

**STUDIES ON ANTIVIRAL ACTIVITY OF SILICA OXIDE
NANOPARTICLE AGAINST WHITE SPOT SYNDROME VIRUS
(WSSV)**

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Science degree in Biotechnology

by

RITHANYA R B (40770045)



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO & CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

JEPPIAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119

MAY 2023



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited "A" Grade by NAAC | 12B Status by UGC | Approved by AICTE
www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this project report is the bonafide work of RITHANYA R B (40770045) who carried out the project entitled "STUDIES ON ANTIVIRAL ACTIVITY OF SILICA OXIDE NANOPARTICLE AGAINST WHITE SPOT SYNDROME VIRUS (WSSV)" under our supervision from November 2022 to April 2023

INTERNAL GUIDE

Dr. V. RAMESH KUMAR

EXTERNAL GUIDE

Dr. K. GOVINDRAJU

HEAD OF THE DEPARTMENT

Dr. V. RAMESH KUMAR

Submitted for viva voice examination held on 08.05.2023

INTERNAL EXAMINER

EXTERNAL EXAMINER

DECLARATION

I, RITHANYA R B (40770045) hereby declare that the Project Report entitled "STUDIES ON ANTIVIRAL ACTIVITY OF SILICA OXIDE NANOPARTICLE AGAINST WHITE SPOT SYNDROME VIRUS (WSSV)" done by me under the guidance of Dr. V. RAMESH KUMAR (Internal) and Dr. K. GOVINDARAJU (External) at Col. Dr. JEPPIAR RESEARCH PARK, CENTRE FOR OCEAN RESEARCH, in Sathyabama Institute of Science and Technology is submitted in partial fulfilment of the requirements for the award of Bachelor of Science Degree in Biotechnology.

DATE: 08.05.2023

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of **SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to Dr. V. Ramesh Kumar., M.Phil., Ph.D., Head of the Department, Department of Biotechnology for providing me with necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Internal Guide Dr. V. Ramesh Kumar and my External Guide Dr. K. Govindaraju for their valuable guidance, suggestions, and constant encouragement that paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

In shrimp aquaculture, the white spot syndrome virus (WSSV) causes mass destruction due to its pandemic spread and lack of effective management method has been implemented at field level, Anti-viral lead compounds are in high priority for the development of anti-WSSV medicine. This study focuses on inhibiting the viral activity of the white spot syndrome virus using silica nanoparticles as an antiviral agent. Silica nanoparticles synthesized from beach sand and the nanoparticles were characterized using techniques including XRD, FT-IR, and TEM. The crystalline nature of silica nanoparticles was confirmed using XRD. The crystalline size of the sample was calculated on average at 39 nm using Debye-Scherrer's equation. In FT-IR spectra of silica nanoparticles, the major peaks occurred between 788, 1097, and 1101 cm^{-1} which is the characteristic of a vibration called "Si-O-Si asymmetric stretching". TEM micrograph revealed that the silica nanoparticles synthesized from beach sand were spherical in shape with an average size of 39nm. In vitro toxicity and anti-viral WSSV activity were carried out using the shrimp-infected hybrid cell line (PmLyo-SF9). The bioactivity of silica oxide nanoparticles against the PmLyo-SF9 cell line infected with WSSV was analysed using dose- dependent and attachment inhibition assays and the virucidal activity at a concentration of 320 $\mu\text{g/mL}$ and it was confirmed by the expression of viral envelope protein VP28.

KEYWORDS: White spot syndrome virus, silica nanoparticle, cytotoxicity, virucidal property.

TABLE OF CONTENTS

S.NO.	TITLE	PAGE NO.
1	ABSTRACT	v
2	TABLE OF CONTENTS	vi
3	LIST OF FIGURES	ix
4	LIST OF ABBREVIATIONS	x
CHAPTER-1		
5	INTRODUCTION	1
6	1.1 CRUSTACEANS	1
7	1.2 SHRIMP	1
8	1.2.1 SHRIMP- <i>Litopenaeus vannamei</i>	2
9	1.3 MARINE VIRUS	2
10	1.4 AQUATIC VIRAL PATHOGEN	3
11	1.5 NANOPARTICLES	5
CHAPTER-2		
12	REVIEW OF LITERATURE	7
13	2.1 SHRIMP AQUACULTURE	7
14	2.2 WHITE SPOT SYNDROME VIRUS	8
15	2.3 INHIBITION USING NANOTECHNOLOGY	11
CHAPTER-3		
16	AIM AND OBJECTIVE	14
17	3.1 AIM	14
18	3.2 OBJECTIVE	14
CHAPTER-4		
19	MATERIALS AND METHODS	15
20	4.1 PREPARATION OF SILICA NANOPARTICLES FROM BEACH SAND	15

S.NO.	TITLE	PAGE NO.
21	4.2 PHYIO-CHEMICAL CHARACTERIZATION OF SILICA NANOPARTICLE	15
22	4.2.1 X-RAY DIFFRACTION (XRD) PATTERN STUDIES	15
23	4.2.2 FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPIC STUDIES	15
24	4.2.3 TRANSMISSION ELECTRON MICROSCOPIC STUDIES	16
25	4.3 CYTOTOXICITY STUDIES OF SILICA NANOPARTICLES	16
26	4.4 VIRUS ISOLATION AND QUANTIFICATION	17
27	4.5 VIRUCIDAL ACTIVITY AND ATTACHMENT INHIBITION ASSAY OF SILICA NANOPARTICLES AGAINST WSSV	17
28	4.6 EXPRESSION AND AMPLIFICATION OF WSSV GENES	18
CHAPTER-5		
29	RESULTS AND DISCUSSION	19
30	5.1 PHYSIO-CHEMICAL CHARACTERIZATION OF BEACH BASED SiO ₂ NPs	19
31	5.1.1 X-RAY DIFFRACTION	19
32	5.1.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR) ANALYSIS OF SILICA NANOPARTICLE	20
33	5.1.3 TRANSMISSION ELECTRON MICROSCOPY ANALYSIS OF SILICA NANOPARTICLE	21
34	5.2 CYTOTOXICITY ASSAY	22
35	5.3 BIOACTIVITY OF SILICA NANOPARTICLE AGAINST WSSV	23
36	DISCUSSION	25

S.NO.	TITLE	PAGE NO.
	CHAPTER-6	
37	SUMMARY AND CONCLUSION	26
38	6.1 SUMMARY	26
39	6.2 CONCLUSION	27
40	REFRENCES	28

LIST OF FIGURES

S.NO	FIGURES	PAGE NO.
1	1.4.1 <i>LITOPENAEUS VENNAMEI</i> INFECTED WITH WHITE SPOT SYNDROM VIRUS	4
2	5.1.1 X-RAY DIFFRACTION (XRD) PATTERN OF BEACH SAND SILICA NANOPARTICLE	19
3	5.1.2 FT-IR ANALYSIS OF SILICA NANOPARTICLE	20
4	5.1.3 TRANSMISSION ELECTRON MICROSCOPIC IMAGE OF SILICA NANOPARTICLE	21
5	5.2.1 MICROSCOPIC IMAGE OF PmLyo-SF9 CELLS TREATED WITH WSSV INACTIVATED USING SILICA NANOPARTICLES AT A CONCENTRATION OF 320µg/mL	22
6	5.2.2 DOSE-DEPENDENT VIRUCIDAL ASSAY OF WSSV INFECTED PmLyo-SF9 CELLS TREATED WITH SILICA NANOPARTICLE	23
7	5.3.1 MICROSCOPIC IMAGE OF ATTACHMENT INHIBITION ANTIVIRAL ACTIVITY OF SILICA NANOPARTICLES SHOWS PmLyo-SF9 CELLS TREATED WITH SILICA NANOPARTICLES AT A CONCENTRATION OF 320µg/mL	24
8	5.3.2 ATTACHMENT INHIBITION ASSAY TO FIND THE ANTIVIRAL ACTIVITY OF SILICA NANOPARTICLES SHOWS PmLyo-SF9 CELLS TREATED WITH SILICA NANOPARTICLES AT A CONCENTRATION OF 320µg/mL	24

LIST OF ABBREVIATIONS

S.NO	ABBREVIATIONS
1	WSSV - White spot syndrome virus
2	IHHNV- Infectious hypodermal and hematopoietic necrosis virus
3	TSV- Taura syndrome virus
4	IMV- Infectious myonecrosis virus
5	YHV-Yellow head virus
6	DNA- Deoxyribonucleic acid
7	RNA- Ribonucleic acid
8	cDNA- Complementary DNA
9	RNAi- RNA interference
10	ORF- Open Reading Frames
11	NP's- Nanoparticles
12	PCR- Polymerase chain reaction
13	RT-PCR – Real time polymerase chain reaction
14	XRD- X-ray Diffraction
15	FT-IR – Fourier transform infrared spectroscopy
16	TEM- Transmission electron microscopy
17	MTT assay – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay
18	IEG- Immediate early gene

CHAPTER-1

1.INTRODUCTION

1.1 CRUSTACEANS

Crustaceans are a wide group of arthropods that inhabit aquatic environments and include species like crabs, lobsters, crayfish, remipedes, branchiopods, amphipods, and shrimp. They are an important source of food that contributes significantly to marine-based industries across the world. They possess a hard exoskeleton that protects their soft bodies, as well as a variety of appendages that help them adapt during times of reproduction, locomotion, and feeding.

