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# *Solanum nigrum* enhancement of the immune response and disease resistance of tiger shrimp, *Penaeus monodon* against *Vibrio harveyi*

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ABSTRACT

The effects of different doses of *Solanum nigrum* extract enriched diets, on the immune response and disease resistance of tiger shrimp, *Penaeus monodon* against *Vibrio harveyi*, were examined. Total hemocyte count (THC) and hyaline cell (HC) of *P. monodon* fed with 0.1% and 1.0% doses of *S. nigrum* extract enriched diets significantly increased to the control against *V. harveyi* whereas semi-granular cell (SGC) and granular cell (GC) at 0.1% dose diet. Phenoloxidase activity, superoxide dismutase activity, glutathione peroxidase activity, and glutathione peroxidase activity significantly enhanced of *P. monodon* fed with 0.1% and 1.0% doses of *S. nigrum* extract enriched diets against *V. harveyi*. However, the respiratory burst activity significantly enhanced of shrimp fed with 0.1% and 1.0% doses on weeks 2 and 4. The phagocytic activity significantly enhanced all the supplementation doses from weeks 1 to 4 compared to control against pathogen. The cumulative percentage mortality was low in 0.1% and 1.0% doses diets with 35% and 30% whereas high in 0.01% dose diet as 50% compared to control (90%) against pathogen. Therefore, diet containing *S. nigrum* extract with 0.1% and 1.0% doses were found to enhance the immunity and disease resistance of *P. monodon* against *V. harveyi*.


## 1. Introduction

Finfish aquaculture is rapidly developing globally. Penaeid shrimp culture, especially black tiger shrimp, *Penaeus monodon* culture, is economically important species and the exports valued in Thailand at US$1.6 billion in 1998 (Thai Department of Business Economics, 1999). However, problems of adverse environmental conditions and disease outbreaks due to several *Vibrio* species has been reported as pathogenic for shrimp aquaculture industry (Lavilla-Pitogo et al., 1990; Pizzuto and Hirst, 1995; Thune et al., 1991) a seriously huge economic losses. Among them, *Vibrio harveyi* is the most frequently detected species that infects *P. monodon* hepatopancreas to cause acute or chronic infections known as vibriosis leading to mass mortality of larvae in hatcheries or juveniles in grow-out ponds (Baticados et al., 1990; Jiravanichpaisal et al., 1994; Lightner, 1993). The traditional approaches, such as chemotherapy for disease prevention and management are prohibited due to the emergence of seafood safety issues caused by drug-resistant micro-organisms, in addition to antibiotic residues in farmed animals and the environment (Weston, 1996). Therefore, finding effective approaches, such as vaccines, probiotics, and immunostimulants, which do not involve drug usage for aquaculture disease control instead of using chemotherapeutic methods, has become an interesting issue (Harikrishnan et al., 2011a; Sakai, 1999).

The medicinal use of herbs has a very long tradition in Asian countries. *Solanum nigrum* Linn. (Solanaceae), commonly known as "black nightshade," has been extensively used in traditional medicine in India and other parts of the world to cure liver disorders, cough, asthma, wounds, ulcers, leprosy, skin diseases, hemorrhoids, dropsy, and inflammations (Warrier et al., 1996). The phytochemical studies revealed the plant contains glycoalkaloids (solanine, solamargine, solanigrine, and solasodine), steroidal glycosides (β-solamargine, solasonine, and α,β-solansodamine), steroidal saponins (diosgenin), steroidal genin (gitogenin), tannin, alkaloid (solanine) polyphenolic compounds (Duke, 1985; Saijo et al., 1982; Son et al., 2003). Several studies indicated that *S. nigrum* exhibited anti-ulcer, anti-tumor, anti-oxidant, anti-microbial, anti-cancer, cytotoxic properties and anti-ulcerogenic and hepatoprotective agents (Prasanth Kumar et al., 2001; Raju et al., 2003; Raju and Bird, 2007; Jainu and Devi, 2004, 2006; Al-Fatimi et al., 2007; Yamada et al., 1997; Shishodia and Aggarwal, 2006; Arulmozhi et al., 2010). One of recent study indicate that aqueous extract of this herb significantly inhibit fish pathogenic bacterium *Aeromonas hydrophila in vitro* and *A. hydrophila* infected *Channa punctatus* and treated with the herb significantly increased the hematological parameters (Rajendiran et al., 2008). It also has the capacity to scavenge hydroxyl radicals (Kumar et al., 2001) by

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inhibiting oxidative damage (Lin et al., 2008). However, there was no report of this herb as immunostimulants in shrimp or fish culture to prevent diseases. Thereafter, *S. nigrum* extract with different doses was incorporated into the diet and fed to *P. monodon* to evaluate its effects on immunomodulation and disease prevention against *V. harveyi*.

