. *Calotropis gigantea* (Yaligar and Pai, 1996), Sulphated polysacheride from marine algae (Takahashi et al., 1998), Fucoidan from *Sargassum polycystum* (Chotigeat et al., 2004) were also tried as natural antivirals against WSSV. Ramesthangam and Ramasamy, 2007 extracted bis (2-methylheptyl) phthalate from *Pongamia pinnata* leaves. *P. monodon* were fed with 200 and 300 µg bis (2-methylheptyl) phthalate /kg body weight of shrimp/day. At highest concentrations they could observe 40-80 % survival in shrimp fed with the extract. 20 species of terrestrial medicinal plants from India were tried against WSSV (Balasubramanian et al, 2007). The protective effect of *Cynodon dactylyon* against WSV was studied in *Penaeus monodon* and the extract could be produced in large scale (Balasubramanian et al, 2008). The protective effect of a probiotic mixture (PM) and antiviral plants against the white spot syndrome virus (WSSV) in *Litopenaeus vannamei*, was evaluated and found that the PM and powdered antiviral plants added to the commercial feed showed an increase in survival and a decrease in the prevalence of WSSV in shrimp. It is important to note that exact mechanisms of antiviral activity in the above mentioned natural products are not well known.

Studies on the use of chemotherapeutics against WSV are few. Park et al. (2004) recommended the use of STEL water for disinfecting sea water and preventing infection. (Central Institute of Fisheries Education) CIFE developed SLC- URINUM, a liquid prepared out of human urine and was used for treating WSSV infected shrimps through diet (Chondar, 1996). The Cidofovir an anti viral drug supplemented with *Spirulina platensis* was found effective in delaying mortality due to WSSV in *Litopenaeus vannamei* (Rahman et al. (2006).

Several products have been tested for the control of viral disease on shrimp due to their potential to stimulate the invertebrate non-specific immune system. Immunostimulant incorporated diet has been found to be effective against WSSV to some extent. B-1, 3 glucan is the most widely used immunostimulant (Chang et al., 1999; 2003). Peptidoglycan from *Bifidobacterium* sp., *Brevibacterium* sp., *and Bacillus* sp have also been found to increase survival rate when shrimps are fed with the peptidoglycan and subsequent challenge with WSSV (Itami et al., 1998; Lee et al., 2004). A lipopolysaccharide from *Pantoea agglomerans* (Takahashi et al., 2000) or extracts of plants like *Cyanodon dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa, Eclipta alba* (Citarasu et al., 2006) were found to increase immunity in shrimps. So far there were no conclusive studies about how these immunostimulants could act in animal body as increased use could lead to negative effects (Horowitz and Horowitz, 2001).

Antimicrobial peptides (AMPs) are part of innate immunity of lower invertebrates like crustaceans. A synthetic antibacterial peptide from *Mytilus galloprovincialis* was found to be reducing mortality due to WSSV in Palaemonid shrimp *Palaemon serratus* (Dupuy et al., 2004; Roch et al., 2008). Yi et al. (2003) claimed that a phage displayed peptide was effective against WSSV in vivo in Cray fish and in vitro in primary culture of lymphoid shrimp.

Other methods like RNA interference (RNAi) were also tried against WSSV. Injection of double stranded RNA (ds RNA) (Robalino et al., 2004),

Short interfering RNA (SiRNA) VP15- SiRNA or VP28- SiRNA (Westenberg et al., 2005), Multiple injection of VP28- SiRNA (Xu et al., 2007) were found according protection against WSSV. Field level application of these technologies is yet to be proved.

It was found that the temperature modulates the WSSV infection. Mortality of shrimps due to WSSV can be controlled by higher (32-33°C) or lower temperature (<15°C) than optimum need for multiplication of WSSV (Vidal et al., 2001; Guan et al., 2003; Jiravanichpaisal et al., 2004; Dupuy et al., 2004; Du et al., 2006 b; Granja et al., 2003). The possible mechanism by which the reduction in mortality might be due to reduced replication, reduction in viral titer, apoptosis, altered gene expression of WSSV, inhibition of replication, activity of heat shock protein (Du et al., 2006 b; Granja et al., 2006; Granja et al., 2003; Reyes et al., 2007; Jiravanichpaisal et al.,2006; Vidal et al., 2001).

1.14 Marine natural products as antiviral molecules

Oceans are unique resources that provide a diverse array of natural products with diverse biological activity with pharmacological importance. Oceans cover around 70% of the earth surface, thus posses a large potential for the discovery of novel molecules (Cragg et al., 1997; Donia et al., 2003). Out of the 36 phyla of life 34 with more than 30, 0000 described species of plants and animals are present in the ocean (Pomponi 1999; Jimeno 2002; da Silva et al., 2006). Marine natural products fascinate researchers to an important area of drug development due to their structural rareness and their diverse biological activities. Today there is a growing attention to isolate biologically active natural products, secondary metabolites with diverse function acting against infection, overgrowth or predation. Production of these factors depends up on chemical ecology of the organisms. Ecological pressures like competition for food, space, fouling of surfaces, predation have lead to the evolution of unique secondary metabolites with various biological activities (Ireland et al., 2000; Donia and Haman et al., 2003). In

contrast to the works with terrestrial natural products the first serious work on marine natural products started just 50 years ago with the pioneering work by Bergman. Compounds isolated from marine sources are often highly complex and the components are usually a part of highly toxic defense mechanisms, which are reflections of the highly competitive solute environment in which the organism resides (Grabley and Thiericke 1999). At present there are a number of compounds from marine origin which are under investigations / or being developed as new pharmaceuticals (Faulkner, 2000a, b; da Rocha et al., 2001; Schwartsmann et al., 2001, Ely et al., 2004). Approximately 16,000 different secondary metabolites have been isolated commonly from marine invertebrates (Kosta et al**.,** 2008). Chemically majority of these molecules are carbohydrates, lipids, proteins alkaloids and phenolic compounds. The most interesting phyla with respect to pharmacologically active marine compounds include Bacteria, Fungi, Algae, Mangroves, Sponges, Soft Corals, Gorgonians, Sea Hares, Nudibranchs, Bryozoans and Tunicates (Faulkner 2000a). A recent review covering 283 literatures published about marine natural products in 2006 reported 779 new compounds (Blunt et al., 2008).

It was thought that it would be difficult to treat viral diseases. However, with the discovery of the first antiviral drug (Idoxuridine) in 1950 and its first clinical use in 1962, it became clear that antiviral drugs could be made a reality (Baurer 1985). After this, several researchers came out with many antiviral agents (Solomon et al., 1966; Gonzales et al., 1987). The work done by Baba et al., (1988a), proved that sulfated polysaccharides present in the algal extract could inhibit both DNA and RNA viruses. Then onwards the marine plants and animals attained great attention among researchers for developing antiviral molecules. Resistance of Viruses to treatment or prophylaxix is a challenging problem for treatment and it make necessary to have more and more diverse group of molecules which can act as antivirals. In this context oceans are unique source of diverse organisms which can produce molecules having unique structures which can be used as antivirals.

Among microbes bacteria, cyanobacteria, microalgae, and fungi were found to be possessing factors which could act against viruses. Molecules such as sulfated polysaccharides from marine *Pseudomonas* against HSV-I and influenza type A virus (Matsuda et al., 1999), Glycosaminoglycan (Ahmad et al., 1999) Cyanovirine –N- (Protein) (Boyd et al., 1997; Gustafson et al., 1996) and Sulfolipids (Gustafson et al., 1989) from Cyanobacteria against HIV, sulfated polysaccharides of marine microalgae *Gyrodinium impudicum* against (EMCV) (Yim et al., 2004), extracellular polysaccharide from microalga *Cochlodinium polykirkoides* against influenza virus type A, and B, Respiratory syncytial virus type A and B (Hasui et al., 1995), calcium spirulan an inhibitor of enveloped viruses from *Spirulina platensis* (Hayashi et al., 1996), Halovirins A-E from a marine fungus of the genus *Scytalidium* (Rowly et al., 2003), Sansalvamide A (cyclic depsipeptide) against MCV (Molluscum contagiosum virus) from *Fusarium* sp. (Hwang et al., 1999,) are some of the other potential antiviral molecules isolated from marine microbes. Myouga et al., 1995, isolated a 52 kDa virucidal agent from *Alteromonas* sp. against Fish viruses.

Seaweeds have been extensively used for studying their anti viral activities (Kathan 1965; Hope et al., 1979; Bhakuni and Dhawan, 1980; Caccamese et al., 1980). Ethanolic extracts of Indian marine algae were tested against Similkiforest virus (SFV), Ranikhet disease virus (RDV) and Vaccinia virus (VV) by Kamat et al. (1992). Blunden et al. (1981) tested 50 species of British marine algae for anti-influenza virus activity based on inhibition on influenza neuraminidase and found the 20 extracts have significant antiviral activity. Zandi et al. (2007) reported anti HSV-1 activity in brown alga *Cystoseirs myrica* from Persian Gulf. Wang, H., et al. (2008) reported anti HSV activity in hot water extracts of Hong Kong Seaweeds. Antivirals isolated from seaweeds include a Sulphated polysaccharide from *Sargassum horneri* (Hoshino et al., 1998) and *Sargassum patens* (Zhu et al., 2006), water-soluble Sulfated Polysaccharides from *Surgassum latifolium* (Asker et al., 2007), fucoidan from brown sea weed (Baba et

al., 1988b) and *Adenocystis utricularis* (Ponce et al., 2003), carrageenan (Neuwshal 1990) sulfated galactan (Witvrouw et al., 1994), Galactan sulfate from *Gracilaria corticata* (Mazumder et al., 2002), Sulphated xylomannan from *Nothogenia fastigiata* (Damonte et al., 1994) Sulfoquinovosyl diacylglycerol from *Gigartina tenella* (Ohta et al., 1998) Peyssonol A and Peyssonol B (Sesquiterpene hydroquinones) from *Peyssonelia* sp. (Loya et al., 1995) , Diterpenens from *Dictyota* (Pereira et al., 2004; Mayer et al., 2007) and Thyrsiferol Ttiterpene from *Laurencia venusta* (Sakemi et al., 1986). These studies clearly indicate that polysaccharides especially sulfated derivatives from marine algae are promising antiviral molecules. The fact is that molecules which are in clinical trials are very less.

Among metazoans, marine sponges have been proved to be very useful in isolating antiviral molecules. da Silva et al.(2006) screened 27 marine sponges for antiviral activity against HSV-1, AdV-5 and RV-SA11, and found that extracts of *Cliona* sp.*, Agelas sp. Tethya* sp. and *Axinella* aff*corrugate* as promising antivirals. Ibisterol sulfate, a poly-sulfated sterol from deepwater sponge *Topsentia* sp. (Mckee et al., 1993, Whitson et al., 2008), adociavirin a protein from *Adocia* sp. (O'keefe et al., 1998), Frondosins A and D, a sesquisterpene hydroquinone derivative from *Eurospongia* sp. (Hallock et al., 1998), ortho sterols and weinbersterols from *Petrosia weinbergi* (Giner et al., 1999) ,Papuamides A –D a depsipeptide (Ford et al., 1999; Andjelic et al., 2008), Crambescidin 826 Alkaloid from *Monanchora* sp. (Chang et al., 2003), Neamphamide A a Depsipeptide from *Neamphius huxleyi* (Oku et al., 2004), Homophymine A, an anti HIV cyclopeptide from *Homophymia* sp.(Zampella et al., 2008) Petrosins an Alkaloid from *Petrosia similis* (Goud et al., 2003), Batzelladine alkaloids from the caribbean sponge *Monanchora unguifera* against HIV(Hua et al.,2007) sesterterpenes from *Lendenfeldia* sp. (Chill et al., 2004), Cyclodepsipeptide, Homophymine A from *Homophymia* sp. are some of the potential anti HIV molecules reported from marine sponges. Mycalamide A from *Mycale* sp. was reported by Perry et al., (1988) and Donia et al (2003). A bromoindole alkaloid dragmacidine F (1) from *Halicortex* sp. was active against

HSV-1 and HIV-1(Cutignano et al.,2000). The most important antiviral molecule of sponge origin is ara-A, a semisynthetic compound based on arabinosyl nucleosides isolated from *Cryptotethia crypta*. Semi synthetic modifications of this molecule like zidovudine, acyclovir and azidothymidine are in clinical use (Donia et al, 2003; De Clerq et al., 2002). Marine sponges are the largest group of organisms from which antiviral molecules have been identified and several molecules like ara-A are in clinical trial. All these developments suggest that marine sponges are good resource for isolating antiviral molecules.

Tunicates (Sea squirts) are another important group of invertebrates as a source of antiviral compounds. Laminarin 2-20 sulfates against HIV (Reddy et al., 1999), DNA polymerase inhibiting compound policitone A (Loya et al., 1999), Didemnins cyclic peptides from *Trididemnum solidum* (Rinehart et al., 1981) are some of the promising antiviral compounds from tunicates. Lindsay et al. (2000) synthesized novel ring- E analogues of ascididemin and studied their antiviral activity against both RNA and DNA viruses. Pan et al. (2000) reported broad antiviral activity in tissues of crustaceans. Chatterji et al. (2002) screened extracts from commercially important Indian marine bivalves against influenza virus type A and type B. A sulfated poly hydroxy steroid from a brittle star against HSV-2, JV (Comin et al., 1999), Gymnochrome D a complex polyketide isolated from a cirinoid inhibit dengue virus (Laille, et al., 1998), hollothurinosides, a non-sulfated triterpenoid glycoside from the sea cucumber *Hollothuria forskalli*, with antitumor as well as antiviral activities (Rodriguez et al., 1991) are some of the important molecules from other marine invertebrates.

The mode of action of marine antiviral molecules varies from preventing viral binding to the host cell, binding to the viral envelop proteins, blocking viral DNA /RNA synthesis, direct virucidal property etc. Sulfated polysaccharieds generally inhibit enveloped viruses by blocking binding of virus to the cells (Baba et al., 1988a, Zhu et al., 2006). Meiyu et al. (2003) suggested that Sulphated polymannuroguluronate interact with HIV-1 rgp 120 and CD4

molecule. Cyanovirin-N protein from cyanobacteria binds to viral surface envelope gp 120 (Boyd et al., 1997). Papuamide A inhibit HIV pseudotype viruses expressing envelope glycoproteins from vesicular stomatitis virus or amphotropic murine leukemia virus indicating the mechanism of viral entry inhibition is not HIV-1 envelope glycoprotein specific (Andjelic et al., 2008). There are also molecules which act on DNA/RNA mechanism. Sansalvamide A inhibits topoisomerase of MCV (Hwang et al., 1999). Sulfoquinovosyldiacylglycerol from *Gigartina tenella* inhibit HIV reverse type -1 (Ohta et al., 1998). Diterpene from *Dictyota menstrualis* inhibit the RNA-dependent DNA polymerase activity of the HIV reverse transcriptase enzyme (Pereira et al., 2004). There are many other molecules whose mode of action on viral infection is to be studied. Only a few molecules reach the clinical trial and many molecules have been discarded due to its adverse effect on host or inability to reproduce the results at various levels. For example polycitone A is an inhibiter of HIV reverse transcriptase and found that this molecule was general inhibitor of DNA polymerase too because of which it could not serve as antiviral drug (Mayer et al., 2002).

Marine natural product leads have been confirmed with many obstacles, like sustainable supply, reproducibility of the activity and toxicity. The strategies to overcome supply include culture of the organism, synthesis, sponge tissue culture, transfer of genetic materials to easily cultivable organism etc (Faulkner 2000a; Donia et al., 2003). A few instances of production of certain metabolites dependent on ecology, cohabiting microbial flora, nutrients available, seasons and other factors. This makes an obstacle to aquaculture production. This will lead to difficulty in reproducing desirable biological activity. Majority of the marine natural products are highly toxic and can lead to less acceptability at later stage of the drug development. However, recent developments in marine biotechnology will overcome these difficulties in future.

1.15 Mangrove plants as a source of antiviral molecules

Mangroves are distributed across tropical and subtropical climates (Bandaranayake 2002; Wu et al., 2008) and act as an ecotone between marine and the terrestrial ecosystem. The potential use of mangrove plants are food, medicine, and for purposes like dwelling, furniture, tools, and weapons. Mangrove plants are being used in folklore medicine and for treatment of several diseases (Kritikar and Basu 1935; Chopra et al., 1956; Dutta and Dutta, 1982). Arabs developed a rich pharmacopoeia from many different species of mangrove and that was why Linn (a Botanist) dedicated one of the most wide spread and important species of mangroves in honor of the famous Arab Doctor Abu Sina (Latinized as *Avicennia*; 980-1036 AD). Mangroves are a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Bandaranayake, 2002). Around 349 metabolites have been isolated from mangrove species (Wu et al., 2008). Extracts from different part of mangroves and mangal associates are widely used through out the world for medicinal purposes (Bandaranayake, 1998). Extracts from mangrove and mangrove dependent species have proven activity against human and animal pathogens, but only limited investigations have been carried out to identify the metabolites responsible for their bioactivities. *Acanthus illicifolius* is one of the most commonly used medicinal plants from the mangrove forest (Bandaranayake 1998, and 2002). *Clerodendron inerme* is recognized for its febrifugal properties as well as larvicidal, antiviral and uterine stimulant property. An antiviral resistance inducing protein polynucleotide-adenosine glycosidase (ribosome-inactivating protein) was found to be responsible for antiviral activity in *Clerodendron inerme* (Olivieri et al., 1996). A novel phorbol ester with anti viral principle was isolated from leaves and stems of *Excoecaria agallocha* collected in northwest Australia (Erickson et al., 1995; Konishi et al., 2000; Bandaranayake 2002). Elanchezhiyan et al., 1993, studied antiviral properties of the seed extract of an Indian medicinal plant *Pongamia pinnata,* found as a mangrove associate. Padmakumar et al., (1993)

tested sixteen different mangrove plants against TMV. Of these, the seed extracts of *Bruguira cylindrica* and leaves of *Excoecaria agallocha* exhibited highest activity. The Inophyllums isolated from *Calophyllum inophyllum* were found to be inhibiting HIV-1 reverse transcriptase (Patil et al., 1993). Another study by Premanathan et al. (1999b) established broad spectrum antiviral activity in bark of *Rhizophora mucuronata* and leaves of *B. ruguira cylindrica* against Newcastle disease virus, Encephalomyocarditis virus Semliki forest virus, human Immunodefficiency virus, Vaccinia virus and Hepatitis B. virus. A polysaccharide extracted from the leaf of *Rhizophora apiculata* was tested for its antiviral activity against HIV and HSV in different cell culture systems and proved its possible use in future drug development. Premanathan et al. (1999a) and He et al., (2007) isolated a pentacyclic triterpens from *Ceriops tagal*. Antiviral property of pentacyclic triterpen was described by several authors (Ryu et al, 1993; Grignon-Dubois and Rezzonico 2007). This indicates the potential of this plant to be used to isolate antiviral molecules and more studies must be done in this direction. From the above examples we could conclude that few attempts have been made to examine the possibility of isolating antiviral molecules from mangroves comparing to terrestrial plants. There is no such report about antiviral activity of the true mangrove species against WSSV so far. On these grounds following objectives have been identified to work on, and the thesis deals with the out come of the endeavor.

- ***Screening mangrove plants for anti WSSV activity***
- ***Confirmation of the anti WSSV activity in aqueous extract of Ceriops tagal and its possible mode of action.***
- ***Extraction and partial purification of the virucidal fractions from the aqueous extract.***

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Chapter 2

SCREENING OF MANGROVE PLANTS FOR ANTI WSSV ACTIVITY

Contents

2.1 Introduction
2.2 Materials and methods
2.3 Results
2.4 Discussion

2.1 Introduction

White spot syndrome virus (WSSV), an enveloped non occluded DNA (300 kb) virus of the family Nimaviride under the new genus *Wispovirus* (Mayo, 2002), is the most devastating shrimp pathogen ever isolated and studied; it causes total mortality to a rearing stock within 3-7 days of infection in a culture system (Lightner, 1996). The virus has a wide host range and has been detected in diverse groups of crustaceans (Lo et al., 1996). Ironically till now no effective treatment or prophylactic measure could be developed to manage the virus. However, different approaches to manage the virus in culture systems have been experimented such as oral administration of peptidoglycan, lipopolysacharides, β-1,3 glucan (Itami et al., 1998; Takahashi et al., 2000; Cheng et al., 2003), vaccination with inactivated viral preparation and viral envelop protein, VP19 and VP 28 (Singh et al., 2005; Namikoshi et al., 200; Witteveldt et al., 2004), feeding with fucoidan extracted from *Sargassm polycysticus* (Chotigeat et al., 2004) and Cidofovir an anti viral drug supplemented with *Spirulina platensis* (Rahman et al., 2006). Besides 20 species of terrestrial medicinal plants from India have also been tried against the virus (Balasubramanian et al., 2007), and the protective effect of *Cynodon dactylyon* has been documented in *Penaeus monodon,* and the extract was produced in large scale (Balasubramanian et al., 2008).

For centuries, mangrove plants found in the tropics have been in focus as source of bioactive molecules (Bandaranayake, 2002) having different dimensions of activities. They have been used in folklore medicine for treatment of several diseases (Kirtikar and Basu, 1935; Chopra et al., 1956; Dutta and Dutta, 1982); extracts from different parts of the plant and their associates being widely used worldwide for medicinal purposes (Bandaranayake, 1998). The most important reports available are the anti Tobaco Mosaic Virus activity from 16 species of mangrove plants (Padmakumar et al., 1993), anti Newcastle Disease Virus, anti Encephalomyocarditis Virus, anti Semliki Forest Virus, anti Human

Immunodeficiency Virus, anti Vaccinia Virus, and anti Hepatitis B Virus activities detected in the leaves of *Bruguira cylindrica,* and the broad spectrum antiviral activity in the bark of *Rhizophora mucuronata* (Premanathan et al., 1999a). However, there has never been any report on the anti WSSV activity from mangrove plants. This prompted us to take up the present investigation. We selected mangrove species because of its greater role in ecology as ecotone between the land ecosystem and marine ecosystem. Due to this uniqueness there remains the chance of metabolites having unique properties.

2.2 Materials and methods

2.2.1 Collection and identification of mangrove plants

Mangrove plants such as *Excoecaria agallocha*, *Acanthus ilicifolius*, *Avicennia* sp., *Rhizophora mucronata*, *Rhizophora apiculata, Sonneratia* sp. and *Ceriops tagal* were collected from different localities in South India (Table 1). The plants were identified following Naskar and Mandal (1999), coded and voucher specimens deposited in the herbarium collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology.

2.2.2 Preparation of aqueous extract from mangrove plants

Leaves were shade dried, powdered and used for the preparation of aqueous extract using a protocol developed at of National Centre for Aquatic Animal Health, Cochin University of Science and Technology. Accordingly, 50 gm mangrove plant leaf powder was soaked in minimum quantity of double distilled water and frozen to -20°C, thawed and frozen repeatedly for three times and extracted to 500 mL final volume of double distilled water in a Waring blender at ambient temperature. The extract was sieved through a fine meshed (100 µm) cloth, centrifuged at 10000 g for 20 minutes and the supernatant maintained at -20°C till used. These preparations were examined for their virucidal activity and for their protective effects from WSSV on oral administration in *P. monodon.*

2.2.3 Preparation of Virus inoculum for challenge

A composite sample of Gills and soft parts of cephalothorax (500 mg) from freshly infected *P. monodon* was macerated in 10 ml cold PBS (NaCl 8g, KCl 0.2g, Na2HPO_4 1.15g, KH_2PO_4 0.2g, double distilled water100 ml) with glass wool to a homogenous slurry using mortar and pestle in ice bath. The slurry was centrifuged at 8200g in a refrigerated centrifuge at 4°C and the supernatant filter sterilized using 0.22µ pore size PVDF membrane filter. The preparation was streaked on ZoBell's agar plates and incubated at 28±2°C for 72 hrs to confirm the absence of bacterial contamination. Viability of WSSV in suspension was checked by injecting 10µl to a batch of apparently healthy shrimp (6nos) and mortality confirmed over a period of 3 to 7 days. The preparation was stored at -80°C till used.

2.2.4 Preparation of mangrove extract coated feed

The aqueous extracts were lyophilized and the dry mass re suspended in the required quantity of distilled water and coated on to feed pellets to arrive at a concentration of 1% w/w. As binder 4% aqueous gelatin was prepared in distilled water and surface dressed at a ratio 5:40 (v/w) to immobilize the plant extract. The above preparation was dried under vacuum and used for oral administration.