1.2 SHRIMP

Shrimp is one of the most significant crustaceans that are widely distributed in both freshwater and saltwater environments. Shrimp has an important economic and cultural value, with the global shrimp industry worth billions of dollars (FAO,2016). They play an important role in the aquaculture industry due to their high nutritional value and economic worth.

Shrimp belong to the family Decapoda, which means "ten feet," which refers to the five pairs of legs. They are further classified into several families based on their morphology, behaviours, and habitat. The most common families of shrimp are Penaeidae, Pandalidae, Alpheidae, and Hippolytidae (Cano-Gómez et al. 2011)

1.2.1 SHRIMP- *Litopenaeus vannamei*

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Order: Decapoda
Family: Penaeidae
Genus: *Litopenaeus*
Species: *L. vannamei*

Litopenaeus vannamei is a species of shrimp that belongs to the family of Penaeidae. It is also known as Pacific white shrimp, which has a high tolerance to a wide range of salinities and temperatures, making it more adaptable to the environment.

1.3 MARINE VIRUS

Marine viruses are inconceivably vast and diverse in the ocean, with an estimated 10^{30} viral particles present in seawater at any given time. These viruses have an eloquent impact on microbial communities, nutrient cycling, and biogeochemical processes. Numerous studies have revealed a complicated and dynamic network of interactions between viruses and the environment. Marine viruses represent a diverse group of living organisms in the ocean, constituting a significant proportion of the biological entities, which indicates their high abundance (Suttle 2007b)

According to studies, bacteriophages make up the majority of marine viruses, and they infect a broad range of bacteria and archaea. Moreover, an eminent proportion of viruses have been observed to infect much eukaryotic phytoplankton such as diatoms, coccolithophores, and dinoflagellates (Suttle 2005c)

Recent developments in metagenomic sequencing techniques have revealed an exceptional diversity of viral communities in aquaculture. This has brought about the discovery of numerous new viral groups and families. Marine viruses have a diversified and intricate ecological role with consequential implications for global biogeochemical cycles and the general functioning of the marine ecosystem. The major critical role of a marine virus is in regulating microbes' population, either by inducing host cell mortality or by the replication and growth of particular microbial groups.

Marine viruses play a major role in bringing down the aquaculture industries through the outbreak of viral diseases in the marine ecosystem. An outbreak of a viral disease caused by the Pacific oyster mortality syndrome virus led to a tragic economic loss in the oyster industry in Australia, New Zealand, and France.

Likewise, the White spot syndrome virus, a highly contagious virus that infects crustaceans like shrimps and other aquatic organisms has caused a markable economic loss in shrimp aquaculture industries in many countries across the world (Lightner 2011)

1.4 AQUATIC VIRAL PATHOGEN

White spot syndrome virus is a highly contagious virus that affects a wide range of crustaceans like shrimp, prawns, crayfish, and crabs. The evolution of WSSV over the years has led to a significant threat to the aquaculture industry across the world. WSSV was first discovered in Taiwan in 1992, where it caused a high rate of mortality in cultured shrimp. Subsequently, it got spread to other shrimp farming regions in Asia, The Americas, and Europe, thereby it has become a huge threat to the sustainability of the global shrimp industry (Oidtmann and Stentiford 2011)

WSSV belongs to the family Nimaviridae, which also has other highly contagious viruses that affect crustaceans at a high rate, such as infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) (OIE, 2019). Nimaviruses are large enveloped viruses with circular double-stranded DNA genomes and are classified in the order of megavirales. The WSSV genome is around 300kb in size and encodes more than 180 open reading frames (ORFs). The genome has a unique arrangement of viral proteins, where the structural proteins are involved in the viral particle formation, and it includes the major envelope protein VP28 and the capsid protein VP19.

Likewise, the non-structural proteins are involved in the replication of viral particles and include DNA polymerase, helicase, and several other enzymes. WSSV infects a wide range of cultured shrimp and other crustaceans, including crayfish and crabs. The virus enters the host through the gills or other epithelial tissues, and it replicates in various organs like lymphoid organs, the hepatopancreas, and muscles. WSSV causes a wide range of clinical signs in the affected shrimp, including lethargy, anorexia, and the characteristic white spots on the exoskeleton.

The virus causes mortality of the shrimp up to 100% in the shrimp population, with death occurring within 3-10 days after infection with the virus (Luca Peruzza et al.,2019)

Shrimp, including *Litopenaeus vannamei*, are known to be prone to a variety of numerous viral and bacterial diseases. It is due to their delicate immune system, which depends on other mechanisms like phagocytosis and melanization to fight the infection. Some of the common viral diseases that affect shrimp include White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), and Taura Syndrome Virus (TSV) (OIE, 2019). These diseases cause high mortality in the shrimp population which also greatly affects the aquaculture industry which uses shrimp farming as a major source of income. The susceptibility of shrimp to these viral particles is influenced by various factors, including genetics, environmental conditions, and management practices.



Figure 1.4.1 *Litopenaeus vannamei* infected by white spot syndrome virus

These viral pathogens are said to be controlled in shrimp farming by focusing on a variety of strategies, including vaccination, breeding for disease resistance, and the use of antiviral agents (Thitamadee Siripong, et al., 2014). Several kinds of research are being conducted to inhibit the activities of these viral pathogens.

1.5 NANOPARTICLES

The aquatic virus causes viral infections that have a devastating impact on the aquaculture industries in the field of production, leading to reduced yields and significant economic losses (OIE,2018).Traditional methods of viral control such as vaccination, antibiotics, and disinfection have limitations and aren't very effective, which leads researchers to explore the potential of nanoparticles as an alternative approach to the inhibition of aquatic viruses (Ying, Shuaixuan, et al., 2022)

Nanoparticles (NPs) have unique physical and chemical properties that make them promising candidates as antiviral agents. Their small size, large surface area, and surface chemistry allow them to interact with viruses and prevent them from infecting host cells. Nanoparticles are used in the inhibition of various types of numerous diseases in aquaculture that is caused by viruses as well as bacteria and fungi (Munawar et al., 2021)

Several kinds of research have been carried out to examine various types of nanoparticles for their potential as antiviral agents. There are several types of nanoparticles have been used for antiviral activity including

- Silver nanoparticle
- Gold nanoparticle
- Zinc oxide nanoparticle
- Silica oxide nanoparticle
- Titanium oxide nanoparticle
- Iron oxide nanoparticle
- Copper nanoparticles

Silica oxide nanoparticles have been studied for their potential as an antiviral agent. Silica oxide nanoparticles possess a porous structure, high surface activity, and adsorption properties, which make them ideal for various applications in the inhibition of viral particles. The goal of the present work was to synthesize and characterize silica nanoparticles to evaluate in vitro anti-viral activity against WSSV using shrimp-insect hybrid cell line PmLyo-Sf9.

CHAPTER-2

REVIEW OF LITERATURE

Aquatic viral pathogens are one of the major threats to the aquaculture ecosystem across the world. These aquatic viral pathogens are responsible for causing significant losses to aquatic animals, including crustaceans, fish, and mollusks, which directly causes a severe economic impact on the aquaculture and marine-based industries.

The viral abundance in aquaculture ranged from 1.6×10^6 to 4.4×10^7 viruses per milliliter, indicating that viruses are one of the most abundant components in the marine ecosystem (Hurwitz and Sullivan 2013). Aquatic viral pathogens are broadly classified into DNA viruses and RNA viruses. Among these, RNA viruses are the most common type of virus in the marine ecosystem which includes a wide range family like Nimaviridae, Flaviviridae, Togaviridae, and Picornaviridae families. DNA viruses include families like Herpesviridae, Adenoviridae, and Papillomaviridae families. Most of these viral pathogens are specific to certain host species and families.

2.1 SHRIMP AQUACULTURE

Shrimp is one of the most commercially important crustaceans that can be found in both freshwater and marine water ecosystem. The Food and Agriculture Organization of the United Nations has listed 343 shrimp species that are potential for economic commercial. Of these, 80% of shrimp belong to the family Penaeidae. Among the other species in the family Penaeidae, eight species of this family have intricate importance in aquaculture.

Those species are: *Penaeus chinensis*, *P. indicus*, *P. japonicus*, *Litopenaeus vannamei*, *Metapenaeus ensis*, *P. stylirostris*, *P. monodon*, *P. merguensis* (Fast and Lester 2013)

Litopenaeus vannamei also known as Pacific white shrimp belongs to the phylum Arthropoda. The life cycle of this species is very complex it takes about 1.5 years to complete its whole life cycle. Though this species takes around 6 months for commercial purposes (Duggasa and Gaetan 2018). Shrimp is notable as a most valuable aquaculture species but at the same time, it is remarkable for causing high economic losses due to diseases (Asche et al. 2021).

The disease outbreaks among the shrimp species have resulted in a high rate of mortality in the aquaculture species. Various influencing factors results in the disease outbreaks including viral pathogens, poor environmental conditions, poor farm management, and the import of viral-infected shrimp. All these factors make the spread of the disease faster and more massive. More than 20 viruses have been reported to infect marine shrimp.

