1.1. Introduction

Penaeid shrimps are the major marine species which are economically important and cultured worldwide. In India, in the last decade, notable increase in shrimp production was observed. It has increased from 35,500 tonnes in 1990-91 to about 2,16,500 tonnes in 2011-12 with an export value of US $ 3.5 billion. Although the production has increased, the annual loss due to diseases met by the nation was estimated and valued at Rs.1022.1 Crores (approximately US $ 165 millions) (Kalaimani, 2013). In the Indian scenario, the epidemic diseases are white spot syndrome virus (WSSV) infection, Loose shell syndrome (LSS), combination of WSSV and LSS, white gut and slow growth syndrome. Of this, WSSV is the prime pathogen that cause high mortality resulting in huge economic loss (Lightner, 1996). Despite the fact that many strategies like immunization with inactivated pathogens, DNA vaccines, siRNA have been used to protect shrimps against WSSV, there is no effective antiviral therapy to curtail the outbreaks and prevalence of WSSV (Jha et al., 2006, Lu et al., 2009, Tharntada et al., 2009, Witteveldt et al., 2004, 2006, Zhao et al., 2009). Antibiotics, which are used in culture ponds can alleviate bacterial and viral infections, but overdose could result to bacterial resistance and development of new diseases (Rodgers, 2009). Lacking an adaptive immune system, shrimps rely completely on the cellular innate immunity to combat invading pathogens (Bachère, 2004). Immunomodulators have been proven to be effective and enhance the immune protection of the animals. Non-specific immune response induced using immunostimulants has been evolving as an alternate approach to curtail diseases in crustaceans. Use of immunostimulants has increased because of the frequent outbreaks of bacterial, viral, parasitic and fungal infections in culture ponds, hatcheries and aquaculture stations (Anderson, 1992). Immunostimulants in field as supplementary diets have been reported to reduce the risk of several pathogenic infections. Several studies have reiterated the use of polyphenols as immunomodulators in shrimps against bacterial and viral infections (Direkbusarakom, 1996; Immanuel, 2004; Micol, 2005; Praseetha, 2005). It has been hypothesized that these bioactive compounds enhance innate immune response and antagonize viral replication in the host cells (Citarasu, 2010). Medicinal herbs
and their products have taken lead role in antiviral research for the past few decades. Extracts of different medicinal herbs showed results against infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), oncorhynchus masou virus (OMV) and yellow head virus (YHV) in fishes and penaeid shrimp (Direkbusarakom el al., 1993, 1998 a and b)

Turmeric (Curcuma longa), a very common kitchen spice has been used in ayurveda since ancient times. The molecular components present in this medicinal plant have multifaceted clinical applications. Even powdered rhizome is used to treat cuts, wounds, bruises and inflamed joints (Surh, 2002). Although, many studies are available on the potential use of curcumin against several human pathological conditions, reports on the application of curcumin to treat shrimp diseases are very scarce. Supamattaya et al., (2005) evaluated the effect of medicinal plants and the in vitro efficacy of one plant, Curcuma longa showed that their extract incorporated in the shrimp feed could eradicate 15 different isolates of shrimp Vibrio spp.

In this context, the present study was carried out to evaluate the immunomodulatory effects of both natural and synthetic immunomodulators like Curcuma longa extract and CpG ODNs in penaeid shrimps respectively. The toxicity of any botanical extract needs to be addressed prior to its application in a living system. This chapter discusses the preparation, characterization of crude C. longa extract and its nanoemulsion and their relative toxicity in brine shrimps.