## 2. Materials and methods

### 2.1. Herbal extract and diet preparation

*S. nigrum* plant was collected from locally and the identification was done by Plant Science Department. The roots were collected from the plants, washed thoroughly with tap water to rid them of dirt. After washing, they were dried under shade to make them suitable for grinding. The dried herbal were grounded in a mechanical grinder and sieved then stored in an air tight container for further use. One hundred grams of coarsely powdered was successively extracted with 85% ethanol and then filtered. The successive extraction was performed by a cold maceration process for seven days with daily agitation twice following Cooper and Gunn (2005) and Singh et al. (2007). The solvent was evaporated using a rotary vacuum evaporator (Buchi, Flawil, Switzerland). The residues obtained after evaporation were stored at −20 °C until used for the experiment. The formulated diet and the ingredients are shown in Table 1. The ingredients of the experimental diet were well mixed and extruded by a pellet extruder (EX 920, Matador, Denmark). Four experimental diets prepared of the pellet with 0%, 0.01%, 0.1%, and 1.0% of *S. nigrum* extracts were sprayed to the basal diet slowly, mixing evenly in a drum mixer, after which it was air dried under sterile conditions for 12 h. The control basal diet was added the same volume of solvent without the extracts. The pellets were dried in an oven at 30 °C for 18 h, packed, and stored in a freezer at −20 °C until used. The proximate composition of the diets were quantified following AOAC method comprised 52.3% crude protein, 8.2% crude lipid, 7.3% crude ash, and 13.7% crude carbohydrate.

### 2.2. Shrimp

Healthy shrimps, *P. monodon* weighing approximately 10.0 ± 0.5 g were purchased from a local shrimp farm and were stocked in cement tanks (1000 l capacity) in the laboratory. The shrimps were acclimatised to ambient laboratory condition. The culture water was first chlorinated with 25 ppm of sodium hypochlorite and dechlorinated and aeration was supplied by a single air-stone to maintain the dissolved oxygen at ≥6 mg/l. Before starting the experiment, the shrimps were examined health status. Shrimp were fed the control diet at 5% of body weight twice a day until the experiment began. Ten percent of water was renewed daily during removal of waste feed and faecal materials. The water quality parameters such as temperature 27 ± 2 °C, pH 8.2 ± 0.2, salinity 28 ± 1.5‰, dissolved oxygen at 6–7 mg $l^{-1}$, and ammonia at <0.1 mg $l^{-1}$ were maintained every day.

**Table 1**
Composition of basal diet for shrimp.

| Ingredients | Composition (g $kg^{-1}$) |
|---|---|
| Brown fish meal | 275 |
| Shrimp head meal | 100 |
| Squid meal | 25 |
| Squid liver powder | 25 |
| Wheat gluten | 65 |
| Wheat flour | 200 |
| Soy bean meal | 100 |
| Broken rice | 100 |
| Fish oil | 20 |
| Vitamin premix | 10 |
| Mineral mix | 40 |
| Other | 40 |

### 2.3. Vibrio harveyi

*V. harveyi* were isolated from diseased shrimps and were confirmed the identity following procedures described by Baumann and Schubert (1984). *Vibrio* strains were cultured in Tryptic soy broth and agar (TSB or TSA) with 2% NaCl (w/v).

### 2.4. Experimental design

Shrimps were divided into four groups of 25 in 60-L plastic aquaria containing 40 L of water at 20‰ and fed with 0%, 0.01%, 0.1%, and 1.0% of *S. nigrum* extract supplementation diets at the rate of 5% of body weight thrice a day (at 8.00, 13.00 and 18.00 h) until the end of the trial. Uneaten food was removed by a siphon after 1 h of feeding. Thus, the experiment was run in triplicate tanks in each group. After 30 days of feeding, all groups were injected intraperitoneally (i.p.) into the ventral sinus of the cephalothorax with 50 μl PBS containing *V. harveyi* at $2.3 \times 10^7$ colony-forming units (cfu) $ml^{-1}$ as the stock bacterial suspension for the challenge trial. On weeks 1, 2, and 4 post-infection, six shrimps were randomly collected from each tank and anaesthetised with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min to collect hemolymph samples were evaluated hematological and immunological immune parameters analyzed included the total hemocyte count (THC) different hemocyte count (DHC), phenoloxidase (PO) activity, respiratory bursts, superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, and phagocytic activity.

### 2.5. THC and DHC measurements

Hemolymph (100 μl) was withdrawn and mixed with 900 μl of an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA, at a pH of 7.55 and with the osmolality adjusted with glucose to 780 mOsm $kg^{-1}$). A drop of the anticoagulant-hemolymph mixture (100 μl) was placed in a hemocytometer to measure the THC and DHC, including hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs) using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany) according to the procedures of Xu et al. (2010).

### 2.6. PO activity assay

PO was spectrophotometrically measured by recording the formation of dopachrome produced from $_L$-dihydroxyphenylalanine ($_L$-DOPA) following the procedures of a previous study (Tseng et al., 2009). The optical density of the shrimp's PO activity was expressed as dopachrome formation in 50 μl of hemolymph.

### 2.7. Respiratory burst assay

Respiratory bursts of hemocytes were quantified using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anion ($O_2^-$) production (Tseng et al., 2009). Respiratory bursts are expressed as NBT-reduction in 10 μl of hemolymph.

### 2.8. Hemocyte lysate supernatants (HLSs)

Diluted hemolymph was prepared as described above and then centrifuged at 500 g at 4 °C for 20 min, and the supernatant was discarded. The hemocyte pellet was washed with a phosphate buffered solution (PBS), and homogenized in PBS on ice. Hemocyte lysate supernatants were then centrifuged at 20,000 g (Hitachi,