2.2.5 Experimental animals

All animals used in this study were single spawner bred, WSSV free juveniles of *Penaeus monodon* grown in a Recirculating aquaculture system at National Centre for Aquatic Animal Health. The shrimps weighing 4-5 g were maintained in 30 liter capacity fiber reinforced plastic tanks (FRP) with diluted sea water at salinity15 ppt. The experiment was repeated 4 times having 5 shrimps in a batch (4x5=20). Uniformly 10% water was exchanged every day to maintain water quality.

2.2.6 Virucidal activity of the aqueous extract in *P.monodon* animal model

The aqueous plant extracts (0.5mL) were mixed with equal volumes of viral suspension and incubated for 3 hours at 28 ^{o}C. The controls included

mixtures of WSSV and PBS (positive control) and PBS alone (negative control). From each of the preparation, aliquots of 10µl each were intramuscularly administered to the animals (5x4 =20 animals each) and monitored for 15 days. Gill tissue was extracted from moribund animals and the controls which survived the 15 day period of experimentation during the course of the experiment. The samples were preserved in 70% ethanol for diagnostic PCR to detect WSSV.

2.2.7 Oral administration of the plant extracts along with diet and challenge with WSSV

All test animals (5x4=20 animals each) were fed with the plant extract coated feed at a rate of 10% of the body weight two times a day. The control animals were fed with the diet coated with 4% gelatin. Feeding continued for 15 days, and the animals were challenged by feeding with freshly generated WSSV infected tissue at a rate of 10% of the body weight, and kept under observation for 15 days on the respective diet. Gill tissue was extracted from moribund/dead animals and from those which survived the challenge with WSSV, and were preserved in 70% ethanol for diagnostic PCR.

2.2.8 Confirmation of anti WSSV activity of the aqueous extract from *C.tagal* through passaging

To confirm the antiviral activity detected in the segregated plant species (*Ceriops tagal*) intramuscular administration of the virus suspension exposed to the plant extract and oral administration of the plant extract and subsequent challenge were repeated in a batch of 24 animals and assayed by way of nested PCR. On completion of experiment, after 15 days, tissue homogenates were prepared from the test and control animals and passaged to a fresh batch of nested PCR negative animals as bio assay to check the presence of virions in survived animals. Presence of WSSV DNA was further examined by way of nested PCR.

2.2.9 Diagnostic PCR of the extracted tissue samples

For diagnostic PCR, DNA from the gill tissue was extracted in DNAzol according to the manufacturer's protocol. A WSSV 2 step nested PCR detection

kit (Bangalore Geni) that yielded 650 and 300 bp WSSV specific amplicons was used for amplification of the viral DNA. Following the instructions given with the kit, the amplified product was generated in a thermal cycler (Ependroff). The PCR products were then analyzed on 1%W/V agarose gels using TAE (1X) buffer (Tris-HCL 0.04 M, EDTA 0.0001M, Glacial acetic acid 5.71%), stained with ethidium bromide and visualized on a gel documentation system, Dolphin-Doc (Weal Tec, USA).

2.2.10 Statistical analysis

The data generated on the survival of shrimp post administration of WSSV exposed to the plant extracts, and challenged with WSSV subsequent to oral administration of the former were statistically analyzed employing $\chi 2$ test. Independent t- Test was performed to the per cent survived shrimp under the above situation with each plant extract separately.

2.3 Results

2.3.1 Collection and identification of mangrove plants

For present study we have collected seven mangrove plants from Vypin and Lakshadweep Islands. Out of the seven plant species 3 plant species *Rhizophora mucronata, Rhizophora apiculata, Ceriops tagal* were belonging to the family Rhizophoraceae. Other plants collected were *Excoecaria agallocha* (Euphorbiaceae)*, Acanthus ilicifolius* (Acanthaceae), *Avicennia* sp. (Avicennia), *Sonneratia* sp. (Sonneratiaceae) Fig.1 & Table 1.

2.3.2 Virucidal activity of the aqueous extract in *P.monodon* animal model

To check the virucidal activity of mangrove plants the aqueous extracts were mixed with WSSV suspension and challenged after incubation for 3 hours at 28 ^{o}C. When shrimps were challenged with WSSV exposed to the extracts from *Rhizophora mucronata*, *Sonneratia* sp. and *Ceriops tagal* significantly higher survival (95%, 100%, 100% respectively) ($P < 0.001$) could be obtained. Meanwhile, with the aqueous extracts from *Excoecaria agallocha*, *Acanthus*

ilicifolius, *Avicennia* sp. and *Rhizophora apiculata* survival of shrimp was significantly lower (0%, 5%, 0% and0% respectively) (P<0.001). Per cent survival of shrimp kept as negative control and the ones administered with WSSV exposed to *Rhizophora mucronata*, *Sonneratia* sp. and *Ceriops tagal* were more or less the same. No instances of mortality was observed in the batches of shrimp which had received WSSV exposed to the extracts of *Sonneratia* sp. and *Ceriops tagal,* even after 15 days of post challenge. Meanwhile, the batches of shrimps injected with WSSV exposed to the aqueous extracts of *Excoecaria agallocha*, *Avicennia* sp., and *Rhizophora apiculata* did not survive alike the positive control (Table 2).

2.3.3 Oral administration of the plant extracts along with diet and challenge with WSSV

To check the efficacy of different mangrove extracts the shrimps were administered with mangrove extract coated feed for 15 days and challenged with WSSV. Shrimps which were fed on the lyophilized aqueous extract of *Ceriops tagal* alone could give 100% survival when challenged with WSSV (P<0.001). Meanwhile, survival of shrimp fed on the extracts from the other plants subsequent to the challenge was comparatively lower (75% with *Excoecaria agallocha* , 50% with *Acanthus ilicifolius,*75% with *Avicennia* sp*.,* 25% with *Rhizophora apiculata,* 40% with *Sonneratia* sp. and no survival of shrimp could be observed on feeding the extract of *Rhizophora mucronata*) (P<0.001) (Table 3).

2.3.4 Comparison of survival of shrimps in two modes of delivery, feeding and injection

To compare the efficacy of different mangrove extracts to accord protection on both the experimental condition the result obtained in both experiments were analyzed using independent t-test, significant variations between the two modes of experiments could be observed between the plant extract from *Rhizophora mucronata* (P<0.001)*, Excoecaria agallocha* (P<0.001), *Acanthus ilicifolius* (P<0.001), *Avicennia* sp. (P<0.05), *Rhizophora apiculata* (P<0.01) and *Sonneratia* sp. (P<0.001) respectively. Whereas in the case of

Ceriops tagal there were significantly higher survival rate under both the conditions and the difference in survival of shrimp between the two experimental conditions were least significant (P>0.05) (Fig.2).

2.3.5 Diagnostic PCR of the extracted tissue samples for presence of WSSV

Virucidal property of the mangrove plant extracts and the protective effects of the same on oral administration and challenge were assessed by amplifying WSSV gene from gill tissue of the experimental animals. The animals which survived (100%) the administration of WSSV exposed to the extract of *C. tagal* were nested PCR positive. Both the dead (5%) and survived (95%) shrimps on challenging with WSSV exposed to the extracts of *R. mucronata* and *Sonneratia* sp., (100% survived) was nested PCR negative. In all other instances the survival rate was very poor and the moribund animals turned out to be PCR positive for WSSV (Fig.3). Meanwhile the animals which survived the oral challenge with WSSV subsequent to feeding with the extract from *C. tagal* (100%) were nested PCR negative to WSSV. The ones which survived challenge with WSSV after receiving diet coated with the extracts from *Sonneratia* sp., (40%) *Avicennia* sp., (75%) *E. agallocha, (75%) A. ilicifolius*, (50%) were also negative to WSSV, and dead animals altogether were positive to WSSV. The survived and dead animals which received the extract from *R. apiculata* and challenged subsequently were first step PCR positive (Fig. 4).

2.3.6 Confirmation of anti WSSV activity of the aqueous extract from *C.tagal* by passaging to apparently healthy shrimp.

The antiviral activity of the aqueous extract of *C. tagal* was reexamined by repeating both the experiments in a batch of 24 animals, and on completion of the experiment after 15 days the animals were nested PCR negative, and when extracted for virus and passaged in to a fresh batch of animals none of them showed any clinical signs of WSSV infection and remained negative to nested PCR. The animals injected with the extract from positive control showed signs of WSSV and were PCR positive to the virus culminating in mortality (Table 4).

2.4 Discussion

Extracts from mangrove plants and associates have been used world wide for medicinal purposes, and having been recorded around 349 metabolites turns out to be a rich source of steroids, diterpenes and triterpenes, saponins, flavanoids, alkaloids and tannins (Bandaranayake, 2002; Pakhathirathien et al., 2005; He et al., 2007; Wu et al., 2008). Pentacyclic triterpenes have been considered as the major bioactive group of compounds which could inhibit tumor cells and induce apoptosis and found useful for antiviral therapy especially for HIV (He et al., 2007).

However, this is the first attempt to look in to the possibilities of using mangrove plants as source of anti WSSV drugs. With this objective the most commonly found 7 mangrove plant species in Indian coastal zones were subjected for extraction for anti WSSV property and generated an aqueous preparation to be applied along with diet as prophylactic. In this study aqueous extracts from *R. mucronata*, *Sonneratia* sp. and *C. tagal* were found contain virucidal property against WSSV. The same on administering along with diet and challenging subsequently with WSSV the preparation from *C. tagal* could accord total protection to shrimp. As the aqueous extract from *C. tagal* alone could give protection to all animals tested against WSSV, under both the experimental conditions, this plant species was identified for further studies. Under the conditions of feeding the aqueous extract coated feed with subsequent challenge, the viral DNA was not detected in the tissue which suggested that the virus was either had not invaded the host tissue and multiplied or it was getting eliminated subsequent to infection.

Several attempts have been made earlier by several workers to detect anti - WSSV property in plants. Citarasu et al., (2006) mixed together in equal proportion methanolic extracts of 5 medicinal plants such as *C.dactylon, Aeglemarnclos, Tinospora cordifolia, Picorhiza kurooa* and *Eclipta alba* and the

combined extracts were supplemented through shrimp diet at different concentrations. In this experiment 74% survival of *P. monodon* was obtained on administering the extract @ 800 mg/kg body weight. In a similar study the percentage survival of shrimp fed on the ethanolic leaf extract of the plant *Pongamia pinnata* was 40% on administering @ 200mg/kg of body weight and 80% on administering @ 300 mg/kg of body weight per day (Rameshthangam and Ramaswamy, 2007). Balasubramanium et al. (2008) on feeding 2% aqueous extract of *C.dactylon* extract coated feed to *P.monodon* could obtain100% survival and the survived animals were PCR negative. There are reports of feeding shrimps with diet containing extracts of herbs which have improved immune system and also accorded protection from WSSV in *P. monodon* (Citarasu et al., 2006). In our study, the percentage of *C.tagal* aqueous extract in the administered feed was nearly 1% of the total feed delivered @ 500mg/kg of body weight per day.

Virucidal property of the aqueous extracts of *R. mucronata*, *Sonnaratia* sp. and *C, tagal* when administered along with WSSV suspension at 1:1 ratio after incubation for 3 hrs at room temperature suggested the presence of molecules in the preparation which could inactivate the virus. In a similar pattern pre incubation of WSSV with a synthetic antibacterial peptide from *Mytillus galloprovincialis* reduced mortality due to WSSV in Palaemonid shrimp *Palaemon* sp. (Dupuy et al., 2004). They suggested that this might be due to contact of virus with Mytillin before injection on to shrimp. PCR analysis showed that the survived animals were not accommodating the viral DNA. The virucidal property of the aqueous extract of *Cereops tagal* was demonstrated through the total survival obtained on challenge with the virus suspension exposed to the extract and the non infectivity of the tissue extracts of the survived ones. Moreover, on oral administration of the extract and subsequent challenge with WSSV the shrimps were found surviving altogether.

This study has brought to light the presence of anti WSV property in *Ceriops tagal,* a mangrove plant in the coastal zones of Indian subcontinent and

found growing in several tropical countries. The plant which has been identified earlier as potential source of antiviral drug (Premanathan et al., 1999a) belongs to the family Rhizophoraceae. Present study also points out that the mangrove plants belonging to family Rhizophoraceae is a potential source of virus inactivating agents. Other than these plants *Sonneratia* sp. also showed protection to shrimp when challenged with WSSV exposed to the extract.

Through this work an appropriate protocol to extract water soluble bioactive metabolites from mangrove plant *C. tagal* which could protect *P. monodon* from white spot syndrome virus could be developed. The present study also could demonstrate the suitability of gelatin as a binder for delivering aqueous extract coated feed to shrimps. This preparation could be effectively delivered to shrimp through diet by coating with 4% aqueous gelatin as demonstrated earlier by Selvin and Lipton (2003) for delivering anti bacterial fractions from *Ulva faciata* and *Dendrilla nigra.* Since the extract is aqueous, chances of leaching out the active fractions into the surrounding water is rather high, and in this context the observation has practical implications, as the only possible way to deliver any drug to shrimp is through oral route. Based on these experimental results further steps were undertaken with the aqueous extract of *Ceriops tagal* for evaluating the mode of action, and purification of the active fractions which possessed the antiviral property. It has been observed that the mangrove plants of family Rhizophoraceae may be useful for screening anti WSSV molecules. The aqueous extract from *C. tagal* needed an in-depth study to evaluate the exact mode of action against WSSV. Bearing this in mind experiments were designed to further confirm the antiviral activity and to evaluate the possible mode of action using molecular tools.

Table 1 Species of mangrove plants used in this study

SI. No	Plant Name	Family
1	*Excoecaria agallocha*	Euphorbiaceae
2	*Acanthus ilicifolius*	Acanthaceae
3	*Avicennia* sp.	Avicennia
4	*Rhizophora mucronata*	Rhizophoraceae
5	*Rhizophora apiculata*	Rhizophoraceae
6	*Sonneratia* sp.	Sonneratiaceae
7	*Ceriops tagal*	Rhizophoraceae

Table 2 Survival of *Penaeus monodon* to challenge with WSV exposed to different mangrove plant extracts

Test	No. of animals used	No. of animals Survived	% of Survival
Excoecaria agallocha	5x4=20	0	0%
Acanthus ilicifolius	5x4=20	1	5%
Avicennia sp.	5x4=20	0	0%
Rhizophora mucronata	5x4=20	19	95%
Rhizophora apiculata	5x4=20	0	0%
Sonneratia sp.	5x4=20	20	100%
Ceriops tagal	5x4=20	20	100%
Positive control	5x4=20	0	0%
Negative control	5x4=20	20	100%

Table 3 Survival of *P.monodon* to challenge with WSV orally after administration of the mangrove plant extracts

Test	No. of animals used	No. of animals Survived	% of Survival
Excoecaria agallocha	5x4=20	15	75%
Acanthus ilicifolius	5x4=20	10	50%
Avicennia sp.	5x4=20	15	75%
Rhizophora mucronata	5x4=20	0	0%
Rhizophora apiculata	5x4=20	5	25%
Sonneratia sp.	5x4=20	8	40%
Ceriops tagal	5x4=20	20	100%
Positive control	5x4=20	0	0%
Negative control	5x4=20	20	100%

Rhizophora mucronata

***Avicennia* sp.**

Excoecaria agallocha

Acanthus ilicifolius

Ceriops tagal

***Sonneratia* sp.**

Rhizophora apiculata

Fig. 1 Mangrove plants used for anti WSSV assay

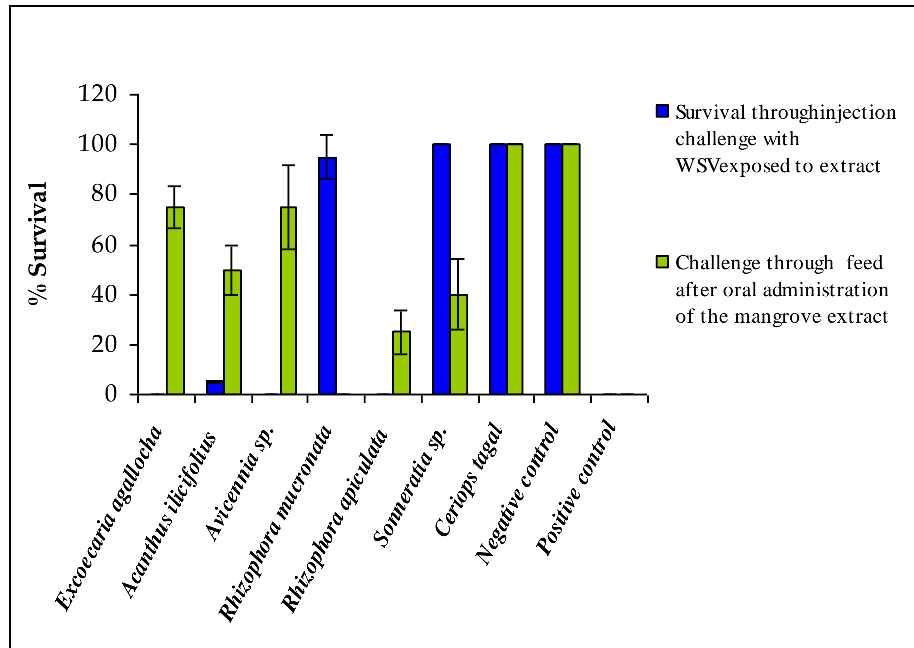
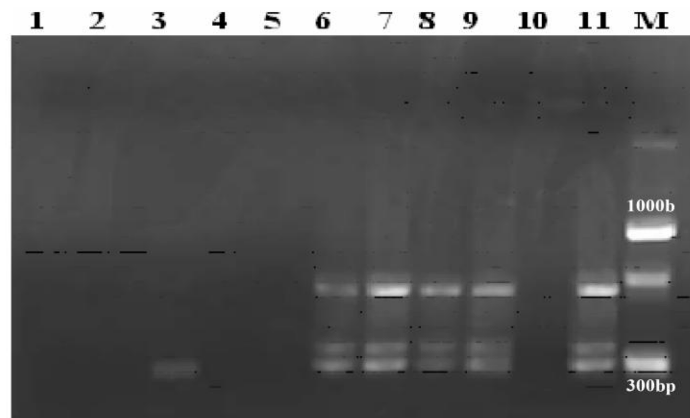
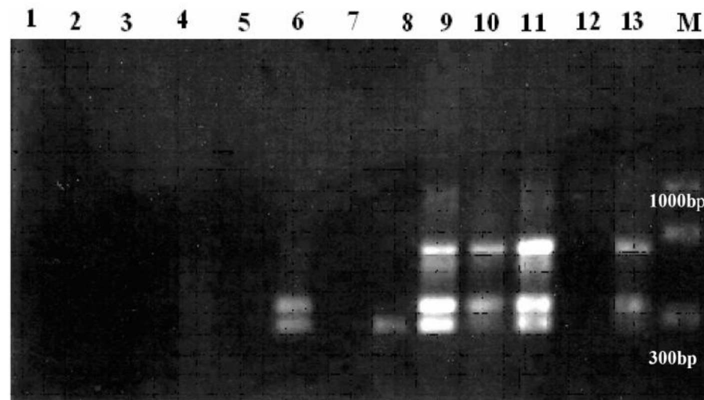


Fig. 2 Percent survival of shrimp challenged with WSSV exposed to mangrove extracts, and challenged with WSSV after oral administration of extracts



Lane 1-4 Live after challenge with WSSV exposed to the *R. mucronata, Sonneratia* sp., *C. tagal* and *A. ilicifolius* respectively. Lane 5-9 dead after challenge with WSSV exposed to the extracts of *R. mucronata, E. agallocha, A. ilicifolius, Avicennia* sp. and *R. apiculata*, Lane 10: Negative control, 11: Positive control, M; Molecular weight marker

Fig. 3 Detection of the virucidal property of aqueous extracts of mangrove species. Each lane represents PCR product of WSSV from shrimp challenged with WSSV exposed to the aqueous extracts



Lane 1-6: Live after challenge with WSSV subsequent to oral administration of the aqueous extracts of C. *tagal, Sonneratia* sp., *Avicennia* sp., *E. agallocha, A. ilicifolius* and *R. apiculata* respectively. Lane 7-11: Dead after challenge with WSSSVsubsequent to oral administration of the aqueous extracts of *E. agallocha Avicennia* sp. *A.ilicifolius R. apiculata* and *R. mucronata,* Lane 12: Negative control, Lane 13: Positive control, M; Molecular weight marker.

Fig.4 Detection of WSSV in *P.monodon* challenged with WSSV after oral administration of the mangrove plant extracts. Each lane represents PCR product of WSSV from shrimp challenged with WSSV after oral administration of aqueous extracts

Table 4. Confirmation of the protective effect of the aqueous extract from *Ceriops tagal* on *P. monodon* from WSSV

SI No	Experimental set up	Survival on feeding with the plant extract and challenge with WSSV	Survival on challenge by injection with WSSV exposed to the plant extract	PCR	Clinical signs on passaging tissue extracts from the experimental animal groups 1, 2 and 3.
1	Test	24/24	24/24	- -	-
2	Positive control	0/24	0/24	+ +	+
3	Negative control	24/24	24/24	- -	-

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Chapter 3

CONFIRMATION OF ANTI WSSV ACTIVITY IN THE AQUEOUS EXTRACT OF *CERIOPS TAGAL* AND ITS POSSIBLE MODE OF ACTION

Contents

3.1 Introduction

3.2 Materials and methods

3.3 Results

3.4 Discussion

3.1 Introduction

Terrestrial plants represented a possible source of new and interesting antiviral drugs; aqueous extracts from such plants were in focus for many years to develop antiviral preparations against viral pathogens (Summerfield et al., 1997; Calderone et al., 1998; Garcia et al., 2006; Roner et al., 2007; Reichling 200;, Álvarez et al., in press,). Thanks to these efforts several reports have appeared in literature over a period of time about the use of aqueous extracts of various plant species against enveloped, non enveloped, DNA/RNA viruses and their mode of action against these pathogens. Summerfield et al. (1997) demonstrated antiviral activity of a distilled water extract from leaves of *Acanthospermum hispidum* against pseudo rabies virus (PRV) and bovine herpes virus (BHV). According to them the mode of action of *Acanthospermum hispidum* extract was inhibition of the virus attachment to and, to a lesser extent, penetration into cells. They also found that viral gene expression was not inhibited by the extract when the extract was added after entry of the virions into the target cells. Aqueous extract of *Artemisia verlotorum* could decrease the virus-induced syncytia of feline immunodeficiency virus (FIV) and could inhibit the viral reverse transcriptase activity and the expression of viral capsid protein P24 (Calderone et al., 1998). Broad spectral antiviral activity in aqueous extracts (AE) of *Achyrocline flaccida* against different members of the herpesvirus family (herpes simplex virus (HSV) type 1 and 2, human cytomegalovirus (HC), bovine herpes virus (BHV) and lower activity with pseudo rabies virus (PRV) could be demonstrated. The extract was found not virucidal and preliminary characterization indicated that an early step of viral replication was affected by the aqueous extract (Garcia et al., 2006). A recent study reported that an aqueous extract of root/stem and bark of *Rhus aromatica* (fragrant sumac extract) was highly active against Herpes Simplex Virus type 1 and type 2 with IC_{50}-values of 0.0005 % for HSV-1 and 0.0043% for HSV-2 as well as high selectivity indices (SI) of 5400 for HSV-1 and 628 for HSV-2 . The extract was

found interacting not only with the viral envelope but also with the surface of the host cells, impairing the ability of HSV to adsorb to and penetrate into the host cells and thus preventing viral infection (Reichling et. al., 2009).

Aqueous extracts of terrestrial plants are rich in proteins, peptides, polysaccharides, phenolics, saponins like triterpene glycosides, flavanoids, carotinoids, and antiviral activities of these compounds have been well studied in many plants. Anti viral property of polysaccharides, especially sulfated polysaccharides isolated from plants are well known. Premanathan et al. (1999a) demonstrated antiviral activity of a polysaccharide isolated from mangrove plant *Rhizophora apiculata* against HIV in MT-4cells. Phenolic compounds are derived from the secondary plant metabolism; many studies have shown a great range of pharmacological potential as antiviral agents (Chávez et al., 2006). Lin et al. (2005) studied anti SARS coronavirus 3C-like protease activity of plant derived phenolic compounds from *Isatis indigotica* roots. African medicinal plant *Pelargonium sidoides* which contained coumarins, simple phenolic structures as well as flavonoid and catechin as major constituents showed inhibitory activity against herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) *in vitro* on RC-37 cells (Schnitzler et al.,2008). Triterpenoid saponins from *Maesa lanceolata*, *Maesa chisia* and *Maesa indica* have reported to exhibit direct virucidal activity against Newcastle disease virus, vaccinia virus and herpes simplex virus (Jassim and Naji, 2003). Roner et al., 2007 reported anti- HIV activity in aqueous extract of *Quillaja saponaria* Molina which contain several physiologically active triterpenoid saponins.