Among those, the major disease-causing viruses are

- 1) White Spot Syndrome virus (WSSV)
- 2) Infectious hypodermal and hematopoietic necrosis virus (IHHNV)
- 3) Yellow head virus (YHV)
- 4) Taura syndrome virus (TSV)
- 5) Infectious myonecrosis virus (IMV)

2.2 WHITE SPOT SYNDROME VIRUS

White spot syndrome virus is one of the most serious and deadliest viruses that infect cultured shrimp in aquaculture. WSSV causes 90 -100% mortality of infected shrimp within 3 to 10 days. The first appearance of WSSV in penaeid shrimp was reported in China and Taiwan in 1992 and it got spread all over the world including Japan (1993), Vietnam (1994), and Malaysia (1995) which continued to America, Brazil, Mexico, and Africa.

WSSV is the only member of the genus *Whispovirus* from the family *Nimaviridae*. It has a double-stranded DNA genome with a size of 80-120×250-380 nm. It has been reported to be approximately 300 to 305 kb in length according to the isolates with 180 open reading frames (ORFs). Structural proteins play an important role in cell targeting, viral entry, and replication of viral particles. Envelope protein has a key role in the viral entry to the host cell. Interactions between the structural proteins and envelope viruses are common in WSSV. It includes nine WSSV virion proteins (VP19, VP24, VP26, VP27, VP28, VP37 or VP281, VP38A or VP38, VP51C or VP51, VP51A OR and WSV010) in which VP19, VP24, VP51A prefers self-interaction. Among the other envelope proteins VP19, VP24, VP26, and VP28 are the main key abundant proteins. VP28 plays an important key role in the early stages of viral infection that binds WSSV viral particles to the shrimp cellular receptors. It is a main adhesion protein that binds the virus to the shrimp cells and enters the cytoplasm during infection along with VP37 which binds the viral particles to haemocytes. VP26 binds to actin or actin-related proteins that help the to translocate to the nucleus. It has been reported that VP26 may be instrumental in trafficking the WSSV nucleocapsid into the host nucleus through interacting with actin or cellular cytoskeleton (Xie & Yang, 2005).

Additionally, the structural proteins of the virion envelope such as VP26, VP31, VP37, VP90, and VP130 interact with integrin receptors to stimulate the binding of viruses to the extracellular matrix (Lee et al. 2022). The biological structure of VP28 plays an important role as an attachment protein. Furthermore (Sritunyalucksana *et al.* 2006) have reported that VP28 could interact with Rab7 protein which mainly functions in the phenomenon of exocytosis and endocytosis in mammalian cells. In addition, antibodies against VP28 have shown a significant delay in shrimp mortality (van Hulten *et al.*, 2001; Yi *et al.*, 2004)

VP664 is a major capsid protein that consists of a remarkably long polypeptide. This huge structural protein tends to form stacked ring structures in the nucleocapsid that are visible under TEM (Leu *et al.* 2005). Time course analysis of VP664 by RT-PCR has reported that this transcript was actively transcribed from 2 hours post-inoculation, reporting that this protein would contribute to the assembly and morphogenesis of the virion (Leu *et al.* 2005).

Several numbers of researches have been carried out to culture shrimp cells inoculated with WSSV for various purposes including understanding the phenomenon behind virus replication, interaction with the host cells, and WSSV genome synthesis. The research has focused on exploring the replication cycle of the white spot syndrome virus (WSSV) in secondary cell culture from the lymphoid organ of *Litopenaeus vannamei*. After the complete layer formation of secondary cells after 24 hrs, and remained viable for 10 days at a 90% survival rate, the WSSV binds to the cells at a high rate at 4°C after 120 minutes.

The virus entered cells through the phenomenon of endocytosis and the colocalization of WSSV was observed after 30 minutes post-inoculation. All the viral particles of WSSV entered the cells between 0 to 60 minutes of post-inoculation. After 1 to 3 hours of post-inoculation, the nucleocapsid protein VP664 and envelope protein VP28 began to synthesize in the cytoplasm, which was transported to nuclei after 3 to 6 hours of post-inoculation. The percentage of the cells that were VP664- and VP28- positive in their nuclei tended to reach the peak at 12 hours post inoculation. After 6 hours post-inoculation, WSSV DNA got synthesized and the progeny WSSV was released from 12 hours post-inoculation and reached its peak at 18 hours post-inoculation. The study came up with a conclusion as secondary cell culture from the lymphoid organ of *Litopenaeus vannamei* is ideal for examining the replication cycle of WSSV (Li et al. 2015).

Crustacean suffering from White spot syndrome disease displays various clinical signs including reduced food consumption, a loosening of the cuticle, discoloration of the hepatopancreas, reduced preening activities, and lethargy. It causes abnormal secretion of calcium salts in the cuticular epidermis or it disrupts the transfer of exudates from the epithelial cells to the cuticle (Wang *et al.*, 1999). Although the high alkalinity and bacterial diseases also cause white spots on the body of the shrimp. WSSV infection occurs in all the tissues of mesodermal and ectodermal origin which includes gills, lymphoid organs, sub-cuticular connective tissues, and cuticular epithelial tissues. In the final stage of infection, there is a destruction of WSSV-targeted tissues. Most of the infected cells have homogeneous hypertrophied nuclei, which shows amphophilic intranuclear inclusions and marginated chromatin (Verbruggen et al. 2016)

2.3 INHIBITION USING NANOTECHNOLOGY

Nanoparticles (NPs) are minute particle substances ranging from 1-100nm, categorized as organic and inorganic nanoparticles. The characteristics of nanomaterials such as their quantum size effect, high surface area, high surface-to-volume ratio, and tunable surface charge have validated their role as excellent carriers of conventional anti-viral therapies. Furthermore, the biomimetic properties of inorganic and metal nanoparticles have inspired researchers to investigate their intrinsic anti-viral activities.

Metal nanoparticles or inorganic nanoparticles include nanoparticles of native metals such as silver (Ag), gold (Au), and tin (Sn) and metal oxide such as silica dioxide (SiO₂), copper (Cu₂O and CuO), Zinc (ZnO), Titanium (TiO₂), Iron (Fe₂O₃ and Fe₃O₄).

Nanoparticles have the potential to be an effective anti-viral agents. The nanoparticles are synthesized using various methods which include chemical reduction, sol-gel synthesis, or electrospinning. The physicochemical properties of the nanoparticles are then characterized using transmission electron microscopy, dynamic light scattering, and X-ray diffraction. Nanoparticles have proven to be a successful and encouraging approach to combat the White Spot Syndrome virus. A recent study emphasizes the significance of silver nanoparticles in promoting the survival of WSSV-infected *Penaeus vannamei* shrimp.

The study found that administering a single dose of metallic silver in the nanomolar range (111nmol/shrimp), which is equivalent to 12ng/ml of silver nanoparticles led to a 20% survival rate in infected shrimps, where it doesn't cause any significant damage to the healthy shrimp when the nanoparticles administered. This survival rate could be attributed to the increased level of the LGBP gene, a key gene in the shrimp immune response, which is most likely induced by the recognition of the silver nanoparticle coating by specific pathogen-associated molecular pattern recognition proteins (PAMPs) of shrimp. The study also observed an increase in the LGBP expression levels even with a 10-fold lower dose of silver nanoparticles (1.2 ng/shrimp, 0.11nmol of metallic silver/shrimp) and even in the

presence of excess iron ions, a condition that favours virus proliferation. These findings in the study have suggested that a slight amount of silver nanoparticles can enhance the shrimp's immune system without any toxic effects and could represent an alternative for the treatment of White spot syndrome virus (Ochoa-Meza et al. 2018)

Another research has been carried out to deliver DNA construct orally using chitosan nanoparticles to protect the shrimp from the white spot syndrome virus. The study aimed to determine the effectiveness of using chitosan nanoparticles to deliver a DNA construct containing the VP28 gene of WSSV orally to black tiger shrimp (*Penaeus monodon*) for protection against WSSV. The results indicated the significant survival of WSSV-affected shrimp at 7, 15, and 30-days post-treatment (85%, 65%, and 50% relative survival) whereas it resulted in 100% mortality of the control shrimp which were given feed containing chitosan/pcDNA or chitosan/PBS complex. The study also examined the chitosan's ability to form a complex with pV28 and stabilize it from endonuclease degradation by agarose gel electrophoresis, as well as the cytotoxicity of chitosan-encapsulated pV28 using MTT assay, which resulted in the 90% of the viability of SISK cells incubated with pV28/chitosan-complexes.

The transcription analysis of the chitosan-encapsulated Pv28 gene in different tissues of DNA-treated shrimp and SISK cell lines was confirmed by an RT-PCR reaction. The results revealed the presence of pV28 in the gut, gill, head muscle tissue, hepatopancreas, and pleopods on the 7th, 15th, and 30th-day post-treatment.

Furthermore, the study examined the changes in the important immunological parameters, such as prophenoloxidase, superoxide dismutase, and superoxide anion in the haemolymph of chitosan-encapsulated VP28 DNA-treated and control shrimp, and correlated the changes with the survival percentage and protective efficacy of the oral route of DNA construct against WSSV in shrimp. The results have shown a higher level of prophenoloxidase and superoxide dismutase level on the 7th day post-treatment and it gradually decreased but didn't return to normal even on the 30th day of the experimental period.

The oral administration of β -1,3 glucan increased the level of prophenoloxidase and superoxide dismutase resulting in the significant survival of WSSV-challenged shrimp (Rajeshkumar, S. et al. 2009).

