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Effects of the water extract of *Gynura bicolor* (Roxb. & Willd.) DC on physiological and immune responses to *Vibrio alginolyticus* infection in white shrimp (*Litopenaeus vannamei*)

Shu-Ling Hsieh [a,*], Chih-Chung Wu [b], Chun-Hung Liu [c], Juang-Lin Lian [a]

[a] Department of Seafood Science, National Kaohsiung Marine University, 142 Haijhuan Rd., Nanzih District, Kaohsiung City 811, Taiwan
[b] Department of Nutrition and Health Science, Chang Jung Christian University, Tainan 711, Taiwan
[c] Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung 916, Taiwan

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ABSTRACT

Gynura bicolor (Roxb. & Willd.) DC is widely distributed in certain areas of Asia and is very popular in vegetarian cuisine in Taiwan. To investigate the regulatory roles of *G. bicolor* in various functions in crustaceans, we examined innate non-specific immune responses (including total hemocyte count (THC), phenoloxidase activity (PO), respiratory bursts (RBs), and superoxide dismutase (SOD) activity), physiological responses (including haemolymph glucose, lactate, and lipids), and gene expressions (including prophenoloxidase (proPO), lipopolysaccharide- and b-1,3-glucan-binding protein (LGBP), and peroxinectin (PE) mRNA transcripts) to the pathogen *Vibrio alginolyticus* in white shrimp (*Litopenaeus vannamei*) that were individually injected with the water extract from *G. bicolor* at 2, 4, and 8 μg g^{-1}. Results indicated that PO, RBs, SOD activity, proPO, LGBP, and PE mRNA transcripts of shrimps receiving the water extract of *G. bicolor* at 2, 4, and 8 μg g^{-1} significantly increased after challenge with *V. alginolyticus* for 96 h. However, no significant difference in the THC was seen at any dose. *L. vannamei* injected with the water extract of *G. bicolor* at all doses respectively maintained lower glucose, lactate, and lipid levels in response to *V. alginolyticus* challenge at 12–36, 24–36, and 24–48 h. Survival rates at 24–72 h of *L. vannamei* that received *G. bicolor* at any dose was significantly higher than those of shrimp that received saline. It was concluded that the water extract of *G. bicolor* can maintain physiological homeostasis and enhance immunity against *V. alginolyticus* infection in *L. vannamei*.

1. Introduction

Natural immunostimulants are biocompatible, biodegradable, cost effective, and safe for the environment [1]. Aquaculture is under pressure to decrease the use of synthetic antibiotics and chemotherapeutics because of the risks posed to humans by chemical residues in foods and by antibiotic resistance being passed on to human pathogens. Consequently, efforts are being made to exploit plants, plant extracts, and natural plant compounds as potential alternatives to synthetic chemicals to stimulate immune responses and disease resistance in shrimp [2]. For example, previous studies showed that non-specific immunity was improved by mixtures of Chinese herbs and plants in shrimp [3,11], and the white shrimp (*Litopenaeus vannamei*) injected with the extract of *Toona sinensis*, *Gelidium amansii*, *Sargassum duplicatum*, and *Gracilaria tenuistipitata* showed increased immunity against *Vibrio* infection [4–7].

Gynura bicolor (Roxb. & Willd.) DC., a perennial plant belonging to the Asteraceae family, is a common vegetable cultivated in Taiwan and Asia. Its leaves are distinctive reddish-purple in color on the abaxial side, contrasting with typical green color on the adaxial side [8]. The leaves of this plant are often consumed in diet. Researches show that the contents of the leaves are nontoxic [9]. The benefits of the traditional use of *G. bicolor* have also been supported by the isolation and identification of several possible flavonoid constituents from this plant. The major constituents of pigment sources and physiological effects of *G. bicolor* are thought to be related to its rich flavonoids including anthocyanins, quercetin, kaempherol, quercitrin, isoquercitrin, and rutin [4,10]. Gynura is usually used as a traditional medicine for the treatment of inflammation, herpes simplex virus, rashes, fever, rheumatism, kidney disease, migraines, constipation, diabetes mellitus, cancer and hypertension [12,13]. For such health-related properties, it is expected to find wide use as a health food material. However, little

* Corresponding author. Tel.: +886 7 3617141x3626; fax: +886 7 3640634.
E-mail addresses: slhsieh@ntu.edu.tw, slhsieh@webmail.nkmu.edu.tw (S.-L. Hsieh).

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effort has been made to determine the impacts of *G. bicolor* application against pathogen infections in shrimp.

Previous research indicated that the total hemocyte count (THC), phenoloxidase (PO) activity, respiratory bursts (RBs), and superoxide dismutase (SOD) activity are commonly used as functional parameters to evaluate the immune potential [14]. PO is the terminal enzyme in the prophenoloxidase (proPO) system, and it acts as both a recognition and effector component of the arthropod defense system [15,16]. On the other hand, when pathogens enter the haemolymph, they are engulfed by hemocytes, and several antimicrobial substances called reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), are generated [17]. The release of superoxide anions is known as the RB, and it plays an important role in microbicidal activity [18]. Although oxygen is an essential element for aerobic cells, it causes potential cytotoxic problems due to the generation of several highly reactive oxygen species in the respiratory process. The effective and rapid elimination of ROS is essential for the proper functioning and survival of organisms. This is performed by antioxidant defense mechanisms, including SOD that scavenges superoxide anions [19].