Information on the use of aqueous plant extracts to control WSSV is scarce. Achuthankutty and Desai (2004) developed and patented an aqueous preparation from a composite mixture of 7 Indian medicinal plants and they could find that the preparation was effective in controlling WSSV at 15 ppm. An aqueous extract from Indian medicinal herb *Cynodon dactylon was* found to be effective in controlling WSSV in *Penaeus monodon* at a concentration of

100mg/kg of animal boy weight (Balasubramanian et al., 2008; Balasubramanian et al., 2007;, Citarasu, 2009). All these studies were focused on the activity and not much attention had been paid on what actually the mechanism was involved in the anti WSSV activity of the extracts.

Ceriops tagal is a true mangrove plant belonging to the family Rhizophoraceae and has been extensively studied for its bioactive potential. Use of this plant as a folk remedy is reported from different parts of the world. In India the decoction of the bark of *C. tagal* is being used to treat hemorrhages and malignant ulcers (Rastogi and Mehrotra, 1991), while in China it is used against sores (Lin and Fu, 1995). Use of this plant in the treatment of malaria is also reported (Duke and Wain, 1981) and its roots are used as a substitute for quinine. This plant is a rich source of tannins and triterpenoids (Ghosh et al., 1985), So far, twenty three diterpenes and twenty nine triterpenes have been reported from the stems, twigs, roots, leaves, hypocotyls and fruits of *C. tagal* and *C. decandra*. The diterpenes that have been obtained can be categorized into four types, viz. dolabrane, kaurane, beyerane, and pimarane, while triterpenes were classified into three types, viz. lupane, dammarane, and oleanane. Obviously, diterpenoids and triterpenoids are the main secondary metabolites of this genus (Wu et al., 2008).

Recently the replication cycle of WSSV and shrimp immune response to WSSV has been demonstrated using molecular tools. The gene involved in WSSV infection cycle include immediate early genes such as *ie 1, ie 2* and *ie 3,* which get activated first during WSSV infection, and subsequently activate STAT (Signal transducer and activator of transcription) (Liu et al., 2009), DNA polymerase (Chen et al., 2002b), Ribonucleotide reductase (van Hulten et al., 2000a, Tsai et al., 200a), thymidine kinase, thymidylate kinase, a chimeric thymidine–thymidylate kinase (Tsai, et al., 2000b), a thymidylate synthase (Li et al. 2004), a dUTPase (Liu & Yang 2005) and two Protein kinases (PK) (van Hulten & Vlak 2001; van Hulten et al. 2001b; Yang et al. 2001) and latency related genes (Khadijah et al., 2003) . The envelop protein Vp28 gene is a late

gene in replication cycle and the protein is able to bind to shrimp cells (van Hulten et al., 2000; Yi et al., 2004; Sritunyalucksana et al., 2006). A few genes involved in the shrimp immune response were also studied; they are proPhenol oxidase, Astakin, Peroxinectin (Jiravanichpaisal et al., 2007), Alpha 2 macroglobulin (Lin et al., 2008), transglutaminase, haemocyanin, penaeidin, and crustin (Jiravanichpaisal et al., 2007). Having all these at the background an investigation was undertaken to evaluate the efficacy of the aqueous extract from *C.tagal* on the expression of the above genes in *P.monodon*.

It has to be emphasized that no report has ever been found on the use of aqueous extracts from the mangrove plant *Ceriops tagal* to manage WSSV in shrimp. In this context the present work was undertaken to delineate the mode of action of the aqueous crude extract on WSSV by adopting a two pronged strategy such as 1. PCR amplification of the viral genes, and 2. PCR amplification of the immune genes.

3.2 Materials and methods

3.2.1 Determination of the viral titer of WSV using *Penaeus monodon* as the animal model

The viral stock prepared from 500 mg freshly infected first step PCR positive tissue in 10 mL PBS was diluted from $1x10^{-1}$ to $1x10^{-6}$ in the same diluent. Apparently healthy shrimps (4 animals in a tank in triplicate) were injected with 10 µl suspension from all dilutions having the negative control maintained by administering with the same quantity of PBS. The animals were observed for mortality every day for 7 days. The highest dilution at which 100% mortality of the test animals was recorded, and the penultimate dilution was selected for application during all assays. The percentage mortality obtained on different dilutions of virus on different days after injection challenge was statistically analyzed by two factor ANOVA and the differences were considered significant at $p \leq 0.05$.

3.2.2 Development of haemocyte primary cell culture

Apparently healthy *P. monodon* were used for haemolymph collection. The animals sacrificed by immersing in crushed ice were surface disinfected in 800 mg/L sodium hypochlorite prepared in ice cold sea water (salinity 15 ppt) for 10 minutes. Subsequently the animals were washed 5 times in sterile ice-cold sea water and dipped in 70% alcohol and rinsed with the ice-cold sea water. Haemolymph was drawn, using capillary tubes containing anticoagulant (Tris HCl 0.01M, Sucrose 0.025 M, Trisodium Citrate 0.1 M) from the rostral sinus and diluted in Modified L-15 (2X) medium (Leibovitz's L-15 (Sigma) 2X supplemented with 2% glucose, 20% FBS, 2.295g/l Tryptose phosphate broth, 10 mM N-phenylethiourea, 100X MEM vitamins, 100 µg/ml streptomycin, 100 IU/ml penicillin, 0.06µg/ml Chloramphenicol and MQ water to make up to 100 ml with a final osmolarity of 720mOsm) (Seena et al., Under publication). Haemocytes in the diluted haemolymph were enumerated using a haemocytometer, and the cell count was adjusted to 10^5 Cells /ml from the preparation, and aliquots of 200 µl were seeded on to 96 well plates and incubated at 25^0C.

3.2.3 Determination of WSSV titer in haemocyte primary culture

Subsequent to seeding the plates with the diluted haemolymph they were incubated at 25^0C for 5 hours. As soon as a monolayer of haemocytes was formed the wells were inoculated with suspensions of WSSV prepared in the modified L-15medium, diluted to 1/10 to 1/5120, and incubated at 25 ^{0}C and monitored for cytopathic effects (CPE). The control wells were inoculated with the medium alone. For each dilution wells in quadruplicate were maintained. After 24 hours the cells were observed under phase contrast microscope (Carl Zeiss, Germany) for CPE and subjected for MTT assay.

3.2.4 MTT Assay

The cell viability is measured based on the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, diphenyl tetrazolium bromide, MTT) with the

mitochondria of metabolically active cells. The nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) reduce the tetrazolium salt and produce insoluble purple formazan crystals, which are later solubilized yielding a purple-colored solution (Mosmann, 1983).

For carrying out MTT assay, the wells after inoculating with WSSV the medium was removed from each well and fresh medium was added. 50 µl of MTT (Sigma) reagent (5mg/ml in PBS; 720mOsm) was added and incubated in dark for 5 hours. Media alone with MTT was kept as control. The supernatant was removed and to each well 200 µl DMSO was added and the formazan crystals dissolved by repeated pipetting and the absorbance measured at 570 nm in a micro plate reader (TECAN Infinite Tm, Australia).

The 50% infectious dose MTT_{50} was determined as the titer at which the average absorbance was 50% of the uninfected cells. This was determined by plotting optical density of each well verse the $-\log_{10}$ of the dilution factor. Following formula was used for determining optical density

$$\text{Optical density} = \frac{\text{Blank absorbance - Well absorbance}}{\text{Blank absorbance}} \times 100\%$$

The blank absorbance is the average absorbance of 12-48 uninfected wells and the well absorbance is the average absorbance of infected wells. From the plot of optical density versus $-\log_{10}$ of the dilution factor, the 50% optical density was determined and converted to milliliter basis and stated as MTT_{50} titer (Heldt et al., 2006).

3.2.5 Preparation of WSSV infected tissue for oral challenge

Apparently healthy *P. monodon* (5-6 gm) was challenged by injecting 10 µl of 10^{-1} dilution of the virus suspension intramuscularly. The animals were monitored for development of clinical signs and mortality for a period of seven

days. All dead or moribund animals were collected and subjected for PCR for WSSV. Here, 50 mg gill tissue was macerated in 1000 µl DNAZOL and centrifuged at 10,000 g for 10 minutes. The DNAZOL supernatant was transferred to a fresh micro centrifuge tube containing 500 µl ethanol (100%). Gently inverted the sample tube several times to mix and incubated at RT for 3 minutes. Centrifuged at 4000 g at RT to palletize the DNA and supernatant removed carefully. 1000 µl 70% ethanol was added to the DNA pellet and centrifuged at 4,000g for 5 minutes at room temperature. The ethanol was tipped off and added fresh 1000µl 70% ethanol and centrifuged at 4,000 g for 5 minutes at room temperature. Supernatant removed and the DNA pellet was allowed to dry for 15 seconds to remove the ethanol fully. The DNA was dissolved in 200 µl MQ water and stored at -20°C till used. A WSSV 2 step nested PCR reaction using primers described by Lo et al., 1996, (146F1, 5'-ACT ACT AACTTC AGC CTA TCT AG-3'; 146R1,5'-TAA TGC GGGTGT AAT GTT ClT ACG A-3' and 146F2, 5'-GTA ACT GCC CCT TCC ATC TCC A-3'; and 146R2, 5’-TAC GGC AGC TGC TGC ACC TTG T-3') that yielded 1447and 941 bp WSSV specific amplicons was used for amplification of the Viral DNA following the instructions given in (Office International des Epizooties)OIE Manual. The PCR reaction mix (25µL) contained 0.5U of Taq DNA polymerase, 200 µM dNTP mix, 10 pmoles of each forward and reverse primer and 1X PCR buffer. The hot start PCR programme used was 94°C for 4 min, followed by 39 cycles of 94°C for 1min (denaturation), 55°C for 1 min (annealing), 72°C for 2 min (extension) followed by 1 cycle of final extension at 68°C for 5 min. The amplification was accomplished in a thermocycler (Ependroff). The PCR products were analyzed on 1%W/V agarose gels using TAE (1X) buffer (Tris-HCl 0.04 M, EDTA 0.0001M, Glacial acetic acid 5.71%), stained by ethidium bromide and visualized in a gel documentation system, Dolphin-Doc (Weal Tec, USA). Those animals which were first step positive were segregated for extracting tissue for challenge. Soft tissues from cephalothorax were minced and stored at -80 ^{o}C in 1gram aliquots.

3.2.6 Toxicity of the crude aqueous extract from *C.tagal* in shrimp animal model

The crude aqueous extract from the leaves of *Ceriposis tagal* was prepared following the method described under section 2.2.3 and lyophilized. From the lyophilized material suspensions were prepared in distilled water having strength 5, 10, 20, 30, 40, 50, 60 mg/mL. From each of the preparations aliquots of 10 µl was administered intramuscularly at the 6th abdominal segment of apparently healthy *P. monodon* (5-8 gm). The control consisted of animals injected with 10µl distilled water. For every strength of the extract 6 animals each were used in triplicates. They were monitored for seven days and subjected for general health assessment following the parameters as shown below. The percentage survival obtained on different dilutions of the extract was statistically analyzed by single factor ANOVA. The Differences were considered significant at $p \leq 0.05$.

Parameters considered for health assessment

i. Characteristic colouration
ii. Feed intake
iii. Moulting
iv. Antenal intactness
v. Necrosis

3.2.7 Determination of cytoxicity of the aqueous extract in haemocyte primary culture

Toxicity of the extracts used for antiviral assay on shrimp haemocytes was determined by performing MTT assay. A stock solution containing 10 mg/mL extract was prepared in the modified L-15 medium, centrifuged at 10000 rpm, filter sterilized and kept at -20^{0}C till use. Monolayer of the haemocytes was exposed to different concentrations of the extract (1000,800,600,400,200, 100 µg/ml) dissolved in the modified L -15 medium and incubated over night (14 hours). Cells with the modified L-15 medium alone were kept as control. Triplicates were maintained for each of the test and controls as well. After 12

hours of incubation the wells were subjected for microscopic observation in a phase contrast microscope (Leica, Germany). Subsequently fresh medium was added and performed the MTT assay. Percentage inhibition of the cells at various concentrations of the plant extract was calculated based on the following formula:

> Percentage of inhibition of haemocyte = [100-(Average absorbance (MTT assay) of haemocytes at a particular concentration of the extract/Average absorbance of control haemocytes without the extract) x100].

The result of cytotoxicity assay was analyzed by probit analysis using the SPSS Software (SPSS Inc., USA), LC_{50} was calculated from the result. The maximum concentration at which no toxicity could be observed was recorded.

3.2.8 Strength of the aqueous extract required for virucidal activity

Various concentrations of the extract (5, 10, 20, 30, 40, and 50 mg/ml) were prepared and mixed with 10^{-1} dilution of the virus suspension at a ratio 1:1 and incubated at 28° C for 3 hours. As negative control PBS alone and as the positive control the virus suspension mixed with PBS at a ratio of 1:1 were also incubated under the same conditions given to the test. After incubation, 10µl aliquots each of the above preparations were injected intramuscularly on to a batch of 6 apparently healthy *P. monodon* (5-6 gm size) in triplicate on 6th abdominal segment. The animals were monitored for clinical signs of WSSV disease and mortality. Percentage survival obtained on different dilutions of the extract was statistically analyzed by single factor ANOVA and the differences were considered significant at $p \leq 0.05$.

3.2.9 Quantitative determination of the aqueous extract from *C.tagal* for according protection to shrimp from WSSV.

To determine the quantity of the aqueous extract required for according protection to shrimp from WSSV, the animals were fed with different concentrations of the extracts for a period of 7 days prior to challenge. To

accomplish this objective shrimp feed (Higashimaru, India (P) Ltd.) was coated with the extracts @1%, 0.5%, 0.25% of the feed. This on administration provided the animals with a quantity of the extract delivered @ 500mg, 250mg, and 125mg/kg body weight/day. The positive and negative control animals received feed without the extract. On eighth day the test and positive control animals were challenged by feeding with freshly generated WSSV positive tissue @ 0.25 mg/ animal, and continued with the feed coated with the extract, and observed for clinical signs of the disease and mortality for 7 days. The percentage survival obtained on different concentrations of the extract was statistically analyzed by single factor ANOVA and the differences were considered significant at $p \leq 0.05$.

3.2.10 Virucidal assay

The aqueous plant extract prepared as described under section 2.2.3 (0.5mL) were mixed with equal volumes of viral suspension prepared as described under section 2.2.4 and incubated for 3 hours at 28 ^{o}C. The controls included mixtures of WSSV and PBS (positive control) and PBS alone (negative control). From each of the preparation, aliquots of 10 µl each were intramuscularly administered to the animals on 6th abdominal segment (5x4 =20 animals each) and monitored for 7 days for clinical signs and mortality. The animal samples for gene expression were collected (3 animals each) from each group on 2nd, 4th and 6th day after challenge and extracted in TriZol (Sigma) reagent for RNA extraction. For histopathology and Indirect Imunoflurescenc Histochemistry, the animals were sacrificed on completion of the experiment and preserved in Davidson's fixative and Neutral buffered formalin respectively; in a similar way the moribund animals were also preserved.

3.2.11 Oral administration of the extract and oral challenge with WSSV

The mangrove extract was mixed with minimum quantity of distilled water and coated on to shrimp feed at the ratio 1% w/w in order to make available the shrimp the extract at a quantity of 500 mg/kg shrimp body weight/ day. The feed

pellets were further coated with 4% aqueous gelatin as binder. For achieving this formulation lyophilized mangrove extract was mixed with feed pellet and 4% gelatin prepared in 5 ml distilled water coated on to the pellet. The above preparation was dried under vacuum and used for feeding. Apparently healthy shrimps of 6 g (5x4 =20 animals each) size were fed with the above extract coated feed for 7 days. On the 8th day the shrimps were challenged orally with freshly generated WSV infected first step PCR positive shrimp tissue @ 10% of the body weight/animal. Controls included shrimp fed on placebo (Positive control) and an unchallenged group (Negative control). Sampling was made from all groups, 3 animals each, on 2nd, 4th and 6th day after the challenge, preserved in the TriZol (Sigma) reagent for RNA extraction, subsequent cDNA synthesis and amplification of WSSV and immune related genes. On completion of the experiment survived animals were sacrificed and fixed for Histopathology and Indirect Imunoflurescenc Histochemistry in Davidson's fixative and Neutral buffered formalin respectively; in a similar way the moribund animals were also preserved when found moribund.

3.2.12 RT-PCR of WSSV genes and immune related genes in *Penaeus monodom*

Shrimp gill tissue 50 (mg) was digested in TriZol reagent (Sigma) and total RNA extracted according to the manufactures protocol and quality and quantity of RNA was checked. In brief Gill (100 mg) tissue was macerated in 1000 µl TriZol reagent. The sample was kept for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2 ml chloroform was added to 1000 µL TriZol reagent and shaken vigorously for 15 seconds and allowed to stand for 15 minutes at room temperature and centrifuged at 12000 g for 15 minutes. Colorless aqueous phase was separated carefully from the three layers formed to a fresh tube. 0.5 ml isopropanol was added, stored for 10 minutes at RT and centrifuged at 12000 g for 10 minutes. RNA was found precipitated on the sides and bottom of the tube after centrifugation at 12000 g for 10 min at 4^0

C. The supernatant was discarded and the pellet washed twice in 74% ethanol. The pelleted RNA was air dried and dissolved in 20 µl DEPC treated water by repeated pipetting at 55^0 C. RNA sample was subjected to DNase treatment with RNase free DNase 1 (New England Biolabs). 0.2 units of enzyme was added to 1 µg of RNA and incubated at 37^0C for 10 minutes. The enzyme was inactivated at 75^0C for 10 minutes. Concentration and quantity of RNA was measured at 260/280 nm on a UN-Visible spectrophotometer. Five µg RNA was subjected to cDNA synthesis with 20 µl reaction mix containing M-MuLV reverse transcriptase (200U), RNase inhibitor (8U), Oligo $(dT)_{12}$ primer (40 pmoles), dNTP mix (1mM), RTase buffer (1X) and $MgCl_2$ (2 mM) at 42°C for 1 hour. All reagents were purchased from New England Biolabs. Subsequently, 8 WSSV genes and 8 immune related genes were amplified by PCR using 1µl cDNA with specific primer sets as given in Table 1. Shrimp ß actin gene was also amplified as a reference. 25µl PCR reaction mix contained 0.5U of Taq DNA polymerase, 200 µM dNTP mix, 10 pmoles each of forward and reverse primers and 1X PCR buffer. The hot start PCR programme used for WSSV genes was 94°C for 2min, followed by 35 cycles of 94°C for 30s, annealing for 30s, 68°C for 30s followed by final extension at 68°C for 10 min. Annealing temperatures were 50°C for endonuclease, 53°C for latency gene 1 and ribonucleotide reductase (rr1), 54°C for DNA polymerase (dnapol), immediate early gene1 (ie1), Vp 28 and thymidine kinase and thymidilate kinase (tk-tmk), and 55°C for protein kinase 1(pk1) and ß actin. For immune related genes also hot start PCR was carried out with 94°C for 2 min, followed by cycles of 94°C for 2 min, annealing for 1min, 72°C for 1min followed by final extension at 72°C for 10 min. Annealing temperature and number of cycles employed for PCR of Astakine and peroxinectin were 56°C and 30 cycles, for prophenoloxidase (Pro PO) and transglutaminase, 56°C and 35 cycles for crustin and penaeidin, 55°C and 35 cycles for alpha 2 macroglobulin, 65°C and 30 cycles, and for haemocyanin the conditions were 56°C and 35cycles. 10 µl each of the PCR products were

analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented.

3.2.13 Histopathology

Tissues were fixed over night in Davidson's fixative and were transferred to 70% ethanol for 24 hours. They were dehydrated in a series of alcohol 70, 80, 90, 95 and 100% and finally dipped in acetone for 2 min and cleared in two changes of xylene for 10 minutes. The tissues infiltrated with paraffin wax through xylene wax mixture of 1:3, and in 3 changes of pure wax and finally embedded in paraffin wax. The tissue blocks were preserved at room temperature till sectioning.

3.2.14 Haematoxylin eosin staining

The tissue blocks were cut into 5 µm thin sections. The sections were transferred to clean microscopic slides previously coated with Chrome Alum Gelatin, de-waxed in two changes of xylene and re-hydrated through 95%, 70% alcohol and finally in distilled water. The sections were stained with haematoxylin and differentiated in acid alcohol. Bluing was done in Scots tap water and counter stained with eosin. Again dehydrated in alcohol and cleared in xylene and mounted in DPX. The sections were observed under a light microscope.

3.2.15 Indirect Immunohistochemistry of the experimental animals

Gill tissues fixed in neutral buffered formalin were embedded in paraffin wax, cut into 5 µm sections, and transferred to microscopic slides coated with Chrome Alum Gelatin and fixed overnight in room temperature. Sections were de-waxed using xylene and re-hydrated through series of alcohol and distilled water. Slides were incubated for 1 hour with 3% BSA in PBS in a humidified chamber to block free sites. Washed in PBS- Tween -20 mixture (0.01%) three times (3 minutes each) and incubated with WSSV specific monoclonal (C 38) antibody (Anil et al., 2002) for 1 hour. After washing the slides in PBS -Tween -20 mixture three times

(3 minutes each) anti mouse IGg FITC conjugate, diluted to 1: 40 dilution (Sigma) in BSA in PBS, was added and incubated for 1 hour in dark in humidified chamber. Washed in PBS -Tween -20 and stained with nuclear stain DAPI (10 µl, 0.02 µg/ml) and incubated for 3 minutes. The slides were rinsed with distilled water, air dried, mounted with mounting media (Vectashield, USA) and observe in a UV fluorescence microscope (Olympus, Germany). DAPI and FITC were viewed under filters with excitation wavelength 360-370 nm and 470-490 nm, respectively. The images were processed and merged using the "Imagepro-express" software (Media Cybernetics Inc, MD, USA)

3.3 Results

3.3.1 Determination of the titer of WSSV stock

3.3.1.1 In shrimp animal model

To determine the virus titer required to cause mortality of shrimp within a period of seven days on intramuscular administration, different dilutions of WSSV in PBS were injected and observed for mortality (n=4). The difference in mortality occurred in shrimp which received varying dilutions of the virus was highly significant (*P<0.001*). Among the dilutions, $1x10^{-1}$ and $1x10^{-2}$ resulted in mortality of all animals within 7days (*P<0.001*), and from the dilution $1x10^{-6}$ onwords no mortality could be registered, and the animals behaved as in the case of PBS injected shrimps (*P<0.001*) (Fig. 1).

3.3.1.2 In haemocyte primary culture

The viral titer was determined in primary haemocyte culture from *P. monodon* and related with the extent of mortality at each titre. The titre at which the entire group of hemocyte perished within seven days was 3.3 $log_{10}MTT_{50}$/ml (*P<0.001*) (Fig**.** 2).

3.3.2 Determination of *in vivo* toxicity of the crude aqueous extract

P. monodon weighing 4-5 g (n=6) were injected with the crude aqueous extract at different concentrations ranging from 5-60 mg/mL and monitored for 7

days (Fig. 3). Response of the animals was more or les the same without any significant mortality even up to a concentration 50 mg/mL ($P<0.05$). However, at 60 mg/mL strength there was significant reduction (77.7% average percentage survival) ($P<0.05$) in survival of shrimps during the experimental period of 7 days.

3.3.3 Determination of toxicity of crude aqueous extract *in vitro* in Primary haemocyte culture from *P.monodon*

The cytotoxicity of the aqueous extract was determined in primary haemocyte culture of *P.monodon*. Minimum concentration at which no toxicity recorded was 100 µg/ml and the LC_{50} calculated by probit analysis was 254.84 µg/ml ($P<0.05$) (Fig. 4). On evaluating the response of the haemocyte to the increasing concentrations of the extract there was reduction in the number of cells as the concentration went up (Fig.5).