Another study (Baozhen Sun et al. 2016) reported the positive effects of chitosan nanoparticles when included in the diet of WSSV-challenged crayfish *Procambarus clarkia*. The inclusion of 10 mg/g of chitosan nanoparticles in their diet increased the survival rate by 65% through modulation of immunological parameters. Furthermore, (Juarez-Moreno et al. 2017) reported that the intramuscular injection of silver nanoparticles in the white shrimp increased the survival rate of WSSV-challenged shrimp to 80% survival rate after 96 hours, without toxicity and without altering the metabolic rate. Altogether, these studies explore the significant inhibition activity of nanoparticles against the white spot syndrome virus (Govindaraju, K. et al. 2019).

CHAPTER-3

AIM AND OBJECTIVE

3.1 AIM:

To inhibit the viral activity of the white spot syndrome virus using silica nanoparticles.

3.2 OBJECTIVE:

- To synthesize silica nanoparticles from beach sand
- To physio-chemical characterize the silica nanoparticles using XRD, FT-IR, and TEM
- To study the in vitro anti-viral activity of the silica nanoparticle in PmLyo-SF9 cells.

CHAPTER-4

MATERIALS AND METHODS

4.1 PREPARATION OF SILICA NANOPARTICLES FROM BEACH SAND

Silica nanoparticles were synthesized from beach sand by modifying the procedure (Meléndez-Villanueva, M.A. et al. 2019). The beach sand was collected from the Akkarai beach, Chennai, and washed with water to remove the unwanted materials and then dried at room temperature for 2 h. Further, dried sand is ground using a pestle and mortar until it gets a powder form. The powdered sand was calcinated at 900° C using a muffle furnace to remove the organic impurities. Finally, calcinated Silica nanoparticles were subjected to a ball milling process [600 rpm for 5 h; 7 g of sample/cylinder/8 balls/ball size 10 mm in diameter] using a planetary ball mill to get nano-sized silica powder.

4.2 PHYSIO-CHEMICAL CHARACTERIZATION OF SILICA NANOPARTICLE

4.2.1 X-RAY DIFFRACTION (XRD) PATTERN STUDIES

X-ray diffraction (XRD) measurements of nanoparticles were performed using a Rigaku smart lab X-Ray powder diffractometer operated at a voltage of 40 kV and a current of 30 mA with a CuK α 1 radiation source.

4.2.2 FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPIC STUDIES

Fourier Transform Infrared (FT-IR) spectra were verified using a Thermo Nicolet Avatar 300 FT-IR spectrophotometer in the range of 4000 – 500 cm⁻¹ employing the KBr pellet technique.

4.2.3 TRANSMISSION ELECTRON MICROSCOPIC STUDIES

The external structure and shape of silica nanoparticles were examined by transmission electron microscope (FEI Technai spirit). The quantitative analysis of nanoparticles was done by FEI QUANTA 200.

4.3. CYTOTOXICITY STUDIES OF SILICA NANOPARTICLES

Silica nanoparticles were tested in shrimp-insect hybrid cell line *PmLyo-SF9* (Anoop et al., 2021) fused using PEG to determine their cytotoxicity using MTT assay. Mature confluent cells were split into 70% SCCM (Shrimp Cell Culture Medium) (Jayesh et al., 2013) supplemented with TNM-FH insect medium (Hi-media, India) and Fetal Bovine Serum (Hi-media, India). Monolayers of cells (1.375×10^5 cells/mL) were seeded 100 μ L each onto a 96-well plate (TPP, Europe) and incubated at 28°C for 24 h. 10 mg/mL stock of lyophilized nanoparticles were prepared in 100 % SCCM augmented with 1% antibiotic solution (Pen-Strep, Gibco). The nanoparticle suspension was sonicated (Oscar Ultrasonics, India) for about 30 min to ensure uniformity, before application onto the cells. Seven concentrations of nanoparticles were then added 100 μ L each as triplicates onto the cultured wells, after removing the media. The selected concentrations were 80 μ g/mL, 160 μ g/mL, 320 μ g/mL, and 640 μ g/mL. Cells incorporated with nanoparticles were then incubated at 28°C for 24 h followed by the addition of a 20 μ L volume of 5mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma Aldrich, Germany) solution. The purple formazan crystals formed after 4 h of incubation were dissolved in 100 μ L dimethyl sulfoxide (HiMedia, India) and the absorbance was recorded at dual wavelengths 570 nm and 690 nm to find the cell viability using a multimode microplate reader (Thermo Scientific, USA).

$$\text{Cell viability \%} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100$$

4.4 VIRUS ISOLATION AND QUANTIFICATION

WSSV stock was collected from National Centre for Aquatic Animal Health, CUSAT was used for infecting *Penaeus monodon*, and the entire work was carried out at the Centre. The infected animal gradually turned lethargic and developed white spots in the body and mortality was observed after 3-4 days of infection. DNA isolation was done by collecting tissue samples from the dead animal and amplification of WSSV viral genes was done by nested PCR (Lo et al., 1996) for confirmation of viral infection. Virus isolation was followed from the gill tissue of the animal. An ample amount of tissue was removed from the animal and macerated with glass fiber wool in 70 % SCCM to form a suspension. After centrifugation under cold conditions, the supernatant was filter sterilized and aliquoted for long-term storage at -80°C deep freezer (New Brunswick Scientific, USA).

StepOnePlus Real-time PCR (Applied Biosystem, USA) was used for real-time quantification and viral titer determination. Power SYBR Green PCR Master Mix (5 µL) (Applied Biosystems, UK), forward and reverse primers of VP 28 (3 µM) were used to amplify the virus. A preliminary holding phase of 95 °C for 10 min was followed by denaturation at 95 °C for the 30s and annealing at 55 °C for 30s, which was repeated for 40 cycles with fluorescence signal detection after each cycle. To guarantee primer specificity and the lack of primer secondary structures, the reaction setup included a melt curve analysis for each sample.

4.5 VIRUCIDAL ACTIVITY AND ATTACHMENT INHIBITION ASSAY OF SILICA NANOPARTICLES AGAINST WSSV

The nanomaterials at an optimum concentration of 320 µg/mL were suspended in 100% SCCM augmented with antibiotics and added to an equal volume of virus inoculum followed by incubation for 4-5 h at 28°C. This step enables a reaction between the virus and the nanoparticles which can lead to inhibitory actions that may inactivate the virus. Monolayers of *PmLyo-SF9* cells (9.17×10^5) were seeded onto 9.2 cm² cell culture dishes (TPP, Europe) in 70% SCCM.

The virus-nanoparticle mixture was added to the cells and incubated at 28°C for 24 h. After documenting the cytopathic effects using an inverted microscope (Leica microsystems, Germany), the cells were fixed in TRI reagent (Sigma Aldrich, Germany).

Cell monolayers were pre-treated with 50 μ L of silica nanoparticles in aqueous media with a 320 μ g/mL concentration. Treated cells were incubated at 28°C for 3-4 h and then 50 μ L of virus solution was added to the cells to determine whether any receptor inhibition happens that prevents virus infection. The cells were observed under the microscope after 24 h to record the cytopathic changes, TRI-fixed, and stored at -80°C. Assays were conducted separately to estimate the virucidal property as well as attachment inhibition following the protocols (Sataporn et al., 1996; Toru et al., 2013).

4.6 EXPRESSION AND AMPLIFICATION OF WSSV GENES

cDNA synthesis was performed using total RNA isolated from WSSV-infected *PmLyo-SF9* cells treated with silica nanoparticles to ascertain the expression of the WSSV viral gene VP 28. The samples fixed in the TRI reagent were phase separated by adding 0.2 mL of chloroform and the upper aqueous phase was pelletized by the addition of isopropanol. The pellet containing RNA was washed twice with 75 % ethanol to remove impurities. The RNA was air-dried and dissolved in MilliQ. 5 μ g of RNA was used as a template for generating cDNA. RNA was denatured and subjected to DNase treatment to ensure the degradation of DNA and protein remnants. To synthesize cDNA, a 20 L reaction mix was incubated at 42 °C for 1 h using the reagents RNase inhibitor, Murine (40,000 units/mL), M-MuLV Reverse Transcriptase (200,000 units/mL), RT buffer (10X), dNTPs (1 mM), Oligo dt (10 M), and MgCl₂ (25 mM). All reagents were purchased from New England Biolabs. Viral envelope protein VP 28 was amplified by Polymerase Chain Reaction with 34 cycles of denaturation at 95°C for 30s primer annealing at 55°C for 30s, and extension at 72°C for 1 min. The reaction mixes included Emerald PCR Master Mix – 5 μ L (Takara Bio, Japan), forward and reverse primers – 0.4 μ L (10 pmol/ μ L each) (Jose et al., 2010; Anoop et al., 2021), Milli-Q water 3.7 μ L and cDNA template – 0.5 μ L. Gene expression was visualized by agarose gel electrophoresis.