1.2. Materials and Methods

1.2.1. Preparation of Curcuma longa extract (CLE)

The dried rhizome of Curcuma longa was crushed to fine powder and the curcuminoids were extracted using acetone. Briefly, 30 g of rhizome powder was immersed in 100 ml of acetone and kept at 80 rpm in an orbital shaker at 28°C for 18 hours. It was then filtered using Whatman No.1 filter paper and the filtrate was evaporated to dryness under vacuum in a rotary evaporator (IKA, Germany).
1.2.2. Preparation of Nanoemulsion

Curcumin nanoemulsion (CNE) was prepared as described by Bouchenal et al., (2004), which involves the preparation of organic phase and aqueous phase. The organic solution composed of 400 mg of α-tocopherol (oil) and 86 mg of Span 80 (a lipophilic surfactant) in 40ml of Acetone (water-miscible solvent). The aqueous phase was prepared with 80 ml of milliQ water and 136mg of Tween 80 (hydrophilic surfactant). To the organic phase 16mg of CLE was added and the organic phase was slowly injected in the aqueous phase under magnetic stirring and the stirring process was maintained for 30min to let the system reach equilibrium. Then, the emulsion was formed instantaneously by diffusion of the organic solvent in the external aqueous phase leading to the formation of nanodroplets.

1.2.3. Fourier Transform Infrared spectroscopy

The FT-IR analysis was carried out to identify the functional groups present in the CLE. The CLE was constituted in methanol and IR spectra was recorded in the spectral range of 400–4000 cm\(^{-1}\) with a FTIR spectrophotometer (Shimadzu, Japan). Curcumin standard was obtained from Sigma Aldrich, USA.

1.2.4. Scanning Electron Microscope

The shape and surface morphology of the CNE was measured by scanning electron microscopy (SEM, Hitachi, S-3400N, Japan) equipped with 5 kV, SE detector with a collector bias of 300V. A small amount of CNE was stuck on a double-sided tape attached on a metallic sample stand, then coated under vacuum with a thin layer of gold before scanning the samples under SEM.

1.2.5. Particle size analyzer

The nanoemulsion droplets size and size distribution, the most important physical characteristics were measured by laser light-scattering particle size analyzer (Zetasizer ZS, Malvern, United kingdom).

1.2.6. Brine shrimp lethality bioassay (BSLB)
Brine shrimp *Artemia salina* (L) also known as sea monkeys, are marine invertebrates about 1 mm in size and are being used widely to assess the toxicity of plant metabolites. Brine shrimp (*Artemia salina*) lethality bioassay technique referring to the modified method of Meyer *et al.* (1982) was followed. Brine shrimps (*Artemia salina, Leach*) nauplii procured from livestock rearing facility, CAS in marine biology, Parangipetttai, were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 h. The mature brine shrimp nauplii were used as the test organisms. The CLE was dissolved in 10% DMSO and the cytotoxic activity was evaluated using Brine shrimp lethality bioassay method where 7 graded doses (15.625, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) were used (Figure 1). DMSO was used as the negative control. The larvae were incubated undisturbed for 24 hours. The animal numbers were counted after 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. They were not fed during the experiment. The dead nauplii in the treatment groups were compared with the control group to ensure that the mortality could be attributed only to CLE and not starvation.

1.2.7. Statistical analysis

The brine shrimp results were expressed as the mean value ± standard error of mean (S.E.M). One way ANOVA was performed using GraphPad Prism software.

1.3. Results

1.3.1. Fourier Transform Infrared spectroscopy

The functional groups present in CLE were identified based on their vibrational frequencies by FTIR analysis using commercial Curcumin (Sigma Aldrich, USA) as the reference compound (Figure 1.1). The IR spectra of the CLE revealed bands at 690.51 cm⁻¹, which could be attributed to the bending vibrations of C-H groups. Absorption bands at 1029 cm⁻¹ and 1114.86 cm⁻¹ indicated the presence of C-O-H and C-O-C groups respectively. Stretching vibrations of -CH₁ and C=C gave characteristic bands at 1456.26 cm⁻¹ and 1664.57 cm⁻¹ respectively. Peaks at 2046.47 cm⁻¹ and 2353.16 cm⁻¹ can be ascribed to CO groups.
Stretching vibrations corresponding to O-H groups in carboxylic acid gave characteristic peaks at 2522.89 cm⁻¹ and 2594.26 cm⁻¹. Peaks at 2833.43 cm⁻¹ and 2943.37 cm⁻¹ were observed which indicated the presence of C-H groups. Absorption bands at 3342.64 cm⁻¹ correspond to the stretching vibrations of O-H groups in phenols.