3.3.4 Strength of the aqueous extract required for virucidal activity

Experiments to determine the virucidal activity was carried out on *P. monodon* weighing 4-5 g (n=6). On administering WSSV at a dilution $1x10^{-1}$ after exposing to different concentrations of the aqueous plant extract at equal proportions for 3 hours at 27°C there were significantly higher ($P<0.001$) survival of shrimps when administered with WSSV suspension exposed to higher concentration of the plant extract such as 30, 40 and, 50 mg/mL. Accordingly, the shrimps were not found getting infected in the above concentrations registering overall total survival. Meanwhile the batches of shrimp administered with 5, 10, and 20 mg/mL extract resulted in a lesser survival of 77.8, 83.3 and 94.4 % respectively. All positive controls succumbed to the virus when administered with the virus exposed to PBS after incubation for 3 hours at 27°C. All negative control animals survived the experimental period (Fig..6).

3.3.5 Quantitative determination of aqueous extract from *C.tagal* for according protection to shrimp from WSSV

To examine the *in vivo* antiviral activity of the aqueous mangrove extract, *P. monodon* weighing 4-5 g (n=6) were fed with 500, 250 and 125mg/kg/body

weight/day for a period of seven days. On challenging all batches of shrimp by oral administration of infected meat all animals which received the extract at a dosage of 500 mg/kg/body weight/day survived, and the differences in survival between different batches were significant (*P<0.001*). Significantly lower average survival of shrimp observed in batches of shrimp which received 250 and 125mg/kg body weight/day is note worthy as the average survival was only 33.3 and 44.4 %. The positive control animals (shrimps administered with normal diet and challenged with WSSV) succumbed to WSSV registering total mortality. All negative control animals survived (Fig.7).

3.3.6 Histopathology of gill tissue of *P.monodon* challenged with WSSV exposed to the aqueous extract and challenged orally with WSSV after administering with the extract

To evaluate the pathological changes, longitudinal sections of secondary non branching gill filaments of the experimental animals were examined under the light microscope. In the positive control groups of animals which received WSSV through injection and the ones challenged orally there were prominent histopathological changes. They included dislodgment of cuticle on the gill filaments with sub-cuticular cells having eosinophilic hypertrophied nuclei, cellular degeneration and shrinkage, multi-focal necrosis and haemocytic infiltration. The necrotic pilaster cells showed eosinophilic hypertrophied nuclei, characteristic of WSSV infection. The pathological changes in the positive control which received WSSV by injection exposed to PBS indicated more severity of the infection than the ones challenged orally. The lacunae occluded by haemocytes showed vacuolization. On the contrary tissues from negative control and the ones administered with WSSV exposed to the extract and challenged with WSSV orally after feeding with extract did not show any clinical pathological changes and they were identical. The gill filaments contained numerous lacunae with sufficiently large number of haemocytes as in the case of apparently healthy animals (Fig.8).

3.3.7 Indirect immuno fluorescence histochemistry of gill tissue

For localization of WSSV in gill tissue of the experimental animals the histological sections were subjected for immunofluorescence assay using WSSV specific monoclonal antibody and the slides were observed under epifluorescence microscope. The positive control animal tissues generated by challenge through the injection and oral administration gave strong positive signals with green florescence of FITC conjugated antibody. The infected cells were characterized by enlarged nuclei with positive signals and the normal nuclei were DAPI stained in blue. In the case of negative control and the experimental animals which received the aqueous extract there were no positive signals with FITC conjugated anti body (Fig.9&10).

3.3.8 Temporal gene expression of viral genes in *P.monodon* in response to WSSV and *C.tagal* aqueous extract.

The expressions of the viral genes on 2nd, 4th and 6th day after the challenge with virus were examined to find out whether the mangrove extract was inhibiting the processes involved in the viral multiplication cycle. The gene expression study was conducted in both the groups of animals such as the ones which received WSSV exposed to the extract and ones which were challenged after oral administration of the extract. Viral genes were not amplified in both the groups of animals and appeared exactly like the negative controls. In the case of positive control (administered with placebo) the viral genes such as immediate early gene ie1, DNA polymerase, thymidine–thymidylate kinase, endonuclease, ribonucleotide reductase, protein kinase, latency related gene, and VP28 were found expressed on 2nd,4th, and 6th day after challenge with WSSV. It was observed that, as the days went by there was increase in the intensity of bands of these genes suggesting more multiplication of the virus in the positive control shrimps. In the case of positive control animals which received virus by injection there was total mortality on 4th day itself and there were no animals available to assay on 6th day (Fig. 11, 12, 13&14).

3.3.9 Temporal gene expression of immune genes and AMPs in *P.monodon* in response to WSSV and feeding of *C.tagal* aqueous extract

Expression of immune related genes and AMPs analyzed in *P.monodon* on 2nd, 4th 6th day after oral administration of *C. tagal* extract and challenge with WSSV is summarized in Fig.15 and 16. Accordingly, prophenoloxidase was found down regulated on 6th day of the challenge in the group of animals fed on the extract. Meanwhile, no variation was observed in Astakine production between the groups during the 6 day experimentation period. No variation in the expression of peroxinectin was observed. Meanwhile transglutaminase was down regulated on the 6th day of challenge. In a similar pattern down regulation of α -2 macroglobulin on the 4th day and 6th day both in the positive control as well in the test group were observed. Interestingly, in the case of haemocyanin two amplicons could be seen uniformly in all instances. Penaeidins did not show any remarkable variation in their expression between the groups over the period of 6 days

3.4 Discussion

Antiviral activities of aqueous extracts from plants are well established (Summerfield et al., 1997; Calderone et al., 1998; Garcia et al., 2006; Roner et al., 2007; Reichling et al., 2009) and this includes reports on the antiviral activity of plant extracts against WSSV too (Takahashi et al., 1998; Chotigeat et al., 2004;, Supamattaya et al., 2005; Citarasu et al., 2006; Balasubramanium et al., 2007; Balasubramanian et al., 2008; Citarasu, 2009). A combination of herbal extracts and probiotics as medicated diet could decrease prevalence of WSSV in *Litopenaeus vannamei* (Gomez et al., 2009). Even though reports are available on the protective effect of plant extracts against WSSV, informations on their mode of action are scanty. In this study antiviral property of an aqueous extract from *C.tagal* was investigated employing molecular tools evaluating their role in protecting shrimps from WSSV.

The titer of WSSV stock used for the experiment was determined both *in vivo* in animal model as well as in *vitro* in shrimp haemocyte primary culture and tittered so as to have adequate virus load to initiate an infection. Accordingly the virus suspension was prepared by macerating 500 mg infected tissue in 10 mL buffer diluting to 10^{-1} to 10^{-6}. Administration of 10 µl of the suspension at 10^{-2} dilution could invariably result in mortality. In all further experiments a titer of 10^{-1} was used. Ha et al., (2008) conducted similar kinds of experiments to determine the dilution of WSSV required to be used for neutralization by WSSV vaccine. According to them a dilution of 10^{-3} could give 100% mortality within 7 days.

Toxicity of the extract was determined both *in vivo* in animal model and *in vitro* in *P.monodon* primary haemocyte culture. The IC_{50} calculated in primary haemocyte culture was 254.84 µg/ml, which was equal to 0.26 µg/µl, and the highest nontoxic concentration worked out was 100 µg/ml. Meanwhile in animal model the highest non toxic concentration went up to 50 mg/ml (50,000 µg/mL) extract of which 10 µl extract was injected to shrimps weighing 5-8g body weight delivering 500 µg per animal, 62. 5 to 100 µg/g animal. Average percent survival of shrimps injected with the aqueous extract was 100% at concentrations 5, 10, 40 and, 50 mg/ml concentrations alike what has been observed in the negative control animals. However, in the batch of animals which received 20 and 30 mg/mL there were only 94.3 % survival. This marginal mortality was due to cannibalism subsequent to moulting. The overall result of the investigation on toxicity of the extract in animal model revealed that the crude extract from *C.tagal was* not toxic to *Penaeus monodon* up to a concentration of 50 mg /mL administered at10 µL/animal. Rahman et al. (2006) injected different concentration of Cidofovir and observed that the antiviral drug was nontoxic to shrimps up to a concentration 200 mg/kg body weight and they could successfully use the same for further assays. Accordingly an experiment was conducted in the present study to determine the lowest concentration of the extract required to exhibit virucidal property against WSSV in *Penaeus monodon.* In this experiment a range of concentrations of the extract

between 5 mg/mL and 50 mg/mLwas chosen and the strength of above 30 mg/ml was found to be effective in exhibiting its virucidal property. In this processes the lowest concentration required for virucidal property was 30 mg/ml. The result indicated that the minimum concentration of the extract required for extending the virucidal activity was less than its *in vivo* and *in vitro* toxic level with high selectivity index, which is the ratio of toxic concentration to the effective concentration, and shows higher antiviral activity at a concentration below the toxic value. This point to the suitability of the aqueous extract to protect shrimps from WSSV. The results generated unequivocally suggest that the virucidal property of the aqueous extract of *C.tagal* is concentration dependent. In a similar pattern on screening 20 Indian medicinal plants, anti WSSV activity was exhibited by the aqueous extract of *Cynodon dactylon* on administering 100 mg/kg animal body weight when injected intramuscularly. Among this the methanolic extract of *Momordica charantia* exhibited protective effect from WSSV when injected along with the virus after incubation. However, the animals which survived the challenge were PCR positive to WSSV (Balasubramanian et al., 2007; Citarasu 2009). Dose dependent antiviral effects against WSSV have been reported in the case of antimicrobial peptide mytilin, and the C10 c fragment of the peptide when injected after incubating with WSSV (Dupuy et al., 2004; Roch et al., 2008). Survival of shrimps when injected with 50µM and 100µM mytilin and C10c respectively was total. It was proposed that the antiviral activity of mytilin was mediated by its binding onto the virus envelop (Dupuy et al., 2004; Roch et al., 2008).

On evaluating effectiveness of orally administered *C.tagal* extract in protecting shrimps from WSSV, none of the test animals exhibited signs of distress during the period of drug administration. Shrimps orally administered with the aqueous extract at a dosage of 125 mg/kg body weight exhibited 33.3% survival, 250 mg/kg body weight/day showed 44.4% and 500 mg /Kg body weight 100% survival on challenging with WSSV infected tissue. In a comparable way administration of peptidoglycan from *Cladosiphon okamuranus* at the rate 100

mg/kg shrimp body weight resulted in 76.2% survival (Takahashi et al., 1998) and administration of fucoidan from *Sargassum polycystum* at 400 mg/kg body weight resulted in 93% survival (Chotigeat et al., 2004). Shrimps fed with 300 mg of *Dunaliella* extract /kg diet showed higher resistance to WSSV infection besides becoming tolerant to stress (Supamattaya et al., 2005). Citarasu et al. (2006) reported 74% increase in survival of *P. monodon* on administering herbal immunostimulant incorporated diet at a concentration of 800 mg/kg feed. However, in the present study the percentage survival of *P.monodon* was 100 when fed with aqueous extracts from *C.tagal* at a concentration of 500 mg /kg body weight. Total survival of the batch of experimented *P. monodon* was reported when fed with *Cynodon daclyone* at a concentration of 2% of the feed (Balasubramanian et al., 2008).

Basis of survival of shrimp was proved as the absence of WSSV in the animals despite challenge with the virus through oral route and by intramuscular injection of the virus suspension exposed to the extract. This conclusion was arrived at through histopathology, indirect imunofluroscent histochemistry, and the absence of any pathological changes accompanied with WSSV infection. Mean while the tissues from positive control animals exhibited hypertrophied nuclei, cellular degeneration and shrinkage of the tissue, multi-focal necrosis and haemocytic infiltration as described earlier by Manjusha et al., 2009. All positive animal tissues gave rise to positive signals for WSSV during indirect immunoflurescent assay. This indicated that the virus could not invade the animals fed on the aqueous extract. Rahman et al., (2006) carried out indirect immunoflurescent histochemical assay to asses the clinical effects of Cidofovir and diet supplemented with *Spirulina platensis* against WSSV. In their study the shrimps that survived as well as dead were found to be positive to WSSV, and they reported that Cidofovir and *Spirulina platensis* supplemented diet could delay mortality to 120 hours of post infection of WSSV. Accordingly, it could be concluded that *C.tagal* was comparatively very much effective in protecting

shrimp from WSSV as there had never been any Indirect Immunofluorescent positive shrimps or any mortality even after 7 days of post infection.

To elucidate the mode of action of the extract in protecting shrimp from WSSV, expression of viral genes was investigated by way of amplifying the genes involved in the multiplication cycle. The genes examined were immediate early gene (*ie1*), DNA polymerase (*dnapol*), Thymidine - thymidilate kinas (*tk – tmk*), Endonuclease (*endonu*), Ribonucleotide reductase (*rr1*), Proteinkinase (*pk1*), VP28, and Latency related genes. Besides the expression profile of a set of genes such as Prophenol oxidase, Astakin, Peroxinectin, Transglutaminase, Alpha 2 macroglobulin, Haemocyanin, Penaeidins and Crustin all involved in shrimp immune mechanism was also looked into. Beta actin gene was used as the internal control in both the case.

Expression profile of the genes involved in WSSV replication cycle proved that none of the genes involved in viral replication could be expressed in the test animals administered with the extract and also in the negative controls. This was alike in both the experimental designs, the ones which were fed on the extract and challenged with WSSV and the ones which received WSSV exposed to the aqueous extract. The striking observation was that even the immediate early genes (*ie*) failed to express in the shrimps challenged. The expression of viral immediate early genes depends on the host cell machinery and occurs independently of any viral de novo protein synthesis (Friesen, 1997). Once expressed, the *ie* gene products may then function as regulatory trans-acting factors and may serve to initiate viral replication events during infection. Viral *ie* genes are expressed immediately after primary infection or as a result of the re activation of the virus. Recently, it was found that white spot syndrome virus (WSSV) used a shrimp STAT as a transcription factor to enhance viral gene expression in the host cells. STAT directly transactivates WSSV *ie1* gene expression and contributes to its high promoter activity (Liu et al., 2008; Liu et al., 2009). JAK-STAT pathway is one among the immune genes activating

signaling pathways in lower animals like insects. (Souza-Neto et al., 2009) The STAT Pathway was found to be mediating Late-Phase Immunity against Plasmodium in the Mosquito *Anopheles gambiae* (Gupta et al., 2009). Like most of the insect baculovirus, WSSV *ie1* transcription is mediated by host RNA polymerase II. This was proved when cycloheximide, a protein synthesis inhibitor treated shrimps did not prevent expression of *ie g*enes when infected with virus. Where as known-immediate early genes like *dnapol*, *rr1*, *pk, tk - tmk*, and *endonu* were inhibited when shrimps were treated with 250 mg/kg CHX (Liu et al., 2005). In the cascade of viral regulatory events, successive stages of virus replication are dependent on the proper expression of the genes in the preceding stage. For example, during infection by the large DNA viruses, such as baculoviruses and herpesviruses, gene expression was regulated such that the immediate-early (*ie* or a) genes were transcribed first, followed by the expression of the early (*e* or h) and late (*l* or g) genes, respectively (Blissard, 1996; Blissard and Rohrmann, 1990; Friesen and Miller, 1986; Honess and Roizman, 1974). In the present study none of these genes, starting from immediate early genes to VP 28, (late stage) were found expressed, suggesting that WSSV was not able to initiate the host RNA polymerase II, which mediated transcription of the immediate early genes in the presence of the aqueous extract. This prevented replication of WSSV due to the inactivation of the virus by the virucidal fractions in the extract. In a similar study, extracts from *Cynodon dactylon* at 2% of the feed (w/w) could protect shrimps from WSSV when challenged orally. They observed that survived animals were negative to WSSV by diagnostic PCR, besides the lack of amplification of VP 28 (Balasubramanian et al., 2008). They suggested that WSSV was not multiplying in shrimps fed with the plant extract inferring viral inactivation and subsequent lack of transcription

On investigating the immune gene expression in shrimp orally administered with the extract and challenging through the same route, the aqueous extract from *C.tagal* was not immunostimulatory to *P. monodon.* This may be due to the failure of

JAK-STAT pathway to activate immune related genes as stated earlier. In lower animals like insects the innate immune response is largely orchestrated by 3 signaling pathways, Toll, Imd and JAK-STAT (Souza-Neto et al., 2009). In a study on shrimp fed with *Dunaliella* extract incorporated diet showed higher resistance to WSSV infection and became tolerant to stress, and there were no significant differences in immune parameters like prophenol oxidase and total haemocyte count (Supamattaya et al., 2005). In the present study it was noticed that prophenol oxidase gene and alpha 2 macroglobuline genes were found to be down regulated on 6th day in *C.tagal* extract administered shrimps. Similarly in a recent study saponin immersion could enhance the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus.* Hyaline cell count, total haemocyte count, specific α 2-Macroglobulin activity, respiratory burst, SOD activity, and GPx activity were directly increasing with the saponin concentration, whereas phenol oxidase activity was inversely related to the saponin concentration. (Su et al., 2007). This suggests that the aqueous extract from *C. tagal* might be containing saponin like compounds having down regulatory effect on phenol oxidase activity.

The over all results suggest that the mode of action of *C.tagal* extract on WSSV is virucidal. This is evident from the indirect immunoflurescent histochemistry, histopathology, and absence of viral gene expression in animals administered with the extract. Based on this evidence we propose that during feeding of the extract and subsequent challenge with WSSV by oral rout, the virus while being in the intestine has been getting inactivated by the virucidal molecules of the aqueous extract. The same pattern of inactivation happens while it is being exposed to the extract *in vitro*. Consequently the inactivated virus fails to multiply in the host and subsequently gets eliminated.

Balasubramanium et al., (2007) proposed 3 possible modes of action of plant extracts against WSSV; 1) Viral inactivation due to interaction between the extract and the envelope protein, 2) Influence of the plant extract on the replication of the virus, which prevents virus replication, and 3) Immunostimulatory activity of the plant

extract. In analyzing the results generated in this study, in the light of the postulates described above, it could be inferred that the virus was getting inactivated by the extract and thus getting prevented from establishing an infection. The observation that none of the viral genes was getting expressed in the group treated with the plant extract supported by the absence of histopathological changes and lack of Indirect Immunofluorescence signals give the strong indication that the virus was getting inactivated in the digestive tract and /or in vitro paving the way for its expulsion from the system. Immune gene expression confirmed that the pathogen was not sensed by the defense mechanism either humoral or cellular. There is the possibility of antigen removal through the action of cellular responses. It is reported that autophagy, a homeostatic mechanism by which cells digest and recycle macromolecules and organelles by lysosomal digestion, might be involved in crustacean immune response (Johnson et al., 2008, Walker and Mohan, 2009). Recent evidences suggest that the antiviral phagocytosis in shrimp may be controlled by a Rab GTPase that is up-regulated in WSSV resistant shrimp and forms a complex with β-actin, tropomycin and the WSSV envelop protein VP 466 (Wu and Zhang, 2007; Wu et al., 2008). The WSSV clearance is also reported in *Macrobrachium rosenbergii* (Sarathi et al., 2008). Further studies are required in this direction to prove the elimination of inactivated virus through cellular immune responses.

In conclusion, through this study we could confirm the antiviral activity of aqueous extract of *Ceriops tagal* and could standardize the quantity of the aqueous extract required to protect shrimps from WSSV infection against a defined titer of virus. We found that the extract was less toxic to the shrimp at concentrations of the extract required for antiviral activity. The mode of action of the extract could be demonstrated through temporal gene expression studies pertaining to both WSSV specific gene and immune gene as well. Absence of virus in the shrimps could be demonstrated through Indirect Immunofluorescence histochemistry and histopathology. The mode of action of the virus was found virucidal; however more studies are required to elucidate further the processes.

Table 1 Primers used for Anti WSSV Study

Sl. No.	Gene	Primer sequence (5'-3')	Size (bp) of PCR product	Reference
		WSSV genes		
1.	Immediate early gene (ie1)	F-GACTCTACAAATCTCTTTGCCA R-CTACCTTTGCACCAATTGCTAG	502	Liu et al., 2005
2	Protein kinase (pk1)	F- TGGAGGGTGGGGACCAACGGACAAAAC R-CAAATTGACAGTAGAGAATTTTGCAC	512	
3	Thymidine kinase and thymidylate kinase (tk-tmk)	F-GAGCAGCCATACGGGTAAAC R-GCGAGCGTCTACCTTAATCC	412	
4	Ribonucleotide reductase (rr1)	F-ATCTGCTAGTCCCTGCACAC R-AAAGAGGTGGTGAAGGCACG	408	
5	DNA polymerase (dnapol)	F-TGGGAAGAAAGATGCGAGAG R-CCCTCCGAACAACATCTCAG	586	
6	Endonuclease	F-TGACGAGGAGGATTGTAAAG R-TTATGGTTCTGTATTTGAGG	408	
7	Vp 28	F-CTGCTGTGATTGCTGTATTT R-CAGTGCCAGAGTAGGTGAC	555	
8	Latency 1	F-CTTGTGGGAAAAGGGTCCTC R-TCGTCAAGGCTTACGTGTCC	647	
		Shrimp immune related genes		
1	Prophenol oxidase (pro PO)	F -TGGCACTGGCACTTGATCTA R -GCGAAAGAACACAGGGTCTCT	590	Jiravanichpaisal et al., 2007
2	Astakine	F -GTCGCGCATTTAACAAGGAG R -CCCTGTGGATTGAGCTCACT	455	
3	Peroxinectin	F -CGAAGCTTCTTGCAACTACCA R -GCAGGCTGATTAAACTGGCTT	547	
4	Alpha 2 macroglobulin	F -ATGGCCAATCCCGAGAGGTACCTACTG R -TGTTGCTGCAGAAGTTTGTTATCCTCAT	345	Lin et al., 2007[a]
5	Haemocyanin	F-GTCGACGAACTTCACTGGGA R-GTTCAGTGTCATCAACGGCA	598	Jiravanichpaisal et al., 2007
6	Transglutaminase	F-TGGGYCTTCGGGCAGTT R-CGAAGGGCACGTCGTAC	627	
7	Crustin	F-GCACAGCCGAGAGAAACACTATCAAGAT R-GGCCTATCCCTCAGAACCCAGCACG	430	
8	Penaeidin-3	F-AGGATATCATCCAGTTCCTG R-ACCTACATCCTTTCCACAAG	240	
9	ß Actin	F-CTTGTGGTTGACAATGGCTCCG R-TGGTGAAGGAGTAGCCACGCTC	520	Zhang et al., 2007

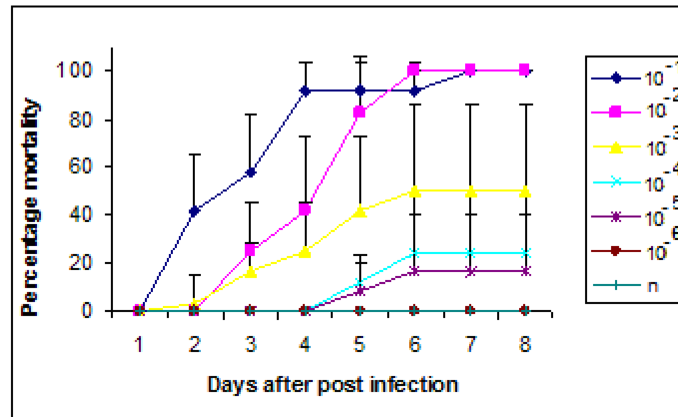


Fig. 1 Injection experiment with different titer of virus

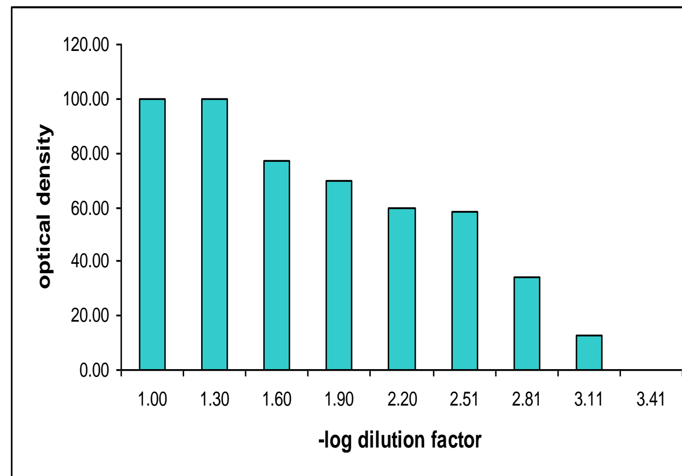


Fig. 2 WSSV titer determined in haemocyte Primary cell culture from *P. monodon* in terms of negative logritham dilution factor