CHAPTER-5

RESULTS AND DISCUSSION

5.1 PHYSIO-CHEMICAL CHARACTERIZATION OF BEACH-BASED SiO_2 NPs

5.1.1 X-RAY DIFFRACTION

The XRD peaks for beach sand were matched with the standard JCPDS and the main phases were found to be Potassium Aluminium Silicate $\text{K}(\text{AlSi}_3\text{O}_8)$ [card number 89-8573], Sodium Silicate $\text{Na}_2\text{Si}_2\text{O}_5$ [card number 76-0784] and Silicon Dioxide [card number 86-2237]

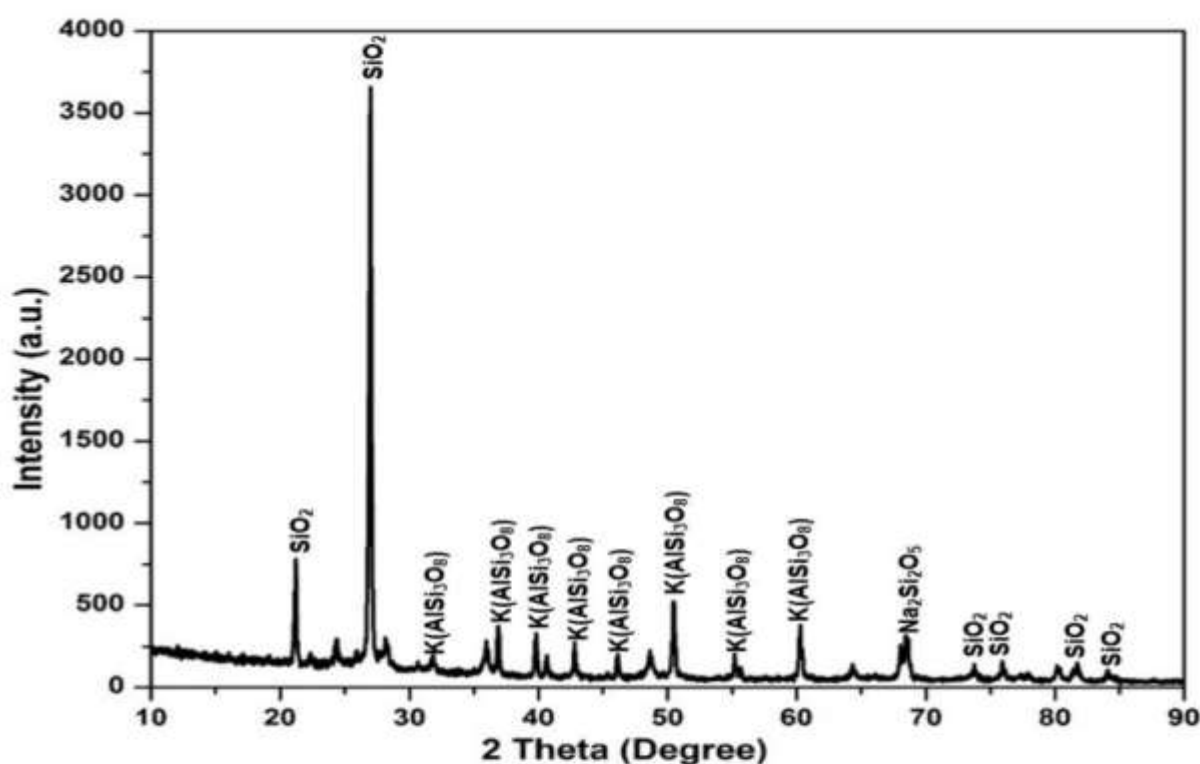


Figure 5.1.1: X-ray Diffraction (XRD) pattern of beach sand silica nanoparticles

5.1.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

ANALYSIS OF SILICA NANOPARTICLE

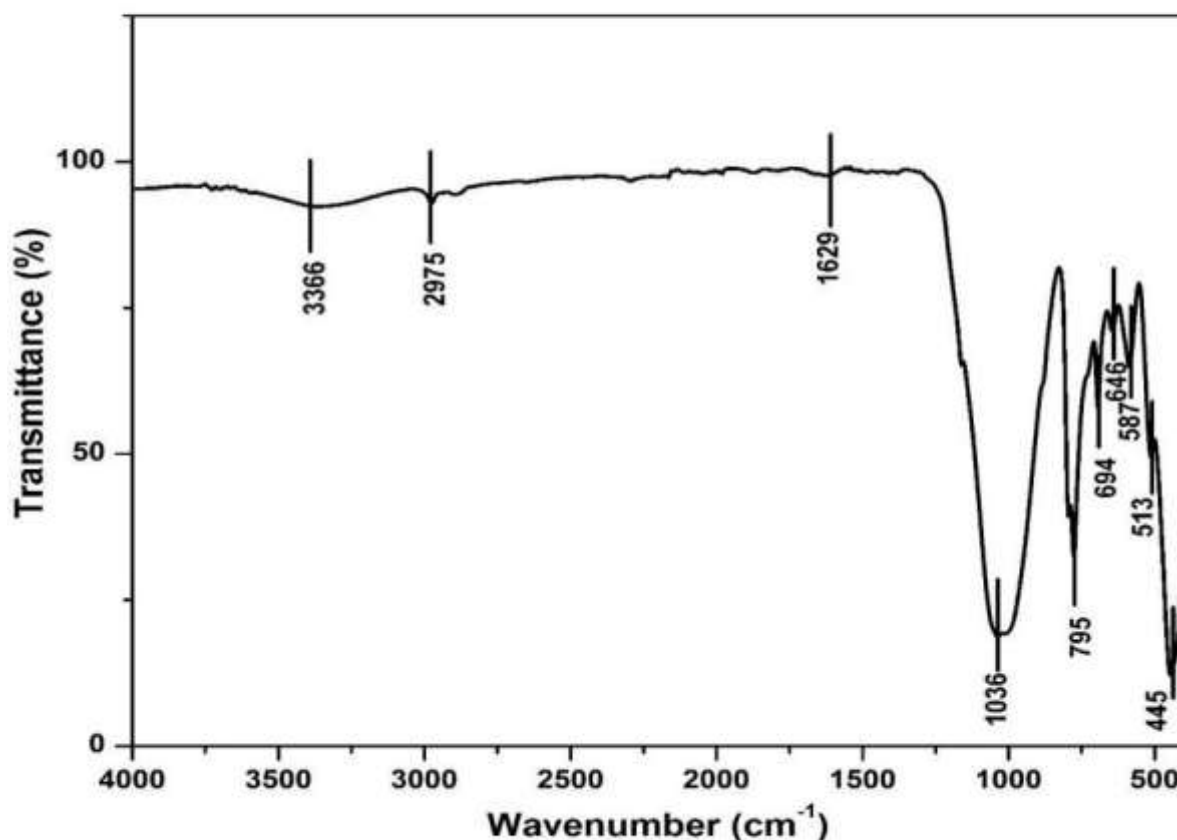


Figure 5.1.2: FTIR analysis of silica nanoparticles

The infrared spectra for the silica nanoparticles prepared from beach sand were showed in Figure 5.1.2. The major peaks occurred between 788, 1097, and 1011 cm⁻¹ characteristic of Si–O–Si asymmetric stretching vibration (Figure 5.1.2) The absorption peaks at 1629 cm⁻¹ [C–N vibration], 2975 cm⁻¹ [Si–CH₃], and 3366 cm⁻¹ [Si–OH].

5.1.3 TRANSMISSION ELECTRON MICROGRAPH ANALYSIS OF SILICA NANOPARTICLES

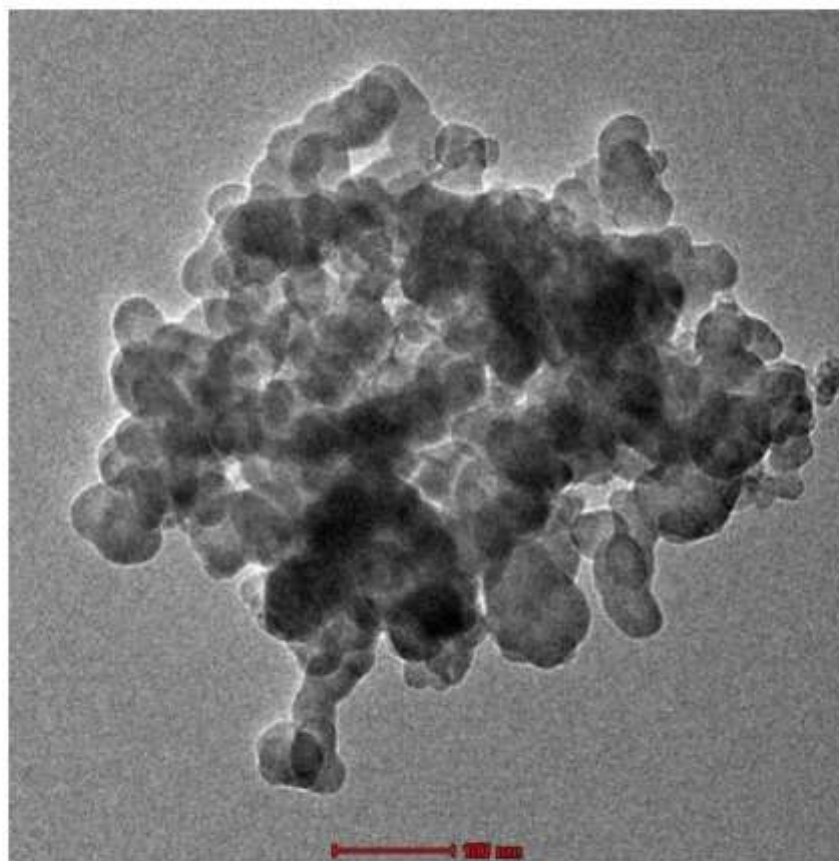


Figure 5.1.3 Transmission electron microscopic image of silica nanoparticles

TEM micrograph revealed that the beach sand-based silica nanoparticles were spherical in shape with an average size of 39 nm and non-uniformity along the grid (Fig.5.1.3)

5.2 CYTOTOXICITY ASSAY

Cytotoxicity of silica nanoparticles towards *PmLyo-SF9* cells was determined by MTT assay. It is a rapid colorimetric assay based on the enzymatic reduction of tetrazolium salt to formazan crystals in live cells. Calculation of the percentage cell viability was done by estimating the optical density of purple formazan crystals. No significant cell death was observed in the wells at lower concentrations. The IC_{50} value of the nanoparticles was calculated as 320 $\mu\text{g/mL}$.