1.3.2. Scanning Electron Microscope

Figure 1.2 shows the SEM image of CNE at 2.00 µm magnification. The fields were randomly selected to observe the particle size. It ranged between 100-200 nm with a spherical morphology. The SEM image clearly depict the prepared emulsion had nano-particles and they had a smooth surface.

1.3.3. Particle size analyzer

Figure 1.3 shows a typical laser graph of nanoparticles size distribution. The CNE sample size distribution is normal and the particles ranging between 10-1000 nm were documented. The mean size of the particles in the emulsion was found to be 571 nm. Few particles ranging between 10-100 nm were also observed confirming the presence of different sized nanoparticles in the emulsion.

1.3.4. Brine shrimp lethality bioassay

The BSLB was performed to evaluate the cytotoxic effect of the CLE. Figure 1.4 illustrates the survival rate of brine shrimp nauplii in response to CLE treatment. The survival rate was found to be 72.22 ± 1.202, 62.22 ± 1.764, 41.11 ± 0.882, 33.33 ± 1.155, 7.78 ± 1.453 at 15.625, 31.25, 62.5, 125 and 250 µg/ml dosage respectively. The mortality rate was observed to be 100 % in 500 and 1000 µg/mL concentrations. The LC₅₀ value was calculated and found to be 47.97 µg/ml.

1.4. Discussion

Immunization using different strategies to combat pathogenic infections in the field of aquaculture has been the topic of debate for the scientific community. Previous reports on the DNA vaccine, siRNA, etc., were found to be good at laboratory scale but the feasibility in the aqua
farming sector in developing nations has yet to be proven. *Curcuma longa*, a potential source of immunomodulator was used in this study to evaluate its cytotoxic effect. Many reports on the extracts of *C. longa* and its derivatives have been attributed with diverse bioactivities which include antioxidant (Menon and Sudheer, 2007), anti-inflammatory (Surh *et al.*, 2001), antiproliferative (Ono *et al.*, 2013), antiprotozoal (Nagajyothi *et al.*, 2012), antimicrobial (Ronita De *et al.*, 2009), anti-allergic (Kurup and Barrios 2008), antiulcer (Tuorky and Karolin, 2009), antidyspeptic (Deitelhofen *et al.*, 2002) and antidepressant (Yu *et al.*, 2002). Keeping this in view, the present study was attempted to evaluate the immunomodulatory effect of CLE against WSSV infection in penaeid shrimps.

Curcumin was extracted from the rhizome of *C. longa* using acetone. Acetone is an ideal solvent to extract curcumin owing to its low boiling point and less toxicity as compared to other organic solvents like ethanol and methanol (Popuri and Pagala, 2013). Revathy *et al.* (2011) have also demonstrated that acetone exhibited maximum yield of curcuminoids. FTIR analysis gives an overview of the functional groups present in a compound. The FT-IR spectra of CLE revealed peaks corresponding to that of the standard curcumin. The results corroborated with the FT-IR spectra of curcumin obtained by Kolev *et al.*, (2005). Presence of vibrational peaks corresponding to phenolic -OH, aromatic C=C, C-O-C and enol C-O groups confirms that CLE contains curcumin as the major component (Shrivastava *et al.*, 2013; Bich *et al.*, 2009; Coates, 2000)