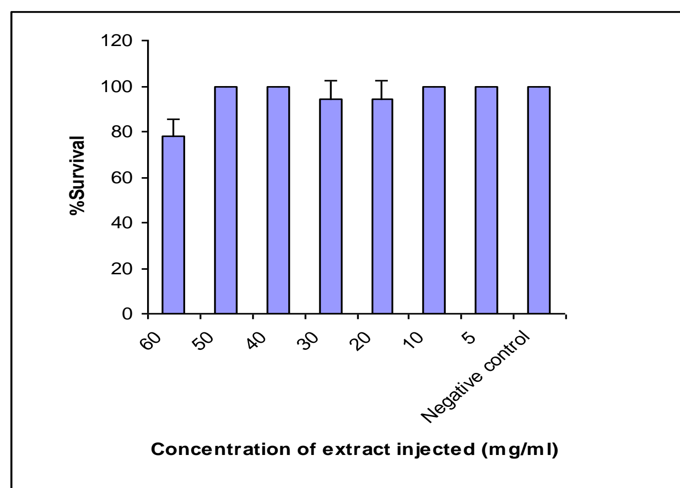


Fig. 3 Toxicity of different concentration of extract in *Penaeus monodon*

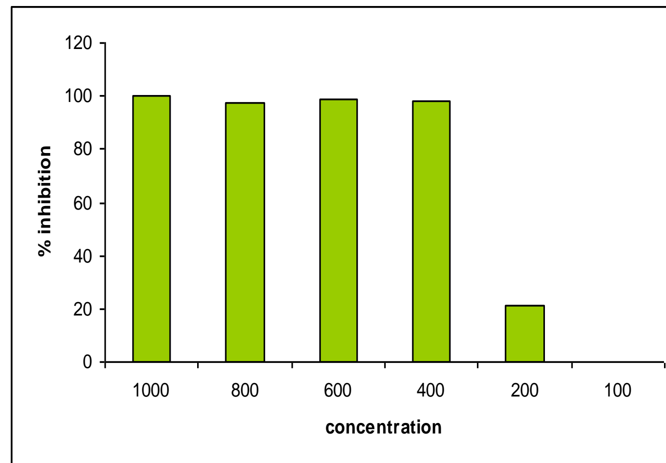


Fig. 4 Cytotoxicity of aqueous extract in haemocyte primary culture form *P. monodon* expressed in terms of % inhibition measured in terms of MTT reduction

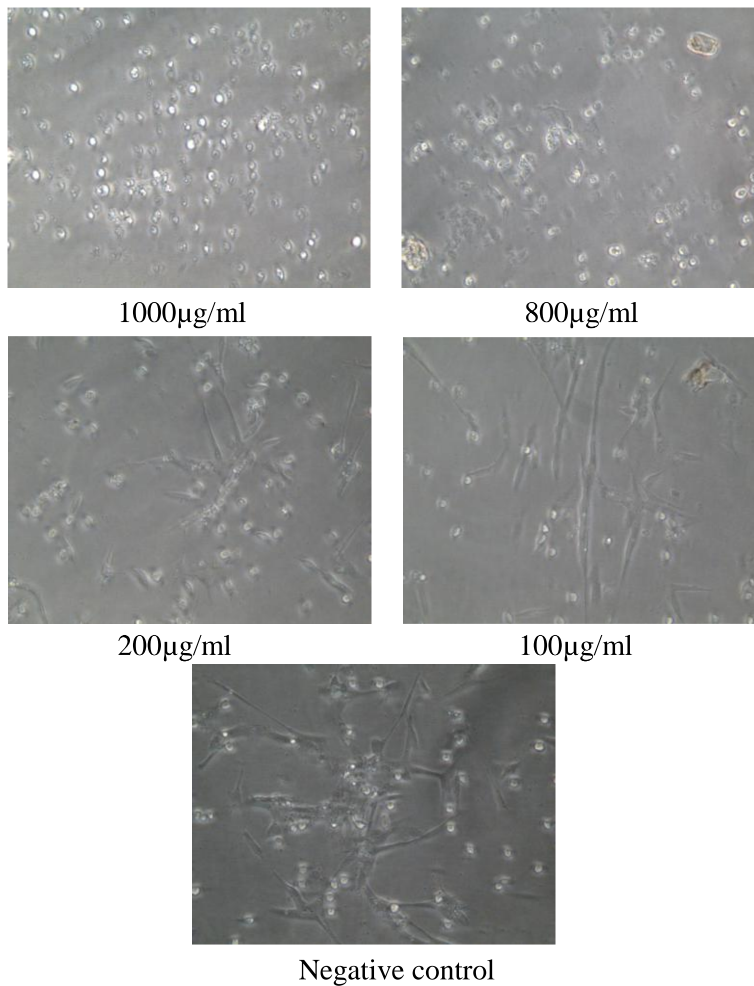


Fig.5 Cytotoxicity of aqueous extract in haemocyte primary culture form *P.monodon*

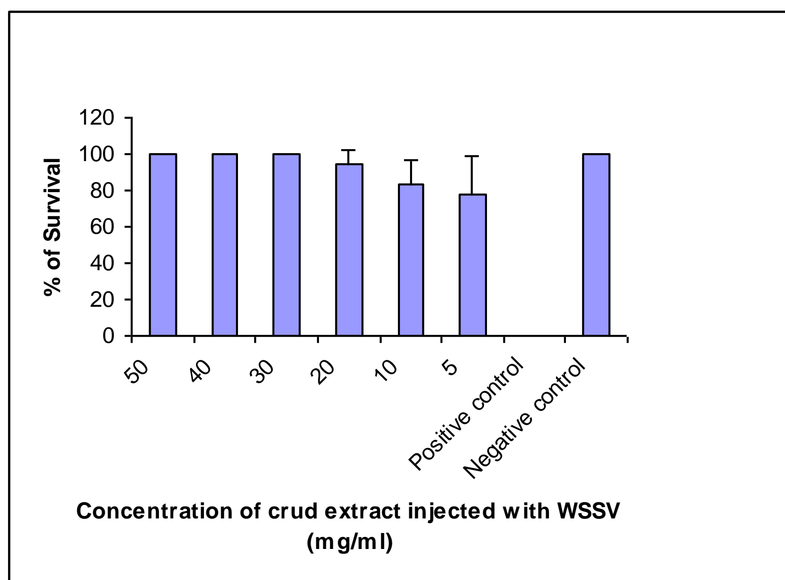


Fig. 6 Virucidal property at different concentration of virus

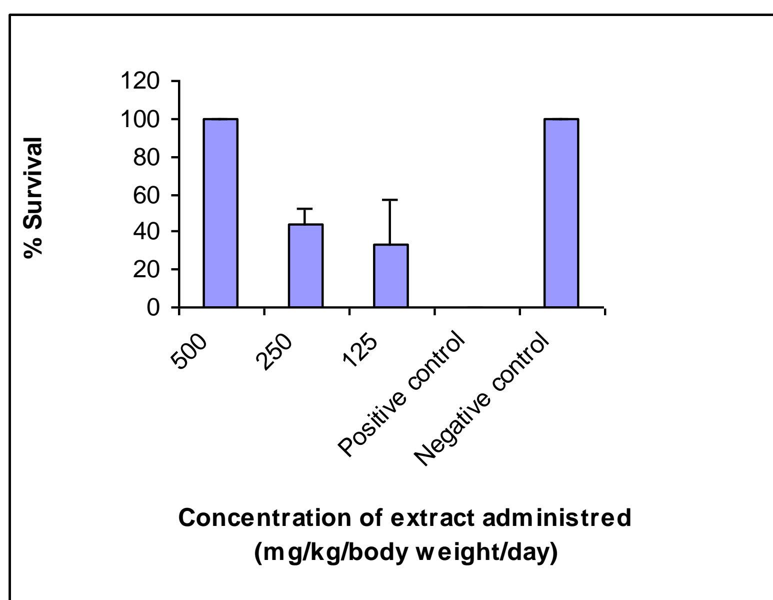
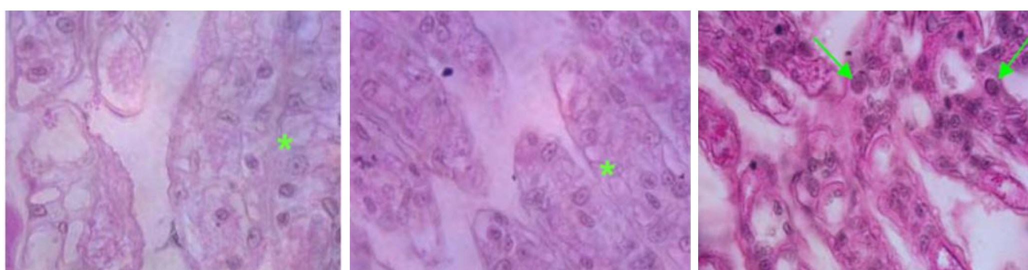


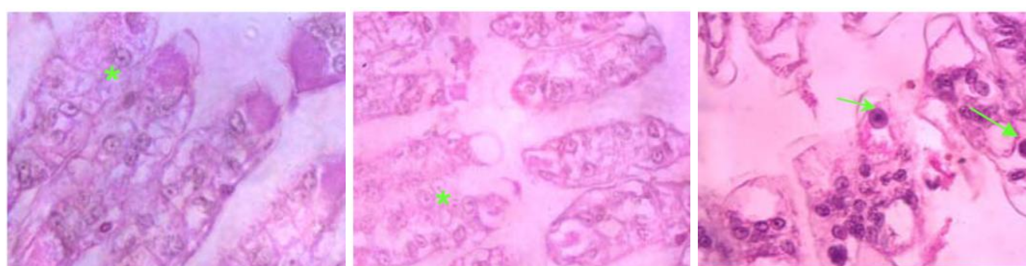
Fig. 7 Efficacy of orally administered extract against WSSV

A. Oral administration of the aqueous extract and oral challenge with WSSV



Negative control (100X) **Test (100X)** **Positive control (100X)**

B. Injection challenge with WSSV exposed to the aqueous extract



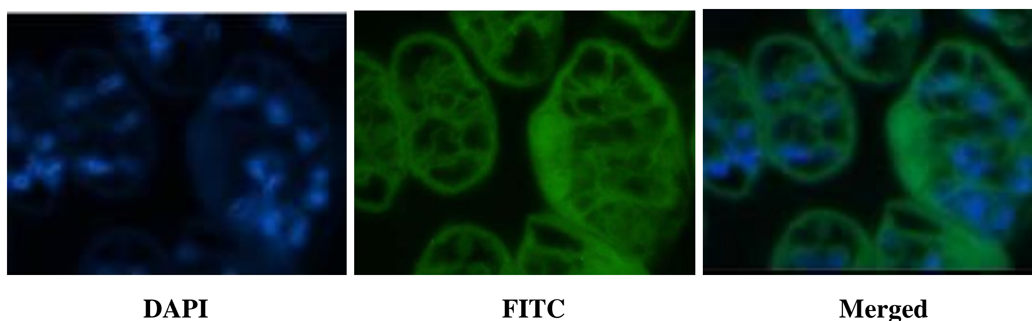
Negative control (100X) **Test (100X)** **Positive control (100X)**

Arrow =Nuclear hypertrophy

*= Normal Cell

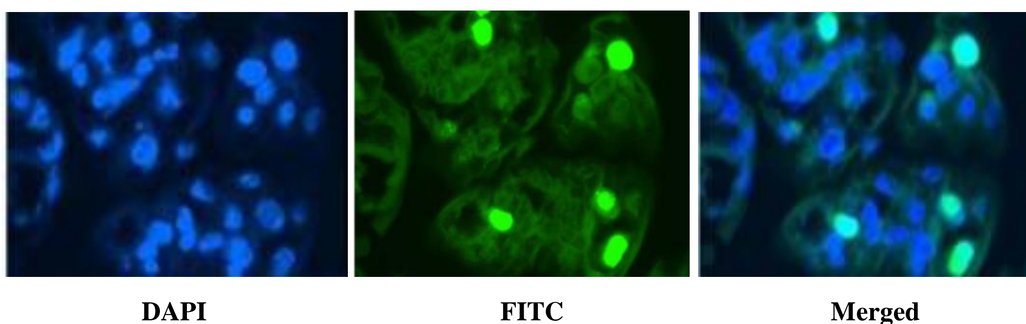
Fig. 8 Protective effect of the aqueous extract from *C. tagal* Histopathology of gill tissue of shrimp challenged with WSSV subsequent to oral administration of the aqueous extract, and the virus exposed to the aqueous extract

A. Negative control tissue- Normal feed with out challenge



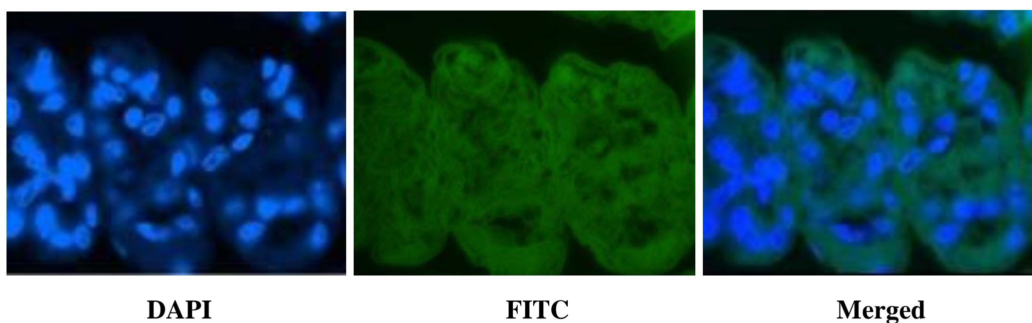
DAPI **FITC** **Merged**

B. Positive control tissue- Normal feed WSSV challenged



DAPI **FITC** **Merged**

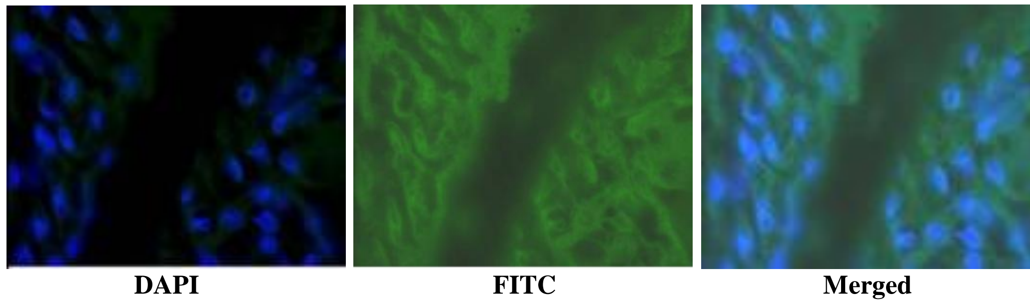
C. Test tissue- Aqueous extract administered and WSSV challenged



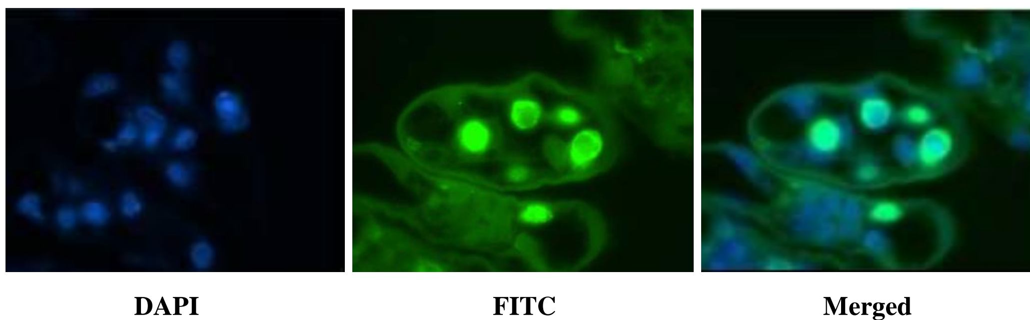
DAPI **FITC** **Merged**

Fig. 9 Indirect Immuno Fluorescence Histology of shrimps survived and died in feeding and WSSV challenge experiment with *C.tagal* aqueous extract

A. Negative control tissue- Administered with PBS



B. Positive control- Challenged with WSSV exposed to PBS



C. Negative control tissue- Challenged with WSSV exposed to the aqueous extract

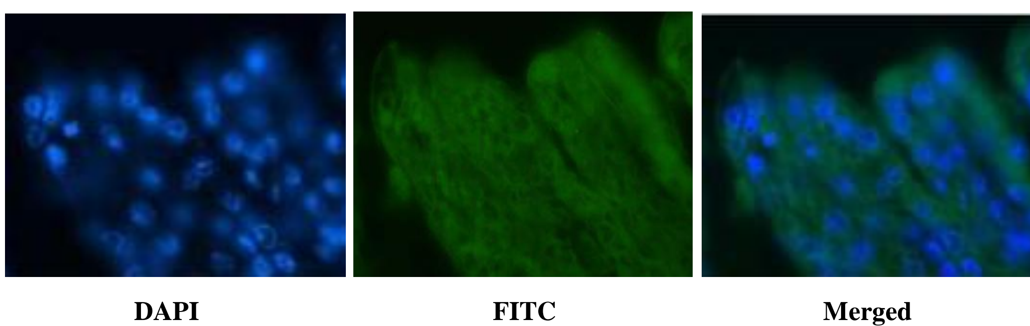
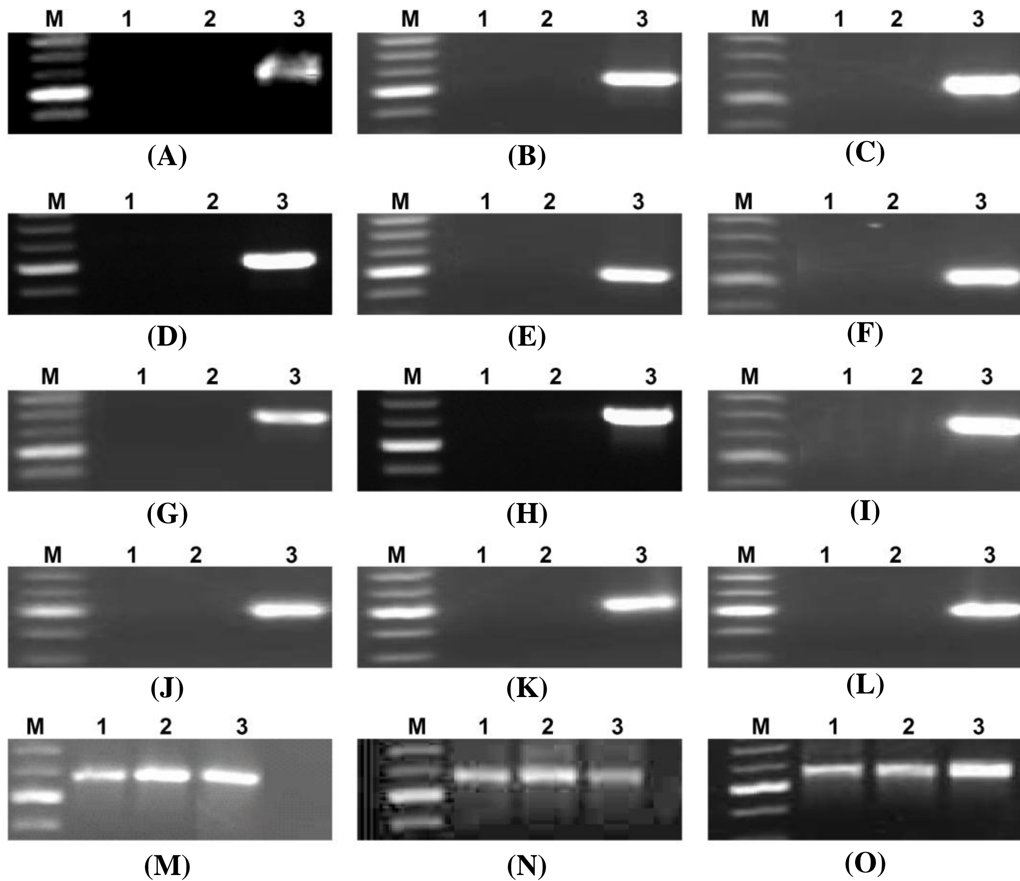


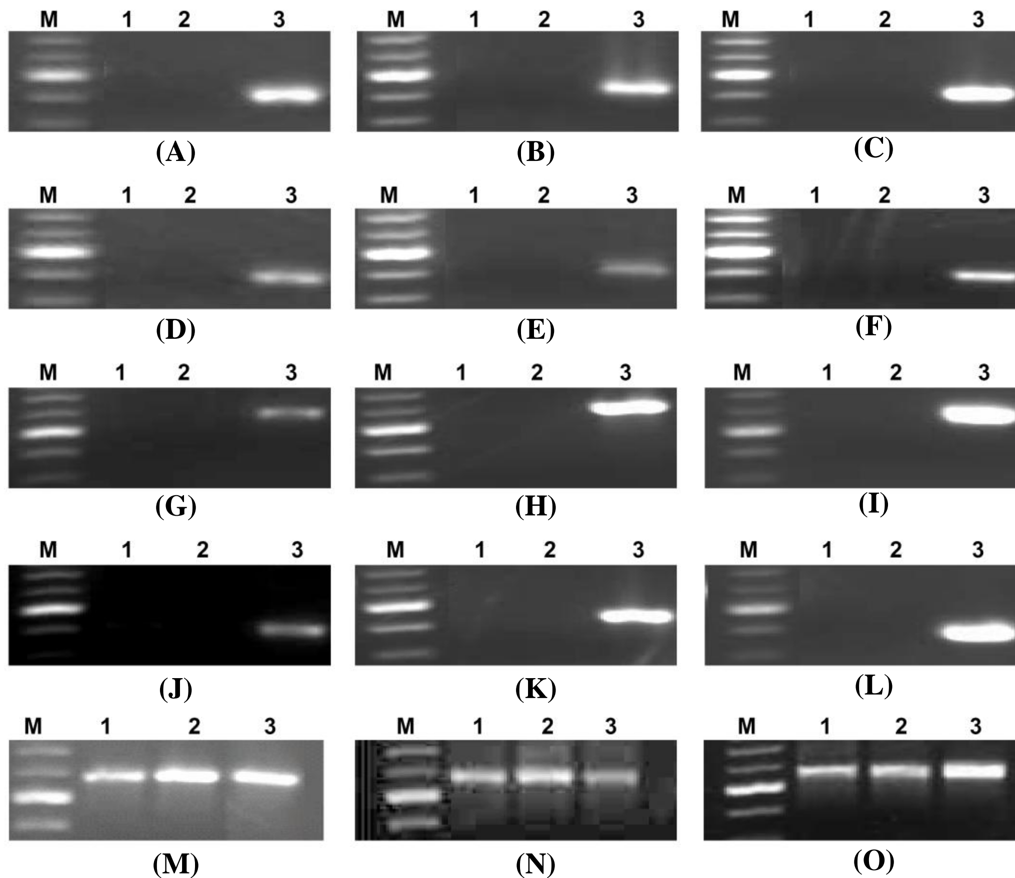
Fig. 10 Indirect Immuno Fluorescence Histology of shrimps survived and died in injection feeding and WSSV challenge experiment with *C.tagal* aqueous extract



***M)Molecular weight marker1) Extract and WSSV Challenge 2) Unchallenged control 3) Control WSSV**

A) VP28 2nd day B) VP28 4th day C) Vp28 6th day D) Immediate early gene 2nd day E) 1mmediate early gene 4th day F) Immediate early gene 6th day G) Latency 2nd day H) Latency 4th day I) Latency 6th day J) Protein kinase 2nd day K) Protein kinase 4th day L) Protein kinase 6th day M) Beta actin 2nd day N) Beta actin 4th day O) Beta actin 6th day