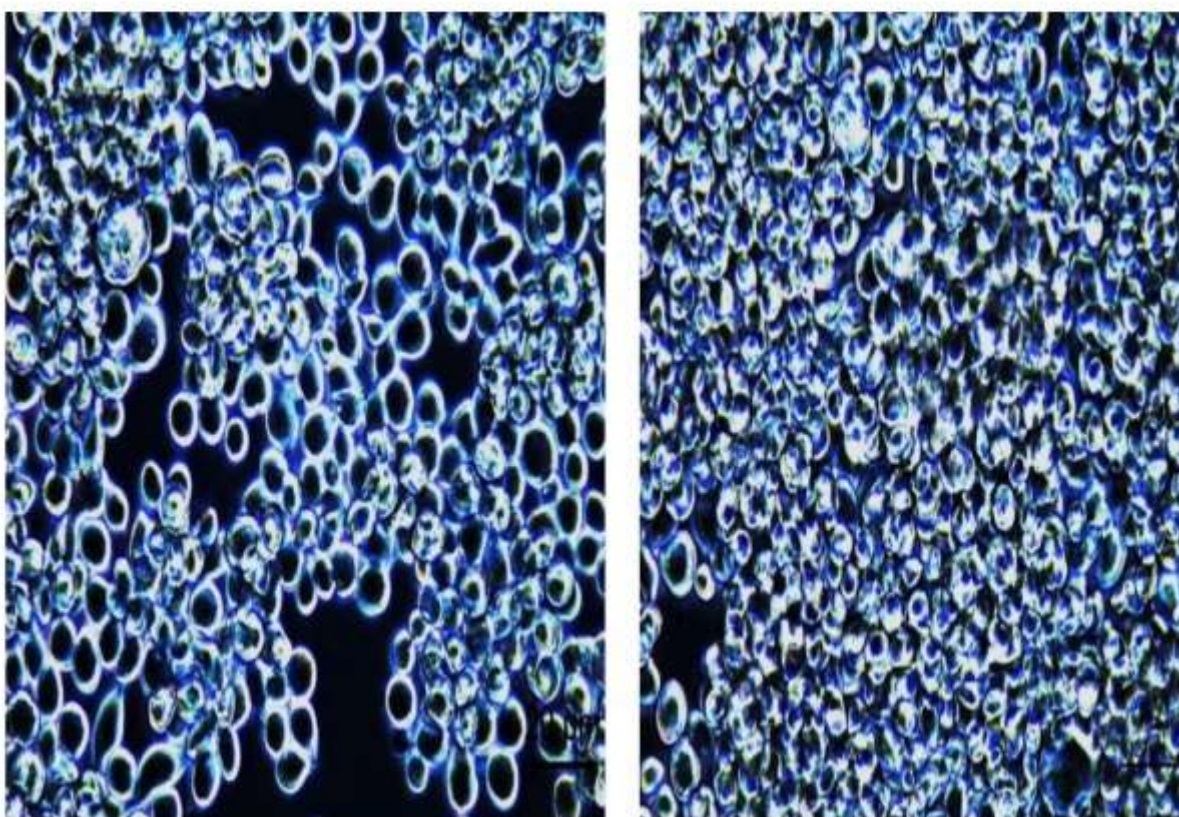


FIGURE 5.2.1 *Microscopic images of PmLyo-Sf9 cells treated with WSSV inactivated using silica nanoparticles at a concentration of 320 $\mu\text{g/mL}$ to determine the virucidal property of the nanoparticles*

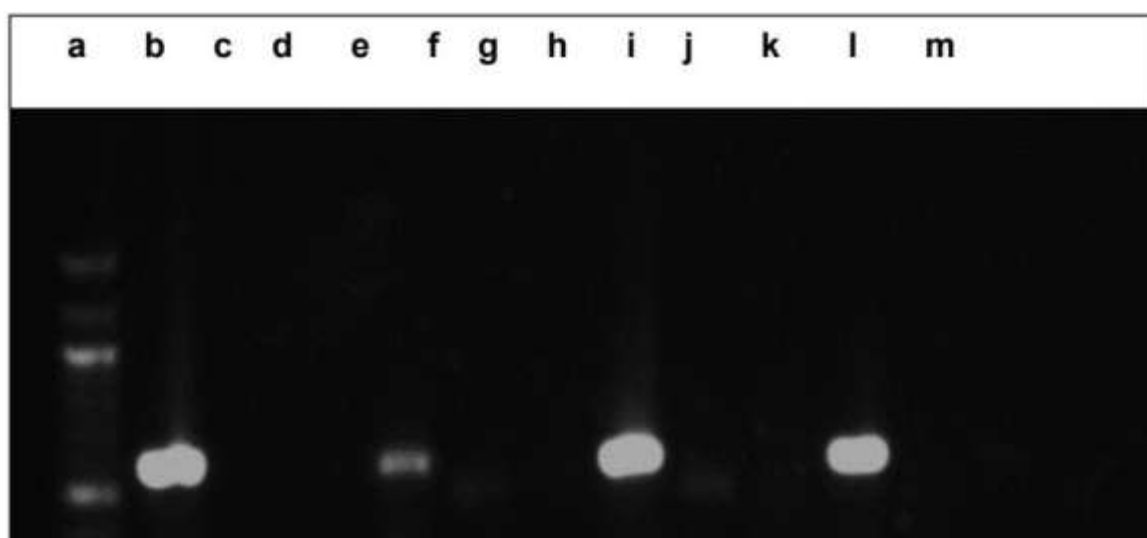


Figure 5.2.2 Dose-dependent virucidal assay of WSSV infected *PmLyo-Sf9* cells treated with silica nanoparticles. (a) 100bp marker; (b, c, d) Genes- β actin, IEG, VP-28 conc: 640 $\mu\text{g/mL}$; (e, f, g) Genes- β actin, IEG, VP-28 Conc: 320 $\mu\text{g/mL}$; (h, i, j) Genes- β actin, IEG, VP-28 Conc: 160 $\mu\text{g/mL}$; (k, l, m) Genes- β actin, IEG, VP-28 Conc: 80 $\mu\text{g/mL}$

5.3 BIOACTIVITY OF SILICA NANOPARTICLES AGAINST WSSV

Assays were performed for determining the virucidal property as well as attachment inhibition properties of silica nanoparticles against WSSV. *PmLyo-SF9* cells incorporated with the virus and nanoparticles were microscopically examined to record the cytopathic changes (Fig.5.3.1) Infected cells treated with nanoparticles showed almost negligible cytotoxicity when compared to the positive control. This clearly showed a possibility for the anti-WSSV activity of the nanoparticles and hence the samples were processed further for viral gene expression studies. The expression of viral gene VP-28 was ascertained by amplifying the gene using forward and reverse primers. WSSV-infected cells were used as positive controls with gene expression. The agarose gel images showed faint bands for the gene for both the assays particularly negligible in the virucidal assay.