Any compound (or) drug from natural source must be evaluated for its toxicity before it is proceeded for translational studies. The BSLB is widely being used to determine the cytotoxic effect of heavy metals, pesticides, natural plant extracts (Price, 1974; Meyer, 1982). The brine shrimp (*A. salina*) is a lower invertebrate of the marine ecosystem. The cytotoxicity test on brine shrimp is an efficient, rapid and inexpensive test which requires very small amount of sample (Devaraj *et al.*, 2013). Dimethyl sulfoxide (DMSO) was reported to be a safer solvent as compared to other organic solvent (Wu, 2014) and hence, the CLE was suspended in 10% DMSO for the cytotoxicity assay. The assay result showed an increase in the toxicity level with increasing concentrations of CLE. The LC$_{50}$ value obtained in this study was found to be 47.97 µg/ml.
Previous studies have reported a lower LC$_{50}$ value with ethanol extract of C. longa as compared to the present study (Khattak et al., 2005; Akter et al., 2012). This indicates the acetone extract of C. longa was found to be relatively less toxic than the ethanolic extract as hypothesized by Popuri and Pagala (2013).

Curcumin is not water soluble and has a very short half life due to the metabolic instability and poor absorption characteristics (Mimeault & Batra, 2011). Though several studies have advocated the role of curcumin as effective immunomodulators, its widespread clinical applications have been limited due to poor aqueous solubility and minimal systemic bioavailability (Aggarwal et al., 2005). This is due to its rapid metabolism in the liver and intestinal walls (Shoba et al., 1998). So to overcome poor absorption and to improve the bioavailability, an alternate option is the preparation of nanoemulsion (Solans et al., 2005).

Nanoemulsions have now become a boon to therapy science, as well as offer a wide range of advantages like high thermodynamic stability, incorporation of both hydrophilic and lipophilic compounds in the same nanoemulsion and efficient transportation of natural bioactive compounds through the cell membranes (Huang et al., 2010). In the present study, curcumin nanoemulsion (CNE) was prepared as previously reported. The validation of the presence of nanomolecules were found evident through SEM and PSA studies.

SEM studies showed the surface morphology of nanoparticles present in the emulsion and PSA supports the presence of nanoparticles in the herbal formulation. The size of random picked nanoparticles in the SEM analysis and the average size of particles in the PSA analysis showed to be similar. Few particles when scanned in the PSA appeared to have a relative particle size ranging between 10-100nm, which clearly depicts that the mixture containing varying size of nanomolecules in the emulsion. Particle size here will depend on the type of methods employed and conditions like time and temperature along with sample properties and composition (Quin and McClement, 2011). The average size in the PSA study from the data showed 571 nm and this is
concordant with the study showing nanoemulsions with mean droplet diameters ranging from 50 to 1000 nm is advisable for delivery (Shah et al., 2010)

Nano sized emulsions are able to easily penetrate the pores of the skin and reach the systemic circulation thus getting channelized for effective delivery and also, larger the particle size of the nanoemulsion higher the chance of successful delivery (Ravi and Padma, 2011). Nanoemulsions may loose their transparency with time as a result of increase in droplet size. Nanoemulsions have a much larger surface area to volume ratio than ordinary emulsions, so phenomena related to deformation of the droplets, such as the elastic modulus, are typically larger for nanoemulsions than ordinary emulsions.

In this study, the CLE was prepared from the rhizome of Curcuma longa with acetone. FTIR results confirmed the presence of curcumin like polyphenols in CLE. The toxicity of the CLE was evaluated using BSLB. The assay showed that the CLE was less toxic with LC50 value of 47.97 µg/ml. Poor bioavailability will be a failure and overdose of C. longa extract can become toxic. Previous studies show that nano-administration of the lead will maintain the bioavailability, and act as adjuvant. The CNE was prepared to increase the bioavailability of CLE. The nanoemulsion was validated using SEM and PSA analyses, which showed that the emulsion contained nanoparticles with smooth morphology. The shape and size of nanoparticles were found to be in the administrable range. All encompassed, these observations suggest that the acetone extract can further be used for immunization studies with penaeid shrimps. The acetone extract of C. longa and curcumin nanoemulsion will be a safer immunomodulator with low or no side effects.