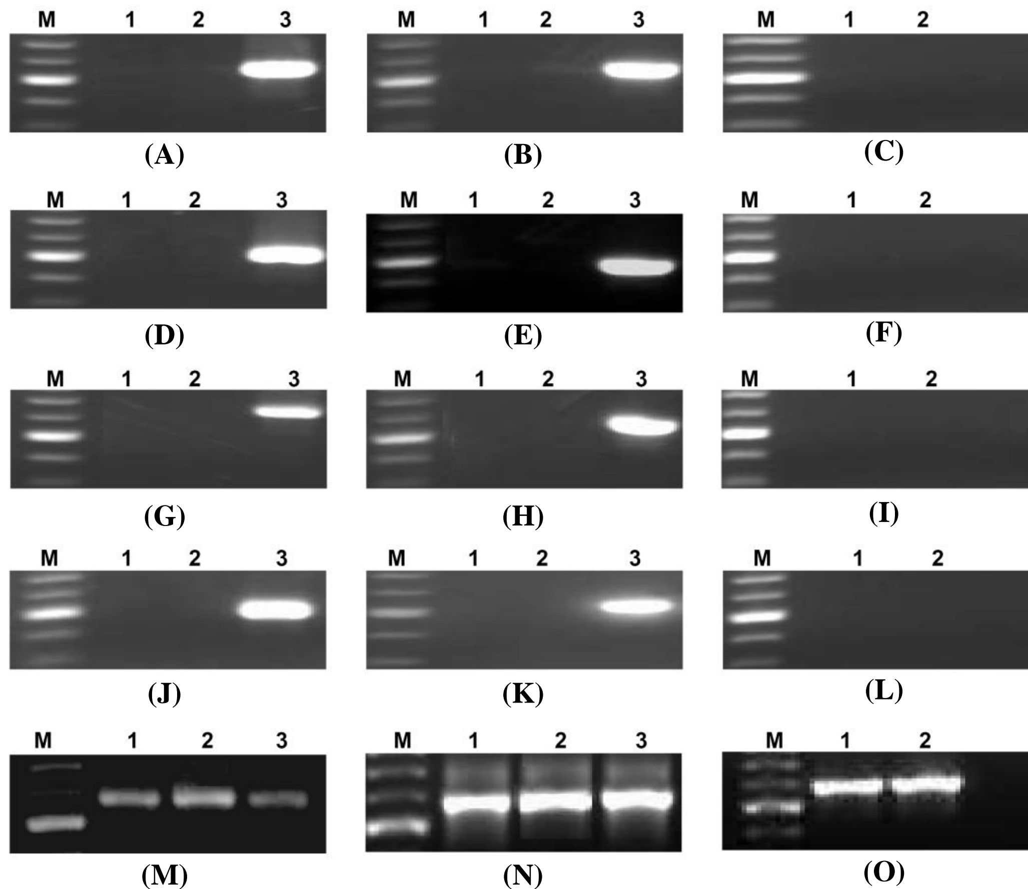
Fig.11 Viral gene expression in *Penaeus monodon* in response to Feeding *C.tagal* extract and WSSV oral challenge



***M)Molecular weight marker1) Extract and WSSV Challenge 2) Unchallenged control 3) Control WSSV**

A) Riboneucliotide reductase 2nd day B) Riboneucliotide reductas 4th day C) Riboneucliotide reductase 6th day D) Endonunuclease 2nd day E) Endonunuclease 4th day F) Endonunuclease 6th day G) DNA polymerase 2nd day H) DNA polymerase 4th day I) DNA polymerase 6th day J) Thymidine kinase-thymidilate kinase 2nd day K) Thymidine kinase-thymidilate kinase 4th day L) Thymidine kinase-thymidilate kinase 6th day M) Beta actin 2nd day N) Beta actin 4th day O) Beta actin 6th day

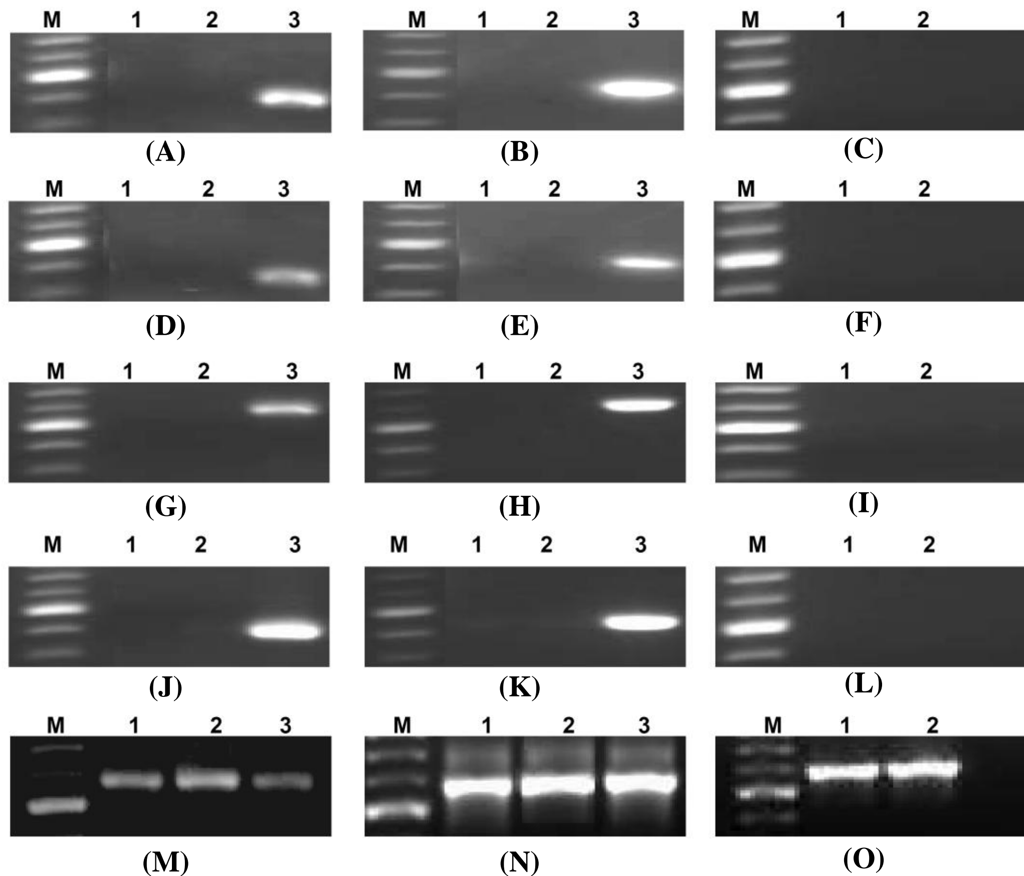
Fig.12 Viral gene expression in *Penaeus monodon* in response to Feeding *C.tagal* extract and WSSV oral challenge



*** M) Molecular weight marker1) Extract and WSSV Challenge 2) Unchallenged control 3) Control WSSV**

A) VP28 2nd day B) VP28 4th day C) Vp28 6th day D) Immediate early gene 2nd day E) 1mmediate early gene 4th day F) Immediate early gene 6th day G) Latency 2nd day H) Latency 4th day I) Latency 6th day J) Protein kinase 2nd day K) Protein kinase 4th day L) Protein kinase 6th day M) Beta actin 2nd day N) Beta actin 4thday O) Beta actin 6th day

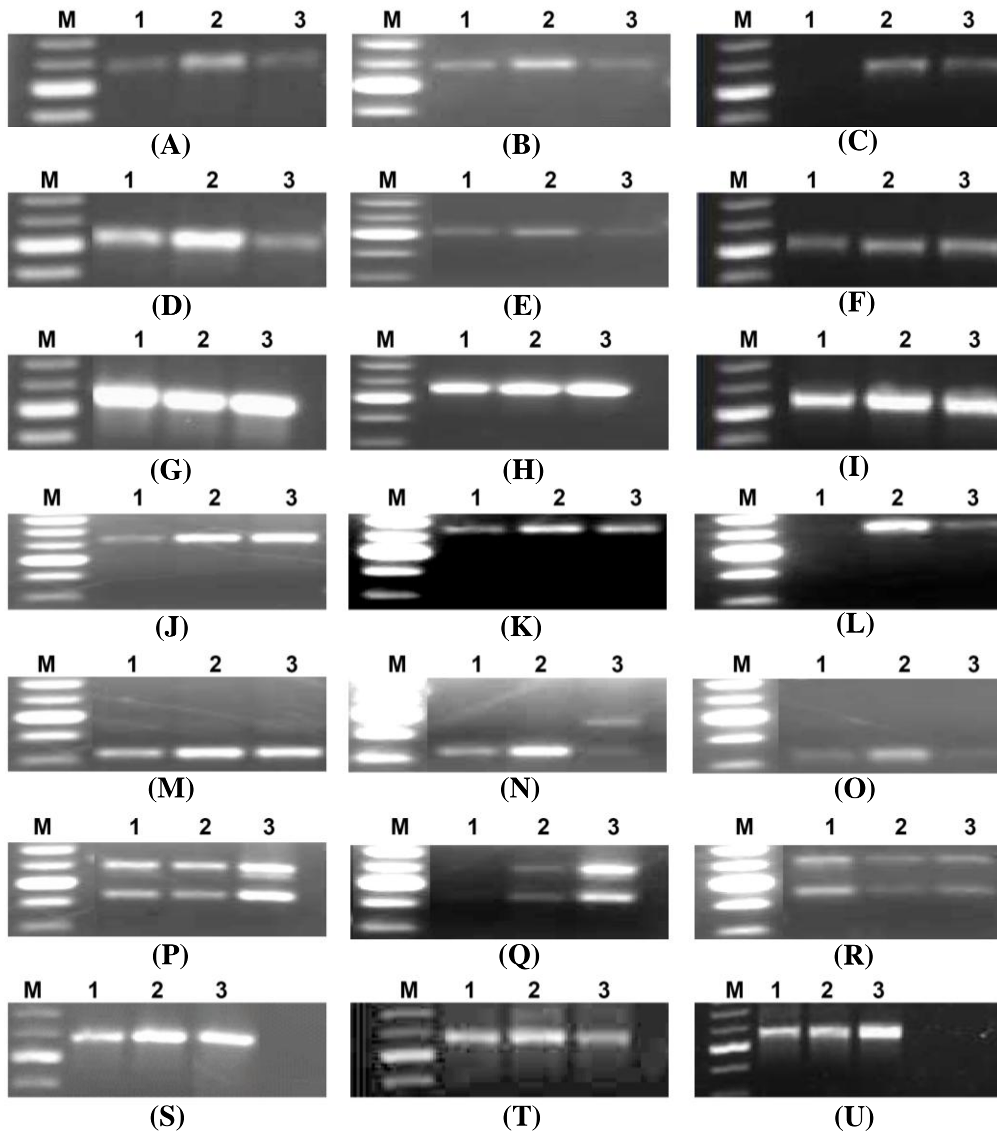
Fig.13 Viral gene expression in *Penaeus monodon* in response to Injecting *C.tagal* extract and WSSV injection



***M) Molecular weight marker1) Extract and WSSV Challenge 2) Unchallenged control 3) Control WSSV**

A)Ribonucleotide reductase 2nd day B) Ribonucleotide reductas 4th day C) Ribonucleotide reductase 6th day D) Endonunuclease 2nd day E) Endonunuclease 4th day F) Endonunuclease 6th day G) DNA polymerase 2nd day H) DNA polymerase 4th day I) DNA polymerase 6th day J) Thymidine kinase-thymidilate kinase 2nd day K) Thymidine kinase-thymidilate kinase 4th day L) Thymidine kinase-thymidilate kinase 6th day M) Beta actin 2nd day N) Beta actin 4th day O) Beta actin 6th day

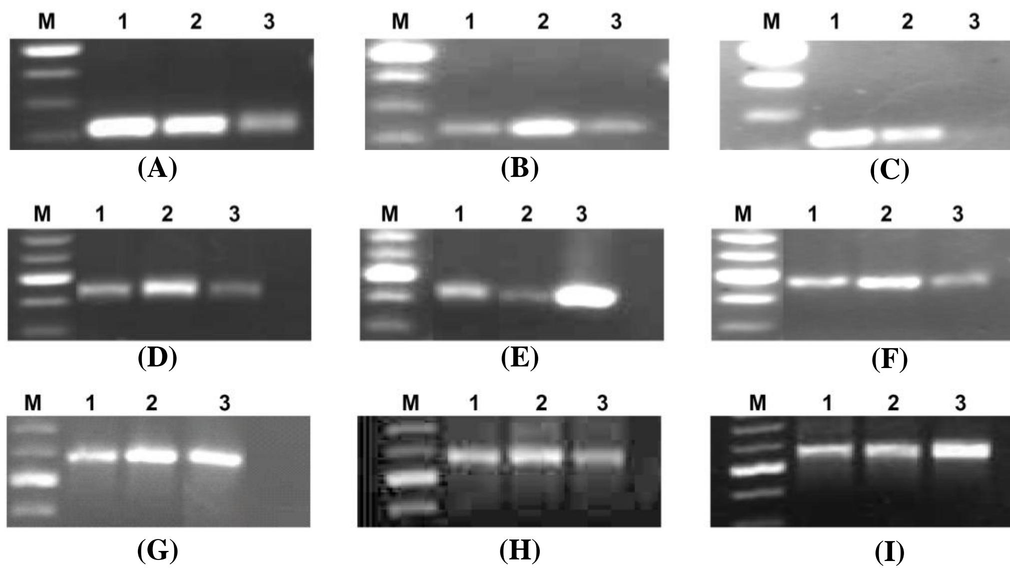
Fig.14 Viral gene expression in *Penaeus monodon* in response to Injecting *C.tagal* extract and WSSV injection



M) Molecular weight marker 1) Extract and WSSV challenge 2) Unchallenged control 3) Control WSSV challenged

A) Prophenol oxidase 2nd day B) Prophenol oxidase 4th day C) Prophenol oxidase 6th day D) Astakine 2nd day E) Astakine 4th day F) Astakine 6th day G) Peroxinectin 2nd day H) Peroxinectin 4th day I) Peroxinectin 6th day J) Transglutaminase 2nd day K) Transglutaminase 4th day L) Transglutaminase 6th day M)α 2-macroglobulin 2nd day N) α 2-macroglobulin 4th day O) α 2-macroglobulin 6th day P) Haemocyanin 2nd day Q) Haemocyanin 4th day R) Haemocyanin 6th day S) β actin 2nd day T) β actin 4th day U) β actin 6th day

Fig.15 Expression of immune genes in *P.monodon* in response to *C.tagal* extract and WSSV



M) Molecular weight marker 1) Extract and WSSV challenge 2) Unchallenged control 3) Control WSSV challenged

A) Penaeidin 2nd day B) Penaeidin 4th day C) Penaeidin 6th day
D) Crustin 2nd day E) Crustin 4th day F) Crustin 6th day
G) β actin 2nd day H) β actin 4th day I) β actin 6th day

Fig. 16 Expression of antimicrobial peptide genes in *P.monodon* in response to *C.tagal* extract and WSSV

Chapter 4

EXTRACTION AND PARTIAL PURIFICATION OF THE VIRUCIDAL FRACTIONS FROM THE AQUEOUS EXTRACT *CERIOPS TOGAL*

Contents

4.1 Introduction

4.2 Materials and methods

4.3 Results

4.4 Discussion

4.1 Introduction

Virucidal molecules in plants have greate potential in treating viral diseases and several plant extracts have been subjected for isolating novel antiviral / virucidal compounds. Polysaccharides from four species of algae were studied for their virucidal property (Harden et al., 2009). Hypericin isolated from the plant St. Johnswort against enveloped and non-enveloped DNA and RNA viruses (Tang et al., 1990), catechins in green tea on influenza virus (Song et al., 2005), the essential oil of *Lippia junelliana* and *Lippia turbinata* against JUNV (Garcia et al., 2003) are some of the virucidal molecules reported from plant sources.

Mangrove plants are a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Bandaranayake, 2002). Over 349 metabolites have been isolated from mangrove species (Wu et al., 2008). Extracts from mangrove and mangrove associated plant species have proven their activity against human and animal pathogens. Only limited investigations have been carried out to identify the metabolites responsible for antiviral activity. Inophyllums isolated from *Calophyllum inophyllum* were found to be inhibiting HIV-1 reverse transcriptase (Patil et al., 1993). A polysaccharide extracted from the leaf of *Rhizophora apiculata* was tested for its antiviral activity against HIV and HSV in different cell culture systems and proved its possible use in future drug development (Premanathan et al., 1999a).

The mangrove plant *Ceriops tagal* is a rich source of biologically active compounds like Triterpenoids, and diterpenoids. Chemical constituents of *C.tagal* include Chlorophyll, carotenoids, proteins, fatty acids, sterols and hydrocarbons (He et al., 2007). So far twenty three diterpenes and twenty nine triterpenes have been reported from *C.tagal* and *C. decandra* (Wu et al., 2008). Both of them are rich source of tannins also (Ghosh et al., 1985; Zhang et al., 2005). He et al. (2007) isolated a pentacyclic triterpens from *C. tagal* having the antiviral property described by several authors (Ryu et al. 1993; Grignon-Dubois and Rezzonico, 2007).

The saponins are naturally occurring surface-active glycosides mainly produced by plants, but also by lower marine animals and some bacteria (Riguera, 1997; Yoshiki et al., 1998). They derived their name from their ability to form stable, soap like foams in aqueous solutions. Saponin mixtures present in plants and plant products possess diverse biological effects when present in the animal body (Francis et al., 2002). Some saponins and sapogenins have been shown to be capable of deactivating viruses; for example, purified saponin mixture from *Maesa lanceolata* (Sindambiwe et al. 1998). Maesasaponins with 21, 22 diacylation had virucidal activity (Apers et al., 2001). The triterpenoid sapogenin oleanolic acid inhibits HIV-1 virus replication probably by inhibiting HIV-1 protease activity (Mengoni et al. 2002). According to a recent study saponin immersion could enhance the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Hyaline cells, the total haemocyte count, specific alpha 2-Macroglobulin activity, respiratory burst, SOD activity, and GPx activity have been found directly increasing with the saponin concentration, whereas pro Phenol oxidase activity was inversely related. (Su et al., 2007). There are no reports available on biological activity of saponins against WSSV and this is the first attempt to study the virucidal property of crude saponins from aqueous exracts of *C.tagal*.

In majority of the previous studies on anti WSSV property of plant extracts there have been very few attempts to purify the components responsible for anti WSSV activity. All these investigations were focused mainly on crude extracts from a single plant or combination of plants (Balasubramanian et al., 2007; Balasubramanian et al., 2008; Citarasu, 2009). This is the first time we report antiviral activity of an aqueous extract from the mangrove plant *C. tagal* against WSSV. In this processes we could partially purify one of the antiviral fractions which was responsible for the anti WSSV activity experimented in *Penaeus monodon*.

4.2 Materials and methods

4.2.1 Preparation of n-Butanol fraction from aqueous extract

The crude aqueous extract (300 ml) prepared from 25g dry leaf powder of *C.tagal* was partitioned with water and n- Butanol at a ratio 1:1 and shaken in a separating funnel and kept for developing two phases. The organic phase which formed the upper layer was collected from the separating funnel. This was repeated 3 times to extract maximum n-Butanol soluble fractions. The n-Butanol fraction was evaporated to dryness in a vacuum rotary evaporator at 40 ^{o}C. The concentrated n- Butanol fraction was stored at -20 ^{o}C till use.

4.2.2 Amberlite XAD-16 Chromatography of n-Butanol fraction

Amberlite XAD-16 (Sigma- Aldrich) was washed in methanol to remove preservatives according to manufactures protocol. A glass column was packed with Amberlite XAD-16 and equilibrated for 12 hours in water before use. The n-Butanol fraction was concentrated to dryness in vacuum at 40^{0}C. An aliquot of 600 mg of the concentrated n-Butanol extract was dissolved in 15 ml distilled water and centrifuged at 10000 rpm for 10 minutes to remove un-dissolved particles. The column was aspirated to remove water and 2 ml of the aqueous n-Butanol fraction was loaded on top of the column. The sample was then washed with water and the fraction collected. The column was then eluted with different gradient of Acetonitrile in water (20% Acetonitrile: water, 40% Acetonitrile: water, 80% Acetonitrile: water and 100% Acetonitrile). Subsequently the column was washed in methanol and saved. Aliquots of 50 ml each of the fractions were collected, dried in vacuum at 40^{0}C and stored at -20^{0}C till used. This was subjected for assaying virucidal property as described in the section 4.2.6

4.2.3 Size exclusion chromatography on Sephadex LH-20

Sephadex LH-20 column was prepared as follows. Slurry of Sephadex LH-20 (Sigma- Aldrich) was prepared in 20 % methanol in water and poured into a

glass column. The Amberlite XAD-16 fraction which exhibited virucidal activity against WSSV (100 mg) was dissolved in 100% methanol (2ml) and applied on top of the column without disturbing the top layer. Fractions were eluted with different gradient of methanol in water (20%, 40%, 60%, 80%, and 100% methanol). Aliquots 50 ml each of the fractions was collected from each gradient, dried in vacuum at 40 ^{0}C and stored at -20 ^{0}C till used. The virucidal property was assayed as described in the section 4.2.6.

4.2.4 Isocratic separation of active fractions in Sephadex LH-20

The active fractions (80%, and 100% methanol) from the previous fractionations in Sephadex LH-20 fractions were pooled together and rechromatographed on Sephadex LH-20 using 80% methanol as solvent. Three fractions were eluted and concentrated to dryness and stored at -20 ^{0}C till used. The virucidal assay was carried out as described in the section 4.2.6.

4.2.5 Extraction and precipitation of crude Saponins from Aqueous extract

The aqueous extract (300 ml) was partitioned with equal volume of ethyl acetate to remove plant pigments. The resultant aqueous extract (300 ml) was again partitioned with n-Butanol. The n-Butanol fraction was concentrated to dryness and dissolved in minimum quantity of methanol. Crude saponins were precipitated with diethyl ether as described by Hostetmann et al., 1991. The precipitated saponins were separated by centrifugation at 10000 rpm. The supernatants removed and the precipitates were pooled. The presence of saponins was confirmed by the frothing test as described by Soetan et al. (2006). Frothing test was done on the basis that aqueous solutions of saponins form very stable foams maintaining by it self for a prolonged period indicating the presence of saponins. To obtain the foaming an aliquot of 1 ml of the concentrated methanolic solution of the extract was shaken with 5 ml distilled water in a test tube. The saponin mixture was kept at -20 ^{0}C till used. This was used for virucidal assay as described under the section 4.2.6.

4.2.6 Virucidal Assay

The fractions were re-dissolved in distilled water at a concentration of 40 mg/ml and used for virucidal assay. This was accomplished by mixing suspension of WSSV with the fractions at a ratio of 1:1 and incubated for 3 hrs at 28±0.5^{0}C. From this preparation 10 µl was injected intramuscularly into apparently healthy shrimps of 5-8g weight each. The positive control was maintained with the virus suspension mixed with distilled water at 1: 1 ratio. Negative control animals were with distilled water administered in the same way. The animals were monitored for clinical signs of the disease and duration was till the positive control animals perished. Animals which survived and died during the challenge experiment with WSSV were subjected for PCR as described in the section 3.2.5.

4.2.7 HPLC analysis of active fractions

All above fractions which exhibited virucidal property were subjected to HPLC analysis in reverse phase column (Acclaim 120, C-18, 5 µm, 4.6x250 mm, Dionex). The sample load was 20 µl dissolved in 50% acetonitrile/water. A binary step gradient 5–80% of acetonitrile/water with a flow rate of 1ml/min for 85 minutes was done. The solvents were used after degassing in a sonicator. The detector wavelength was set at 220 nm. The peak pattern of each of the fraction which showed virucidal property against WSSV was analyzed using Chromeleon 6.8 software (Dionex USA).

4.3 Result

4.3.1 Virucidal activity of n-Butanol extract in *P.monodon*

The n-Butanol fraction prepared from the aqueous extract of *C. tagal* was assayed for Virucidal activity against WSSV in *P. monodon* animal model. All animals challenged survived, where in the positive control animals experienced total mortality within 7 days of experimental period and no mortality was observed in negative contro1 (Table 1). On subjecting the survived and dead

animals, the survived ones were negative to diagnostic PCR and the dead ones found positive (Fig.1).

4.3.2 Virucidal activity of XAD Amberlite-16 fractions generated from active n-Butanol fraction

On further fractionation of n-Butanol fraction in XAD Amberlite-16 column chromatography we could obtain 5 fractions. These fractions were assayed for virucidal property individually. Among the 5 fractions, fraction B and C (40% and 80% acetonitrile) showed virucidal property having reported no mortality while injecting with the virus exposed to the fraction (Fig.2). This was further confirmed through diagnostic PCR (Fig.3). Where as when the fractions A (20% Acetonitrile) gave a survival of 90%, D (100% Acetonitrile) and E (H_2O) provided 50% each survival respectively. On HPLC analysis of the fractions, 12 major peaks in 40% and 19 in the case of 80% acetonitrile fractions could be observed (Fig.5).