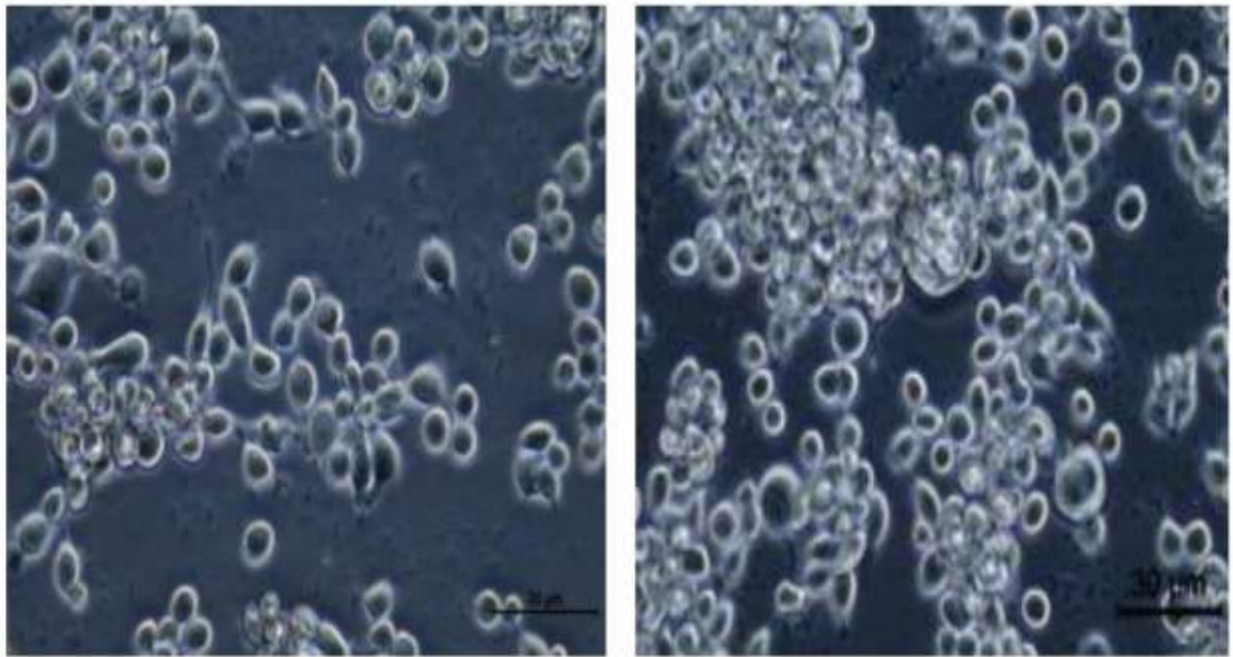


Figure 5.3.1 *Microscopic image of attachment inhibition antiviral activity of silica nanoparticles shows PmLyo-Sf9 cells treated with silica nanoparticles at a concentration of 320 μg/mL. Scale bar 30 μm.*

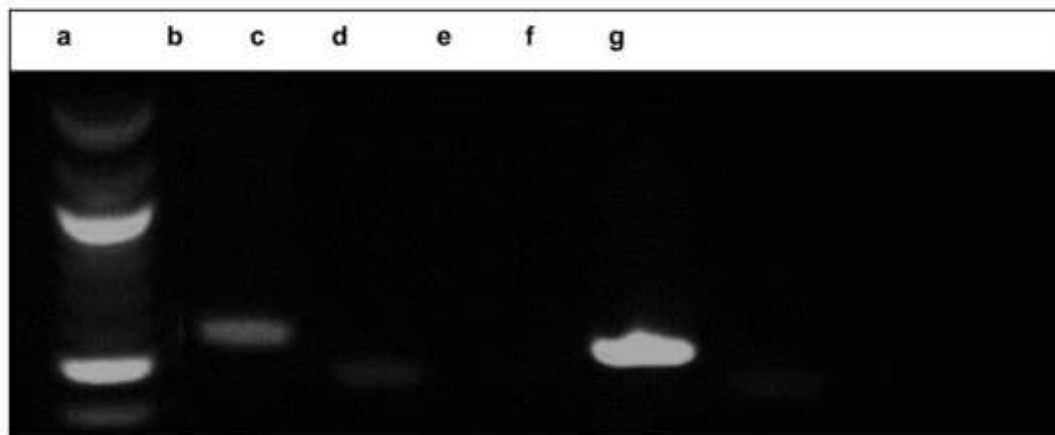


Figure 5.3.2 *Attachment inhibition assay to find the antiviral activity of silica nanoparticles shows PmLyo-Sf9 cells treated with silica nanoparticles at a concentration of 320 μg/mL. (a) 100 bp marker; (b, c, d) Genes β actin, IEG, VP-28 attachment inhibition assay; (e, f, g) Genes β actin, IEG, VP-28 virucidal assay.*

DISCUSSION

This confirmed the activity of silica nanoparticles against the virus, and it is more successful in inactivating the virions. Various metal and metal oxide nanomaterials have been used as potential antiviral agents. Gold nanoparticles stand out among other nanomaterials for their distinctive physiochemical properties. A special phenomenon associated with plasmonic (noble metal) nanoparticles results in powerful electromagnetic fields on the particle surface. Concerning the previous literature, gold nanoparticles inhibit various viral pathogens such as herpes simplex virus type 1&2 (Dhanasezhian et al., 2019; Paradowska et al., 2021), Measles virus (Meléndez-Villanueva et al., 2019), influenza viruses (Babaei et al., 2021), SARS-CoV-2 (Chang et al., 2021) and adenoviruses (Chaika et al., 2021). The literature and experimental results inferred that actinobacteria synthesized gold nanoparticles possess interaction and block the virus attachment and entry responsible for antiviral activity.

CHAPTER-6

SUMMARY AND CONCLUSION

6.1 SUMMARY

White spot syndrome virus is one of the highly contagious viruses that infect crustaceans including shrimp, crayfish, prawns, and crabs. The outbreak of the disease has caused a significant economic loss in the aquaculture industry across the world. Several types of research have been carried out to inhibit the activity of the virus using traditional methods like immune stimulation antibiotics, probiotics, and RNA interference (RNAi). These methods can be replaced by using nanomaterials which act as effective therapeutic agents.

This research is carried out to inhibit the viral activity of wssv by nanoparticles. The study aims to inhibit the viral activity of the white spot syndrome virus using silica nanoparticles using nanoparticles as an antiviral agent. The silica nanoparticles were synthesized from the beach sand and the characterization was conducted using analytical techniques including XRD, FT-IR, and TEM. The FT-IR spectra confirmed the major peaks occurred between 788, 1097, and 1011 cm^{-1} which is the characteristic of Si-O-Si asymmetric stretching vibration. TEM micrograph revealed that the silica nanoparticles synthesized from beach sand were spherical in shape with an average size of 39nm. The antiviral activity of silica nanoparticles has been examined in vitro by inoculating with hybrid cell line PmLyo-SF9 extracted from the wssv infected shrimp. The cytotoxicity of silica nanoparticles toward the PmLyo-SF9 cells was determined by using the MTT assay. In this study there isn't any significant cell death was observed in the wells at lower concentrations of the silica nanoparticle and the IC_{50} value was calculated at 320 $\mu\text{g/mL}$.

The virucidal property of the nanoparticle was determined in the study as well as the attachment inhibition property of silica oxide nanoparticles against WSSV. With that, the expression of the viral gene VP28 was also examined. The viral envelope protein VP28 is amplified using a polymerase chain reaction and the gene expression was visualized by agarose gel electrophoresis.

The agarose gel images showed faint bands for the gene for both assays, particularly negligible in the virucidal assay. The result shows that the VP28 gene was reduced in the infected cells treated with silica nanoparticles compared to untreated infected cells. This study clearly shows that the silica nanoparticles have a potential anti-viral effect against the white spot syndrome virus.

6.2 CONCLUSION

Outbreaks of disease caused by a highly contagious virus-like white spot syndrome virus which causes 100% mortality in cultured shrimp and also leads to significant economic loss in aquaculture across the world can be controlled and inhibited using the nanotechnology method. This research has shown that the silica nanoparticles act as an effective anti-viral agent against the white spot syndrome virus. Hence the silica oxide nanoparticles can be formulated as an anti-viral agent against aquatic viral pathogen in efficient disease control.

REFERENCES

1. Babaei, S. M. Mousavi, M. Ghasemi, N. Pirbonyeh, M. Soleimani, A. Moattari, 2021. Gold nanoparticles show potential *in vitro* antiviral and anticancer activity. *Life Sciences* 284, 119652.
2. Dhanasezhian, S. Srivani, K. Govindaraju, Parija, Preetam Sasikala, S. Kumar, M.R. Ramesh, Anti-Herpes Simplex Virus (HSV-1 and HSV-2) activity of biogenic gold and silver nanoparticles using seaweed *Sargassum wightii*, *Indian Journal of Geo-Marine Sciences*, 48:1252-1257.
3. Abad-Rosales, Selene María, Martín Gabriel Frías-Espericueta, Oscar Guadalupe Romero-Bernal, Rodolfo Lozano-Olvera, Silvia Alejandra García-Gasca, Leobardo Montoya-Rodríguez, and Domenico Voltolina. "White spot syndrome virus (WSSV) infection and immunity responses in white shrimp (*Litopenaeus vannamei*) exposed to sublethal levels of metals." *Aquaculture Research* 50, no. 3 (2019): 758-764.
4. Anoop, B.S., Puthumana, J., Vazhappilly, C. G., Kombiyil, S., Philip, R., Abdulaziz, A., Bright Singh, I.S., Immobilization of shrimp lymphoid cells by hybridizing with the continuous cell line Sf9 leading to the development of PmLy-Sf9, *Fish & Shellfish Immunol.* 113 (2021) 196-207.
5. Asche, Frank, James L. Anderson, Robert Botta, Ganesh Kumar, Eirik B. Abrahamsen, Ly T. Nguyen, and Diego Valderrama. "The economics of shrimp disease." *Journal of invertebrate pathology* 186 (2021): 107397.
6. Cano-Gomez, Ana, Lone Høj, Leigh Owens, and Nikos Andreakis. "Multilocus sequence analysis provides basis for fast and reliable identification of *Vibrio harveyi*-related species and reveals previous misidentification of important marine pathogens." *Systematic and applied microbiology* 34, no. 8 (2011): 561-565.
7. Dugassa, Hailu, and De Gyrse Gaetan. "Biology of white leg shrimp, *Penaeus vannamei*: Review." *World Journal of Fish and Marine Sciences* 10, no. 2 (2018): 5-17.