Several immune-related genes have been successfully cloned from crustaceans and used to investigate the immunostimulatory mechanism of immunostimulants in terms of gene expression [20]. The proPO system has a role in recognizing infections and defending against disease [21]. It is prevalent in crustaceans and allows them to resist many possible foreign particles that enter their bodies by promoting cell-to-cell communication and subsequently eliminating the particles. Specific recognition proteins, including β-1,3-glucan-binding protein, lipopolysaccharide (LPS)-binding protein, peptidoglycan-binding protein, and LPS- and β-1,3-glucan-binding protein (LGBP), which recognize and respond to intruders, were reported in several crustacean species including penaeid shrimp [22,23]. In addition, peroxinectin (PE) functions as a cell adhesion and encapsulation factor and also as an opsonin in promoting phagocytosis and shows peroxidase activity in removing H_2O_2 [24,25].

All forms of stress activate responses through diverse physiological processes, and among them, energy metabolism is of prime importance for physiological compensation by organisms [17,26]. Stress can also suppress the defense system to such an extent that susceptibility to disease is increased [27]. Since aquatic organisms are constantly subjected to environmental fluctuations and under challenge from potential pathogens in the aquatic environment, reciprocal changes in the physiological and immune processes are anticipated. Previous research indicated that glucose, lactate, and lipid contents are commonly used as functional parameters for evaluating the energy metabolism and physiological potential [4].

The Pacific white shrimp, *L. vannamei*, is naturally distributed along the Pacific coasts of Central and South America, and is commonly caught or farmed for food in many countries. Aquaculture production of *L. vannamei* in Taiwan reached 8782 metric tons in 2011 [28]. As the major species of farmed shrimp, the shrimp culture industry has often suffered economic losses attributed to outbreaks of infectious viral and bacterial diseases [29]. Thus, enhancement of immunity and disease resistance in the Pacific white shrimp has become very crucial to the continued and sustained growth of the shrimp culture industry.

This study was undertaken to examine the physiological and immune parameters of shrimp and its resistance to *Vibrio alginolyticus* after receiving the water extract of *G. bicolor*, a common plant cultured in Taiwan. Physiological parameters such as haemolymph glucose, lactate, and lipids, and immune parameters including THC, PO, RBs, and SOD activity of *L. vannamei* were examined. In addition, immune gene expressions, including proPO, LGBP, and PE mRNA transcripts, of *L. vannamei*, and the susceptibility to *V. alginolyticus* when shrimp received the water extract of *G. bicolor* were also monitored.

2. Materials and methods

2.1. Shrimp

L. vannamei shrimp averaging 10.45 ± 0.8 g (mean ± SD) were supplied by the Department of Aquaculture, National Pingtung University of Science and Technology. Upon arrival, they were acclimated to laboratory conditions for 7 days in indoor fiberglass-reinforced plastic tanks and fed a commercial diet (Shye-Yih, Kaohsiung, Taiwan). In all tests, shrimp were fed twice daily with a formulated shrimp diet (Shinta Feed Company, Pingtung, Taiwan). During the experimental period, the water temperature was maintained at 27 ± 1 °C, pH at 7.8–8.0, and salinity at 32‰.

2.2. Gynura bicolor extract

G. bicolor (Roxb. & Willd.) DC was purchased from Yuan-Shan Village's farm cooperative (Ilan, Taiwan). The water extract of *G. bicolor* was prepared based on a method described by Fujiki et al. [30]. Briefly, leaves of *G. bicolor* were washed with water and dried naturally at room temperature before being lyophilized and milled. Five hundred grams of milled leaves was added to 500 mL of deionized water and stirred for 5 min. The suspension was centrifuged at 3500× *g* for 10 min at 4 °C, and the filtrate was concentrated under reduced pressure. The harvested weight of the water extract obtained from 500 g of milled leaves of *G. bicolor* was 5.55 g. The water extract was prepared with phosphate-buffered saline (PBS) to make final concentrations of 1, 2, and 4 mg mL^{-1} as test solutions.

2.3. Culture of V. alginolyticus

The bacterium, *V. alginolyticus* (CH003), was isolated from diseased *L. vannamei* [24]. A known pathogenic strain of *V. alginolyticus* (CH003), which had been isolated from infected *L. vannamei* in Pingtung, Taiwan, was used for the study. Stocks were cultured on tryptic soy agar (TSA; supplemented with 2% NaCl, Difco, Detroit, MI, USA) for 24 h at 28 °C and then transferred to 10 mL of tryptic soy broth (TSB; supplemented with 2% NaCl, Difco) for 24 h at 28 °C. The broth culture was centrifuged at 7155× *g* for 20 min at 4 °C. The supernatant was removed, and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at 10^7 colony-forming units (CFU) mL^{-1} for the susceptibility test.

2.4. Experimental design

Two studies were conducted. In the first set of experiments to determine the susceptibility of *L. vannamei* to *V. alginolyticus*, the treatment and control groups were comprised of 10 shrimp each in triplicate. *L. vannamei* shrimp were individually injected into the ventral sinus of the cephalothorax with 1, 2, and 4 mg mL^{-1} of the *G. bicolor* solution (around 20 μL) to achieve doses of 2, 4, and 8 μg g^{-1} body weight, respectively, on day 1. The challenge test was conducted on day 2 by injecting 20 μL of a bacterial suspension (1×10^7 CFU mL^{-1}) resulting in 2×10^5 CFU $shrimp^{-1}$ through the ventral sinus of the cephalothorax. Shrimp that received no *G. bicolor* extract and then received *V. alginolyticus* at 2×10^5 CFU $shrimp^{-1}$ served as the challenged control, while those that received no *G. bicolor* extract and then received saline (20 μL) served as the unchallenged control (Table 1). There were therefore five treatments in total, the treatment and control groups were comprised of 10 shrimp each in triplicate. Each treatment was conducted with a