4.3.3 Virucidal activity of Sephadex-LH 20 fractions generated from active XAD Amberlite-16 fraction

On running the 40% acetonitrile fraction on Sephadex-LH 20 using gradient of methanol, five fractions could be resolved and assayed for virucidal activity. Administration of WSSV exposed to the fractions Fr- 4 & Fr- 5 (80% and 100% methanol) could not cause any mortality to the experimental group (Fig. 6). On diagnostic PCR the survived animals which received the virus exposed to the Fr – 4 turned out to be nested PCR negative and the other nested PCR positive. In all other fractions (Fr -1, Fr – 2, Fr – 3) mortality was observed (100, 17 and 17% respectively) and all moribund animals were reported either first step or nested PCR positive to WSSV (Fig.7). The fractions, Fr -4&5, on subjecting to HPLC generated identical peaks (6 major peaks) (Fig.8 & Fig.9), and there fore these two fractions were pooled and rechromatographed on Sephadex-LH 20 in isocratic mode in 80% methanol.

4.3.4 Virucidal activity of Sephadex-LH 20 fractions after rechromatography in isocratic mode in 80% methanol

The 80% Acetonitrile fraction and 100% Acetonitrile fractions were pooled together since they had similar HPLC pattern and were rechromatographed on Sephadex-LH20 using 80% methanol as solvent. In this processes 3 fractions could be obtained, out of which fraction - 2 exhibited virucidal property by protecting the shrimp from getting infected on administering with WSSV exposed to it (Fig.10). Those animals were found to be negative to diagnostic PCR (Fig.11). HPLC provided 3 major peaks out of this fraction (Fig.12).

4.3.5 Virucidal activity of crude saponins precipitated from n-Butanol fraction of *C.tagal*

Saponins extracted and precipitated from n-Butanol fraction of *C. tagal* provided stable form when shaken in aqueous solution (Fig.15). The saponin fraction exhibited virucidal property as those animals which received the virus after exposure to could survive the challenge (Table 2). The survived animals were found second step positive to WSSV (Fig.13). On HPLC analysis the saponin fraction gave a large single peak (Fig.14).

4.4 Discussion

In an attempt to purify the active antiviral components from the aqueous extract of *C. tagal,* the active fraction could be partitioned into n-Butanol and was proved to retain the virucidal property. Similar fractionation using n-Butanol have been carried out by several researchers to separate plant based natural products (Norberg et al., 2004; Zhu et al., 2004; Saxena and Albert 2005; Rinaldo et al., 2006; Xu and He 2007;Yang et al., 2007) having biological activity. The biologically active molecules which got isolated from n-Butanol fractions of plant extracts included saponins, phenolic compounds, flavonoids,, isoflavones etc (Norberg et al., 2004; Zhu et al., 2004; Saxena and Albert 2005; Rinaldo et al., 206; Xu and He 2007; Yang et al., 2007). Rhyncosides A-F, Phenolic constituents have

also been isolated from n-Butanol fraction from Chinese mangrove plant *Bruguiera sexangulata* var. *rhynchopetala* (Bao et al., 2007).

The active n-Butanol fraction was subjected for desalting in XAD-Amberlite-16 as the mangrove plants were found to have higher salt content, and in this processes two fractions such as, 40% and 80% acetonitrile, could be obtained having virucidal property. The 40% acetonitrile fraction was selected for further purification since it gave better peak separation in HPLC analysis and its yield was higher than that of 80% acetonitrile fraction. On running the 40% acetonitrile fraction on Sephadex-LH 20 using gradient of methanol the fractions Fr- 4 & Fr- 5 (80% and 100% methanol) were found to retain the virucidal property as supported by diagnostic PCR. These fractions on subjecting to HPLC generated identical peaks (6 major peaks) and hence were pooled and rechromatographed on Sephadex-LH 20 in isocratic mode in 80% methanol. In this processes 3 fractions could be obtained, out of which fraction - 2 exhibited virucidal properties by protecting the shrimp from getting infected on administering with WSSV exposed to it. Those animals were found negative to diagnostic PCR, and HPLC provided 3 major peaks in it. It could be noticed that on each chromatographic step there was decrease in the number of peaks indicating that the fractions were getting purified as proceeded on to each separation.

In another attempt to extract known antiviral molecules from the plant, saponins were extracted and precipitated from the n-Butanol fraction and were re-dissolved in water. Presence of saponin was confirmed through the frothing test which on maintenance retained stability for over15 minutes, an indication of the presence of saponins (Fransworth, 1966; Hostetmann et al., 1991; Soetan et al., 2006). On assaying for anti WSSV activity crude saponin extract was virucidal extending total protection of shrimp from WSSV. On comparing the HPLC profiles of both the active fractions, a. obtained from Sephadex LH-20 on rechromatography of the pooled fractions (80 & 100% methanol fractions) and b. the saponin fraction, the major peaks have been found eluted with in the same

duration (between 2 and 5 minutes) suggesting that one of the active ingredients in the antiviral fraction of the aqueous extract of *C.tagal* is saponin. It is hypothesized that saponin might be one of the compounds which possessed the virucidal property in the aqueous extract.

The antiviral activity of saponins has been well established through several studies. Triterpenoid - saponins from *Maesa lanceolata*, *Maesa chisia* and *Maesa indica* have been reported to exhibit direct virucidal activity against Newcastle disease virus, vaccinia virus and herpes simplex virus (Jassim & Naji, 2003). Another saponin from *Pontentilla anserine* L has shown to have antiviral property against Hepatitis –B virus (Zhao et al., 2008). The presence of triterpenoids have been well established in *C. tagal* having many triterpenoids and diterpenoids been isolated by (He et al. 2007 and Wu et al. (2008). This indicates the possibility of the active fraction obtained in the aqueous extract of *C. tagal* be triterpenoid - saponins. Saponins offer novel mechanisms of antiviral action which include interactions with viral envelopes leading to their destruction, interactions with host-cell membranes leading to a loss of virus binding sites and coating of cells to prevent virus binding (Amoros et al., 1987; Tokuda et al., 1988; Apers et al., 2001; Gosse et al., 2002; Roner et al., 2007). The probable mode of action evaluated in the present study points to the above possibilities. There has never been any report on saponins proved to have anti – WSSV property. However, saponin immersions have enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus.* Hyaline cells, total haemocyte count, specific α 2-Macroglobulin activity, respiratory burst, SOD activity, and GPX activity could directly be increased with the saponin application, whereas Phenol oxidase activity was inversely related (Su and Chen, 2007). This is important in the present context that the animals administered with the aqueous extract expressed down regulation of proPhenol oxidase gene on 6th day of challenge with WSSV, which might be due to the influence of saponins in the extract.

The presence of saponins in *C. tagal* opens up a new avenue for research as it can be used as adjuvant to WSSV vaccine. The saponins have been used as adjuvant in human and animal vaccines and triterpenoid saponins have been shown to exhibit strong adjuvant activity (Dalsgaard, 1974; Kensil, 1996; Wu et al., 1992).

The information gathered here warrants in depth studies on the active fraction to elucidate its structure, interaction with WSSV and the susceptible cells, and to develop a viable process of commercial production to have field level application.

Table 1 Response of *P.monodon* administered with WSSV exposed to n - Butanol fraction

	MNG-7 n- Butanol fraction + WSSV	WSSV+PBS	PBS+0
Number of animal injected	**10**	**10**	**10**
Dead	**0**	**10**	**0**
Live	**10**	**0**	**10**

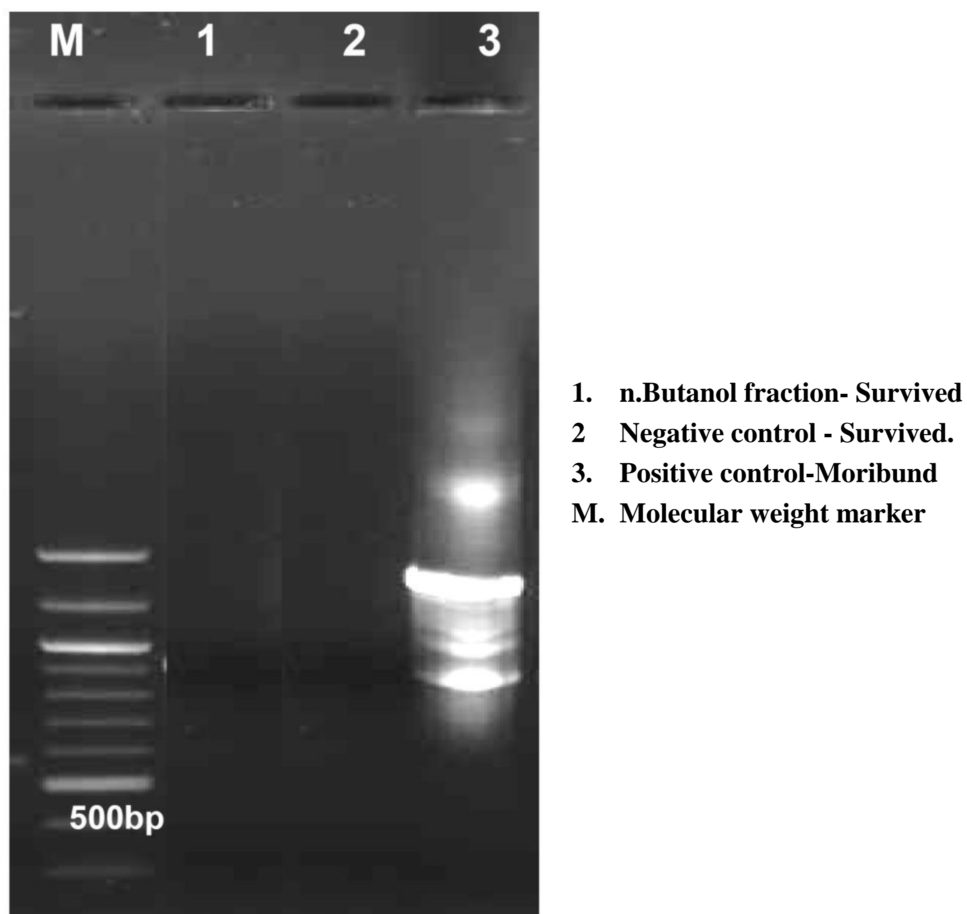


Fig.1 PCR of shrimp administered with WSSV exposed to n- Butanol fraction.

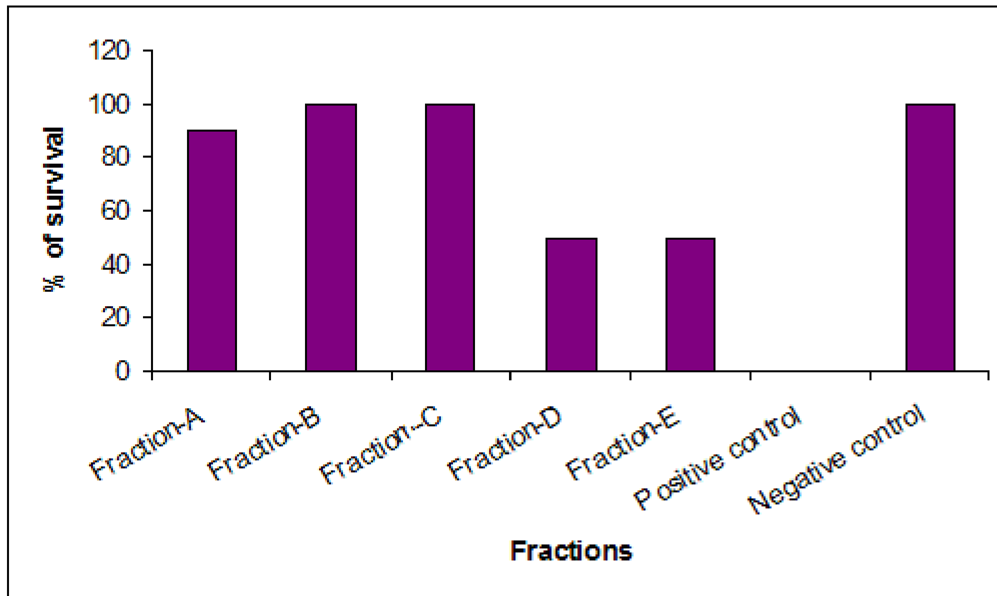


Fig.2 Percentage survival of shrimp administered with WSSV exposed to different XAD fractions.

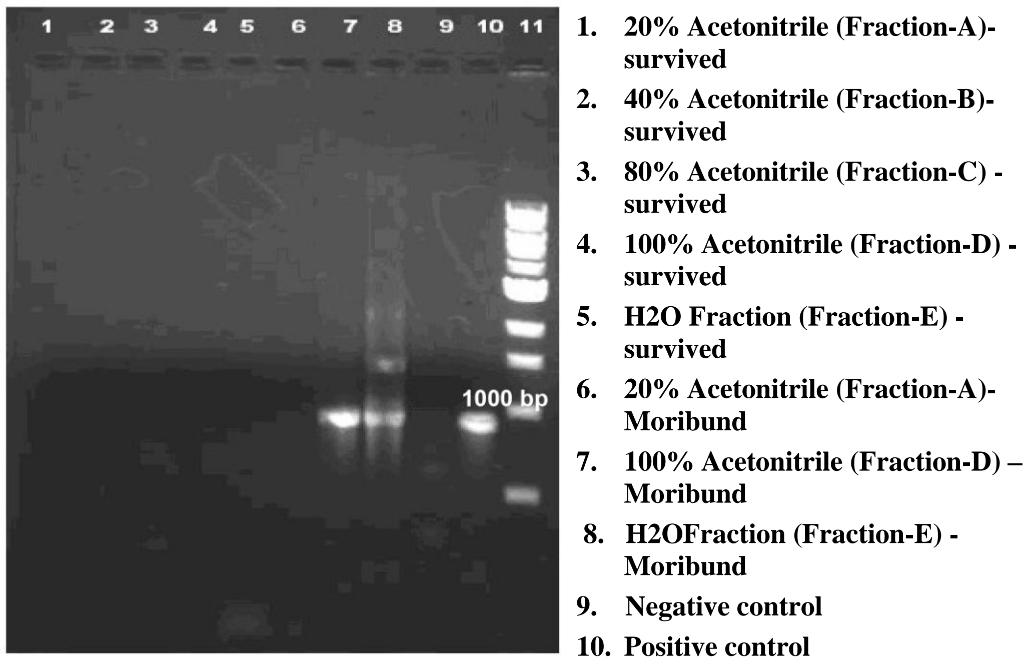


Fig.3 PCR result of shrimp administered with WSSV exposed to different XAD Amberlite – 16 fractions.

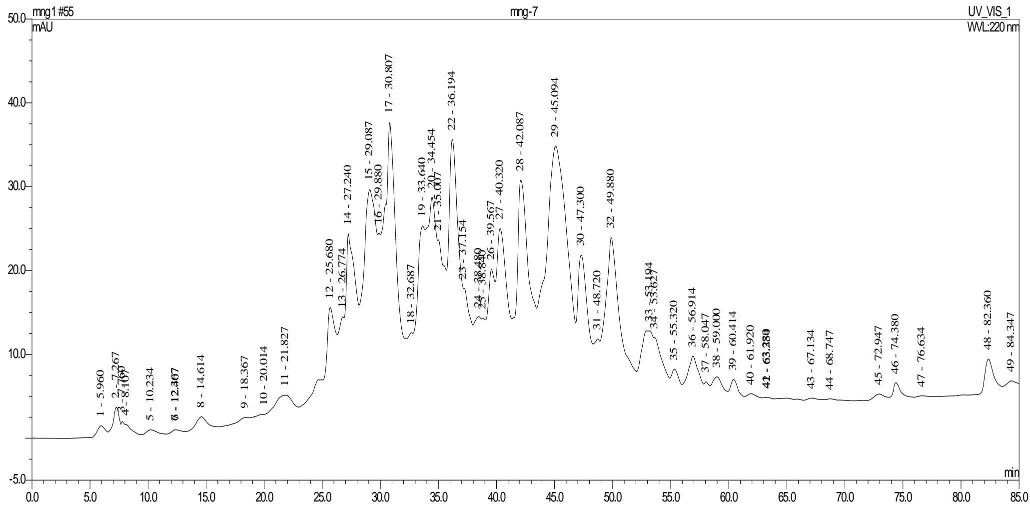


Fig. 4 HPLC chromatogram of XAD-16 (80% Acetonitrile fraction)

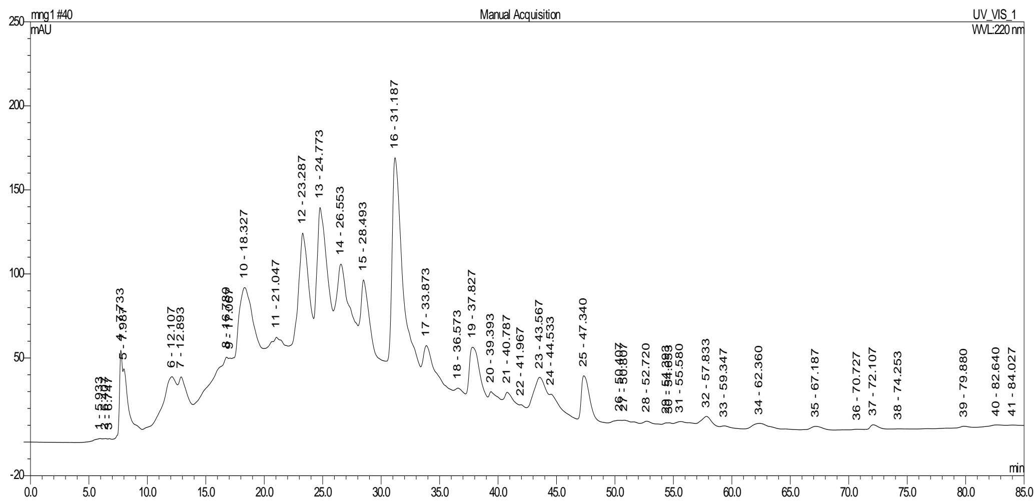


Fig. 5 HPLC chromatogram of XAD-16 (40% Acetonitrile fraction)

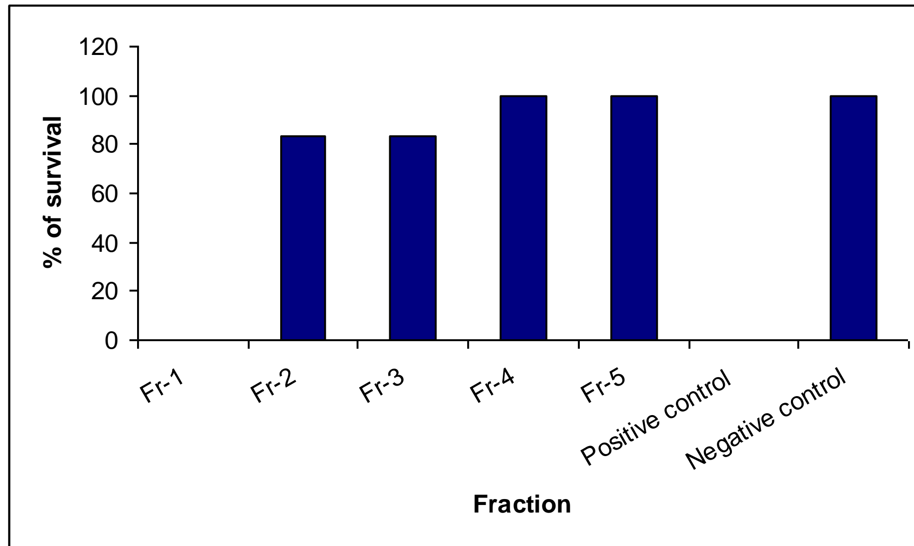


Fig.6 Percentage of survival of shrimp administered with WSSV exposed to different Sephadex LH-20 fractions.

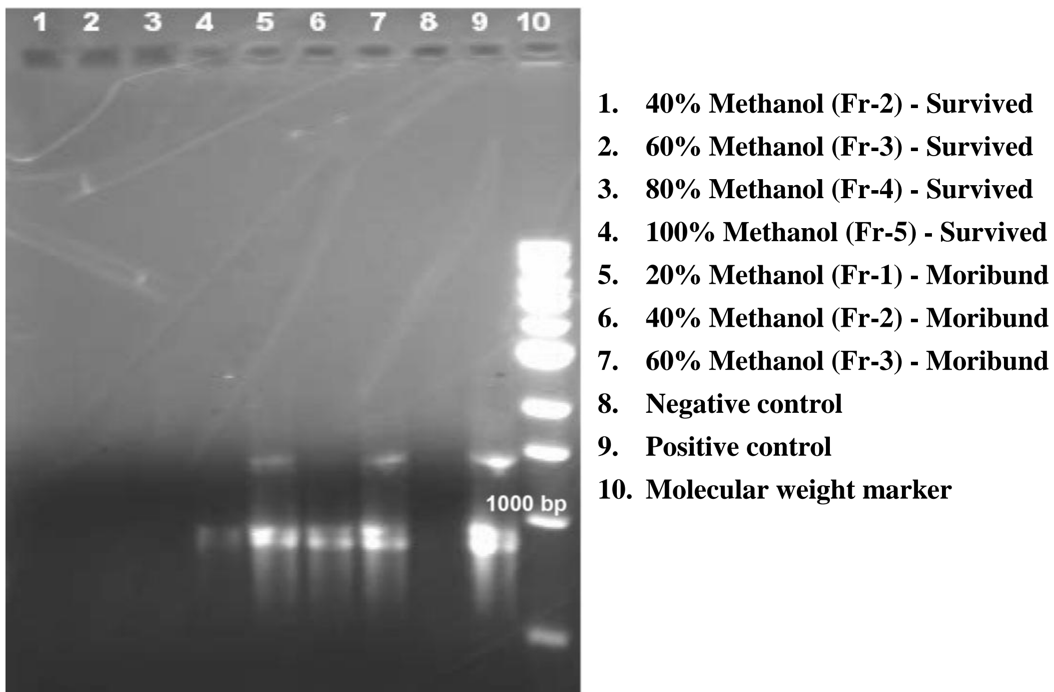


Fig.7 PCR of shrimp administered with WSSV exposed to Sephadex LH-20 fractions.

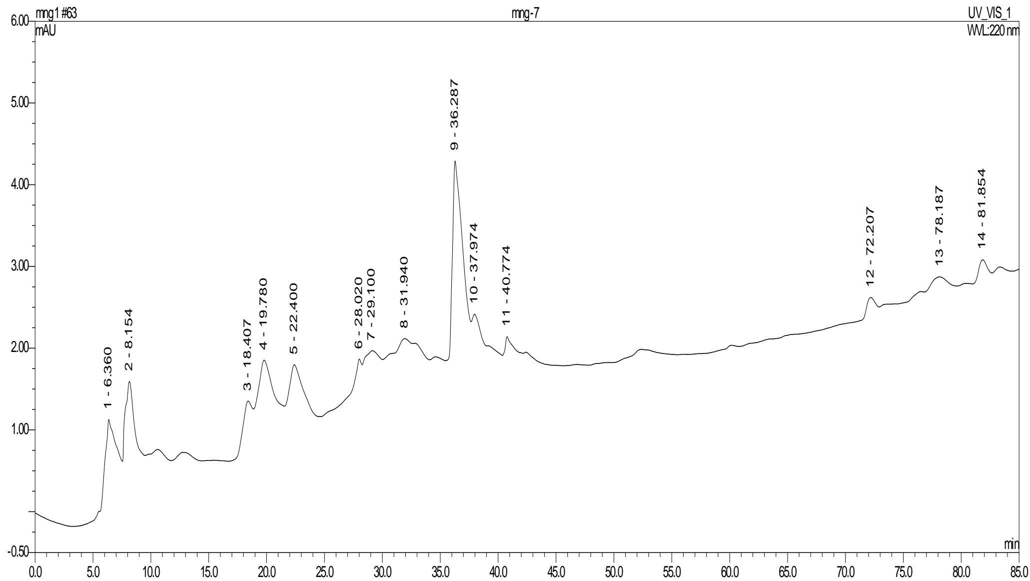


Fig.8 HPLC chromatogram of Sephadex LH-20 (80% Methanol fraction) (Fr-4).