8. E. Paradowska, M. Studzińska, A. Jabłońska, V. Lozovski, N. Rusinchuk, I. Mukha, N. Vitiuk, Z.J. Leśnikowski, 2021. Antiviral effect of nonfunctionalized gold nanoparticles against Herpes Simplex Virus Type-1 (HSV-1) and possible contribution of near-field interaction mechanism. *Molecules* 26: 5960.
9. Fast, Arlo Wade, and L. James Lester, eds. *Marine shrimp culture: principles and practices*. Elsevier, 2013.
10. Govindaraju, K., Prerna Dilip Ittroutwar, V. Veeramani, T. Ashok Kumar, and S. Tamilselvan. "Application of nanotechnology in diagnosis and disease management of white spot syndrome virus (WSSV) in aquaculture." *Journal of Cluster Science* 31 (2020): 1163-1171.
11. Hurwitz, Bonnie L., and Matthew B. Sullivan. "The Pacific Ocean Virome (POV): a marine viral metagenomic dataset and associated protein clusters for quantitative viral ecology." *PloS one* 8, no. 2 (2013): e57355.
12. Jayesh, P., Jose, S., Philip, R., Bright Singh, I.S., A novel medium for the development of in vitro cell culture system from *Penaeus monodon*. *Cytotechnology* 65 (2013) 307–322.
13. Jose, S., Mohandas, A., Philip, R., Singh, I.S.B., Primary hemocyte culture of *Penaeus monodon* as an *in vitro* model for white spot syndrome virus titration, viral and immune-related gene expression and cytotoxicity assays. *J. of Inverte. Pathol.* 105 (2010) 312-321.
14. Juarez-Moreno, Karla, Claudio Humberto Mejía-Ruiz, Fernando Díaz, Horacio Reyna-Verdugo, Ana Denisse Re, Edgar F. Vazquez-Felix, Edna Sánchez-Castrejón, Josué D. Mota-Morales, Alexey Pestryakov, and Nina Bogdanchikova. "Effect of silver nanoparticles on the metabolic rate, hematological response, and survival of juvenile white shrimp *Litopenaeus vannamei*." *Chemosphere* 169 (2017): 716-724.
15. Lee, Dain, Young-Bin Yu, Jae-Ho Choi, A-Hyun Jo, Su-Min Hong, Ju-Chan Kang, and Jun-Hwan Kim. "Viral shrimp diseases listed by the OIE: A review." *Viruses* 14, no. 3 (2022): 585.

16. Leu, Jiann-Horng, Jyh-Ming Tsai, Han-Ching Wang, Andrew H-J. Wang, Chung-Hsiung Wang, Guang-Hsiung Kou, and Chu-Fang Lo. "The unique stacked rings in the nucleocapsid of the white spot syndrome virus virion are formed by the major structural protein VP664, the largest viral structural protein ever found." *Journal of virology* 79, no. 1 (2005): 140-149.
17. Li, Wenfeng, Lowiese MB Desmarets, Gaëtan MA De Gryse, Sebastiaan Theuns, Vo Van Tuan, Khuong Van Thuong, Peter Bossier, and Hans J. Nauwynck. "Virus replication cycle of white spot syndrome virus in secondary cell cultures from the lymphoid organ of *Litopenaeus vannamei*." *Journal of General Virology* 96, no. 9 (2015): 2844-2854.
18. Lightner, D. V. "Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review." *Journal of invertebrate pathology* 106, no. 1 (2011): 110-130.
19. Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H., White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Dis. Aquat. Org.* 27 (1996) 215-225.
20. M. Chaika, K. Naumenko, Y. Pankivska, O. Povnitsa, I. Mukha, N. Vityuk, A. Dorovskih, V. Lozovski, V. Lysenko, M. Lokshyn, N.M. Rusinchuk, S. Zagorodnya. Size- and concentration-dependence of antiviral and virucidal activity of Au nanoparticles against adenoviruses and influenza viruses H1N1. *Beilstein Archives* 2021, 202192.
21. M.A. Meléndez-Villanueva, K. Morán-Santibañez, J.J. Martínez-Sanmiguel, R. Rangel-López, M.A. Garza-Navarro, C. Rodríguez-Padilla, D.G. Zarate-Triviño, L.M. Trejo-Ávila¹, Virucidal activity of gold nanoparticles synthesized by green chemistry using garlic extract. *Viruses*. 2019, 11: 1111.
22. Munawar, Noshaba. "Interaction and applications of nanoparticles in fishes and aquaculture." *Sch Bull* 7, no. 6 (2021): 150-155.

23. Ochoa-Meza, Alba R., Ana R. Álvarez-Sánchez, Carlos R. Romo-Quíñonez, Aarón Barraza, Francisco J. Magallón-Barajas, Alexis Chávez-Sánchez, Juan Carlos García-Ramos et al. "Silver nanoparticles enhance survival of white spot syndrome virus infected *Penaeus vannamei* shrimps by activation of its immunological system." *Fish & Shellfish Immunology* 84 (2019): 1083-1089.
24. Oldtmann, Birgit, and Grant D. Stentiford. "White spot syndrome virus (WSSV) concentrations in crustacean tissues—a review of data relevant to assess the risk associated with commodity trade." *Transboundary and Emerging Diseases* 58, no. 6 (2011): 469-482.
25. Peruzza, Luca, M. S. Shekhar, K. Vinaya Kumar, A. Swathi, K. Karthic, Chris Hauton, and K. K. Vijayan. "Temporal Changes in Transcriptome Profile Provide Insights of White Spot Syndrome Virus Infection in *Litopenaeus Vannamei*." *Scientific Reports* 9, no. 1 (September 18, 2019).
26. Rajeshkumar, S., C. Venkatesan, M. Sarathi, V. Sarathbabu, John Thomas, K. Anver Basha, and AS Sahul Hameed. "Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV)." *Fish & Shellfish Immunology* 26, no. 3 (2009): 429-437.
27. S.Y. Chang, K.Y. Huang, T.L. Chao, H.C. Kao, Y.H. Pang, L. Lu, C.L. Chiu, H.C. Huang, T.J. R. Cheng, J.M. Fang, P.C. Yang, 2021. Nanoparticle composite TPNT1 is effective against SARS-CoV-2 and influenza viruses. *Scientific Reports*, 11:8692.
28. Sataporn, D., Angkana, H., Mamoru, Y., Yoshio, E, Antiviral Activity of Several Thai Traditional Herb Extracts against Fish Pathogenic Viruses, *Fish Pathology* 31(4) (1996) 209-213.
29. Sritunyalucksana, Kallaya, Wanphen Wannapapho, Chu Fang Lo, and Timothy W. Flegel. "PmRab7 Is a VP28-Binding Protein Involved in White Spot Syndrome Virus Infection in Shrimp." *Journal of virology* 80, no. 21 (2006): 10734-10742.

30. Sun, Baozhen, Haizhi Quan, and Fei Zhu. "Dietary chitosan nanoparticles protect crayfish *Procambarus clarkia* against white spot syndrome virus (WSSV) infection." *Fish & Shellfish Immunology* 54 (2016): 241-246.
31. Suttle, Curtis A. "Marine viruses—major players in the global ecosystem." *Nature reviews microbiology* 5, no. 10 (2007): 801-812.
32. Suttle, Curtis A. "Viruses in the sea." *Nature* 437, no. 7057 (2005): 356-361.
33. Thitamadee, Siripong, Prachumwat, Anuphap, Srisala, Jiraporn, Jaroenlak, Pattana, Salachan, Paul V., Sritunyalucksana, Kallaya, Flegel, Timothy W., and Ornachuma Itsathitphaisarn. "Review of current disease threats for cultivated penaeid shrimp in Asia." *Aquaculture* 452, (2016): 69-87
34. Toru, I., Thommas, M.M., Ken, W., Nobuyuki, K., Evaluation of antiviral activity of Oligonol, an extract of *Litchi chinensis*, against betanodavirus, *Drug Discoveries & Therapeutics*. 7(6) (2013) 254-260.
35. van Hulten, Mariëlle CW, Jeroen Witteveldt, Marjolein Snippe, and Just M. Vlak. "White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp." *Virology* 285, no. 2 (2001): 228-233.
36. Verbruggen, Bas, Lisa K. Bickley, Ronny Van Aerle, Kelly S. Bateman, Grant D. Stentiford, Eduarda M. Santos, and Charles R. Tyler. "Molecular mechanisms of white spot syndrome virus infection and perspectives on treatments." *Viruses* 8, no. 1 (2016): 23.
37. Wang, Y. G., M. D. Hassan, M. Shariff, S. M. Zamri, and X. Chen. "Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation." *Diseases of aquatic organisms* 39, no. 1 (1999): 1-11.
38. Xie, Xixian, Hongyan Li, Limei Xu, and Feng Yang. "A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles." *Virus research* 108, no. 1-2 (2005): 63-67.

39. Yi, Guohua, Zhimin Wang, Yipeng Qi, Lunguang Yao, Juan Qian, and Longbo Hu. "Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells." *BMB Reports* 37, no. 6 (2004): 726-734.
40. Ying, Shuaixuan, Guan, Zhenru, Ofoegbu, Polycarp C., Clubb, Preston, Rico, Cyren, He, Feng, and Jie Hong. "Green synthesis of nanoparticles: Current developments and limitations." *Environmental Technology & Innovation* 26, (2022): 102336.