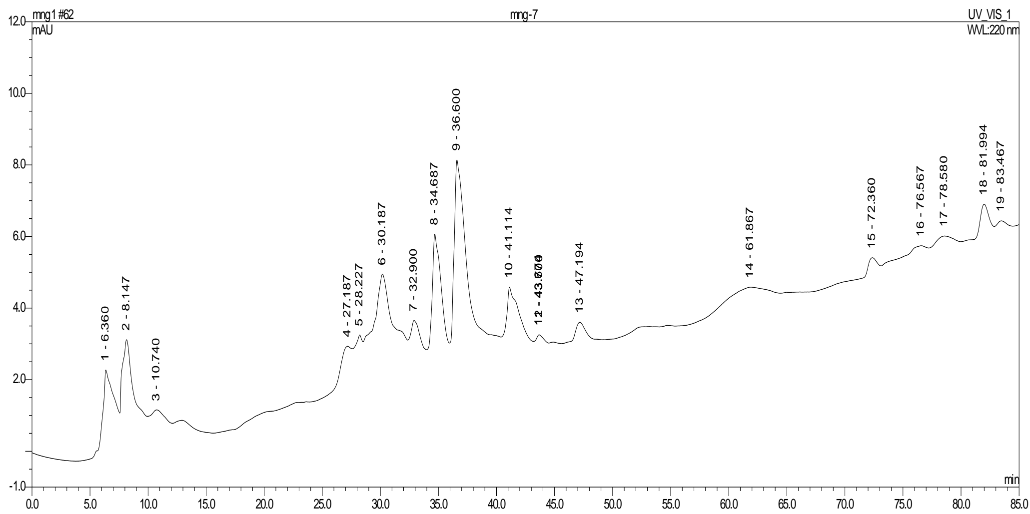


Fig.9 HPLC chromatogram of Sephadex LH-20 (100% Methanol fraction) (Fr-5).

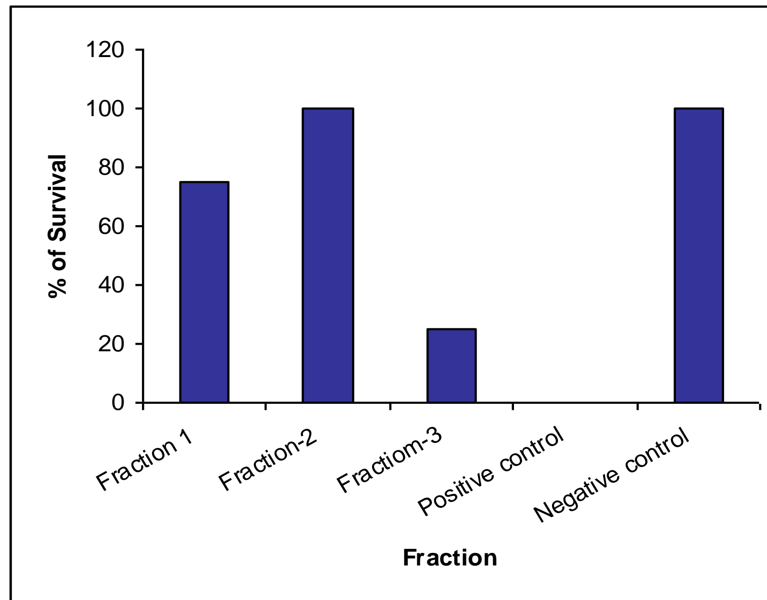


Fig. 10 Percentage survival of shrimp administered with WSSV exposed to different fractions obtained from rechromatography on Sephadex LH-20 in isocratic mode

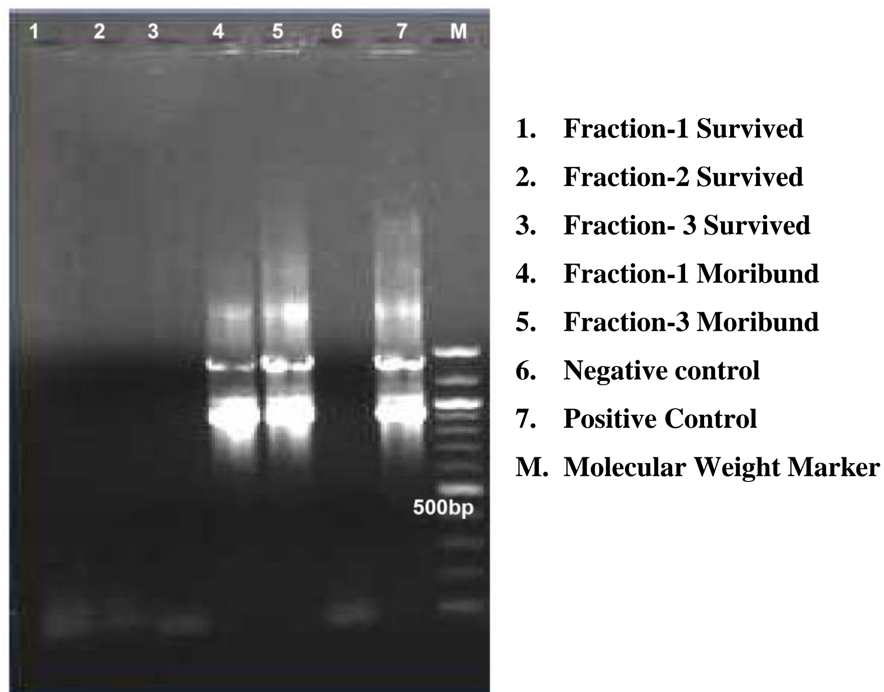


Fig. 11 PCR Result of shrimp administered with WSSV exposed to different fractions obtained from rechromatography on Sephadex LH-20 in isocratic mode

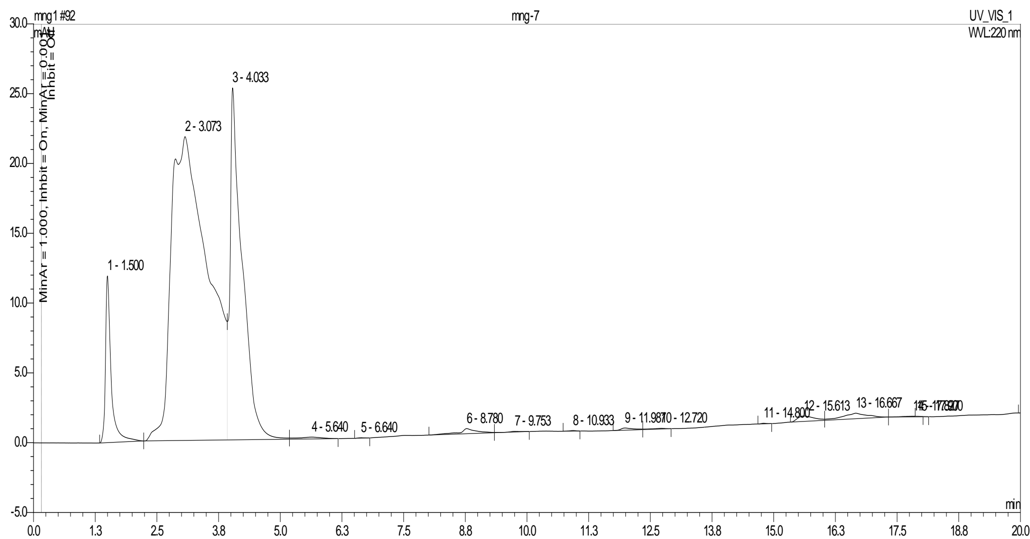
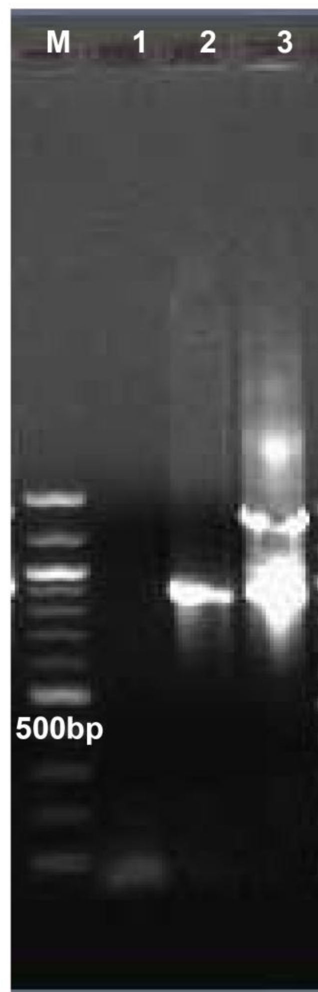


Fig. 12 HPLC chromatogram of rechromatographed Sephadex LH-20 active fraction.

Table 2 Response of the animals administered with WSSV exposed to Saponin fraction

	Saponin fraction + WSSV	**WSSV+PBS**	**PBS+0**
Number of animal injected	10	10	10
Dead	0	10	0
Live	10	0	10



M. Molecular weight marker
1, Negative control-Survived
2. Saponin administered –Survived
3. Positive control- Moribund

Fig.13 PCR of shrimp administered with WSSV exposed to crude saponin fraction from *C.tagal*

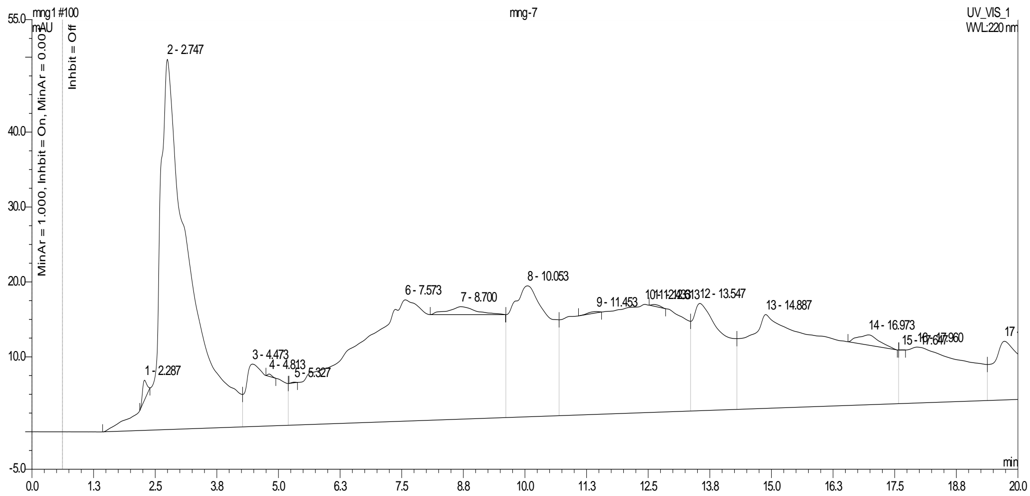


Fig. 14 HPLC chromatogram of active saponin fraction

Fig. 15 Demonstration of frothing test to prove the presence of saponins

Chapter 5

CONCLUSION AND SCOPE FOR FUTURE RESEARCH

White spot syndrome virus (WSSV) is the deadliest virus among crustaceans ever discovered having several unique and novel features. Recent developments in genomics and proteomics could elucidate the molecular process involved in the WSSV infection and the host pathogen interaction to some extent. Until now no fool proof treatment or prophylactic measure has been made available to control WSSV out breaks in culture system. Even though there are technologies like application of immunostimulants, vaccines, RNAi and several antiviral natural products none of them has been taken to the level of clinical trials. However, there are several management options such as application of bioremediation technologies to maintain the required environmental quality, maintenance of zero water exchange systems coupled with application of probiotics and vaccines which on adoption shall pave way for successful crops amidst the rapid spread of the virus. In this context the present work was undertaken to develop a drug from mangrove plants for protecting shrimp from WSSV.

Mangroves belong to those ecosystems that are presently under the threat of destruction, diversion and blatant attack in the name of so called 'developmental activities'. Mangrove plants have unique ecological features as it serves as an ecotone between marine and terrestrial ecosystem and hence possess diversity of metabolites with diverse activities. This prompted them being used as remedial measures for several ailments for ages. Among the mangrove plants *Ceriops tagal,* belonging to the family Rhizophororaceae was in attention for many years for isolating new metabolites such as triterpenes, phenolic compounds, etc. Even though there were attempts to study various plant extracts to develop anti-viral preparations their activity against WSSV was not investigated as yet. Considering this situation the present work was undertaken with the following objectives:

- Screening mangrove plants for anti WSSV activity
- Confirmation of the anti WSSV activity in aqueous extract of *Ceriops tagal* and its possible mode of action.

- Extraction and partial purification of the virucidal fractions from the aqueous extract.

Overall achievements in this work are summarized as given below:

1. Among seven mangrove plants such as *Excoecaria agallocha*, *Acanthus ilicifolius*, *Avicennia* sp., *Rhizophora mucronata*, *Rhizophora apiculata, Sonneratia* sp. and *Ceriops tagal* were screened for their anti WSSV property, and the aqueous extract from *C. tagal* was segregated based on the potency to protect shrimp from WSSV.
2. The aqueous extract was prepared as follows: Leaves were shade dried, powdered and 50 gm soaked in minimum quantity of double distilled water and frozen to -20°C, thawed and frozen repeatedly for three times and extracted to 500ml final volume of double distilled water in a Warring blender at ambient temperature. The extract was sieved through a fine meshed (100µm) cloth, centrifuged at 10000 g for 20 minutes and the supernatant maintained at -20°C till used. These preparations were examined for their virucidal activity and for their protective effects from WSSV on oral administration in *P. monodon.*
3. The antiviral property was further investigated by employing molecular tools.
4. Through out the study titred virus was used both in shrimp animal model as well as in haemocyte culture.
5. Through a series of experiments 10^{-2} dilution of WSSV suspension prepared from 500 mg infected tissue macerated with 10 mL PBS injected at an aliquot of 10 µL was found to cause mortality invariably in all challenged shrimp. However, for all practical purposes a dilution of 10^{-1} was used.
6. As the next step toxicity of the extract was determined both *in vivo* in animal model and *in vitro* in *P.monodon* primary haemocyte culture. The IC_{50} calculated in primary haemocyte culture was 254.84 µg/ml,

which was equal to 0.26 µg/µl, and the highest nontoxic concentration worked out was 100µg/ml. Meanwhile in animal model the highest non toxic concentration went up to 50 mg/ml (50,000 µg/mL) extract of which 10 µl extract was injected to shrimps weighing 5-8 g body weight delivering 500 µg per animal. The overall result of the investigation on toxicity of the extract in animal model revealed that the crude extract from *C.tagal was* not toxic to *P. monodon* up to a concentration of 50 mg /mL administered at 10 µl/animal.

7. The experiments conducted to determine the lowest concentration which exhibited virucidal property against WSSV in *Penaeus monodon* revealed that the strength of the extract above 30 mg/mL was sufficient enough to extend its virucidal property. The result indicated that the minimum concentration of the extract required for extending the virucidal activity was less than its *in vivo* and *in vitro* toxic level with high selectivity index (the ratio of toxic concentration to the effective concentration) and shows higher antiviral activity at a concentration below the toxic value. This points to the suitability of the aqueous extract to protect shrimps from WSSV. The results generated unequivocally suggest that the virucidal property of the aqueous extract of *C.tagal* is concentration dependent.

8. On evaluating effectiveness of orally administered *C.tagal* extract in protecting shrimps from WSSV, none of the test animals exhibited signs of distress during the period of drug administration. Shrimps orally administered with the aqueous extract at a dosage 500 mg /Kg body weight demonstrated 100% survival on challenging with WSSV infected tissue.

9. Basis of survival of shrimp was proved as the absence of WSSV in the animals despite challenge with the virus through oral route and by intramuscular injection of the virus suspension exposed to the extract.

This conclusion was arrived at through histopathology, indirect imunofluroscent histochemistry, and the absence of any pathological changes accompanied with WSSV infection.

10. Compared to the earlier reports on other plant extracts, it could be concluded that *C.tagal* was comparatively very much effective in protecting shrimp from WSSV as there had never been any Indirect Immunofluorescent positive shrimps or any mortality even after 7 days of post infection.
11. To elucidate the mode of action of the extract in protecting shrimp from WSSV, expression of viral genes was investigated by way of amplifying the genes involved in the multiplication cycle. The genes examined were immediate early gene (*ie1*), DNA polymerase (*dnapol*), Thymidine - thymidilate kinas (*tk – tmk*), Endonuclease (*endonu*), Ribonucleotide reductase (*rr1*), Proteinkinase (*pk1*), VP28, and Latency related genes. Besides, the expression profile of a set of genes such as Prophenol oxidase, Astakine, Peroxinectin, Transglutaminase, Alpha 2 macroglobulin, Haemocyanin, Penaeidins and Crustin involved in shrimp immune mechanism was also looked into.
12. Expression profile of the genes involved in WSSV replication cycle proved that none of the genes involved in viral replication could be expressed in the test animals administered with the extract and also in the negative controls. This was alike in both the experimental designs, the ones which were fed on the extract and challenged with WSSV and the ones which received WSSV exposed to the aqueous extract.
13. The striking observation was that even the immediate early gene (*ie 1)* failed to express in the shrimps challenged. The expression of viral immediate early genes depends on the host cell machinery and occurs independently of any viral de novo protein synthesis. Once expressed, the *ie* gene products function as the regulatory trans-acting factors and

may serve to initiate viral replication events during infection. Viral *ie* genes are expressed immediately after primary infection or as a result of the re activation of the virus. Recently, it was found that white spot syndrome virus (WSSV) used a shrimp STAT as a transcription factor to enhance viral gene expression in the host cells. STAT directly transactivates WSSV *ie1* gene expression and contributes to its high promoter activity. JAK-STAT pathway is one among the immune genes activating signaling pathways in lower animals like insects.

14. In the cascade of viral regulatory events, successive stages of virus replication are dependent on the proper expression of the genes in the preceding stage. For example, during infection by the large DNA viruses, such as baculoviruses and herpesviruses, gene expression was regulated such that the immediate-early (ie or a) genes were transcribed first, followed by the expression of the early (e or h) and late (l or g) genes, respectively. In the present study none of these genes, starting from immediate early genes to VP 28, (late stage) were found getting expressed, suggesting that WSSV was not able to initiate the host RNA polymerase II, which mediate transcription of the immediate early genes in the presence of the aqueous extract. This prevented replication of WSSV due to the inactivation of the virus by the virucidal fractions in the extract.

15. On investigating the immune gene expression in shrimp orally administered with the extract and challenged through the same route, the aqueous extract from *C.tagal* was not immunostimulatory to *P. monodon.* This may be due to the failure of JAK-STAT pathway to activate immune related genes as stated earlier. In lower animals like insects the innate immune response is largely orchestrated by 3 signaling pathways, Toll, Imd and JAK-STAT.

16. It was noticed that proPhenol oxidase gene and alpha 2 macroglobuline genes were found down regulated on 6th day in *C.tagal* extract administered shrimps. Earlier studies suggested this as the property of saponins indicating that the aqueous extract from *C. tagal* might be containing saponin like compounds having down regulatory effect on phenol oxidase activity.
17. Over all results suggest that the mode of action of *C.tagal* extract on WSSV is virucidal. This is evident from the indirect immunoflurescent histochemistry, histopathology, and absence of viral gene expression in animals administered with the extract. Based on this evidence it has been proposed that during feeding of the extract and subsequent challenge with WSSV by oral rout the virus must be getting inactivated by the virucidal molecules of the aqueous extract. The same pattern of inactivation happens while it is being exposed to the extract *in vitro*. Consequently the inactivated virus fails to multiply in the host and subsequently gets eliminated.
18. The observation that none of the viral genes was getting expressed in the group treated with the plant extract supported by the absence histopathological changes and lack of Indirect Immunofluorescence signals give the strong indication that the virus was getting inactivated in the digestive tract and /or in vitro paving the way for its expulsion from the system.
19. Immune gene expression confirmed that the pathogen was not sensed by the defense mechanism either humoral or cellular. There is the possibility of antigen removal through the action of cellular responses. It is reported that autophagy, a homeostatic mechanism by which cells digest and recycle macromolecules and organelles by lysosomal digestion, might be involved in crustacean immune response.

20. In an attempt to purify the active antiviral components from the aqueous extract of *C. tagal,* the active fraction could be partitioned into n-Butanol and was proved to retain the virucidal property.

21. The active n-Butanol fraction was subjected for desalting in XAD-Amberlite-16 as the mangrove plants were found to have higher salt content, and in this processes two fractions such as, 40% and 80% acetonitrile, could be obtained having virucidal property. The 40% acetonitrile fraction was selected for further purification since it gave better peak separation in HPLC analysis and its yield was higher than that of 80% acetonitrile fraction.

22. On running the 40% acetonitrile fraction on Sephadex-LH 20 using gradient of methanol the fractions Fr- 4 & Fr- 5 (80% and 100% methanol) were found to retain the virucidal property as supported by diagnostic PCR.

23. These fractions on subjecting to HPLC generated identical peaks (6 major peaks) and hence were pooled and rechromatographed on Sephadex-LH 20 in isocratic mode in 80% methanol. In this processes 3 fractions could be obtained, out of which fraction - 2 exhibited virucidal properties by protecting the shrimp from getting infected on administering with WSSV exposed to it. Those animals were found negative to diagnostic PCR, and HPLC provided 3 major peaks in it.

24. It could be noticed that on each chromatographic step there was decrease in the number of peaks indicating that the fractions were getting purified as proceeded on to each separation.

25. In another attempt to extract known antiviral molecules from the plant, saponins were extracted and precipitated from the n-Butanol fraction and were re-dissolved in water. Presence of saponin was confirmed through the frothing test which on maintenance retained stability for

over15 minutes, an indication of the presence of saponins. On assaying for anti WSSV activity crude saponin extract was virucidal extending total protection of shrimp from WSSV.

26. On comparing the HPLC profiles of both the active fractions, a. obtained from Sephadex LH-20 on rechromatography of the pooled fractions (80 & 100% methanol fractions) and b. the saponin fraction, the major peaks have been found eluted with in the same duration (between 2 and 5 minutes) suggesting that one of the active ingredients in the antiviral fraction of the aqueous extract of *C.tagal* is saponin. It is hypothesized that saponin might be one of the compounds which possesed the virucidal property in the aqueous extract.

27. The presence of triterpenoids has been well established in *C. tagal* indicating the possibility of the active fraction as triterpenoid - saponins.

28. Saponins offer novel mechanisms of antiviral action which include interactions with viral envelopes leading to their destruction, interactions with host-cell membranes leading to a loss of virus binding sites and coating of cells to prevent virus binding. The probable mode of action evaluated in the present study points to the above possibilities. There has never been any report on saponins proved to have anti – WSSV property. However, saponin immersions have enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus.*

29. The presence of saponins in *C. tagal* opens up a new avenue for research as it can be used as adjuvant to WSSV vaccine. The saponins have been used as adjuvant in human and animal vaccines and triterpenoid saponins have been shown to exhibit strong adjuvant activity.

30. Through this work an appropriate protocol to extract water soluble bioactive metabolites from mangrove plant *C. tagal* which could protect *P. monodon* from white spot syndrome virus could be developed. The present study also could demonstrate the suitability of gelatin as a binder for delivering the aqueous extract by coating on to shrimp feed.
31. Since the extract is aqueous, chances of leaching out the active fractions into the surrounding water is rather high, and in this context this observation has practical implications, as the only possible way to deliver any drug to shrimp is through oral route.
32. The information gathered here warrants in depth studies on the active fraction to elucidate its structure, interaction with WSSV and the susceptible cells, and to develop a viable process of commercial production to have field level application.
33. Considering the importance of mangrove plants, conservation of mangrove ecosystems has to be augmented as a commercial venture.

Scope for future Research

1. Further resolution of the active fractions employing HPLC using different chemistry to obtain pure compound having the anti WSSV activity.
2. Structural elucidation of the purified compound employing LC / MS. NMR, FTIR.
3. Development of a viable process of extraction of the active molecule for commercial application
4. Determination of the interaction of the active molecule/compound with the virus particles and the cell surface receptors.

5. Up take of the active molecule in to shrimp animal system and its interaction with signaling path ways such as JAK-STAT and other immune related pathways.
6. Evaluation of the ranges of application such as through diet to protect the animal from horizontal transmission, in to the spawning tank to inhibit the virions released during spawning (in case of spawners which are positive to WSSV) and also in the treatment of WSSV positive post larvae.

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